The Role of Dietary Fat and Adipose Tissues in the Aetiopathogenesis and Treatment of Crohn's Disease

by

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Declaration

I declare that the content of this thesis entitled “The Role of Dietary Fat and Adipose Tissues in the Aetiopathogenesis and Treatment of Crohn’s Disease” was undertaken and completed by myself, unless otherwise acknowledged and has not been submitted in support of an application for another degree or qualification in this or any other university or institution.

Sarah Ajabnoor

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Abstract

Treatment of Crohn's disease (CD) is challenging. The use of enteral nutrition (EN) as primary therapy in adult patients with CD is not yet supported by high quality evidence. The fat composition of EN has been suggested to be a key factor in controlling the inflammation in CD. Moreover, deep understanding of disease pathogenesis is lacking. In CD, the mesentery attached to the inflamed intestine is often focally thickened: the phenomenon known as “fat-wrapping”, but the reasons for this are unknown. Additionally, the alteration in tissue remodelling of intestinal epithelium by high fat intake is a newly suggested cellular mechanism for intestinal diseases. It is predicted that improved patient outcomes will come from novel nutritional therapies resulting from an improved understanding of the disease pathogenesis.

The aims of my PhD research were to investigate novel mechanisms of action of dietary fatty acids in CD patients’ mesenteric pre-adipocytes and epithelial cells which could benefit the development of optimized lipid formulation of enteral feeds.

Here, in a systematic review of previous clinical trials in CD, we demonstrated that high remission rate is significantly associated with the intake of exclusive enteral nutrition (EEN) feeds that have a high *n-6*:*n-3* ratio. The amount of medium chain triglyceride (MCT) in the feeds was also positively correlated with the remission rate but without statistical significance. lower remission rates were non-significantly associated with higher intakes of feeds enriched with long chain triglycerides (LCTs) or monounsaturated fatty acids (MUFA).

In CD mesenteric adipose tissue (MAT) I have identified several abnormalities in their gene expression profile. Unlike typical adipose tissue, MAT in CD was associated with defective adipogenesis via reduced expression of leptin and CEBPα, and was associated with a low anti-inflammatory profile via decreased expression of M2 macrophage markers. Moreover, in *in-vitro* study I showed that mesenteric pre-adipocytes have an increased adipogenic response to oleic acid, linoleic acid, and α-linolenic acid, which was predominantly modulated via CEBPα. Finally, in cell

culture study I have shown that low concentration of lipids can modulate the physiology of the gut epithelium by reducing colonic crypt proliferation.

In conclusion, my data indicate that the fatty acid composition of EEN can play a key role in improving clinical outcomes in CD. According to my findings, the mechanism of this action can be mediated via MAT function and possibly through intestinal stem cell function. Overall, the findings of this PhD research provide important insights into a new mechanism of action which can be exploited to target future therapeutic approaches in CD and to help optimize the lipid formulation of enteral feeds used in its treatment.

List of publications and conference papers

These publications have been realised during the pursuit of this PhD thesis:

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AA	Arachidonic acid
ALA	α-linolenic acid
ATG16L1	Autophagy 16-like 1
BHT	Butylated hydroxytoluene
BMI	Body mass index
BODIPY	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (a neutral lipid-specific dye)
BSA	Bovine serum albumin
CD	Cluster of differentiation 68, 3, 206, 163
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
cDNA	Complementary DNA
CEPBα	CCAAT-enhancer binding protein alpha
CLA	Conjugated linoleic acid
CRC	Colorectal cancer
CRP	C-reactive protein
CT	Computed tomography
Ct	Cycle threshold
DHA	Decosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetra-acetic acid
EdU	5-ethynyl-2′-deoxyuridine
EEN	Exclusive enteral nutrition
EN	Enteral nutrition
EPA	Eicosapentaenoic acid
ESPEN	European Society for Clinical Nutrition and Metabolism
FABP4	Fatty acid binding protein 4
FAMEs	Fatty acid methyl esters

FBS	Fetal bovine serum
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
GIT	Gastrointestinal tract
GLC	Gas-liquid chromatography
GUSB	Glucuronidase Beta
GWAS	Genome-wide association studies
H_2SO_4	Sulphuric acid
HBI	Harvey-Bradshaw index
HEPES	N2-2-hydroxyethylpiperazine-N2-ethanesulphonic acid
IBD	Inflammatory bowel disease
IBMX	Isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
ISCs	Intestinal stem cells
KCl	Potassium Chloride
$KHCO_3$	Potassium bicarbonate
LA	Linoleic acid
LCT	Long chain triglyceride
LPL	Lipoprotein lipase
MAT	Mesenteric adipose tissue
MCP1	Monocyte chemoattractant protein 1
MCT	Medium chain triglyceride
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
MUFA	Monounsaturated fatty acid
NH_4Cl_2	Ammonium chloride
NNUH	Norwich and Norfolk University Hospital
NOD2	Nucleotide-binding oligomerization domain 2
NSAIDs	Non-steroidal anti-inflammatory drugs
OCP	Oral contraceptive pills
PBS	Phosphate buffered saline

PFA	Paraformaldehyde
PN	Parenteral nutrition
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
RCT	Randomized clinical trial
RNA	Ribonucleic acid
RPL13a	Ribosomal Protein L13a
RR	Remission rate
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFA	Saturated fatty acid
SIBDQ	Short Inflammatory Bowel Disease Questionnaire
SVF	Stromal-vascular fraction
TGF-β	Transforming growth factor-β
TLC	Thin layer chromatography
TNFα	Tumour necrosis factor α
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule-1

1 Chapter 1. Introduction: Crohn's disease, therapeutic lipids, the fat-wrapping phenomenon, and intestinal epithelium healing

1.1 Background of Crohn's disease

CD is a chronic inflammatory bowel disease (IBD). Unlike ulcerative colitis (UC), CD can affect any segment of the gastrointestinal tract (GIT) from mouth to anus, and usually in a discontinuous and transmural pattern of inflammation, affecting all layers of the gut from mucosa to serosa. Therefore, the disease is associated with the development of fistula and intestinal perforation. The terminal ileum and colon are typically affected in CD. Patients with CD usually experience unpredictable events of relapse and remission. Thus, the main goal of disease management is to induce clinical remission and to maintain it using drug therapy. However, when the disease becomes complicated and drug resistance develops, most CD patients will require surgical intervention. The prevalence of CD varies across the world. The incidence rate is greater in the north and west of Europe and North America, while it is low in Asia, Africa, and South America (Hart and Ng, 2015). CD can present at any stage of life, but is usually diagnosed between the age of 10 and 40 years (Hart and Ng, 2015). However, the number of cases is currently increasing, particularly in younger and non-western populations.

Until recently, CD was considered to be an idiopathic disorder. However, it is now believed that CD may be caused by a combination of genetic and environmental factors. The role of different risk factors in modulating the chronic inflammatory response of CD is complex, and there are gaps in the existing knowledge of disease aetiology and pathogenesis. Future medical therapies targeting new plausible mechanisms are currently under investigation.

1.2 Epidemiology

CD incidence varies according to geographical location. Generally, the incidence rate of the disease is high in North America, northern regions of Europe, and United Kingdom (Vind et al., 2006, Yapp et al., 2000, Loftus Jr et al., 1998). Nevertheless, the incidence and prevalence rate of CD have changed over the last decades; it started to increase not only in the western countries but also in eastern countries like Japan, Hong Kong, and Korea (Thia et al., 2008). Epidemiological

studies across eight countries in Asia and Australia found that the incidence of CD was 0.54 per 100,000 individuals which is significantly lower than in western countries (Ng et al., 2013). The disease is still considered uncommon in African and South American countries, however, recent studies suggest a rising number of new cases in these regions (Archampong and Nkrumah, 2013, Ukwenya et al., 2011). An increasing number of CD cases have been reported in Saudi Arabia, with similar morbidity as in the western countries (Al-Mofarreh and Al-Mofleh, 2013). Furthermore, the role of environmental risk factors in increasing the incidence of CD is supported by studies on migrants, which aim to monitor disease incidence and behavior in migrant populations and their offspring, during their transition from developing countries to developed countries. Data from studies in Sweden suggested that the incidence of the disease in migrants coming from West or South Asia increased in second generation migrants while it was low in first generation migrants (Li et al., 2011). While another Canadian study comparing the incidence of the disease between migrants and their Canadian-born children, with the Canadian population found that the lower the age of migrants coming to Canada, the higher the risk of developing the disease; furthermore, children of migrants (coming from selected countries) who were born in Canada had a higher incidence of the disease, suggesting that disease risk factors could be influenced by earlier exposure in life to environmental triggers (Benchimol et al., 2015). Overall, such studies have provided a deeper understanding of the relationship between environmental factors and disease characteristics.

CD is more commonly diagnosed at a younger age; the disease is considered a disease of the youth. Data from population-based studies have shown that the median age of patients at the time of diagnosis is between 29.5 and 31years (Ramadas et al., 2010, Vind et al., 2006, Loftus Jr et al., 1998). The majority of the studies have also shown that CD predominates in females, unlike UC, which is slightly more prevalent in males (Forbes, 2001, Loftus Jr, 2004). However, the CD incidence can be also vary by race and ethnicity. For instance, the risk of the disease is higher in Jewish populations from America and Europe, while it is low in African-American and Hispanic populations (Ananthakrishnan, 2015).

1.3 Aetiology and pathogenesis

Over the past years, CD has been considered as an idiopathic disease. Several risk factors are implicated in its pathogenesis but with unclear mechanisms of action. The combination of genetic predisposition, environmental factors, changes in gut microbiome, and the resulting host immune response have been suggested to trigger chronic inflammation in the gut, causing the disease.

1.3.1 Genetic factors

Convincing evidence from family and twin studies have supported the derived heritability in CD before genetic confirmation existed; the risk of IBD is greater in first degree relatives of patients with CD (Halme et al., 2006) and is approximately 50% in identical twins. The genome-wide association studies (GWAS) have identified many susceptibility genes and loci for IBD, which greatly help in understanding the molecular mechanism of the disease (McGovern et al., 2015). Most of these genes are involved in the maintenance of intestinal barrier function, immune responses, autophagy, tissue repair, and in immune reactions to foreign microorganisms and antigens (Chan et al., 2015, Ananthakrishnan, 2015). However, several of the identified genes could be influenced by the presence of certain environmental triggers (Guerreiro et al., 2009). Most of the identified genes are associated with both UC and CD with similar outcomes; however, the expression of the NOD2 and ATG16L1 genes is considered to be more specific to CD only, while other genes are associated with UC (Jostins et al., 2012).

1.3.2 Environmental factors

The rising incidence rate of CD in countries adopting a western lifestyle, and among migrants living in western countries strongly support the association between environmental factors and disease pathogenesis (Chan et al., 2015). Over the past years, several risk factors have been identified by epidemiological studies. Some were found to occur consistently between the studies, while other risk factors were debated with inconclusive evidence and lack of certainty about their effect.

Smoking has been consistently recognized as a risk factor for CD. According to a systematic review, the estimated rise in CD risk among smokers was found to be twofold (OR 1.76, 95% CI 1.40–2.22) (Mahid et al., 2006). However, the effect of smoking on CD risk seems to be dose dependent, and the earlier the exposure to smoking in life, the higher the disease risk (Ko et al., 2014). Smoking was also found

to be correlated with early onset of the disease and with a high dependency on immunosuppressive drugs, particularly in females (Cosnes et al., 2004). It is also associated with disease worsening and poor prognosis, as the requirement for surgical resection, and the rate of disease relapse and recurrence increased significantly in CD patients who smoke (Hovde and Moum, 2012). The mechanism of action of smoking on disease pathogenesis is not completely understood. Nevertheless, studies have shown that smoking could play a role in changing the smooth muscles that stimulate endothelial function, in reducing intestinal epithelial integrity, in increasing the level of oxidative stress, and impacting the gut microbiome (McGilligan et al., 2007).

The association of diet and early life feeding patterns with CD risk has been supported by many epidemiological studies. A protective effect of breastfeeding against CD development has been reported by several studies. An earlier meta-analysis founded that breastfeeding is associated with lower risk of CD, however, only few of the included studies were graded as high quality (Klement et al., 2004). Moreover, the evidence also seems to be contradictory, as a recent large prospective cohort study of U.S. women did not find a significant correlation between early life factors, including being breastfed, and the risk of CD (Khalili et al., 2013). Further studies of a higher methodological quality are needed for clarifying this association.

Studies have found that the risk of CD is usually high in people following a western diet, which is high in refined and processed foods that are high in fat and protein, but low in fruit and vegetable. Scientists have explained the association between diet and CD risk using several theories. For example, the presence of antigens, alteration in gut microbiota, over nutrition, and changes in prostaglandin levels are proposed as possible biological mechanisms behind this association (Hou et al., 2011). However, the evidence for these mechanisms is contradictory. For instance, the practice of excluding food antigens from diet (exclusion diet) is not supported by adequate evidence, as there has been an apparent similarity of the efficacy of the elemental enteral diet and the polymeric enteral diet in patients with active CD, which will be highlighted later in this report (Zachos et al., 2007b).

The effect of macronutrients intake on increasing the risk of the disease has been investigated by many case-control and large cohort studies, which can be associated with several limitations. The effect of excessive carbohydrate intake is

suggested to be relevant to inducing the development of CD by either affecting gut microbiota composition or by leading to obesity, which could influence the expression of markers predisposed to intestinal inflammation (Chan et al., 2014b). However, limited findings from earlier studies have shown inconsistent results, and a recent large prospective cohort study has found no association between total carbohydrate and total sugar intake with CD, suggesting that future epidemiological studies should address the individual effects of each type of carbohydrate that could be considered an actual risk factor (Chan et al., 2014b). For the effect of protein intake, it is mostly limited to the intake of animal protein, as studies have shown a positive correlation between the rising incidence of CD and increased meat intake in Japan and European countries (Shoda et al., 1996, Jantchou et al., 2010). High meat consumption was suggested to increase CD risk due to an increase in toxic, metabolic end product production in the colon by the local microbiota (e.g. hydrogen sulphide) (Jantchou et al., 2010).

Out of all the macronutrients, fat has been the most interesting dietary factor to investigate in CD, mostly because of the suggested involvement of some types of fat in the inflammation process, consequently augmenting the risk of CD. A resent systematic review of case-control and cohort studies has shown that the risk of both types of IBD is greater in individuals with a history of high total fat, polyunsaturated fatty acid (PUFA) and meat intake; whereas high intake of dietary fibre and fruits were correlated with lower CD risk, and high vegetable intake was associated with lower UC incidence (Hou et al., 2011). In this systematic review, there was a great level of heterogeneity between the included studies and the retrospective nature of many case-control studies that may have further limited the quality of the results, due to the possibility of a recall bias of dietary intake. Nevertheless, a large prospective study from the Nurse Health Study has shown inconsistent findings; insignificant correlation was found between the intakes of total fat, saturated/unsaturated fatty acids, or specific polyunsaturated fatty acids (PUFAs) with the risk of CD (Ananthakrishnan et al., 2014). However, only a significant association was found between high, long-term intake of *n*-3 fatty acid and reduced risk of UC, and between the excessive intake of trans-fatty acids and increased incidence of UC. Although, the second study has many strengths with regard to its design, it is still limited by the inclusion of only white women who might have specific traits for later disease onset; thus, we cannot

generalize its findings (Chan et al., 2015). As current evidence in the literature is limited and contradictory, future epidemiological studies with better design are still needed to clearly understand the role of various dietary components in triggering CD.

The effect of selected micronutrients on CD risk have been reported by several studies. Vitamin D was found to be linked to CD pathogenesis. Vitamin D deficiency was found to be highly prevalent in patients diagnosed with IBD (Abraham et al., 2014). Yet, it is not clear whether low vitamin D levels are a result of the disease itself or if it is a causative factor in disease onset. Current evidence from observational studies is inconclusive and contradictory in supporting the association between vitamin D deficiency and CD risk (Del Pinto et al., 2015). However, the evidence associating vitamin D deficiency with disease outcomes and progression is more convincing. Vitamin D deficiency in CD patients is considered multifactorial; it could be related to low level of sun exposure, low intake, malabsorption secondary to small intestinal resection, altered enterohepatic circulation, and increased intestinal vitamin losses as a result of protein-losing enteropathy (Ulitsky et al., 2011). Additionally, iron intake has been linked to CD risk. Iron could stimulate intestinal inflammation via specific signaling pathways, via oxidative stress, and by altering the composition of gut microbiota (Carrier et al., 2001, Khalili et al., 2017). One epidemiological study has shown a relationship between iron enriched drinking water and higher CD risk (Aamodt et al., 2008). However, findings from cohort studies have shown no association between dietary iron intake (i.e. total and heme iron) and CD risk (Khalili et al., 2017). Yet, the association between iron and disease onset and pathogenesis needs further validation. Moreover, zinc intake might be related to CD risk by its impact on autophagy, innate and adaptive immunity, and on the regulation of the gut barrier function. According to one epidemiological study, dietary zinc was inversely correlated with CD risk (Ananthakrishnan et al., 2015).

The level of exposure to infections, antibiotic use, and the use of drugs such as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), and oral contraceptive pills (OCP) are other identified environmental factors that could trigger the development of CD. The use of antibiotics, especially during the first year of life, was highly associated with CD incidence rate. According to a cohort study, 58% of IBD diagnosed pediatric patients had received antibiotics in the first year of life, while only

39% did not receive antibiotics (Shaw et al., 2010). The relationship between NSAID use and IBD risk was found to be stronger with larger doses and longer duration of use, and was the same for both types of IBD (Ananthakrishnan et al., 2012). In addition, results from a meta-analysis showed that OCP use raised the incidence rate of CD in a dose-response manner, while OCP withdrawal decreased the risk of disease development (Cornish et al., 2008).

1.3.3 Immunological factors

Disturbed immune response to the normal intestinal microbiota, and the irregular balance of pro-inflammatory and anti-inflammatory cytokines are both considered contributing factors in CD pathogenesis. The inflammatory responses in CD are characterized by the high expression of pro-inflammatory markers like Tumour necrosis factor-α (TNF-α), Interleukin-6 (IL-6), IL-8, and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Overall, maintenance of gut homeostasis needs an adequate balance between pro-inflammatory and anti-inflammatory cytokines, which are controlled by T-cells. An imbalance in these pathways may result in an interrupted immune response to intestinal bacteria leading to CD development (Round and Mazmanian, 2009).

1.4 Clinical symptoms and complications

The symptoms for CD are dependent on the location and the severity of the inflammation. Generally, the clinical symptoms for patients diagnosed with CD usually involve: diarrhea, abdominal pain, thickening of the intestinal wall, which causes intestinal stricture and obstruction, abscess formation, and fistula development between the skin and internal organs. Progressive disease can lead to nutrient malabsorption and steatorrhea.

Other extraintestinal manifestations of the CD inflammatory response include: low-grade fever, weight loss (because of reduced food intake and malabsorption), delayed growth in children, large joint arthritis, eye complications (e.g. uveitis, iritis, and episcleritis), and skin lesions (e.g. erythema nodosum and pyoderma gangrenosum) (Gajendran et al., 2018).

1.5 Diagnosis

CD is associated with transmural inflammation, where all layers of the intestinal wall can be affected. CD can affect any part of the GIT starting from the mouth to the anus. Gajendran et al. have reported the prevalence of colonic, ileal and colonic-ileal CD at about 25%, 25% and 50%, respectively. (Gajendran et al., 2018). Overall, the key aspects of CD diagnosis depend on an evaluation of clinical symptoms and also on laboratory, radiographic, endoscopic, and pathological findings which indicate focal, asymmetric, transmural or granulomatous characteristics. Computed tomography scanning (CT scan) of the intestine is the most commonly used radiographic method for assessing CD, while magnetic resonance imaging (MRI) is also used, and is associated with reduced exposure to ionizing radiation. Nevertheless, endoscopies remain the gold standard for assessing disease activity and progress, and also for measuring the degree of mucosal healing, particularly in clinical studies.

1.6 Assessment of disease activity

Several scoring systems have been established for the clinical assessment of disease activity in CD patients. This include the Crohn's Disease Activity Index (CDAI) which is the most commonly used criteria, the Harvey-Bradshaw index (HBI), the Montreal classification system, the Short Inflammatory Bowel Disease Questionnaire (SIBDQ), and the Lehmann score (Gajendran et al., 2018). Most of these criteria will assess disease activity based on: subjective measures, such as the disease's affect on the patient's life (e.g. symptoms, quality of life, and fatigue), objective or quantifiable measures (e.g. C-reactive protein (CRP) level), and disease severity measures (e.g. history of bowel resection, number of relapses, or presence of any extraintestinal disease features) (Peyrin-Biroulet et al., 2016).

1.7 Management

1.7.1 Medical therapy

The primary goal of medical therapy in CD patients is to induce and maintain remission, restore the damaged mucosa, and to improve the patient's overall quality of life. The choice of therapy usually depends on the severity and the location of the disease. Several therapeutic agents have been used over the past years. The use of corticosteroids is now disputed as it is probably only effective in reducing symptoms, while it is inefficient in maintaining remission (Dignass et al., 2010, Forbes et al., 2011). 5-aminosalicylic containing drugs are not effective in maintaining remission

and their use in CD has been controversial (Akobeng and Gardener, 2005). Immunomodulators are, nevertheless, effective in acute intervention and as a maintenance therapy. Other emerging therapies that modulate key aspects of the different inflammatory processes in CD have been investigated for their efficacy. Although, there is strong evidence supporting the benefit of using these medications, the number of associated side effects cannot be overlooked. In situations where the disease becomes more complicated or when there is resistance to drug therapy, surgical intervention will be required. This kind of intervention is more common when the disease involves the ileum. It has been estimated that about 50% of CD patients will undergo at least one operation during the first 10 years of diagnosis, while 40% of patients will need additional surgery in the first 10 years of diagnosis (Hart and Ng, 2015).

1.7.2 Nutritional therapy

Poor nutritional status is commonly seen in CD patients, especially in individuals with affected small bowel. The prevalence of malnutrition in CD are attributed to the following reasons: the malabsorption of nutrients, intestinal losses, anorexia, vitamin deficiencies, catabolic effects of inflammation, poor appetite as a result of gastrointestinal symptoms (abdominal pain and diarrhoea), and the side effect of certain drugs. Therapeutic approaches in CD should also target disease-related malnutrition, which would eventually improve the overall patient prognosis. Artificial nutrition support has been more favoured over the past years in CD. The rational for using tube feeding or parenteral nutrition (PN) in CD is basically two-fold; first, it can treat malnutrition, secondly, it can modify the underlying disease pathogenesis aiming to achieve a high rate of remission, via controlled delivery of exogenous dietary substances to the gut (Forbes et al., 2011).

Several dietary therapies have been identified for managing patient symptoms and prolonging remission. For example, a gluten-free diet, a diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (the low FODMAP diet), or following a diet rich in *n*-3 PUFAs, have been increasingly recommended over the past few decades. Nevertheless, clinical trials are limited and the evidence is yet inconclusive in showing the potential benefits of such dietary modifications in CD patients. According to a recent IBD guideline published by the European Society for

Clinical Nutrition and Metabolism (ESPEN), no specific diet can be generally recommended for inducing disease remission.

1.8 Enteral nutrition in the treatment of Crohn's disease

The efficacy of nutritional support, especially that of EN in adult patients, has only been supported by limited evidence, which provided limited assumptions about its mechanism of action. Therefore, the current published guidelines state that EN in adults is less efficient than steroids in inducing remission, but it might be recommended in special situations, such as when other primary therapies become unfeasible, or when patients refuse the use of medication (Lomer, 2014, Narula et al., 2018). Contrarily, evidence from paediatric studies are relatively consistent in recommending EN as a primary therapy; such a practice originates from the observation that EN administration resulted in a significant improvement in growth, while growth failure and poor bone health occurred with the continuous use of steroids.

The effect of EN in CD has been observed by different types of formulations: whole protein-based feeds with high fat (polymeric feeds), glucose and amino acid-based feeds with low fat (elemental feeds), and peptide-based feeds (semi-elemental feeds). The low antigenic profile of elemental formulae was initially proposed as a reason for why these feeds are preferable. However, this idea was later challenged by the findings of an updated meta-analysis of clinical trials, that found similar effects of polymeric and elemental feeds in CD patients (Zachos et al., 2007b, Narula et al., 2018). Due to the proposed role of lipids in manipulating the inflammatory response, some trials have specifically compared the effect of different feeds consisting of differing fat compositions, in CD patients, which generated inconsistent interpretations of the effect of lipids.

1.9 The role of lipids in Crohn's disease

1.9.1 Lipid classes

Generally, lipids are classified into fatty acids, triacylglycerol, phospholipid, glycerol, steroid and wax groups. Fatty acids are considered the building blocks of lipids. The chemical structure of fatty acids consist of long hydrocarbon chains ending with carboxyl group. Fatty acid chains can be either short (< 6 carbon atoms), medium (between 6 and 12 carbon atoms), or long (> 14 carbon atoms). Another classification of fatty acids is based on the presence of double-bonded carbon atoms. Unsaturated

fatty acids contain double bonds in the carbon chain, while saturated of fatty acids do not.

Fatty acids are important for human health, and include essential fatty acids that cannot be synthesised by the human body, therefore needing to be obtained through one's diet. This includes *n*-6 PUFA (linoleic acid) and *n*-3 PUFA (α-linolenic acid), both of which have at least two double bonds and are vital for maintaining health. The metabolism of α-linolenic acid results in the production of two essential unsaturated fatty acids, which are eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) (Figure 1). The main dietary source for EPA and DHA is fish and fish oils. Over the past decades, several health benefits have been suggested to be associated with the high intake of EPA and DHA.

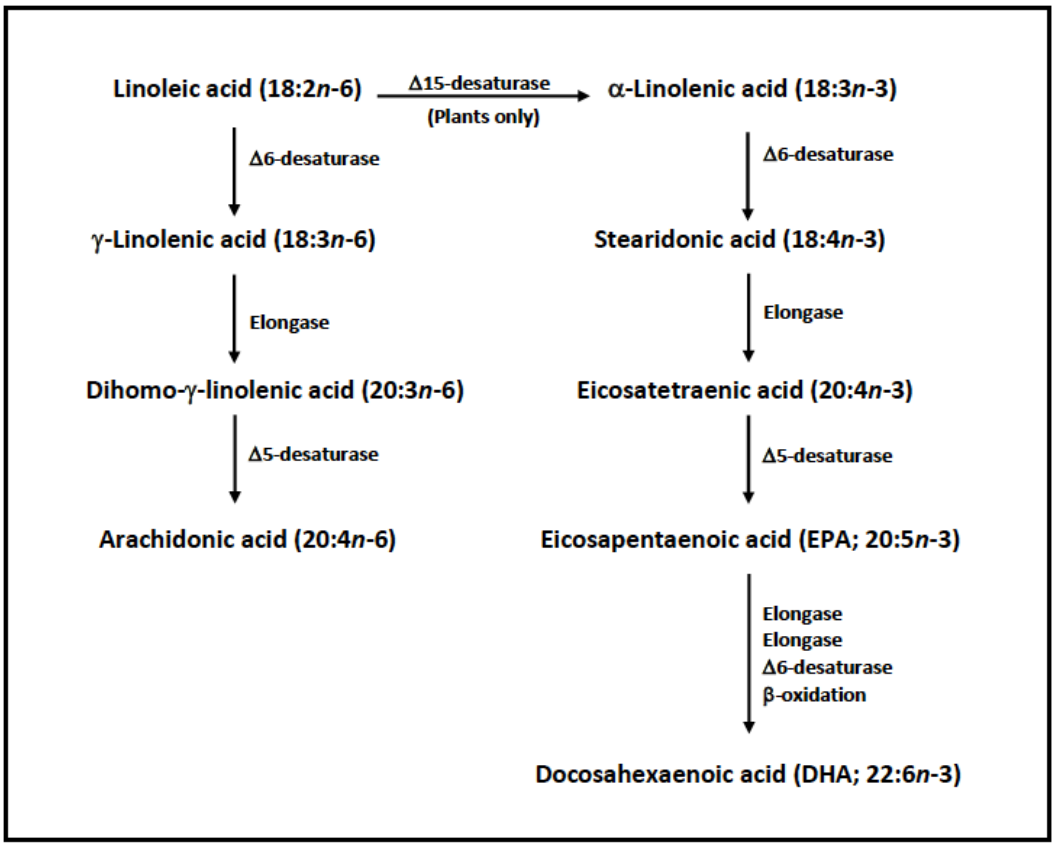


Figure 1: The conversion of plant-derived essential *n-6* and *n-3* PUFA to their longer chain, more unsaturated derivatives.

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

1.9.2 Lipids as pro-inflammatory mediators

n-3 PUFAs have several anti-inflammatory functions. They have the ability to reduce leucocyte chemotaxis, the degree of adhesion molecule expression and leucocyte-endothelial adhesive interactions, the release of eicosanoids (i.e. prostaglandins and leukotrienes) from the *n*-6 PUFA metabolism, the release of inflammatory cytokines, and the proliferation of T-cells (Calder, 2013). On the other hand, *n*-6 PUFA has been associated with many inflammatory effects, where its derivative, "arachidonic acid" is considered a precursor for key pro-inflammatory mediators. (Innes and Calder, 2018). However, current evidence is still inconclusive in confirming the inflammatory effect of *n*-6 PUFA. Also, the balance between *n*-3 and *n*-6 PUFAs, and the communication between their lipid mediators during inflammation is complex and unclear.

1.9.3 Role of dietary and therapeutic lipids

Due to the proposed role of lipids in controlling the inflammatory response, several studies have examined the effect of different fat compositions in nutritional feeds that have been prescribed for CD patients as EN. Polymeric, protein-based feeds with high fat content have been compared with low fat, glucose and amino acid-based feeds, and with oligomeric peptide-based feeds (Fernandez-Banares et al., 1995, Griffiths et al., 1995, Messori et al., 1996, Zachos et al., 2007a), but without compelling evidence that one is better than another (Zachos et al., 2007a). One meta-analysis found a negative correlation between long chain triglyceride (LCT) content and remission rate (RR) (Middleton et al., 1995), and a second found comparable, but non-significant trends favouring low LCT and low overall fat content (Zachos et al., 2007a). Regarding the effect of feeds high in medium chain triglyceride (MCT) on modulating the inflammatory response in CD, the evidence is yet not conclusive. An earlier meta-analysis of randomized clinical trials (RCTs) has shown a significant reduction in the levels of inflammatory markers observed with MCT-rich feed, suggesting that there is an anti-inflammatory effect of MCT oil, while a later study found no significant difference between low MCT feed and high MCT feed (70% MCT) on the clinical RR in CD patients (Middleton et al., 1995, Sakurai et al., 2002). Given the potential aetiopathogenic relevance of lipids to CD (due to a higher disease prevalence in populations on high fat Western diets) and the curious phenomenon of fat-wrapping (almost pathognomonic of CD), further investigation in this area is

warranted, partly because of the inability of the other meta-analyses to provide a verdict on this issue.

The anti-inflammatory effect of *n-3* in CD has been strongly supported by animal and *in-vitro* studies, while the currently available clinical trials provide conflicting results. Most of the available trials assessed the effect of *n*-3 PUFA in CD patients by using fish oil dietary supplements, while only few studies assessed its effect within therapeutic EN feeds. A recent systematic review of RCTs did not show any positive effect of using *n*-3 PUFA in maintaining remission in CD patients (Lev-Tzion et al., 2014). The major limitation of this review was the heterogeneity of the included clinical trials, such as variations in *n*-3 PUFA formulations and in routes of delivery. Although supplementation with *n*-3 PUFA has always been considered safe, an analysis from this review has shown that adverse events of gastrointestinal symptoms, like diarrhoea, are more prevalent in the intervention group than in the control group. Several mechanisms have been proposed for the role of *n*-3 PUFA in treating CD patients. A high intake of *n*-3 PUFA can reduce the availability of arachidonic acid, leading to a lower production of eicosanoids, and consequently, a decreased expression of pro-inflammatory markers. It also can reduce the synthesis of pro-inflammatory cytokines, such as TNF-α (Gorard, 2003).

1.10 Mesenteric adipose tissues in Crohn's disease

1.10.1 Fat-wrapping: a characteristic feature in Crohn's disease

In 1932, the fat-wrapping phenomenon in CD was first identified and was characterized by a hypertrophy of the MAT adjacent to the inflamed intestines (Crohn et al., 1932). It is considered a specific feature to CD. However, only few studies have speculated the effect of fat-wrapping on disease activity (i.e. the degree of ulceration, transmural inflammation, and inflammatory response), suggesting that it could be a predictor of more chronic disease (Sheehan et al., 1992, Desreumaux et al., 1999). Nonetheless, later studies have started to focus on the pathogenic mechanism behind this phenomenon, proposing a potential role of MAT in CD pathogenesis (Zulian et al., 2012, Peyrin-Biroulet et al., 2012).

1.10.2 Histology

Only a few studies have tried to investigate the morphology and the cellular compartment of fat-wrapping in CD. Histological findings from earlier studies found that morphological features of the MAT of CD patients differ from other patients. The

size of adipocytes in the MAT of CD patients was found to be significantly smaller, but higher in number compared to mesenteric adipocytes derived from control cases (Peyrin-Biroulet et al., 2007). Conversely, hypertrophied MAT is caused by an increase in adipocyte size in obesity. Moreover, the inflamed mesenteric fat in CD is significantly infiltrated with inflammatory cells. Compared to non-inflamed MAT, the expression of macrophages and T lymphocyte markers (i.e. CD68 and CD3 positive cells) were found to be significantly higher in hypertrophied MAT in CD (Yamamoto et al., 2005). The evidence is still inconclusive in determining whether adipocyte hyperplasia is primarily responsible for activating inflammation in the hypertrophied fat in CD, or whether this is a secondary outcome resulting from the transmural inflammation of the diseased intestine.

1.10.3 Expression of inflammatory adipocytokines in mesenteric fat

Adipocytes are considered important factors in adipose tissue inflammation, as inflammatory mediators are synthesized by these cells. In mesenteric adipocytes, alteration in the balance of pro-inflammatory and anti-inflammatory adipocytokines was reported (Paul et al., 2006). Smaller adipocytes tend to secret higher amounts of adiponectin than larger adipocytes (Yamamoto et al., 2005). The secretion of other adipocytokines including leptin, resistin, IL-6, and Monocyte chemoattractant protein 1 (MCP1) by CD mesenteric fat has also been reported.

1.10.4 Mesenteric fat as a source of CRP

CRP is an acute-phase protein marker that is synthesized primarily by the liver. It has a key role in innate immunity. Due to its fast response and short half-life, CRP has been used as a clinical indicator for assessment of infections and inflammation (Henriksen et al., 2008). Although CRP is not considered a specific marker for intestinal inflammation in the context of CD, several clinical outcomes reflecting disease activity were shown to correspond to CRP levels. For example, CRP level was shown to correlate with the CD endoscopic score and with clinical relapse rate (Chang et al., 2015). Interestingly, the secretion of CRP by MAT has been recently investigated in CD. The mesenteric fat depot can be an important source of CRP in CD, which eventually could contribute to the overall status of inflammation. Furthermore, recent findings have identified bacterial translocation and local inflammation as triggers to CRP production in MAT (Peyrin-Biroulet et al., 2012).

1.10.5 Mesenteric fat as a target for bacterial translocation

Generally, CD is associated with enhanced gut permeability and bacterial translocation. However, the association between bacterial translocation and the accumulation of mesenteric fat in CD has been recently investigated. Studies have shown that adipocytes can respond to bacterial stimuli (Batra et al., 2009). Recent findings have shown that bacterial translocation into mesenteric adipocytes results in the inflammatory response in CD via CRP synthesis (Peyrin-Biroulet et al., 2012). Other findings from animal studies on experimental colitis have shown that the accumulation of MAT could protect against bacterial translocation via phagocytosis (by adipocytes) and via the increased production of cytokines and infiltration of immune cells, thus mediating chronic inflammation in order to act as a defense mechanism (Batra et al., 2012). Yet, the various proposed theories regarding the involvement of the MAT in CD pathogenesis require further validation.

1.10.6 Fatty acid profile of mesenteric fat

Adipose tissue has always been recognized as an important organ in regulating lipid storage and metabolism. Adipose tissue acts as an energy reservoir by storing lipids. In addition, mesenteric adipocytes that are adjacent to lymph nodes have an important role in paracrine interactions. Adipocytes can supply fatty acids to its surrounding lymph nodes, which makes the MAT that contains lymph nodes work as a link between the human diet and gut epithelial immunity (Pond, 2009). Generally, the immune system depends on fatty acids for energy provision and for cell membrane functions (especially *n*-3 and *n*-6 PUFAs). However, in the case of chronic inflammation, studies in animals with experimental colitis have found that the fatty acid profile of lymph node-containing MAT is high in PUFA (predominantly *n*-6 PUFA) (Acedo et al., 2011). While, another study on human CD tissues showed that MAT and the attached lymphoid tissues have more saturated fatty acids and less PUFA than controls, the study also found that the ratio of *n*-6: *n*-3 fatty acids is high in the MAT, while it was low the in the adjacent lymphoid tissues (Westcott et al., 2005). The author has suggested that interrupted paracrine interaction between adipose and lymphoid tissues in CD is responsible for the abnormal fatty acid composition in lymphoid cells. Moreover, similar findings were observed in an earlier study, but with a different design, in which the fatty acid profile of plasma phospholipids and subcutaneous adipose tissue for CD patients were investigated: a significant reduction

in the sum of *n*-3 and *n*-6 PUFA was found, and the author speculated that the abnormal fatty acid profile in such patients was a result of altered fat metabolism, rather than intestinal fat malabsorption (Geerling et al., 1999). Given the fact that there are differences in the nature of the disease between human CD and the animal CD models, the findings from the above studies should be carefully interpreted. Further investigations of the role of CD mesenteric fat in paracrine interaction are warranted. Understanding more about such defective lipid metabolism would help in identifying the underlying mechanisms between the MAT and intestinal inflammation in CD.

1.10.7 Therapeutic approaches targeting mesenteric fat in Crohn's disease

Interestingly, the presence of hypertrophied mesenteric fat in CD patients is currently considered a working model for ongoing IBD research. Some of the current applied therapeutic approaches for CD have been investigated in relation to the fat-wrapping phenomenon. A recent trial has found a relationship between exclusive enteral nutrition (EEN) therapy and the alteration of MAT in patients with active CD; changes in adipocyte morphology accompanied with a reduced production of TNF-α and leptin, and an increased secretion of adiponectin was observed in EEN treated CD patients (Feng et al., 2014). However, another study with retrospective design has reported a correlation between high visceral fat area and reduced intestinal mucosal healing following anti-TNF therapy (infliximab) in CD patients, suggesting the earlier utilization of high doses of anti-TNF therapy among CD patients, especially in those with a higher ratio of visceral fat (Shen et al., 2018). Also, other studies have shown the effect of steroids, methotrexate, and infliximab therapies on the mesenteric fat production of inflammatory markers (Schäffler et al., 2006, Thomaz et al., 2009, Clemente et al., 2012). Therefore, drug therapy could modify the inflammatory response of mesenteric fat in CD.

1.11 Intestinal epithelium remodelling by lipids

The intestinal epithelium, considered the fastest self-renewing tissue in the body, is a key player in controlling inflammation. Intestinal stem cells (ISCs) help maintain the rate of self-renewal for proper tissue homeostasis in the gut. Digested nutrients in the gut can lead to alteration in the circulating factors that stimulate the biology of ISCs, which could affect the pattern of tissue remodeling and renewal in mucosal epithelium (Luo and Puigserver, 2016). Animal studies have shown that animals fed a high fat diet had a significantly higher rate of ISC proliferation, which

resulted in an elongation of the crypts (the lower part of the epithelium structure that resides in the ISCs), thus predisposing the animals to intestinal cancers (Beyaz et al., 2016). Interestingly, the resulted changes in the pattern of ISCs were produced by specific fatty acids in the interventional high fat diet. Although the findings of such studies focused on the effect of dietary fat on the tumorgenicity of the gut, rather than the inflammatory response, it does suggest a new mechanism of action of intestinal handling of dietary fats, which could be applicable in the aetiopathogenesis of CD.

1.12 Research gaps

The findings from previous clinical trials of EN in CD are conflicting, regarding which fat composition performs better. Critical analysis and interpretation of the available evidence is required to help understand more about the limitations and the strengths of each study finding. Moreover, a deep understanding of the pathogenesis of CD is lacking. The role of the fat-wrapping phenomenon in CD, and whether it is a pre-illness condition or a secondary phenomenon as a consequence of the disease, is still not supported by enough evidence. A thorough characterization of this type of tissue in CD patients is needed. Moreover, the intestinal epithelium is a key player in controlling inflammation. ISCs help maintain the rate of self-renewal for proper tissue homeostasis in the gut. Digested nutrients, like lipids, in the gut can lead to an alteration in the circulating factors that stimulate the biology of ISCs, which could affect the pattern of tissue remodeling and renewal in the mucosal epithelium (Luo and Puigserver, 2016). This suggests a new mechanism of action of the intestinal handling of dietary fats, which could be applicable in the aetiopathogenesis of CD. Overall, the modification of dietary fatty acid intake by CD patients could improve their clinical course, via modulation of the hypertrophic MAT and the adjacent intestinal epithelium, a proposal that, nevertheless, needs further investigation.

1.13 Objectives and hypothesis

The objectives of this research project are to:

- Critically reanalyze and interpret the evidence and the available RCTs, specifically to evaluate the association between the nutrient fat content of nutritional feeds and their response rates in CD patients.
- Characterize the MAT in CD, to investigate the physiological behavior of these tissues by identifying and measuring adipogenic and inflammatory gene expression and their fatty acid profile.

- Investigate the *in-vitro* effect of different mixtures of fatty acids on the adipogenesis of mesenteric pre-adipocytes, using human cultured mesenteric pre-adipocytes isolated from CD and control patients.
- Investigate the *in-vitro* effect of different fatty acid mixtures on gut epithelial physiology in CD and control tissues (e.g. on the proliferation of intestinal epithelial cells) by using the human cultured intestinal crypt model.

The hypothesis of this project is that the lipid composition of therapeutic feeds has an influence on controlling the inflammatory response in CD. First, this will be tested by conducting a systematic review to evaluate the relationship between the fatty acid content of enteral feeds and RRs in CD. Secondly, the hypothesis will be tested by characterizing and studying the *in-vitro* effect of fat on the molecular behavior of MAT in CD and the adjacent intestinal epithelium. The methodology will be described in detail in the following chapters.

2 Chapter 2. Effect of fat composition in enteral nutrition for Crohn's disease in adults: a systematic review

2.1 Introduction

2.1.1 Enteral nutrition guidelines in Crohn's disease

As well as having a probable role in pathogenesis, nutrition has been identified as a key mediator in established CD, such that, in paediatrics at least, defined EN is the treatment of first choice for many patients. However, as is often the case in clinical nutrition, the evidence base is not as strong as might be wished. Several meta-analyses have been conducted, but it remains difficult to judge the true effectiveness of EN in patients with CD. The collected evidence supports a superior effect of corticosteroids over EN in adults with CD, but many adult clinicians and most paediatricians believe that EN is an appropriate and evidence-based primary therapy in CD. This belief rests on the positive results from studies of paediatric and malnourished CD patients, which confirm beneficial effects of EN in improving growth and nutritional status, but which also indicate mucosal healing, and of course a favourable risk profile compared to pharmacological options.

EN comprises, however, a broad range of options, and the limited comparative evidence prevents confidence that the best choice(s) can currently be made. Polymeric, protein-based feeds with high fat content have been compared with low fat, glucose and amino acid-based feeds, and with oligomeric peptide-based feeds (Fernandez-Banares et al., 1995, Griffiths et al., 1995, Messori et al., 1996, Zachos et al., 2007a), but without compelling evidence that one is better than another (Zachos et al., 2007a). At present a single EN formula is licenced and marketed specifically for inflammatory bowel disease in adults. This is a casein-based polymeric feed rich in transforming growth factor-β (TGF-β), but there is little evidence to support any particular efficacy (Triantafillidis et al., 2010, Kanwar et al., 2016).

2.1.2 Effect of lipid compositions of enteral feed

Due to the physiological role of lipids in manipulating the inflammatory response, some trials have specifically compared the effect of different enteral feeds with different fat compositions in CD, which overall generated inconsistent

interpretations of the effect of lipids. PUFAs are known to have an anti-inflammatory and pro-inflammatory effect in several inflammatory diseases. However, in inflammatory intestinal diseases, several mechanisms of action for the impact of PUFA have been suggested. This include regulation of pro-inflammatory mediators, stimulation of anti-inflammatory cytokines, and restoration of intestinal epithelium via enhancing the process of wound healing (Scaioli et al., 2017).

2.1.3 Research gaps

Meta-analysis shows a weak and non-significant positive association between the protein content of feeds and their associated clinical RR. One meta-analysis found a negative correlation between LCT content and RR (Middleton et al., 1995), and a second found comparable but non-significant trends favouring low LCT and low overall fat content (Zachos et al., 2007a). Given the potential aetiopathogenic relevance of lipids to Crohn's disease (more disease in populations on high fat Western diets) and the curious phenomenon of fat wrapping (almost pathognomonic of Crohn's), further investigation in this area appears readily justifiable despite and partly because of the inability of the other meta-analyses to provide a verdict on this issue.

2.1.4 Hypothesis

The available evidence is not clear regarding the effect of fat composition in enteral feeds in CD, indicating the need for detailed analysis of the current literature in EEN in CD. Such hypothesis will be studied by conducting a systematic review of all the available RCTs concerned with EEN in CD, which will be examined in details to help elucidate the potential effect of lipid composition on disease outcomes.

2.1.5 Objectives

The aim of this systematic review has been to reanalyse the findings of the older studies and to combine these with the findings of those more recently published, specifically to evaluate the relationship between nutrient fat content and response rates in the treatment of patients with CD. Conscious that currently reported evidence is inconclusive and aware that many authorities consider the case for EN so weak as to argue robustly against it in the treatment of CD, we have approached this in a different and we hope more exploratory fashion than previous reviews. We focus on specific fatty acids, not just on lipid class, and on the ratios of individual fatty acids to each other, as well as to other macronutrients and to their relative contributions to energy

provision. The work of this chapter was originally published as a paper (Ajabnoor and Forbes, 2017).

2.2 Materials and methods

The PRISMA checklist and guidelines were used for this systematic review (Appendix 1). The study is registered with the PROSPERO database of systematic reviews, registration number: CRD42016033857.

2.2.1 Search strategy

A computer-based systematic search was undertaken using the Medline database (1946 to present) and the Embase database via OVID. The search strategy was customized for each database and applied to titles and abstracts of papers. For text terms related to enteral nutrition we used: "enteral", "elemental", "polymeric", "whole protein", "amino acid based", "peptide based", "low fat", or "high fat"; these terms were all combined with "nutrition", "feeding", "diet", or "feed". For disease-related text terms we used "Crohn's disease", or "inflammatory bowel disease". Also, we searched "enteral nutrition" and "Crohn disease" as index terms (MeSH) and exploded them as appropriate. The searches were limited to studies that involved humans, adults (18-plus years), clinical trials, controlled clinical trials, randomized controlled trials, meta-analyses, and systematic reviews. The searches were not restricted to the English language. In addition, a manual search of the reference lists of previously published papers was carried out, looking specifically for clinical trials investigating the effect of EN in adult patients with active CD.

2.2.2 Selection criteria

The selection of studies was determined by two reviewers following set criteria. The studies included were required to be prospective clinical trials in adults with CD (including controlled and uncontrolled trials). The EN intervention was to have been given exclusively for a defined period of time without any food intake (only water and sugar/milk-free beverages were allowed). The response rate must have been measured as a primary or secondary outcome, according to clearly stated criteria. The enteral feed used had to be clearly defined (i.e. name and type of feed, oil source, and fatty acid composition). Studies were removed from consideration if EN was given together with oral food intake, the study was retrospective, or performed in a paediatric population. Trials that did not provide a defined RR for CD, and trials that

investigated the effect of EN in combination with other medical therapies (e.g. with non-absorbable antibiotics or with erythropoietin) were also excluded. Studies where the full identity of the lipid content was not published were excluded only after application to researcher and/or manufacturer had failed to provide this information. When studies were published initially as interim reports our analysis used data only from the later full article.

2.2.3 Data extraction

For each eligible study, a detailed review was undertaken using a report form, looking for the type and quantity of fatty acids in the enteral feeds, the RR achieved by EN, which was calculated on the basis of a "per protocol" analysis, and selected characteristics related to study design (e.g. duration of intervention, criteria for remission, geographical location, number of patients). The gender and age of patients, and the anatomical location and duration of their disease were recorded. Any apparent discrepancies in the data extracted were discussed and resolved between the two reviewers.

Most papers did not provide sufficient detail of the fat composition in the enteral feeds for our purposes. These deficits have been addressed as follows. Where the formula was described by a proprietary name the manufacturer's data sheet has been interrogated. Where no proprietary name was provided a query was sent to the primary investigator of the study concerned. In each case our analysis was based on the fatty acid content of the feed used. In the great majority of cases this information was not provided either by authors or by manufacturers. However, the nature and proportion of the oils in the feeds was generally available or possible to estimate from the information given. The fatty acid profile of each oil was then drawn from a thorough published analysis (Dubois et al., 2007). One additional and unexpected problem arose from the fact that the composition of some feeds has been modified within the last fifteen years. Care was therefore taken to ensure that the analysis of the lipid content referred to that of the feed available at the time of the study.

2.2.4 Quality assessment

The quality of the included studies was judged according to the Downs and Black quality checklist on reporting, external validity, internal validity (study bias), and confounding (selection bias) (Downs and Black, 1998), with Livingston's amendment for assessment of power (Livingston et al., 2012). This is considered a

reliable assessment tool for both randomized and non-randomized clinical trials: the higher the score the better the quality of the methods.

2.2.5 Data synthesis and statistical analysis

The primary aim of this review has been to review and interpret the available evidence in order to test the potential correlation between the fat composition of enteral feeds and the resultant RR. Scatter plots were used to identify trends. The significance of possible relationships was tested by the Pearson correlation test (SPSS Statistics for Windows, Version 22.0, released 2013. IBM Corp., Armonk, NY, USA). Subgroup analysis was also conducted which stratified RR by the different levels of fats in EEN feeds (e.g. low vs. moderate vs. high MCT) and by the different levels of response rate (i.e. low RR <70% vs. high RR >70% response rate).

2.3 Results

2.3.1 Literature search

The electronic searches yielded 63 articles and the manual search from previous meta-analyses and reviews identified an additional 14 articles. Initial screening of the 77 articles comprised examination of title and abstract in the context of our selection criteria. Forty articles were judged relevant and were further assessed for eligibility. In each case the full paper was read (professionally translated if necessary) and checked against our selection criteria. Joint decisions on selection were made by the two reviewers, following discussion if any initial discrepancy arose. Ultimately our systematic review was based on 29 pertinent papers (Figure 2).

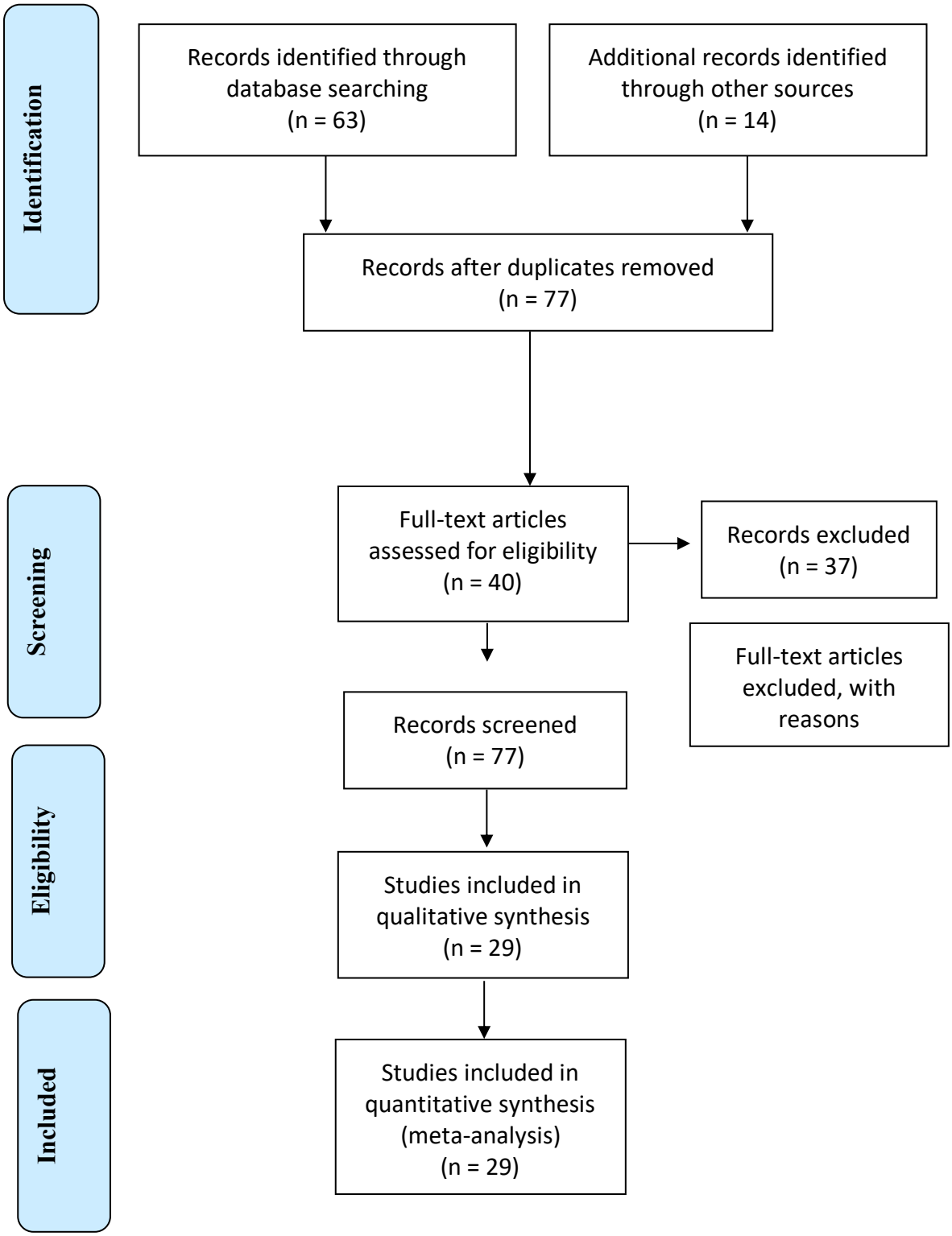


Figure 2: PRISMA 2009 flow diagram demonstrating the search and selection strategy.

PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

2.3.2 Study characteristics

From the total of 29 studies, 24 were controlled trials and 5 were uncontrolled. Among the controlled trials: 10 compared the efficacy of EN against drug therapy; 2 compared EN with PN; and 8 investigated the effect of the type of EN by comparing elemental feeds with non-elemental feeds (which include polymeric and semi-elemental, oligomeric feeds). Only 4 trials specifically addressed the effect of fat composition; these trials compared similar types of feeds but with different fat composition. The study design, patient characteristics, and criteria used to measure RR in the papers considered by this review are provided in Appendix 2-3.

2.3.3 Quality of studies

The quality of the included studies was highly variable. The study with the highest quality (Gassull et al., 2002) scored 26 (out of 28) by the assessment tool (Downs and Black, 1998, Livingston et al., 2012), while the lowest quality study scored only 10 (Mantzaris et al., 1996). Poor (or unknown) representativeness of study subjects and the lack of power calculations were the commonest defects overall, and in the controlled trials, there were high risks of performance and detection bias due to the lack of blinding, and high risk of selection bias due to the lack of allocation concealment during randomization (Appendix 7).

2.3.4 Characteristics of identified enteral feeds

No fewer than 29 distinct enteral feeds have been used in the published studies. We have excluded one study (Rigaud et al., 1991), and therefore data on two formulae, because patients who were randomized to receive polymeric feeding were prescribed one or other of the two formulae depending on availability, but the RR was provided only as a combined rate for the two formulae. Therefore, the final number of reviewed formulae is 27: 4 elemental formulae and 23 non-elemental preparations. The fatty acid composition of these formulae with reference to RR is demonstrated in Table 1. More detailed fat composition data are provided in Appendix 4-6.

Table 1: Fat composition and remission rate for enteral nutritional formulas

Reference	Type of enteral nutrition	Energy Kcal/day	Fat% of total calories	Source of oil	LCT% of total calories	LCT% of total fat	MCT% of total calories	MCT% of total fat	SFA% of total calories	SFA% of total fat	MUFA% of total calories	MUFA% of total fat	PUFA% of total calories	PUFA% of total fat	Total n-6% of total fatty acids	Total n-3% of total fatty acids	n-6:n-3 Ratio	RR%
Bamba *et al.* (2003)(Bamba et al., 2003)	Elemental, Low fat (6 packs of Elental + 6 packs of dextrin)	2400	1.15	Soybean oil	1.15	100	0	0	1.19	16.8	0.27	23.9	0.68	59.3	0	7.7	6.70	80
	Elemental, Medium fat (6 packs of Elental + 3 packs of dextrin+ 3 packs of C-1 dextrin)	2400	6.21	Soybean oil	6.21	100	0	0	1.04	16.8	1.48	23.9	3.68	59.3	51.6	7.7	6.70	40
	Elemental, High fat (6 packs of Elental + 6 packs of C-1 dextrin)	2400	11.27	Soybean oil	11.27	100	0	0	1.89	16.8	2.69	23.9	6.68	59.3	51.6	7.7	6.70	25
Gassull *et al.* (2002)(Gassull et al., 2002)	Polymeric, high in n-9 MUFA	2307	32	Synthetic Trioleate	30.17	94.28	1.83	5.71	5.16	16.11	25.28	79	2.56	8	6.5	1.5	4.33	27
	Polymeric, high in n-6 PUFA	2266	32	Corn oil	30.17	94.28	1.83	5.71	7.94	24.8	9.02	28.2	14.91	46.6	45	1.6	28.13	63
Giaffer *et al.* (1990)(Giaffer et al., 1990)	Elemental (Vivonex)	2500	1.3	Safflower oil	1.3	100	0	0	0.12	9.1	0.18	13.9	1	77.3	76.5	0.8	95.63	86
	Polymeric (Fortison)	2500	36	Vegetable oil (canola & sunflower)	36	100	0	0	3.74	10.4	15.26	42.4	17.55	48.75	43.6	5.2	8.38	42
Leiper *et al.* (2001)(Leiper et al., 2001)	Polymeric, 5% LCT	-	34.8	Soybean & coconut oils	5	13.8	29.8	86.2	30.69	88.2	1.18	3.4	2.92	8.4	7.4	1	7.40	46
	Polymeric, 30% LCT	-	34.8	Palm, Canola, and coconut oils	30	84.7	4.8	15.3	18.72	53.8	12.35	35.5	3.72	10.7	9.5	1.2	7.92	45
Mansfield *et al.* (1995)(Mansfield et al., 1995)	Elemental (E028)	2250	16	Arachis oil	16	100	0	0	2.72	17	8.96	56	4.16	26	20	1	20.00	42
	Semi-elemental (Pepti-2000 LF liquid)	2250	9	Corn (50%) & MCT oils	4.5	50	4.5	50	5.22	58	0.99	11	2.79	31	28.05	0.5	56.10	42
Middleton *et al.* (1995)(Middleton et al., 1995)	Elemental (E028)	-	15.6	Arachis oil	15.6	100	0	0	2.65	17.1	8.88	56.9	4.06	26	20	1	20.00	92
	Elemental (E028), High LCT	-	35.6	Safflower & canola oils	35.6	100	0	0	3.95	11.1	23.92	67.2	7.73	21.7	17	4.5	3.78	55
	Elemental (E028), High MCT	-	31.6	Safflower, canola, and coconut oils	20.5	64.9	11.1	35.1	13.9	44	13.27	42	4.42	14	11	3	3.67	92
	Semi-elemental (Peptide 2+)	-	33.2	Corn & coconut oils	21.6	65	11.6	34.9	21.71	65.4	3.88	11.7	7.6	22.9	22.5	0.4	56.25	87
Park *et al.* (1991)(Park et al., 1991)	Elemental (E028)	2266	16.47	Arachis oil	16.47	100	0	0	3.01	18.3	8.17	49.6	5.07	30.8	30.2	0.4	75.50	50
	Polymeric (Enteral 400)	2289	36	Arachis (75%) & MCT oils	27	75	9	25	14	38.9	13.57	37.7	8.42	23.4	22.9	0.3	76.33	83
Raouf *et al.* (1991)(Raouf et al., 1991)	Elemental (EO28)	-	16.5	Arachis oil	16.5	100	0	0	2.82	17.1	9.39	56.9	4.29	26	20	1	20.00	75
	Polymeric (Triosorbon)	-	36	Sunflower (22%) & MCT oils	7.9	22	28.1	78	29.09	80.8	1.76	4.9	5.22	14.5	14.4	0.1	144.00	73
Rigaud *et al.* (1991)(Rigaud et al., 1991)	Elemental (Vivonex HN)	2286	0.8	Safflower oil	0.8	100	0	0	0.07	9.1	0.11	13.9	0.62	77.3	76.5	0.8	95.63	71
Royall *et al.* (1994)(Royall et al., 1994)	Elemental (Vivonex-TEN)	-	3	Safflower oil	3	100	0	0	0.27	9.1	0.42	13.9	2.32	77.3	76.5	0.8	95.63	84
	Semi-elemental (Peptamen)	-	33	Sunflower (30%) & MCT oil	10	30.3	23	69.7	24.37	73.84	2.22	6.72	6.53	19.8	19.68	0.15	131.20	75
Sakurai *et al.* (2002)(Sakurai et al., 2002)	Elemental, Low fat (Elental)	-	1.5	Soybean oil	1.5	100	0	0	0.24	15.7	0.36	24.2	0.9	59.8	52.1	7.8	6.68	67
	Semi-elemental, High MCT (Twinline)	-	25	Safflower & MCT oil (tricaprilin)	7	28	18	72	18.64	74.54	0.97	3.89	5.41	21.64	21.42	0.22	97.36	77
Verma *et al.* (2000)(Verma et al., 2000)	Elemental	2500	17	NS	11.05	65	5.95	35	6.63	39	7.82	46	2.55	15	12	-	-	80
	Polymeric	2500	17	NS	11.05	65	5.95	35	6.63	39	7.82	46	2.55	15	12	-	-	67
Gonzalez-Huix *et al.* (1993)(Gonzalez-Huix et al., 1993)	Polymeric (Edanec HN)	2800	32	Olive oil (55%) & milk fat	27.8	87	4.2	13	13.12	41	13.12	41	5.76	18	-	-	-	80
Lindor *et al.* (1992)(Lindor et al., 1992)	Semi-elemental (Vital HN)	-	9	Safflower (55%) & MCT (45%)	4.95	55	4.05	45	4.68	52.04	1.1	12.32	3.26	36.3	36.08	0.3	120.27	60

Lochs *et al.* (1991)(Lochs et al., 1991)	Semi-elemental (Peptisorb)	-	8	Soybean oil (50%) & MCT	4	50	4	50	4.62	57.85	0.97	12.1	2.39	29.9	26.05	3.9	6.68	60
Malchow *et al.* (1990)(Malchow et al., 1990)	Semi-elemental (Survimed)	-	10	Sunflower	10	100	0	0	1.28	12.8	2.24	22.4	6.6	66	65.6	0.5	131.20	96
Greenberg *et al.* (1988)(Greenberg et al., 1988)	Polymeric (Precision-Isotonic)	-	28	Soybean oil	28	100	0	0	4.4	15.7	6.8	24.2	16.7	59.8	52.1	7.8	6.68	58
Kobayashi *et al.* (1998)(Kobayashi et al., 1998)	Elemental (Elental)	-	1.5	Soybean oil	1.5	100	0	0	0.24	15.7	0.36	24.2	0.9	59.8	52.1	7.8	6.68	70
	Polymeric (Clinimeal)	-	28	Corn & coconut oils	19.3	69	8.7	31.15	15	53.7	4.8	17.1	8.23	29.5	28.95	0.55	52.64	67
Mantzaris *et al.* (1996)(Mantzaris et al., 1996)	Polymeric (Nutrison HE)	-	36	Corn, palm, & coconut oils	34.17	94.92	1.9	5.27	13.45	37.41	11.35	31.54	11.35	31.24	30.63	0.61	50.21	40
O'moráin *et al.* (1984)(O'moráin et al., 1984)	Elemental (Vivonex)	-	2.5	Safflower	2.5	100	0	0	0.23	9.1	0.35	13.9	1.9	77.3	76.5	0.8	95.63	100
Gorard *et al.* (1993)(Gorard et al., 1993)	Elemental (Vivonex TEN)	2100	2.5	Safflower	2.5	100	0	0	0.23	9.1	0.35	13.9	1.9	77.3	76.5	0.8	95.63	77
Okada *et al.* (1990)(Okada et al., 1990)	Elemental (Elental)	-	1.5	Soybean	1.5	100	0	0	0.24	15.7	0.36	24.2	0.9	59.8	52.1	7.8	6.68	80
Bodemar *et al.* (1991)(Bodemar et al., 1991)	Polymeric (Semper lowfat)	-	20	Soybean	20	100	0	0	3	15.7	5	24.2	12	59.8	52.1	7.8	6.68	90
Coyle and Sladen (1989)(Coyle and Sladen, 1989)	Polymeric (Enteral 250)	2000-3000	28	Corn oil	28	100	0	0	4	14.8	8	28.1	16	57.1	56.1	1	56.10	67
Riordan *et al.* (1993)(Riordan et al., 1993)	Elemental (E028)	-	15.6	Arachis oil	15.6	100	0	0	2.82	17.1	9.39	56.9	4.29	26	20	1	20.00	84
Guo *et al.* (2013)(Guo et al., 2013)	Polymeric (Nutrison Fiber)	1500-2000	34	Sunflower, canola, & MCT oils	29	84.6	5	15.4	8.7	25.6	19.2	56.4	6.12	18	-	-	-	85
Zoli *et al.* (1997)(Zoli et al., 1997)	Semi-elemental (Peptamen)	-	33	Sunflower (30%) & MCT oil	10	30.3	23	69.7	24.37	73.84	2.22	6.72	6.53	19.8	19.68	0.15	131.20	80
Hu *et al.* (2014)(Hu et al., 2014)	Semi-elemental (Peptisorb liquid)	-	15	Soy oil % MCT oil	7.95	53	7.05	47	8.3	55.3	1.9	12.8	4.8	31.7	27.6	4.1	6.73	71
Zhu *et al.* (2013)(Zhu et al., 2013)	Polymeric (Nutrison Fibre)	2037	34	Sunflower, canola, & MCT oils	29	84.6	5	15.4	8.7	25.6	19.2	56.4	6.12	18	-	-	-	67

2.3.5 Correlation between fat composition and remission rate

2.3.5.1 Total amount of fat

Eight studies have compared a pair of feeds with different nutrient composition (e.g. polymeric versus elemental or semi-elemental versus elemental). It is difficult to determine the effect of fat content from these comparisons, as their composition for other nutrients was not standardised. Only two studies have specifically examined the effect of the amount of total fat. High and low fat feeds (fat mainly in the form of LCT) were compared. The earlier study showed that the feed with a low percentage of fats (15.6% of total calories) achieved a higher RR (92%), than the high fat feed (35.6% of total calories), which achieved a RR of 55% (Middleton et al., 1995). The later study indicated that a very low fat feed (1.15% of total calories) achieved a significantly higher RR (80%) than a modest fat feed (11.27% of total calories), which achieved a RR of (25%) (Bamba et al., 2003). It will be noted that the amount of fat in this higher fat feed was barely distinguishable from that of the low fat feed of the earlier study and yet the clinical effects were hugely different. Overall we find no significant correlation or trend between total fat content and RR ($r= 0.176$, $p= 0.252$) (Figure 3A).

2.3.5.2 Medium chain triglycerides (MCT)

Varying MCT content does not have a consistent strong effect. A single study which compared a feed with added MCT against a feed with no MCT, generated significantly different RRs of 77% and 67% respectively (Sakurai et al., 2002). However, the high MCT feed was semi-elemental and the low MCT feed was elemental, which precludes any firm conclusions about the contribution of the lipid to the observed differences. Our quantitative analysis, which is based on results from all studies, finds a weak non-significant positive trend between MCT delivery as a percentage of the total energy provision and RR ($r = 0.072$; $p = 0.643$) where the range was from 0 to 30% of total energy supply (Figure 3B). The apparent outlier to the upper left of the plot comes from Leiper's study (Leiper et al., 2001) in which there was a particularly high concentration of MCT (>86% of all fat) with a high proportion of MUFA (29%) and a low n-6:n-3 ratio (see below) amongst the fats that were LCTs.

2.3.5.3 Long chain triglycerides (LCT)

The effect of undifferentiated LCTs has been addressed by comparing feeds with similar amounts of total fat but with different percentages of LCT. One study

(already mentioned above) compared four feeds: elemental, elemental with added LCT, elemental with added MCT, and semi-elemental (Middleton et al., 1995). The feed with high LCT was associated with the lowest RR (55%), while the elemental feed with added MCT performed best, with a RR of 92%. However, a second study found no significant difference in RRs between use of feed with 5% LCT and an isocaloric feed with 30% LCT (Leiper et al., 2001). Our quantitative analysis of all the reported studies of all feedings reveals a non-significant negative trend between LCT provision and RR ($r = -0.254$; $p = 0.096$) where the range was from 4 to 35% of total energy supply and where in most cases the predominant lipids were of the n-6 class (where not, the relative excess came from n-9 lipid which we also consider disadvantageous (Figure 3C and see below).

2.3.5.4 Saturated fats

No single study has directly compared feeds with different levels of saturated fatty acids. We found no significant correlation or trend between the amount of saturated fat and the RRs ($r = -0.007$, $p = 0.964$) where the range was from trace amounts to over 30% of total energy supply (Figure 3D).

2.3.5.5 Olive oil/MUFA

Only a single study has compared two feeds with the same amount of total fat but with different amounts of oleic acid (balanced by linoleic acid) (Gassull et al., 2002). The feed with higher oleic acid content (79% of total fat) was significantly less effective (RR = 27%) than the feed with lower oleic acid (28%) and higher linoleic acid (45%), which achieved a RR of 63%. Although there are no other specific studies addressing MUFAs, our overall quantitative analysis is concordant, showing disadvantage from monounsaturated fatty acids (MUFA) with no statistical significance ($r = -0.23$, $p = 0.13$) with a range from trace amounts to about 25% of total energy supply (Figure 3E).

2.3.5.6 *n-6* and *n-3* PUFAs

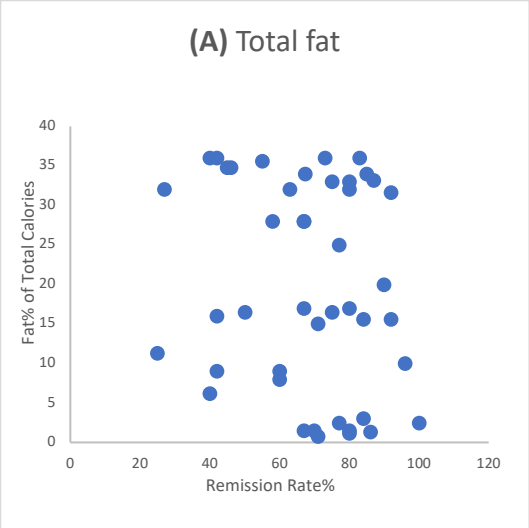
Only the study by Gassull et al. has directly investigated the effect of an *n-6*-rich feed (specifically linoleic acid), in which a significantly higher RR was achieved than with a lower n-6 content (Gassull et al., 2002). No study of non-elemental formulae readily allows assessment of the individual effects of an n-3-rich approach.

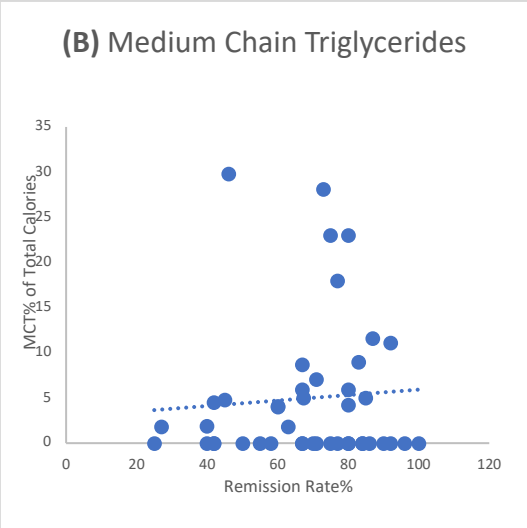
In our quantitative analysis a very weak non-significant negative correlation was found between the amount and proportion of PUFA (of all types) and the response

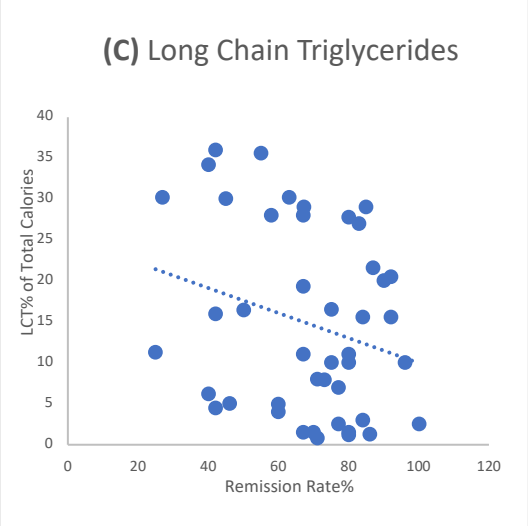
rates from all feeds (r = -0.157, p = 0.308) (Figure 3F) as was also the case for n-3 fatty acids (r = -0.166, p = 0.313) (Figure 3H).

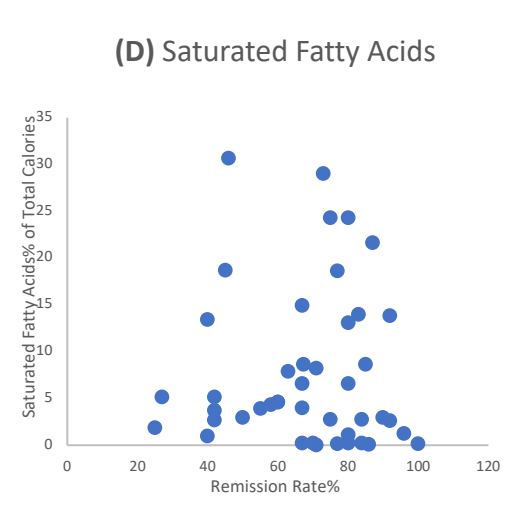
However, there was a weak positive correlation between the total n-6 fatty acid content and response rates (r = 0.253, NS) (Figure 3G), statistical significance (r= 0.378, p = 0.018) which remained significant after correction for multiple tests (Figure 3I). In the subgroup analysis (Table 2), when RR was stratified by the level of *n-6*:*n-3*, significant difference (p = 0.011) was reported in the pooled RR between EEN feeds with moderate n-6:n-3 (58.94% RR) (95% CI 48.99, 68.9) versus feeds with high *n-6*:*n-3* (79.91% RR) (95% CI 72.31, 87.51).

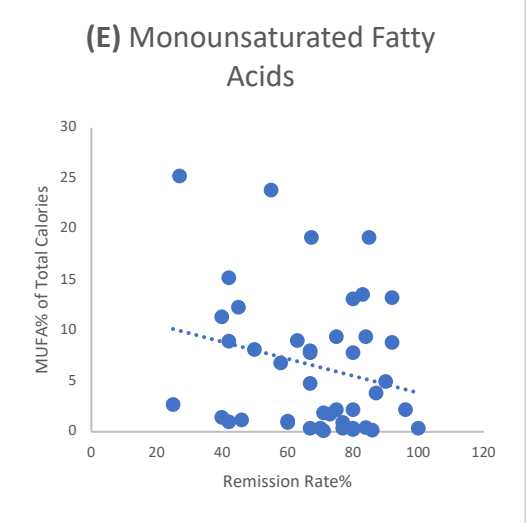
When patients exposed to only a single oil are considered (informal subgroup analysis) then the use of safflower oil is favoured, with a mean (median) response rate of 83.6% (84%) compared to the overall average response of 68.1% and mean (median) values for isolated exposure to soybean or arachis oil of 63.7% (68.5%) and 68.6% (75%) respectively.

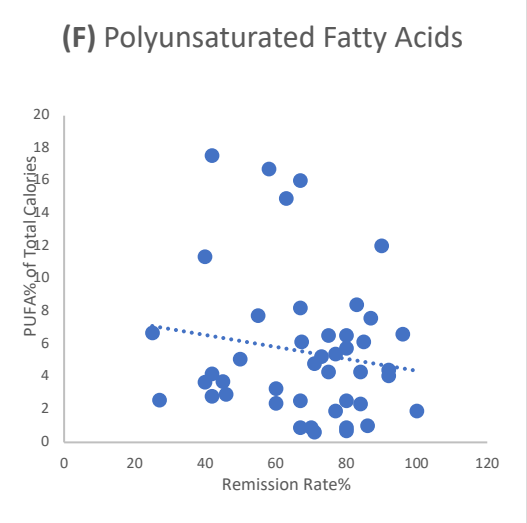

(A) Total fat
Fat% of Total Calories
Remission Rate%


(B) Medium Chain Triglycerides
MCT% of Total Calories
Remission Rate%


(C) Long Chain Triglycerides
LCT% of Total Calories
Remission Rate%


(D) Saturated Fatty Acids
Saturated Fatty Acids% of Total Calories
Remission Rate%


(E) Monounsaturated Fatty Acids
MUFA% of Total Calories
Remission Rate%


(F) Polyunsaturated Fatty Acids
PUFA% of Total Calories
Remission Rate%

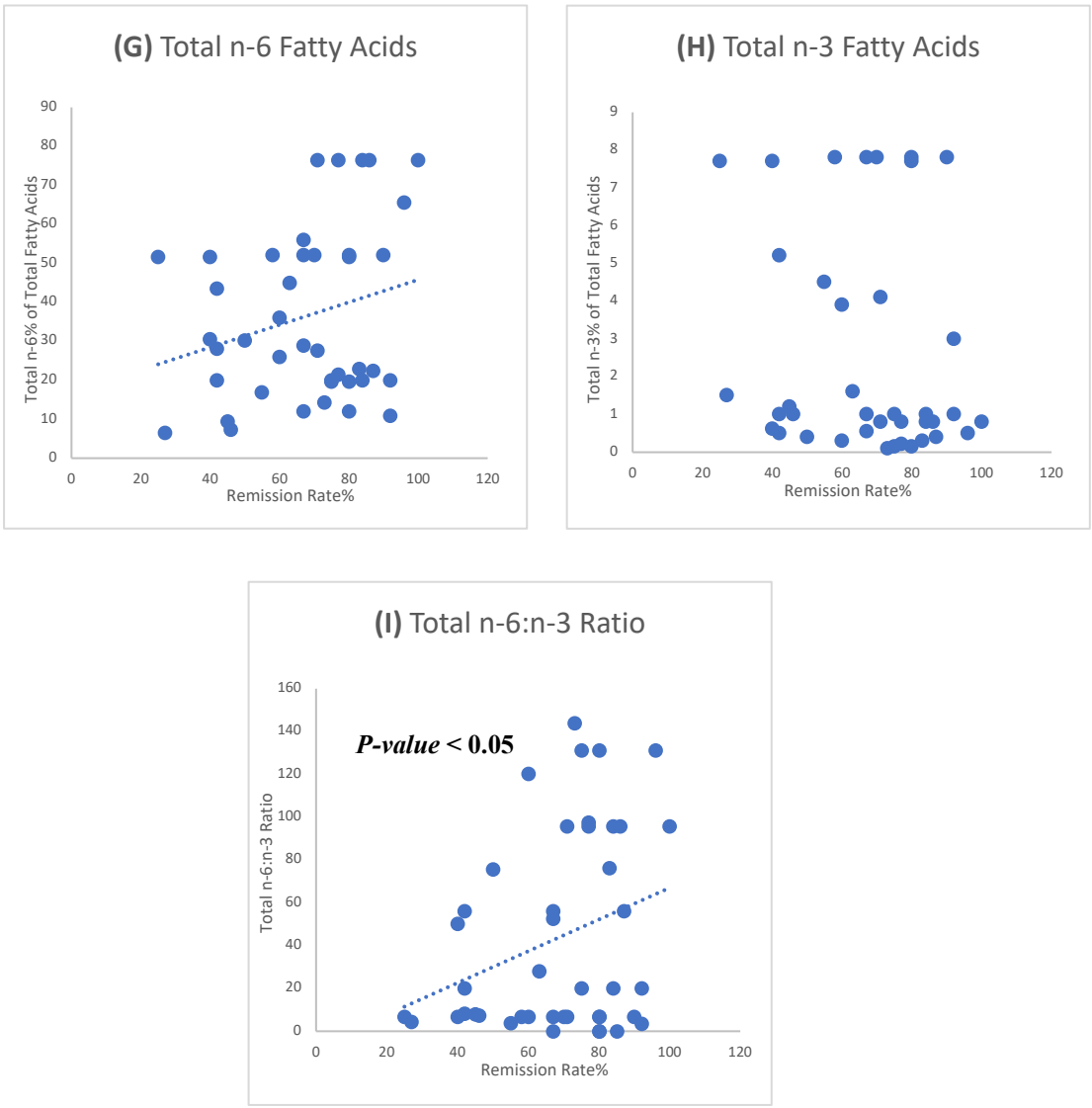


Figure 3: The association between fat composition of enteral nutritional feeds and remission rates (calculated based on per protocol analysis) in patients with Crohn's disease.

Pearson correlation test was used to measure the strength of the correlation. **(A)** Total fat percentage (r= -0.176, P-value= 0.252). **(B)** Medium chain triglycerides (MCT) percentage (r= 0.072, P-value= 0.643). **(C)** Long chain triglycerides (LCT) percentage (r= -0.254, P-value= 0.096). **(D)** Saturated fatty acids (SFA) percentage (r= -0.007, P-value= 0.964). **(E)** Monounsaturated fatty acids (MUFA) percentage (r= -0.23, P-value= 0.13). **(F)** Polyunsaturated fatty acids (PUFA) percentage (r= -0.157, P-value= 0.308). **(G)** Total linoleic acid (n-6) percentage (r= 0.253, P-value= 0.110). **(H)** Total linolenic acid (n-3) percentage (r= -0.166, P-value= 0.313). **(I)** Total n-6:n-3 ratio (r= 0.378, P-value= 0.018*).

Table 2: Subgrouping analysis for the effect of fat composition of enteral nutritional feeds on CD remission rate stratified by the level of lipid class

Factor assessed	Subgroup	Number of comparisons (compared enteral feeds)	Pooled RR (95% CI)
Total fat level	Low fat	11	74.09 (63.63, 84.56)
	Moderate fat	21	66.9 (57.53, 76.28)
	High fat	12	64.86 (53.34, 76.38)
MCT level	No MCT	22	69.59 (60.59, 78.59)
	Moderate MCT	10	56.93 (43.8, 70.06)
	High MCT	12	74.83 (67.32, 82.35)
LCT level	Low LCT	11	74.27 (64.1, 84.45)
	Moderate LCT	22	70.55 (62, 79.09)
	High LCT	11	57.21 (45.35, 69.07)
SFA level	Low SFA	11	77.36 (66.55, 88.18)
	Moderate SFA	22	62.83 (54.36, 71.31)
	High SFA	11	69.55 (57.47, 81.62)
MUFA level	Low MUFA	11	77.45 (70.25, 84.65)
	Moderate MUFA	21	65.29 (56.53, 74.05)
	High MUFA	12	64.61 (50.74, 78.48)
PUFA level	Low PUFA	12	76.83 (70.03, 83.63)
	Moderate PUFA	20	65.17 (56.02, 74.31)
	High PUFA	12	64.42 (50.46, 78.37)
Total n-6 level	Low n-6	11	65.45 (52.23, 78.68)
	Moderate n-6	18	61.11 (51.29, 70.93)*
	High n-6	12	78.83 (70.7, 86.97)*
Total n-3 level	Low n-3	10	72.3 (60.26, 84.34)
	Moderate n-3	19	67.84 (58.07, 77.62)
	High n-3	10	60.7 (45.96, 75.44)
n-6:n-3 level	Low n-6:n-3	10	67.9 (54.06, 81.74)
	Moderate n-6:n-3	18	58.94 (48.99, 68.9)*
	High n-6:n-3	11	79.91 (72.31, 87.51)*

Low level (lower quartile range); moderate level (interquartile range); high level (upper quartile range); RR (remission rate); MCT (medium chain triglycerides); LCT (long chain triglycerides); SFA (saturated fatty acids); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids); One-way ANOVA with multiple correction test have been used to test the significance of difference in RR between the subgroups; *Difference between subgroups is significant (P-value<0.05).

Table 3: Subgrouping analysis for the correlation between the fat composition of enteral nutritional feeds and CD remission rate stratified by the level of remission rates achieved

Factor assessed	Subgroup	Number of comparisons (compared enteral feeds)	r (95% CI)	P-value
RR for total fat correlation	Low RR < 70%	20	-0.03 (-0.46, 0.42)	0.91
	High RR ≥ 70%	24	-0.00 (-0.41, 0.40)	0.99
RR for MCT correlation	Low RR < 70%	20	0.05 (-0.39, -0.48)	0.83
	High RR ≥ 70%	24	-0.28 (-0.62, 0.14)	0.18
RR for LCT correlation	Low RR < 70%	20	-0.05 (-0.49, 0.39)	0.81
	High RR ≥ 70%	24	0.27 (-0.15, 0.60)	0.21
RR for SFA correlation	Low RR < 70%	20	-0.00 (-0.44, 0.44)	>0.99
	High RR ≥ 70%	24	-0.21 (-0.57, 0.21)	0.32
RR for MUFA correlation	Low RR < 70%	20	-0.16 (-0.56, 0.31)	0.51
	High RR ≥ 70%	24	0.23 (-0.19, 0.58)	0.29
RR for PUFA correlation	Low RR < 70%	20	0.13 (-0.33, 0.54)	0.57
	High RR ≥ 70%	24	0.24 (-0.18, 0.59)	0.26
RR for n-6 correlation	Low RR < 70%	19	0.19 (-0.29, 0.59)	0.44
	High RR ≥ 70%	22	0.19 (-0.26, 0.56)	0.41
RR for n-3 correlation	Low RR < 70%	18	-0.08 (-0.53, 0.39)	0.74
	High RR ≥ 70%	21	-0.13 (-0.53, 0.32)	0.59
RR for n-6:n-3 correlation	Low RR < 70%	18	0.30 (-0.19, 0.67)	0.22
	High RR ≥ 70%	21	-0.01 (-0.44, 0.43)	0.98

r (Pearson correlation coefficient); RR (remission rate); MCT (medium chain triglycerides); LCT (long chain triglycerides); SFA (saturated fatty acids); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids).

2.4 Discussion

The wide range of patient characteristics, the low number of participants in each study, and varying study designs obstruct the route to confident and generalizable conclusions. We deliberately used results taken from observations on patients who followed treatment protocols (rather than intention to treat), but although biologically justifiable this will be of limited clinical value if a future "optimal" formula is not tolerated and thus the treatment plan is not completed. Fortunately, the compliance/acceptance of the many different formulae did not appear systematically different according to the particular lipid profiles. This may have been obscured however by the range of duration of the intended therapies. The duration of intervention in most of the trials examined was between 3 and 8 weeks, 12 weeks in one trial (Hu et al., 2014), and only 2 weeks in 3 studies (Bodemar et al., 1991, Riordan et al., 1993, Zoli et al., 1997).

No fewer than eight different sets of criteria have been utilised to define response. Some were strict and binary (e.g. complete steroid withdrawal) and associated with relatively low response rates (Giaffer et al., 1990, Mansfield et al., 1995), while others were more qualitative (subjective). It should not have had a major effect on our interpretations since a full analysis performed on this basis provides the same qualitative results (data not shown).

Our methods may not have been sufficient to overcome bias introduced by the differing anatomical location of the CD (small bowel, large bowel, or both). The trials with the highest proportions of patients with small bowel CD (50% and 52%) also had amongst the highest RRs (86% and 75% respectively)(Giaffer et al., 1990, Royall et al., 1994), a linkage already well recognized in the literature, and perhaps a confounder despite apparently well-matched controls.

It has been thought that EN is more effective in those with early, purely inflammatory disease. Although not all evaluated studies provided the duration of the disease, the shortest and longest mean disease durations (1.3 and 18 years) were associated with similar and very respectable RRs of 90% and 80% respectively, suggesting that this effect is not profound (Okada et al., 1990, Bodemar et al., 1991).

Considerable differences were observed in respect of sex ratio (0-89% male (Sakurai et al., 2002), (Park et al., 1991)), but although prognosis of CD may differ

between the sexes (Park et al., 1991) a systematic bias could not be detected within our analysis (Zelinkova and van der Woude, 2014).

The divergence between the different types of unsaturated LCTs (*n-3*, *n-6* and *n-9*) and outcome appear at first surprising, but are fully consistent with the negative results from supplementary fish oil in CD (Lev-Tzion et al., 2014). In terms of specific oil content, interpretation is clouded by the number of feeds which contain multiple oils. However the numerical advantage to safflower oil is very much in line with the overall conclusion that high *n-6*:*n-3* ratio is advantageous and low proportion of MUFA could be relatively effective as well, given the relative paucity of MUFA in safflower oil (13.9% compared to 23.9% in soy and 56% in arachis oil) and its n-6:n-3 ratio, which, at over 90, is the highest of all the dietary oils. It has been more difficult still to link interpretation to individual fatty acids, but linoleic acid is favoured, and oleic acid as the only n-9 fatty acid in artificial feeds is targeted for avoidance.

There is a little supportive evidence also for our hypothesised complementary combination of safflower oil and MCT. One of the highest response rates in the literature (92% (Middleton et al., 1995)) was in patients on this combination, and only the study of Lindor *et al* appears to point in the opposite direction, this being a small study in which the comparator was steroid therapy (Lindor et al., 1992).

2.5 Conclusions

The fat content of EN formulae and its influence on controlling the inflammation of CD has generated interest, but its true role has remained unclear. Given its potential importance it is surprising that most authors have not thought it worthwhile or necessary to disclose the lipid analysis of the formulae used in their study. This systematic review has dissected the previously very broad classification of lipids in order to try to assess the effects of individual dietary oils and their fatty acids. It is recognised that definitive analysis is not possible given, on the one hand, the incomplete comparative information available, and, on the other, the inevitable complexity introduced by the replacement of one lipid with another and/or by different total fat content in different feeds. We manifestly lack sufficiently robust clinical trials in this area (Forbes et al., 2016).

However, our results expose significant results from individual studies, and, as well as several suggestive trends, support significant advantage from a high *n-6* to *n-3* ratio and perhaps from avoidance of MUFA. The various trends are, moreover,

not mutually exclusive despite the considerable variation in study design and response rates. Aiming for a relatively low total LCT content and proportionately high MCT content, with a relative low MUFA and high *n-6*:*n-3* fatty acid ratio can now be argued to offer an optimised approach. This might most easily and effectively be achieved by development of feeds based on a combination of safflower oil and MCT.

3 Chapter 3. Investigating the fatty acid profile and adipocytokine gene expression in mesenteric adipose tissues of Crohn's disease patients and control

3.1 Introduction

3.1.1 Mesenteric adipose tissues as an emerging role in CD

Chronic inflammation is part of the pathogenesis of many human diseases, including intestinal diseases like CD. In the visceral fats (mesenteric adipose tissues - MAT), chronic and low-grade inflammation can create a local microenvironment for inflammatory diseases as in CD. A potential role for MAT in CD pathogenesis is suggested by previous studies (Zulian et al., 2012, Peyrin-Biroulet et al., 2012). In CD, the mesentery attached to the inflamed intestines is usually thickened due to unknown reasons, the condition being called the "fat-wrapping" phenomenon, which represents a hallmark of the disease. Histologic investigations of the mesenteric fat of patients with fat wrapping demonstrated alterations in inflammatory markers (adipocytokines). Combining these data with the emergence of adipose tissue as an endocrine organ suggest a key role of visceral fat in CD pathophysiology.

3.1.2 Inflammation in mesenteric adipose tissues

In obesity, the accumulation of visceral adipose tissues stimulates the activation of the pro-inflammatory pathway, while in CD the hypertrophied MAT is associated with the increased production of both pro-inflammatory and anti-inflammatory mediators (Kredel and Siegmund, 2014). The complex inflammatory profile shown in CD mesenteric fat has been investigated by a few studies with inconsistent findings. One study has shown that the MAT in CD demonstrate increased M2 macrophages infiltration, which is associated with anti-inflammatory phenotype, therefore suggesting a protective function of this type of tissue in CD pathogenesis (Kredel et al., 2013). However, another study has shown that the mesenteric fat depot can be a great source of CRP in CD, which eventually could contribute to the overall inflammation in CD. Furthermore, bacterial translocation and local inflammation have been identified as triggers to CRP production in MAT (Peyrin-Biroulet et al., 2012). Yet, it is unknown if the accumulation of mesenteric fat in patients with CD is a pre-

illness condition or a secondary phenomenon resulting from local inflammation of the adjacent intestine. Further characterization of the inflammatory profile of this type of adipose tissue is thus required.

3.1.3 Lipids in mesenteric adipose tissues

Adipose tissue acts as an energy reservoir by storing lipids, and has always been recognized as an important organ in lipid storage regulation and metabolism. The cellular uptake and storage of dietary fatty acids in the adipose tissue is controlled by adipocytes. In a situation where adipocyte function is disrupted due to impaired adipose tissue accumulation and increased pro-inflammatory response, as observed in visceral obesity, the release of fatty acids from this tissue will increase, possibly leading to insulin resistance (Siegmund, 2012). In CD, the observed MAT hypertrophy results in a complex inflammatory response, and one proposed mechanism has associated this phenomenon to the abnormal composition of fatty acid in these tissues. The MAT contains lymphoid tissues for facilitating triglyceride entry into the blood and they are also capable of the uptake and retention of selected fatty acids (Camell and Smith, 2013, Pond, 2017). It has be shown that fatty acid precursors levels of eicosanoid and docosanoid are reduced in CD MAT lymphoid cells, which could be due to the interrupted interaction between the adipocytes and lymphoid cells within these tissues leading to defective fatty acid transport (Westcott et al., 2006). Understanding more about such defective lipid metabolism would help in identifying the underlying mechanisms between MAT and intestinal inflammation in CD.

3.1.4 Research gaps

Our understanding of how the intestinal tissues in CD interact with the diet and lead to a relapsing inflammatory response is still poorly understood. Dietary fatty acids have been suggested to play a key role in modulating intestinal inflammation in CD; yet, their mechanism of action is unclear. However, the abnormal accumulation of mesenteric fats that is exclusive to CD does provide an aetiopthogenic relevance of lipids in the disease's behavior. Fatty acids are considered important regulators of adipose tissue function and have been demonstrated to stimulate adipocytes via innate immunity receptors, which subsequently facilitate the secretion of pro-inflammatory cytokines (Shi et al., 2006). Characteristics of the fatty acid profile of the mesenteric fat depot in CD, in relation to their inflammatory gene expression is poorly investigated, thereby warranting further studies in this area.

3.1.5 Hypothesis

An abnormal inflammatory gene expression and fatty acid profile is associated with the fat-wrapping phenomenon observed in CD, which could be a key factor in controlling chronic intestinal inflammation. This will be tested by measuring fatty acids levels in CD against a control. Additionally, the expression levels of adipocytokines and genes involved in inflammation and adipogenesis of the MAT in CD will be also measured against a control.

3.1.6 Objectives

The primary objective of this study is to better characterize the inflammatory gene expression profile and the fatty acids composition of the MAT in patients with and without CD.

3.2 Methods

3.2.1 Human mesenteric adipose tissue collection

Ethical approval for collecting human tissue samples was obtained from the Faculty of Medicine and Health Sciences Research Ethics Committee of the University of East Anglia (UEA) (Norwich, UK) (reference: 20152016 66 HT). The Norwich Biorepository (Norwich and Norfolk University Hospital (NNUH), UK) patient information sheet and consent form were used to obtain the participants' consent (Appendix 8).

Eligible patients were mainly identified from a theatre list of colorectal surgeons, which was checked regularly with the help of the surgeons' secretaries. Patients were then approached on the day of surgery. All patients provided informed consent prior to surgery at the same day in the admission unit. Information about patients' medical history was collected pre-operatively. This included: disease type, surgical procedure, comorbidities, drug history, smoking history, age, gender, BMI, Montreal classification data (for IBD patients only), and biochemical data.

MAT was collected from colorectal surgical patients with confirmed diagnosis of either IBD or colorectal cancer (control group) who were having bowel resection involving the small bowel. The inclusion criteria for this study were: 1) patients older than 18 years, 2) patients diagnosed with moderate to severe CD affecting either the small bowel or both, the small and large bowel, and undergoing intestinal resection, 3) patients undergoing intestinal resection surgery for UC (as it is usual for a small

cuff of healthy ileum to be included in the resection specimen) and, 4) patients diagnosed with colorectal cancer (CRC) (control group) who were undergoing right hemicolectomy, which is a procedure involving resection of the right side of the colon and at least 10 cm of the distal terminal ileum – which is the same portion of the small bowel mainly affected in CD. The exclusion criteria excluded: 1) patients undergoing surgical resection that only involved the colon (e.g. anterior resections), 2) patients with multiple or serious comorbidities, 3) pregnant women and 4) patients unwilling to provide informed consent.

Specimens were collected as follows: once the bowel tissue was resected, the operating theatre immediately sent the specimen in a sterile pot to the histopathology lab, where a specialized GI histopathologist cut the required MAT samples and handed them to myself. All specimens were collected from MAT close to the small intestinal wall as shown in Figure 4. For this study, two specimens of healthy MAT (100 mg each) were collected from CRC patients, while four specimens (two of healthy MAT and two of inflamed MAT) were collected from IBD patients, if possible. Specimens were collected in sterile cryovials and immediately frozen in liquid nitrogen and stored in the Biorepository at -80^{o}C until laboratory analysis, which included RNA extraction for gene expression studies and lipid extraction for fatty acid analysis. In addition, one separate specimen (2 gm, approximately 4 x 4 cm in size) of fresh MAT was also collected if possible and placed immediately in a sterile collection tube, which was immediately sent to the lab for pre-adipocyte isolation and culture study (Chapter 4). The time between the surgical resection of the bowel and the sample collection was variable as this mainly depended on the nature of each surgery. Moreover, tissue collection was successful from the majority of morning cases, while collection from late afternoon cases was difficult because of the limited service of the histopathology lab at that time.

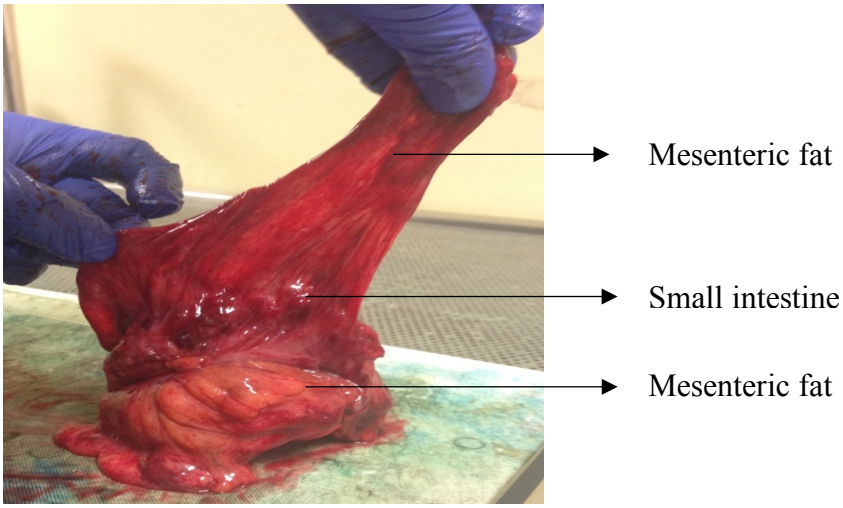


Figure 4: A diagram illustrating the intestinal fat-wrapping in Crohn's disease.

The surgical specimen show the fold of mesenteric adipose tissue that cover the intestinal surface.

3.2.2 RNA extraction and Quantitative real-time PCR (qRT-PCR)

3.2.2.1 RNA extraction

For measuring the level of gene expression, RNA was extracted from frozen MAT by using the ReliaPrep RNA tissue extraction kit (Promega Z6111). Briefly, 60 mg of tissue was homogenized in 500 µl of BL+TG lysis buffer with stainless steel beads for 10 minutes at 50 Hz using the TissueLyser II (Qiagen). The homogenized sample was then centrifuged at 12,000 g for 10 minutes and at room temperature to separate the fat layer, which was removed, and the supernatant collected in a 1.5 ml centrifuge tube where the required volume of 100% isopropanol was added. The lysate was then transferred to the extraction kit mini-column and centrifuged at 14000g for 30 seconds. RNA Wash solution (500 µl) was added to the mini-column and centrifuged at 14000g for 30 seconds. Next, DNase I incubation mix was prepared by mixing 24µl of Yellow Core Buffer, 3µl 0.09M $MgCl_2$ and 3µl of DNase I enzyme and then added to each mini-column and incubated at room temperature for 15 minutes. 200µl of column wash solution was then added to the mini-column and centrifuged at 14000g for 15 seconds, followed by 500µl of RNA wash solution, which was again centrifuged at 14000g for 30 seconds. The mini-column was thereafter placed into a new collection tube where 300µl of RNA wash solution was added and centrifuged at 14000g for 2 minutes. Finally, the mini-column was transferred to an

elution tube, and 15µl nuclease-free water was added directly onto the membrane and centrifuged at 14000g for 1 minute to elute the RNA. The extracted RNA was then stored at -20°C until further analysis. RNA concentration and purity was measured using a NanoDrop 2000 Spectrophotometer (ThermoScientific, UK). RNA solutions with a A260/A280 ratio of 1.9-2.1 was considered pure and of acceptable quality.

3.2.2.2 cDNA synthesis

The reverse transcription reaction for cDNA synthesis was done using the qPCRBIO cDNA synthesis kit (PCR Biosystems, London, UK). To make a 10 µl reaction of cDNA, 2 µl of 5x cDNA Synthesis Mix and 0.5 µl of 20x RTase were added to 7.5 µl of nuclease-free water that carried up to 100ng RNA in PCR tubes. The tubes were then incubated in the Thermocycler (Bio-Rad, Watford, UK) at 42°C for 30 minutes and at 85°C for 10 minutes to denature RTase, and then kept at 4°C for up to three hours or stored at -20°C. Before performing gene expression by qRT-PCR, the cDNA samples were diluted 1:5 or 1:10 in nuclease-free water.

3.2.2.3 Gene expression using quantitative real-time PCR (qRT-PCR)

qRT-PCR was done using the qPCRBIO SyGreen Mix (PCR Biosystems, London, UK). For a 10 µL reaction, 5 µL of SyGreen Mix and 1 µl of forward and reverse primer mix were added to 4 µL of diluted cDNA. All the samples were then run in duplicates on a 96-well white PCR plate (STARLAB). In addition, a negative control without cDNA sample was prepared along with every assay. The predesigned qRT-PCR primers for the selected genes for this study were purchased from Sigma (KiCqStart® SYBR® Green Primers) and are listed in Table 4. These primers were dissolved in nuclease-free water as specified by the manufacturer's technical sheet, which were then briefly vortexed and stored at -20°C until future use. The cDNA samples were run on a LightCycler 480 (Roche Life Science, Burgess Hill, UK) and the following cycles were performed: the pre-amplification step (95°C for 2 minutes), followed by cDNA amplification over 45 cycles (95°C for 15 seconds, 60°C for 10 seconds and 72°C for 10 seconds), which was then followed by a melting curve analysis step (95°C for 5 seconds, 65°C for 1 minute and 97°C continuous), which was performed to confirm product specificity and to detect any formation of primer dimers which could give false positives if unidentified. Finally, the reactions were cooled at 40°C for 30 seconds.

3.2.2.4 qRT-PCR data analysis

A cycle threshold (Ct) value was measured and generated at the end of each qRT-PCR reaction. Data was normalized against the housekeeping genes Ribosomal Protein L13a (RPL13a) and Glucuronidase Beta (GUSB) which were selected for their most stable gene expression levels amongst the mesenteric MAT samples (Appendix 9). The mRNA expression level for each sample was calculated using the Delta Ct method (Delta Ct = Ct target gene – Ct housekeeping gene), where Ct represents the number of cycles required to generate a fluorescent signal above a pre-defined threshold in qRT-PCR. All mRNA expression levels for target genes in this study are presented as relative to the geometric mean of the housekeeping genes RPL13a and GUSB. However, the Delta Ct method was used for qRT-PCR data analysis in Chapter 4, where a change in gene expression was expressed as a fold change relative to control groups (equivalent to $2^{-(\text{Delta Ct treated-Delta Ct control})}$).

Table 4: Primers used for mRNA expression analysis using qRT-PCR

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
ACTB (Beta actin)	GACGACATGGAGAAAATCTG	ATGATCTGGGTCATCTTCTC
RPL13A	GTCTGAAGCCTACAAGAAAG	TGTCAATTTTCTTCTCCACG
GUSB	ACTGAACAGTCACCGAC	AAACATTGTGACTTGGCTAC
GAPDH	ACAGTTGCCATGTAGACC	TTGAGCACAGGGTACTTTA
RN18S1	ATCGGGGATTGCAATTATTC	CTCACTAAACCATCCAATCG
ADIPOQ (Adiponectin)	GGTCTTATTGGTCCTAAGGG	GTAGAAGATCTTGGTAAAGCG
Leptin	TCAATGACATTTCACACACG	TCCATCTTGGATAAGGTCAG
PPARG	AAAGAAGCCAACACTAAACC	TGGTCATTTCGTTAAAGGC
CEBPA (CEBP4)	AGCCTTGTTTGTACTGTATG	AAAATGGTGGTTTAGCAGAG
FABP4	CCACCATAAAGAGAAAACGAG	AGTTGCTTGCTAAATCATGG
IL-6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
CD68	GTACTGAACCCCAACAAAAC	ATGTAGCTCAGGTAGACAAC
CCL2 (MCP1)	AGACTAACCCAGAAACATCC	ATTGATTGCATCTGGCTG
CD163	ATGAGTCCCATCTTTCACTC	CTATGTCCCAGTGAGAGTTAC
MRC1 (CD206)	AAATTTGAGGGCAGTGAAAG	GGATTTGGAGTTTATCTGGTAG
IL-10	GCCTTTAATAAGCTCCAAGAG	ATCTTCATTGTCATGTAGGC
ARG1 (Arginase)	ACTAGGAAGAAAGAAAAGGC	TCTTCTGTGATGTAGAGACC

Gene name, forward and reverse primer sequences are shown in the table.

3.2.3 Lipid extraction and fatty acid analysis

3.2.3.1 Total lipid extraction

Lipid extraction from and fatty acid analysis of the MAT samples were conducted in collaboration with James Dick from the Nutrition group at the Institute of Aquaculture in the University of Stirling (UK). The method is based on a previous protocol by Folch (Folch et al., 1957). First, the total lipid content was extracted from

the snap frozen, pre-weighed MAT samples (detailed in section 3.2.1) with 20 volumes of ice-cold chloroform: methanol mixture (2:1 v/v) in 50 ml Quickfit stoppered tubes, and then homogenized using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) in the fume cupboard. To isolate non-lipid impurities, potassium chloride (KCl) at 0.88% (w/v) was subsequently added to the homogenized sample followed by centrifugation at 1500 rpm for at least 5 minutes; the top (aqueous) layer was discarded while the lower solvent layer containing the lipids was transferred to a pre-weighed 15 ml Quickfit stoppered tube and filtered through a pre-washed (with chloroform/ methanol (2:1 v/v)) Whatman no.1 filter paper. Lipid extracts were then dried under a stream of oxygen-free nitrogen on a nitrogen evaporator and resuspended in 2:1 v/v chloroform: methanol with 0.01%, w/v butylated hydroxytoluene (BHT) (Sigma-Aldrich, Dorset, UK) to a final concentration of 10 mg/ml and transferred to 2 ml glass vials, which were stored under nitrogen at -20°C until further analysis. Two replicates of lipid extractions were performed on each tissue sample.

3.2.3.2 Fatty acid analysis: FAME analysis and Gas Chromatography

Fatty acid methyl esters (FAMEs) from total lipid extracts were prepared by acid-catalyzed transesterification of total lipid according to Christie's method (Christie, 2003). Total lipid samples (normally 1 mg) were placed into a 15 ml Quickfit test tube and free fatty acid standard (Heptadecaenoic acid 17:0 at 1 mg/ml in 2:1 v/v chloroform: methanol) was added at 10% of the total lipid mass. The organic solvent was then evaporated under nitrogen and 2 ml of methylation reagent solution (1% sulphuric acid (H_2SO_4) in methanol) was then added to the samples for overnight incubation at 50°C in a heating block. The samples were then cooled to ambient temperature the following day and 2 ml of aqueous potassium bicarbonate $KHCO_3$ (2% (w/v) was added to neutralize the samples. 5 ml of iso-hexane: diethyl ether (1:1, v/v) with 0.01% (w/v) BHT was added thereafter and the contents were mixed by inverting the tube followed by centrifugation at 1500 rpm for 2 minutes. After centrifugation, the upper organic layer was transferred to a clean test tube using a Pasteur pipette. A further 5 ml of iso-hexane: diethyl ether (1:1, v/v) with 0.01% (w/v) BHT was added to the original tube, mixed and re-centrifuged as previously; the upper organic layer was transferred to the other tube as before. The combined organic extracts were evaporated under nitrogen and re-dissolved in 100 µl of iso-hexane. The

samples were then loaded onto thin layer chromatography (TLC) silica plates (20 x 20 cm) using a glass Hamilton syringe, not exceeded 1.5 mg/cm per sample. A methyl ester standard was also loaded to the left side of every plate. The plate was then chromatographed in 90:10:1 iso-hexane: diethyl ether: acetic acid eluent for an hour. After the plate was dried in the fume cupboard, they were sprayed with 1% (w/v) iodine in chloroform to visualize the FAMEs. The FAME bands were all marked with a pencil and scraped from the TLC plates into test tubes using a straight edged scalpel blade or razor blade. The FAMEs were then extracted from the silica by adding 10 ml of iso-hexane: diethyl ether (1:1, v/v) + 0.001% (w/v) BHT followed by mixing and centrifugation at 1500 rpm to sediment the silica, where after the supernatant was carefully transferred to a clean test tube with a glass pasteur pipette and the solvent was evaporated under nitrogen. The samples then were transferred into 2 ml glass vials dissolved in 1 ml iso-hexane. The FAMEs were stored under nitrogen at -20°C or less until gas-liquid chromatography (GLC) analysis.

GLC was performed on a Thermo Fisher Trace GC 2000 (Thermo Fisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZBWax, 60 m x 0.32 x 0.25 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at 40°C/min and then to 195°C at 1.5°C/min and finally to 220°C at 2°C/min. Individual methyl esters were then identified according to previously published data (Tocher and Harvie 1988). Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy).

3.2.4 Statistical analysis

GraphPad Prism software (Version 7.0, GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Results are presented as mean ± standard error of the mean (SEM). The non-parametric Mann-Whitney U test was used to compare differences in the gene expression level and the fatty acid level between IBD and CRC MAT samples. For paired IBD samples (i.e. inflamed versus non-inflamed samples) the non-parametric Wilcoxon signed-rank test was used. $P < 0.05$ was considered statistically significant. Moreover, the significance of correlation between fatty acids level and level of gene expression was tested by the Pearson correlation test

3.3 Results

3.3.1 Study subjects' characteristics

Surgical specimens of MAT were obtained during intestinal resection of 15 patients with CRC (mean age 78.5 ± 5.24 years, mean BMI 26.65 ± 4.21 kg/m^2) and 8 patients with IBD (6 patients were diagnosed with CD and 2 patients with UC, mean age 43.5 ± 6.36 years, mean BMI 25.51 ± 1.22 kg/m^2). Patient characteristics are shown in Table 5. The mean age and mean BMI were not significantly different between CRC and IBD patients ($p > 0.05$).

Table 5: Characteristics of study subjects

	CRC	**IBD**
n	15	8 (6 CD; 2 UC)
Male (%)	6%	12%
Female (%)	33%	75%
Age (years)	78.5 ± 5.24	54 ± 18.73
BMI (kg/m^2)	26.65 ± 4.21	25.51 ± 1.22
Type of surgery:		
Right hemi-colectomy	14	
Panproctocolectomy	1	2
Ileocecal resection		3
Ileal resection		1
Small bowel resection		1
Open ileorectal anastomosis		1

Data are presented as percentages mean ± SEM. BMI, body mass index. *$p < 0.05$ CRC vs IBD.

3.3.2 Expression of adipokines and adipogenesis genes in CRC and IBD mesenteric adipose tissues

Different types of adipokines and adipogenesis genes expressed differently in the MAT. As seen in Figure 5, adiponectin was highly expressed in these tissues (1.41 ± 0.27 for CRC and 1.98 ± 0.42 for IBD) compared to leptin (0.19 ± 0.04 for CRC and 0.18 ± 0.09 for IBD). Also, the gene expression level for FABP4 was the highest among the three key markers for adipogenesis (26.49 ± 5.01 for CRC and 16.65 ± 2.81 for IBD), while the gene expression levels for PPARγ and CEPBα were 0.11 ± 0.01 and 0.21 ± 0.03 for CRC, and 0.12 ± 0.03 and 0.08 ± 0.01 for IBD.

The difference in gene expression between the different types of MAT samples (CRC vs. CD) is shown in Figure 6 and Figure 7. There was a significant difference in the leptin mRNA level between CRC (0.19 ± 0.04) and CD (0.05 ± 0.01) MAT (p= 0.03), but not in adiponectin mRNA levels (CRC 1.41 ± 0.27 and CD 1.68 ± 0.51, p= 0.89) (Figure 6A-B). Furthermore, adipogenesis specific markers expressed differently in the MAT samples. The difference in CEPBα mRNA levels in CRC and CD MAT were statistically significant (CRC 0.21 ± 0.03 and CD 0.07 ± 0.01, p= 0.003) (Figure 7B). However, the difference in gene expression for PPARγ and FABP4 was not significant in the tissue samples; the relative mRNA level for PPARγ was 0.11 ± 0.1 in CRC samples and 0.10 ± 0.03 in CD samples (p= 0.38), while the mRNA level for FABP4 was 26.49 ± 5.01 in CRC samples and 15.04 ± 3.38 in CD samples (p= 0.15) (Figure 7A and C). Overall, these results indicate a trend for reduced gene expression of adipocyte specific markers in inflamed CD MAT compared to healthy CRC MAT.

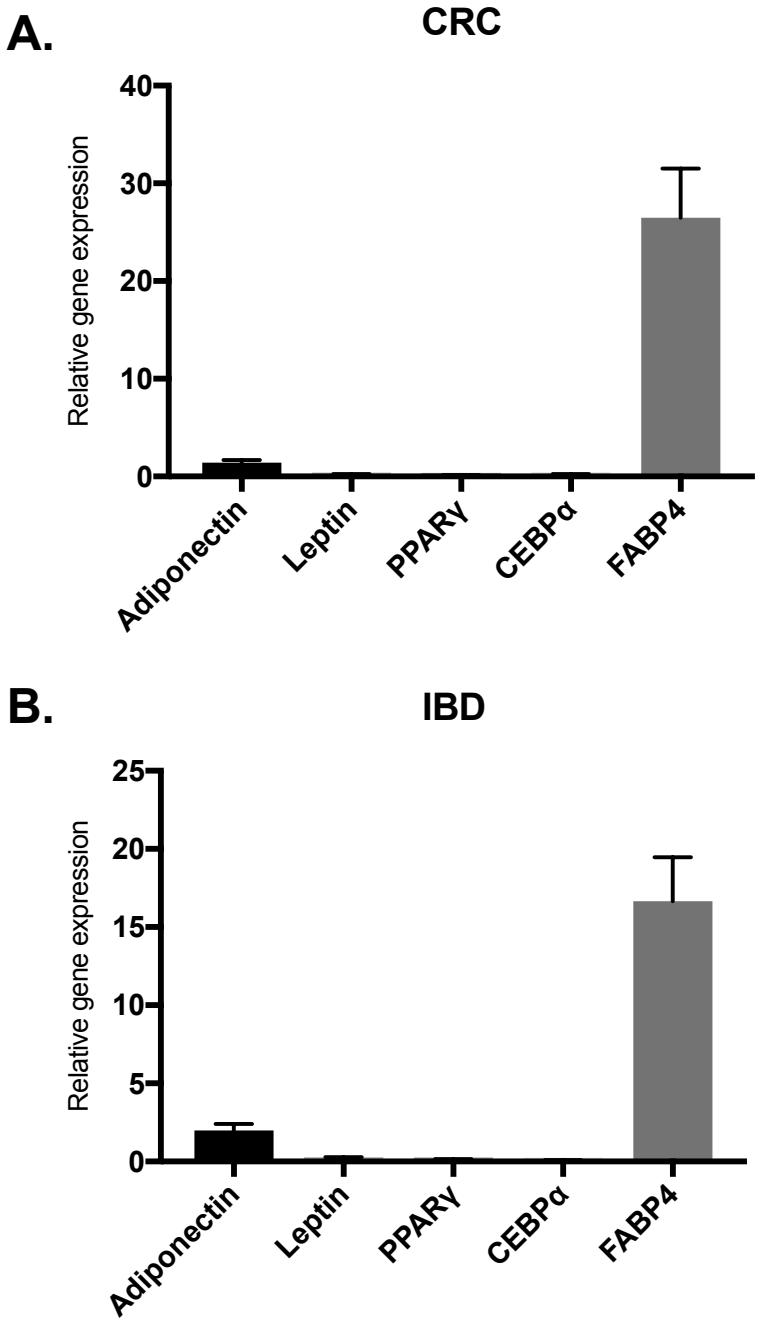


Figure 5: Bar graphs showing the relative increase in gene expression across adipocytokines and adipogenesis genes in CRC and IBD mesenteric fat samples.

Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC and n= 8 for IBD (including CD and UC).

Adipocytokines genes

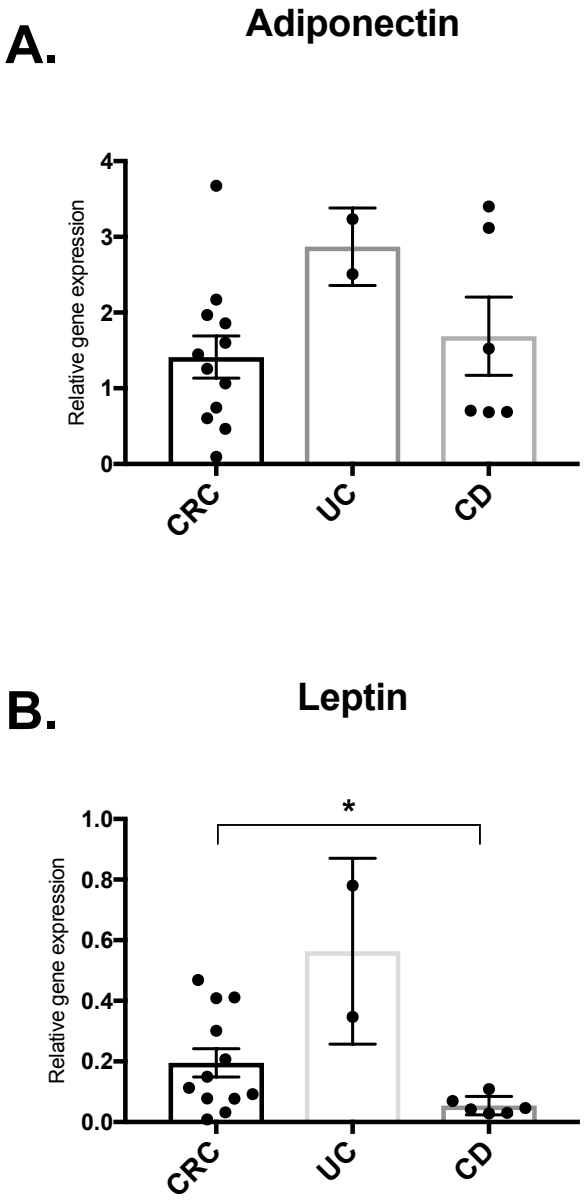


Figure 6: Bar graphs comparing the difference in the relative gene expression for adipocytokines genes between CRC and IBD mesenteric fat samples.

Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC, n= 6 for CD, and n= 2 for UC. Differences in the mRNA expression between the CRC and CD groups were tested using Mann-Whitney U test. *$p < 0.05$.

Adipogenesis genes

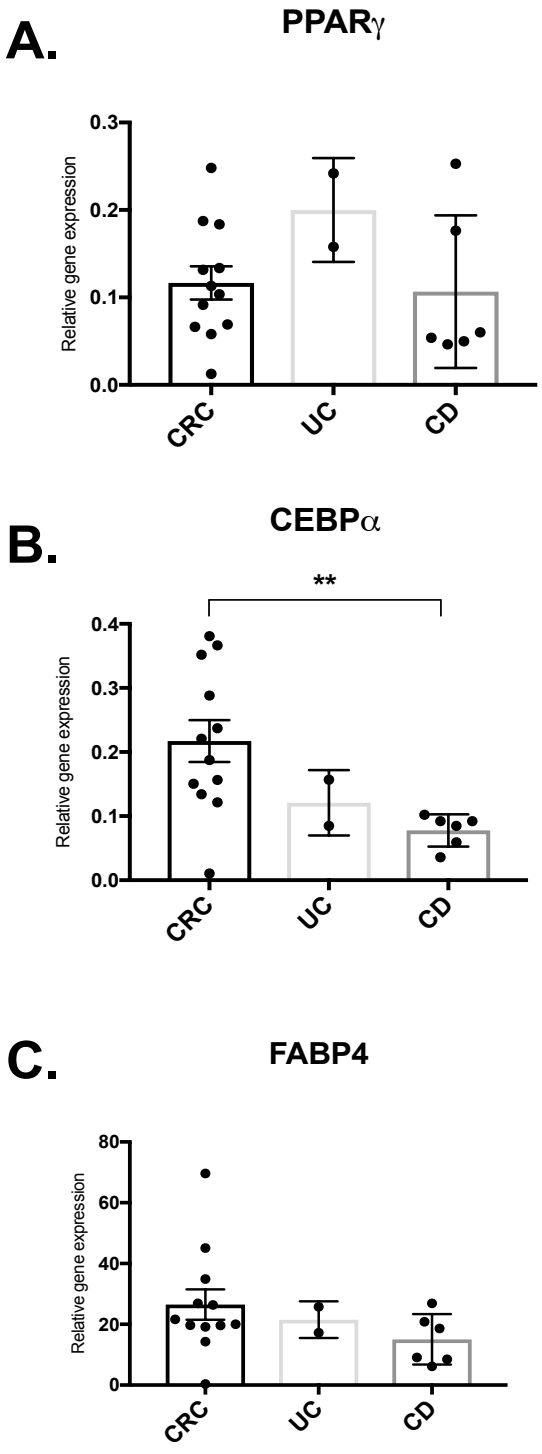


Figure 7: Bar graphs comparing the difference in the relative gene expression for adipogenesis genes between CRC and IBD mesenteric fat samples.

Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC, n= 6 for CD, and n= 2 for UC. Differences in the mRNA expression between the CRC and CD groups were tested using Mann-Whitney U test. **$p < 0.01$.

3.3.3 Expression of inflammatory genes in CRC and IBD mesenteric adipose tissues

mRNA expression levels of inflammatory markers (including macrophage-specific markers) were investigated in MAT samples. According to Figure 8, expression levels varied for all the inflammatory markers. For pro-inflammatory specific markers, IL-6 mRNA was highly expressed (0.17 ± 0.09 for CRC and 0.11 ± 0.03 for IBD) compared to CD68 (0.05 ± 0.01 for CRC and 0.04 ± 0.01 for IBD). Amongst anti-inflammatory markers, MCP1 mRNA was the most highly expressed (0.11 ± 0.06 for CRC and 0.08 ± 0.03 for IBD) followed by CD163 (0.05 ± 0.02 for CRC and 0.01 ± 0.006 for IBD) and CD206 (0.03 ± 0.01 for CRC and 0.01 ± 0.002 for IBD). mRNA levels for IL-10 and arginase were very low (for some samples the levels of IL-10 and arginase were under the detection limits of qRT-PCR). Moreover, due to technical problems with TNF-α and CRP primers, measurement of their gene expression levels in mesenteric fat was not feasible.

Comparisons between CRC and CD groups revealed no significant difference in mRNA expression levels for the pro-inflammatory markers (IL-6 and CD68); the relative gene expression level for IL-6 was 0.17 ± 0.09 for CRC and 0.08 ± 0.03 for CD ($p= 0.75$), while the gene expression level for CD68 was 0.05 ± 0.01 for CRC and 0.04 ± 0.01 for CD ($p= 0.35$) (Figure 9A-B). However, expression levels of the CD163 and CD206 anti-inflammatory markers in CD MAT was lower compared to CRC MAT, showing a statistically significant difference: CD163 expression in CD was 0.01 ± 0.008 compared to CRC (0.05 ± 0.02, $p= 0.04$) (Figure 10B); CD206 expression level in CD was 0.01 ± 0.003) versus CRC (0.03 ± 0.01, $p= 0.01$) (Figure 10C). Both are specific markers for M2 macrophages. The mRNA expression levels for the other investigated anti-inflammatory markers (MCP1, IL-10, and arginase) didn't show any significant difference (Figure 10A, D, and E).

Overall, these results indicate a trend for reduced gene expression of selected anti-inflammatory markers in inflamed CD MAT compared to healthy CRC MAT, while no difference was observed in the gene expression of the pro-inflammatory markers between the two types of samples investigated in this study.

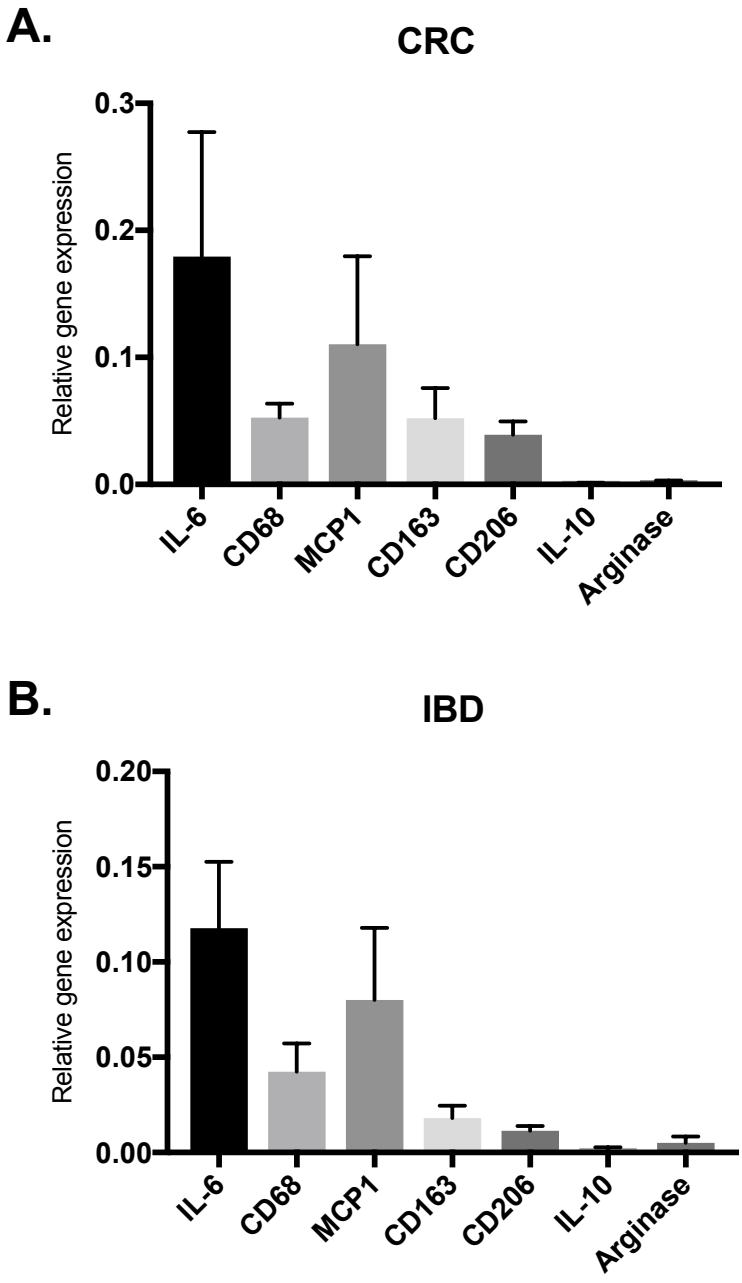


Figure 8: Bar graphs showing the relative increase in gene expression across inflammatory genes in CRC and IBD mesenteric fat samples.

Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC and n= 8 for IBD (including CD and UC).

Pro-inflammatory genes

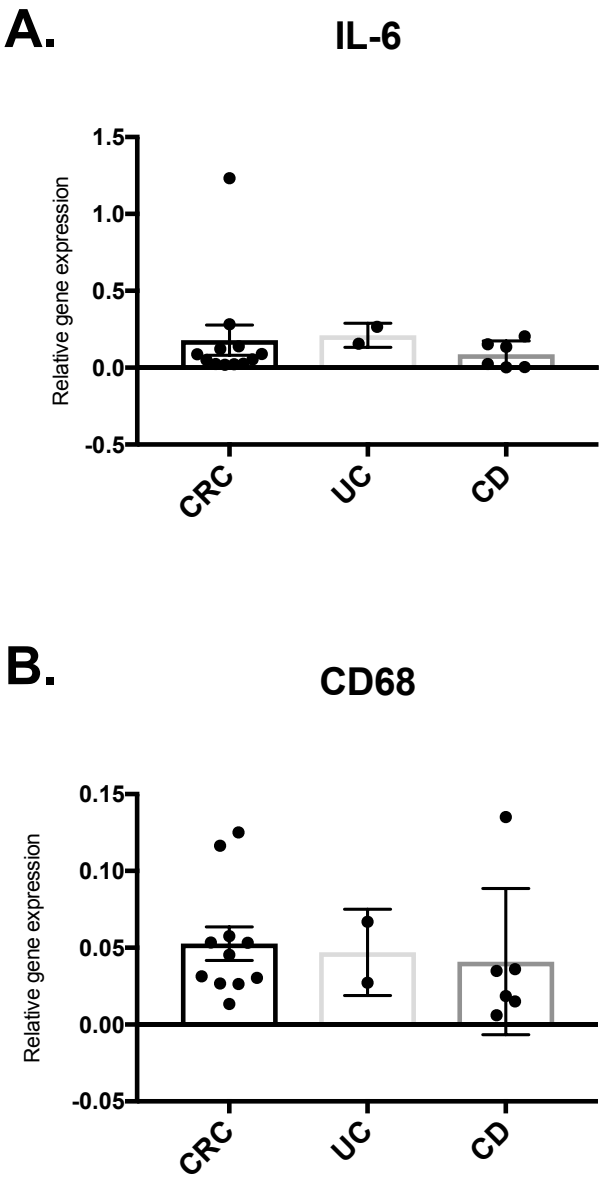
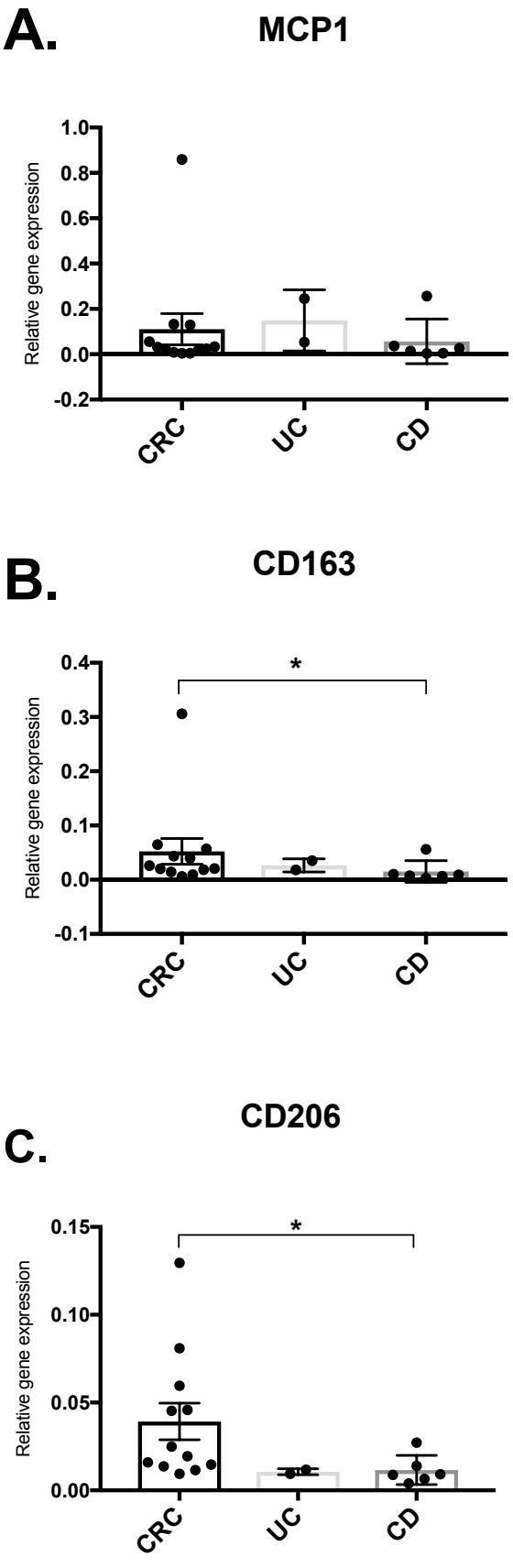


Figure 9: Bar graphs comparing the difference in the relative gene expression for pro-inflammatory genes between CRC and IBD mesenteric fat samples.

Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC, n= 6 for CD, and n= 2 for UC. Differences in the mRNA expression between the CRC and CD groups were tested using Mann-Whitney U test.

Anti-inflammatory genes



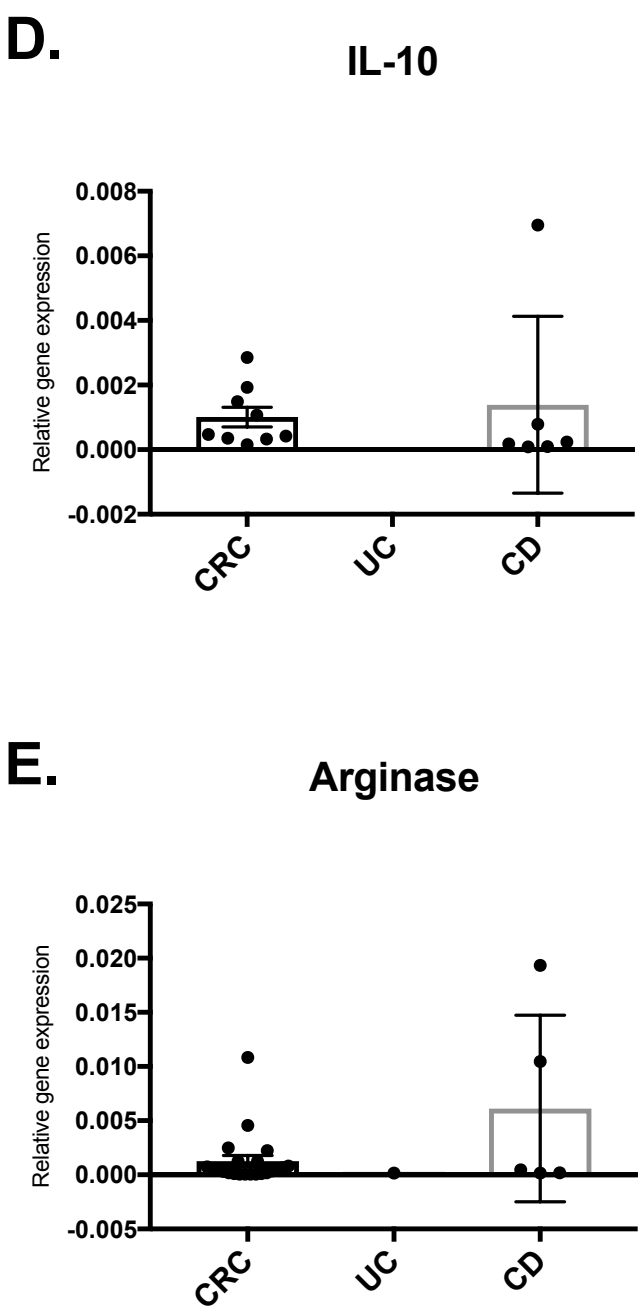


Figure 10: Bar graphs comparing the difference in the relative gene expression for anti-inflammatory genes between CRC and IBD mesenteric fat samples.

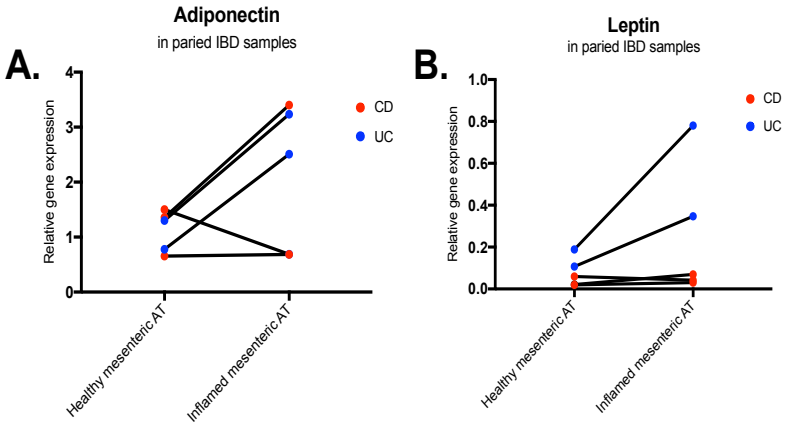
Data is presented as relative quantity of mRNA normalised to the reference genes RPL13a and GUSB. Values are expressed as mean ± SEM. n= 12 for CRC, n= 6 for CD, and n= 2 for UC. For some samples the levels of IL-10 and arginase were under the detection limits of the qRT-PCR (these samples were taken out from the analysis). Differences in the mRNA expression between the CRC and CD groups were tested using Mann-Whitney U test. *$p < 0.05$.

3.3.4 Expression of adipokines and adipogenesis genes in healthy and inflamed IBD mesenteric adipose tissues

This comparison aimed to better characterise the mesenteric fat depots (normal vs. inflamed or hypertrophied tissues) in IBD patients only. Unfortunately, because of the insufficient amount of resected normal intestinal tissues after the operation, collection of healthy mesenteric fat tissues was not always feasible from all IBD cases, which limited this type of analysis. Paired samples were only obtained from 3 CD patients and 2 UC patients. These data are presented in Figure 11 as individual and mean values of relative mRNA in normal MAT and hypertrophied (inflamed) mesenteric fats from the same patients.

The gene expression for adiponectin (2.1 ± 0.59) and leptin (0.25 ± 0.14) in inflamed MAT was higher than those in paired normal mesenteric samples (adiponectin 1.11 ± 0.16, leptin 0.07 ± 0.03) from the same IBD patients (Figure 11A-B). Similarly, PPARγ mRNA was upregulated in inflamed samples (0.15 ± 0.04) and downregulated in healthy mesenteric samples (0.08 ± 0.01) (Figure 11C). Overall, the trend for increased upregulation of these genes seems to be observed mainly in the UC samples. All the inflamed MAT of UC cases showed a consistently increased expression of adiponectin, leptin, and PPARγ with no statistical significance but further analysis may validate this, while the inflamed MAT of CD cases didn't show any significant changes in upregulation or downregulation. For CEBPα and FABP4 gene expression, no significant trends were identified between inflamed MAT and normal MAT samples from the same IBD patients (Figure 11D-E).

Adipocytokines genes



Adipogenesis genes

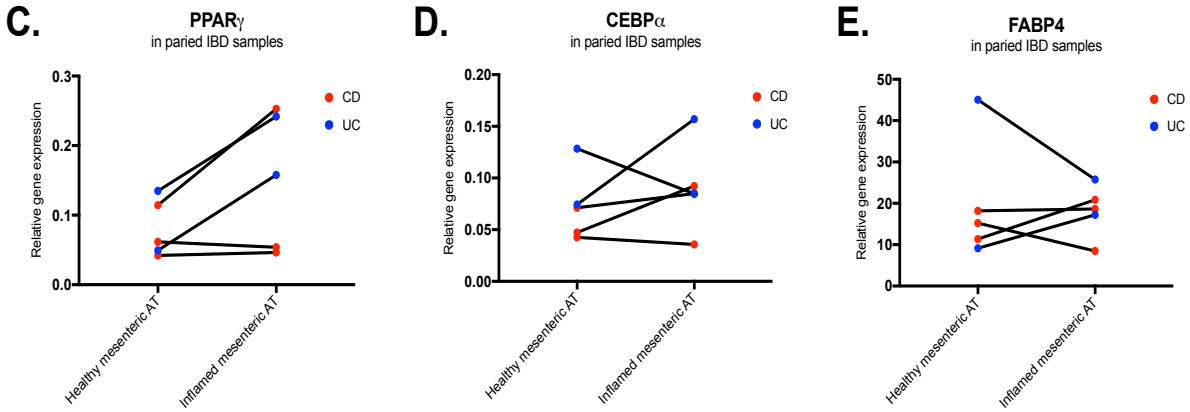


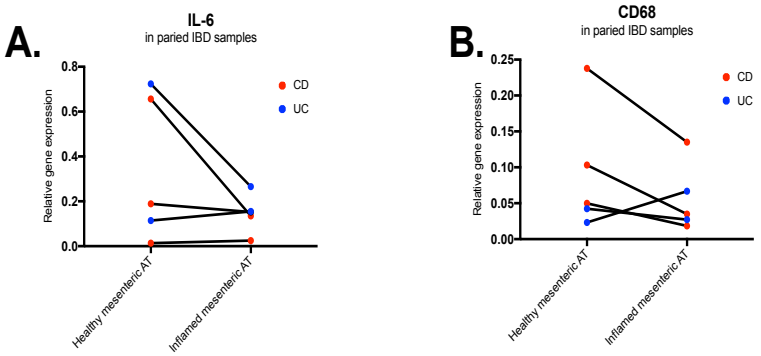
Figure 11: Gene expression level for adipokines and adipogenesis genes in paired IBD mesenteric AT.

Data are individual and mean values (horizontal bars) of relative mRNA in normal mesenteric adipose tissues of CD and UC patients and hypertrophied (inflamed) mesenteric adipose tissues of CD and UC patients from the same patients.

3.3.5 Expression of inflammatory genes in healthy and inflamed IBD mesenteric adipose tissues

Downregulation of both anti-inflammatory and pro-inflammatory markers was observed in inflamed MAT compared to their paired normal fat samples in the same IBD patients. The gene expression for the anti-inflammatory markers IL-6 and CD68 was lower in inflamed fat samples (0.14 ± 0.03 and 0.05 ± 0.02) than those in paired healthy fat samples (0.33 ± 0.14 and 0.09 ± 0.03) (Figure 12A-B). Similarly, the mean expression levels for MCP1, CD163, and CD206 was lower in inflamed MAT (0.12 ± 0.05, 0.02 ± 0.009, and 0.01 ± 0.003), and higher in normal fat tissues (0.28 ± 0.12, 0.07 ± 0.02, and 0.04 ± 0.01) (Figure 12C-E). Data on IL-10 expression trends were available from the paired inflamed and normal MAT samples of only 2 CD patients, as their levels were below qRT-PCR detection limits; lower IL-10 levels were observed in inflamed versus normal MAT samples (Figure 12F). Due to the limited sample size, further analysis would be needed to validate this observation. Data on arginase expression levels could also not be obtained due to the above stated limitation.

Pro-inflammatory genes



Anti-inflammatory genes

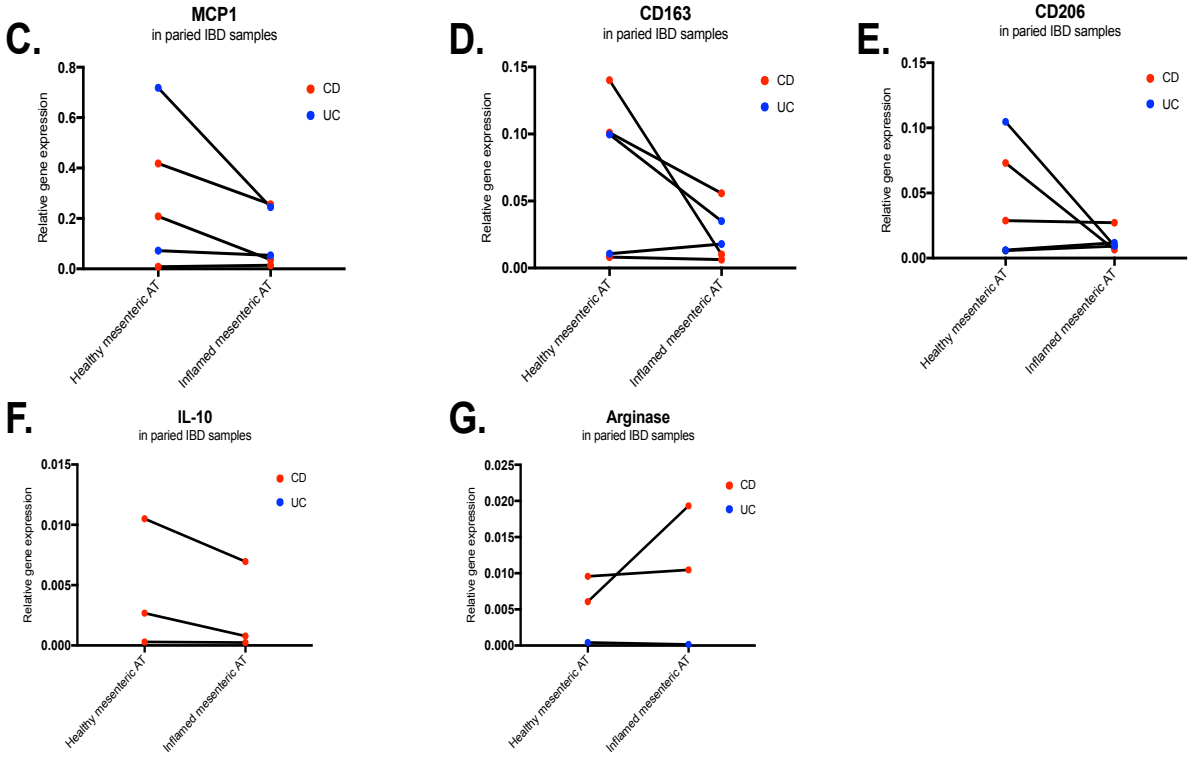


Figure 12: Gene expression level for pro-inflammatory and anti-inflammatory genes in paired IBD mesenteric AT.

Data are individual and mean values (horizontal bars) of relative mRNA in normal mesenteric adipose tissues of CD and UC patients and hypertrophied (inflamed) mesenteric adipose tissues of CD and UC patients from the same patients. For some samples the levels of IL-10 and arginase were under the detection limits of the qRT-PCR (these samples were taken out from the analysis).

3.3.6 Fatty acid composition in mesenteric adipose tissues

The fatty acid profile was investigated in CRC and IBD mesenteric fat samples. Overall, MUFAs constituted the highest percentage of lipids (57%) in the mesenteric fat depot, followed by saturated fatty acids (SFAs) and PUFAs at 29% and 13%, respectively. Table 6 and Figure 13 show the fatty acid composition of the MAT for CRC and IBD patients. Statistical analysis showed that the differences in fatty acid compositions of CRC and CD MAT samples were not statistically significant. The percentage of total SFA was 28.65 ± 0.79% in CRC mesenteric fat and 29.41 ± 2.20% in CD (p= 0.84). Total MUFA was 57.90 ± 0.89% in CRC and 57.51 ± 1.19% in CD mesenteric fats (p= 0.60). While total PUFA composition was 13.45 ± 0.51% in CRC and 13.08 ± 1.11% in CD (p= 0.77). n-6 fatty acids constituted most of the PUFAs in MAT (12.11 ± 0.42% for CRC and 11.89 ± 1.02% for CD, p= 0.65), whereas n-3 fatty acid levels were 10 times lower (1.34 ± 0.12% in CRC and 1.20 ± 0.17% in CD). EPA levels were negligible in MAT samples from both CRC (0.10 ± 0.0%) and CD (0.02% ± 0.02%), while DHA levels were 0.23% ± 0.04 in CRC and 0.16% ± 0.02 in CD. The n-6: n-3 fatty acid ratios were similar for CRC (9.78 ± 0.74) and CD MAT samples (10.89 ± 1.53).

Table 6: Fatty acid composition (% of total fatty acids) of the mesenteric adipose tissues of CRC and IBD patients

Fatty acid composition	CRC	UC	CD	*P*-value CRC vs. CD
Total saturated fatty acids %	**28.65 ± 0.79**	**31.03 ± 1.96**	**29.41 ± 2.20**	**0.84**
12:0	0.39 ± 0.05	0.38 ± 0.07	0.65 ± 0.24	0.44
14:0	2.62 ± 0.17	3.14 ± 0.46	3.12 ± 0.67	0.84
15:0	0.28 ± 0.02	0.37 ± 0.04	0.28 ± 0.05	0.85
Iso 16:0	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.26
16:0	20.99 ± 0.57	21.01 ± 0.73	20.34 ± 1.03	0.44
Ante Iso 17:0	0.13 ± 0.01	0.20 ± 0.03	0.14 ± 0.03	0.64
Iso 17:0	0.18 ± 0.01	0.25 ± 0.02	0.18 ± 0.03	0.92
18:0	3.79 ± 0.24	5.34 ± 0.58	4.43 ± 0.51	0.28
20:0	0.22 ± 0.02	0.27 ± 0.03	0.21 ± 0.02	0.98
22:0	0.06 ± 0.0	Nil	Nil	0.55
Total monounsaturated fatty acids %	**57.90 ± 0.89**	**55.85 ± 0.41**	**57.51 ± 1.19**	**0.60**
14:1	0.30 ± 0.02	0.28 ± 0.02	0.37 ± 0.09	0.70
16:1n-9	0.61 ± 0.04	0.45 ± 0.03	0.55 ± 0.03	0.58
16:1n-7	5.35 ± 0.34	3.80 ± 0.23	4.69 ± 0.57	0.54
16:1	0.08 ± 0.0	Nil	0.02 ± 0.02	0.98
17:1	0.29 ± 0.02	0.27 ± 0.02	0.26 ± 0.03	0.28
18:1n-9	48.11 ± 0.82	47.57 ± 0.47	48.60 ± 1.59	0.90
18:1n-7	2.19 ± 0.12	2.04 ± 0.12	2.00 ± 0.13	0.34
18:1	0.11 ± 0.0	0.13 ± 0.03	0.12 ± 0.02	0.41
20:1n-11	0.13 ± 0.02	0.18 ± 0.04	0.13 ± 0.01	0.64
20:1n-9	0.86 ± 0.03	1.11 ± 0.09	0.82 ± 0.12	0.26
22:1n-11	0.01 ± 0.01	Nil	Nil	0.99
22:1n-9cis	0.10 ± 0.01	0.04 ± 0.04	0.10 ± 0.01	0.88
Total polyunsaturated fatty acids %	**13.45 ± 0.51**	**13.12 ± 1.55**	**13.08 ± 1.11**	**0.77**
18:2n-6 (LA)	10.99 ± 0.45	10.60 ± 1.43	10.91 ± 1.00	0.77
CLA	0.23 ± 0.02	0.27 ± 0.01	0.23 ± 0.05	0.45
18:3n-6	0.08 ± 0.0	0.05 ± 0.05	0.10 ± 0.01	0.66
20:2n-6	0.18 ± 0.01	0.22 ± 0.06	0.18 ± 0.03	0.40
20:3n-6	0.25 ± 0.02	0.24 ± 0.07	0.20 ± 0.04	0.38
20:4n-6	0.32 ± 0.03	0.31 ± 0.05	0.27 ± 0.03	0.45
22:4n-6 (AA)	0.15 ± 0.01	0.12 ± 0.12	0.15 ± 0.02	0.64
Total n-6 PUFA %	**12.11 ± 0.42**	**11.80 ± 1.67**	**11.89 ± 1.02**	**0.65**
18:3n-3 (ALA)	0.88 ± 0.07	0.70 ± 0.04	0.86 ± 0.10	0.88
18:4n-3	0.11 ± 0.01	Nil	0.02 ± 0.02	0.88

20:3n-3	Nil	Nil	0.06 ± 0.0	0.07
20:4n-3	0.09 ± 0.0	Nil	0.07 ± 0.0	0.99
20:5n-3 (EPA)	0.10 ± 0.0	0.05 ± 0.05	0.02 ± 0.02	0.81
22:5n-3	0.26 ± 0.02	0.33 ± 0.01	0.22 ± 0.04	0.29
22:6n-3 (DHA)	0.23 ± 0.04	0.25 ± 0.09	0.16 ± 0.02	0.58
Total n-3 PUFA %	**1.34 ± 0.12**	**1.33 ± 0.12**	**1.20 ± 0.17**	**0.99**
n-6:n-3	**9.78 ± 0.74**	**9.08 ± 2.05**	**10.89 ± 1.53**	**0.71**

Data are presented as mean ± SEM for n= 14 CRC patients, n= 2 UC patients, and n= 6 CD patients. Differences in the fatty acid level between the CRC and CD groups were tested using Mann-Whitney U test (*$p < 0.05$). LA, linoleic acid; CLA, conjugated linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, decosahexaenoic acid.

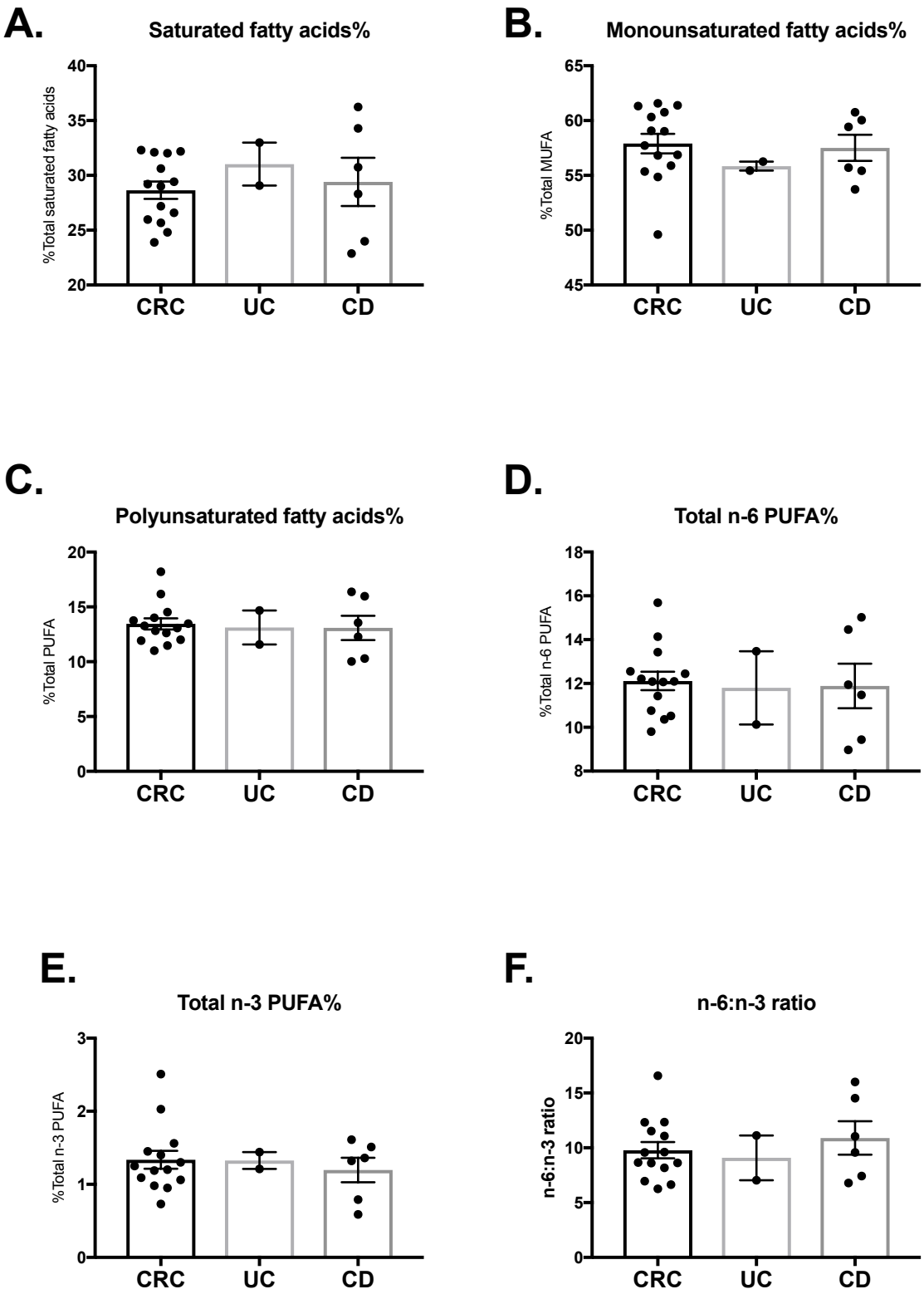


Figure 13: Bar graphs comparing the difference in the fatty acids composition between CRC and IBD mesenteric fat samples.

Values are expressed as mean ± SEM. n= 14 for CRC, n= 6 for CD, and n= 2 for UC. Differences in the fatty acid level between the CRC and CD groups were tested using Mann-Whitney U test. *$p < 0.05$.

3.3.7 Fatty acid composition in healthy and inflamed IBD mesenteric adipose tissues

Table 7 compares the fatty acid composition between paired samples of normal and inflamed MAT in IBD patients. Paired normal and inflamed samples were obtained from 3 CD patients and 2 UC patients. The fatty acid profiles for SFAs, MUFAs, and PUFAs in both, healthy and inflamed MAT were similar ($p > 0.05$) for total (combined) IBD sample, as well as for CD sample.

Table 7: Fatty acid composition (% of total fatty acids) in paired IBD mesenteric adipose tissue sample

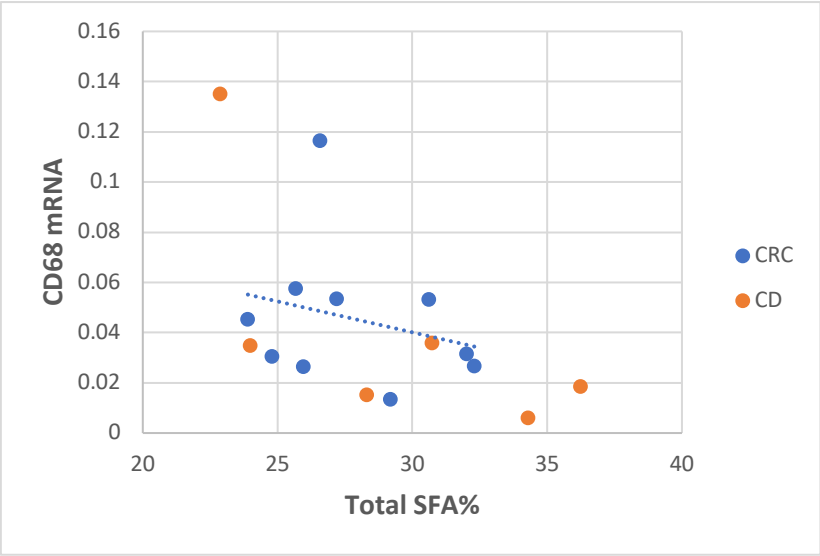
	Total IBD			CD			UC		
Fatty acid composition	**Healthy mesenteric AT**	**Inflamed mesenteric AT**	***P*-value**	**Healthy mesenteric AT**	**Inflamed mesenteric AT**	***P*-value**	**Healthy mesenteric AT**	**Inflamed mesenteric AT**	***P*-value**
Total saturated fatty acids %	29.83 ± 2.1	29.03 ± 2.55	0.31	28.96 ± 3.68	27.7 ± 4.28	0.5	31.13 ± 0.84	31.03 ± 1.96	NA
Total monounsaturated fatty acids %	56.38 ± 1.06	57.24 ± 1.35	0.62	56.9 ± 1.84	58.18 ± 2.23	0.75	55.61 ± 0.47	55.85 ± 0.4	NA
Total polyunsaturated fatty acids %	13.79 ± 1.31	13.72 ± 1.25	0.99	14.14 ± 2.25	14.13 ± 2.05	0.99	13.27 ± 1.29	13.12 ± 1.55	NA
Total n-6 PUFA %	12.55 ± 1.22	12.5 ± 1.14	0.99	13.03 ± 1.98	12.97 ± 1.77	0.99	11.84 ± 1.54	11.8 ± 1.67	NA
Total n-3 PUFA %	1.23 ± 1.19	1.22 ± 1.16	0.99	1.11 ±0.29	1.15 ± 0.29	0.99	1.43 ± 0.25	1.33 ± 0.12	NA
n-6:n-3	11.04 ± 1.53	10.96 ± 1.46	0.99	12.56 ± 1.64	12.21 ± 1.94	0.99	8.75 ± 2.59	9.08 ± 2.05	NA

Data are presented as mean ± SEM of fatty acid% in normal mesenteric adipose tissues and hypertrophied (inflamed) mesenteric adipose tissues of the same IBD patients. n= 5 for total IBD sample, n=3 for CD sample, and n= 2 for UC sample. Differences in the fatty acid level between the paired samples were tested using the non-parametric Wilcoxon signed-rank test (*$p < 0.05$). NA: not applicable.

3.3.8 Mesenteric adipose tissue fatty acid status and inflammatory genes expression

To study the effect of fatty acid enrichment of MAT on the adipogenesis and the inflammatory response of this tissue, the correlation between SFA, MUFA, and PUFA levels and the expression levels of several adipogenesis and inflammatory genes was investigated for all samples using the Pearson's correlation coefficient. %SFA was found to be negatively associated with the expression of the pro-inflammatory marker CD68 ($r = -0.53$, $p < 0.05$) (Figure 14A), while no correlation was observed between its composition and the level of expression of adipogenesis and anti-inflammatory genes. %MUFA was positively associated with the expression of CD68 ($r = 0.51$, $p < 0.05$) (Figure 14B), with no correlation observed with expression levels of adipogenesis and anti-inflammatory genes. %PUFA was positively correlated with the expression of the anti-inflammatory marker MCP1 ($r = 0.49$, $p < 0.05$); this correlation was also significant for total n-6 PUFA ($r = 48$, $p < 0.05$) (Figure 14C-D). There was no correlation found between total PUFA% and adipogenesis and pro-inflammatory markers expression. Total n-3 PUFA correlated negatively with mRNA levels of the adipogenesis marker PPARγ ($r = -0.50$, $p < 0.05$) (Figure 14E), and no association was found with the expression of inflammatory or pro-inflammatory markers. No correlation was found between the n-6: n-3 ratio and the expression levels of all the investigated adipogenesis and inflammatory markers. Overall, these findings indicate an association of the various fatty acid levels with only a few genes involved in the adipogenesis and inflammatory response of CRC and CD MAT.

A.


0.16
0.14
0.12
0.1
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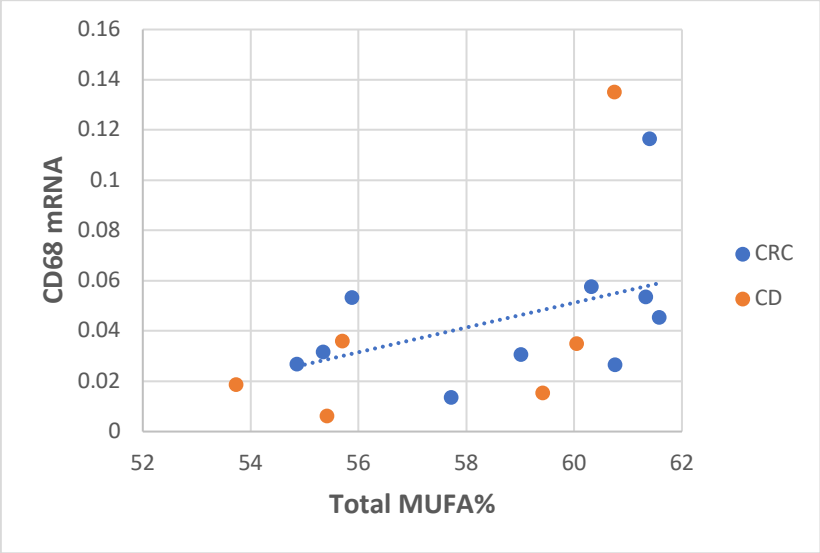
CD68 mRNA
20
25
30
35
40
Total SFA%
CRC
CD

B.


0.16
0.14
0.12
0.1
0.08
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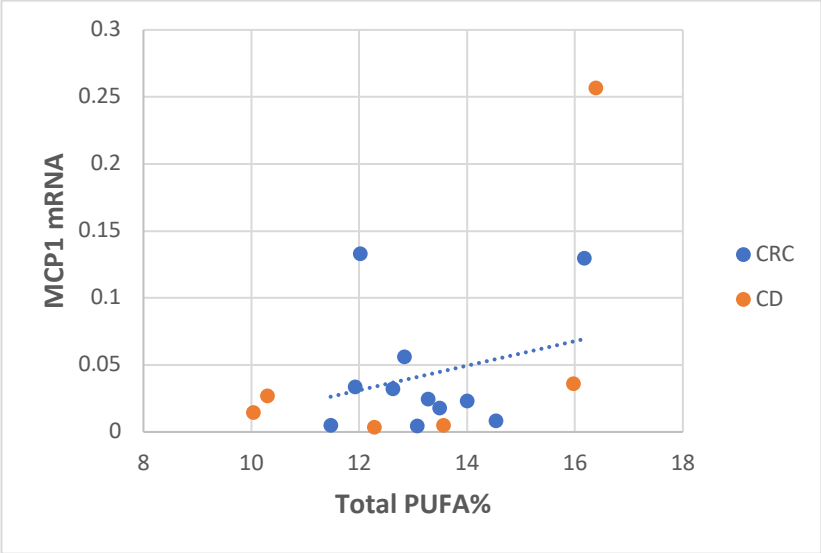
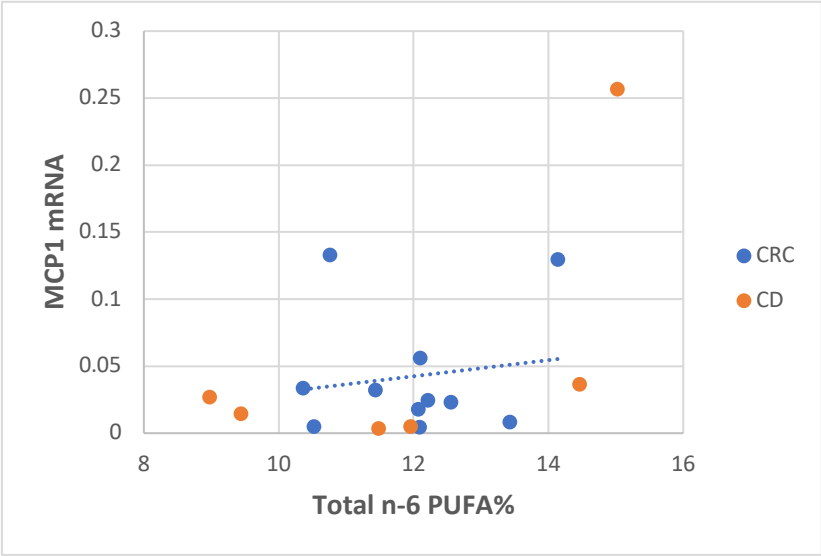
0
CD68 mRNA
52
54
56
58
60
62
Total MUFA%
CRC
CD

C.


0.3
0.25
0.2
0.15
0.1
0.05
0
MCP1 mRNA
8
10
12
14
16
18
Total PUFA%
CRC
CD

D.



E.

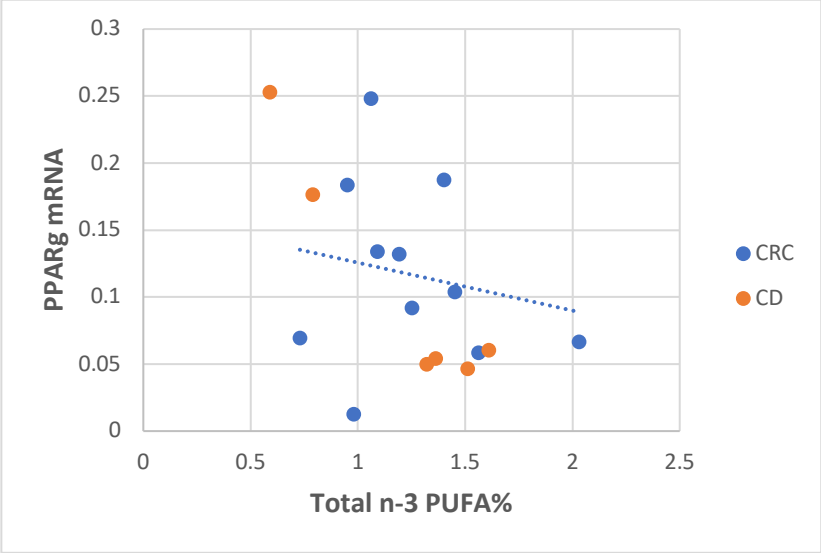


Figure 14: Correlation between CD68, MCP1, and PPARγ mRNA expression levels and fatty acid composition in mesenteric adipose tissues.

n= 11 for CRC patients and n= 6 for CD patients. **A)** Correlation between total SFA% and CD68 (r= -0.53, p= 0.03). **B)** Correlation between total MUFA% and CD68 (r= 0.51, p= 0.04). **C)** Correlation between total PUFA% and MCP1 (r= 0.49, p= 0.04). **D)** Correlation between total n-6 PUFA% and MCP1 (r= 48, p= 0.04). **E)** Correlation between total n-3 PUFA% and PPARγ (r= -0.50, p= 0.03). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.3.9 Summary of the results

The level of leptin mRNA was significantly reduced in CD mesenteric samples compared to CRC samples, while there was no difference in adiponectin mRNA levels. The expression of adipogenesis marker CEBPα was significantly reduced in CD mesenteric fat tissues compared to CRC fat tissues, whereas no differences in the levels of the other two adipogenesis specific markers (PPARγ and FABP4) were observed between the two disease cohorts. Regarding the inflammatory profile of the MAT, a pattern of significant reduction of selected anti-inflammatory mediators (CD163 and CD206) in CD mesenteric samples was identified; both CD163 and CD206 are specific markers for M2 macrophages. Nevertheless, no significant difference in the gene expression of pro-inflammatory markers (IL-6 and CD68) was reported for CD and CRC MAT samples. Results from the paired IBD samples (normal versus inflamed mesenteric fats) showed a reduced expression of anti- and pro-inflammatory markers (IL-6, CD68, MCP1, CD163, and CD206) in inflamed versus normal MAT samples; further analysis would be needed to validate this observation. However, differences in the expression of adipocyte markers (adiponectin, leptin, and PPARγ) between the paired samples were not statistically significant. For the fatty acid composition findings, no differences were found between MAT fatty acid levels of CRC and CD samples. Also, the level of fatty acid was similar between healthy and inflamed MAT from the same IBD patients. Interestingly, correlation analysis indicated a relationship between the level of each fatty acid and the gene expression levels of selected adipogenesis and inflammatory response markers in the MAT: SFA was associated with reduced gene expression of the CD68 pro-inflammatory marker, while MUFAs were associated with its increase. Total PUFA and n-6 fatty acid levels were associated with an increased gene expression of MCP1. An association was found between n-3 fatty acid levels and the reduced expression of PPARγ.

3.4 Discussion

This study aimed to characterize the MAT in CD and CRC patients. The findings have shown few abnormalities in the gene expression profile of CD hypertrophied mesenteric fat, while no differences in the fatty acid profile for this type of adipose tissue was found in comparison to the control. Nevertheless, simple correlation analysis suggested few associations between fatty acids levels and mRNA levels of selected genes related to adipogenesis and inflammatory response in the mesenteric fats of CD and CRC patients.

Abnormal production of adipokines in inflamed mesenteric fats in CD patients has been reported by previous studies (Zulian et al., 2012, Yamamoto et al., 2005). In this study, the level of leptin mRNA was significantly reduced in CD mesenteric samples compared to CRC samples. A previous study by Oliver et al. found similar results, where leptin expression was very low in human CD hypertrophied MAT compared to the normal mesenteric and subcutaneous fat depots (Olivier et al., 2011). The same study also found a similar pattern of a lowered expression of leptin and other adipose tissue markers, like hormone-sensitive lipase, perilipin, and adiponectin in hypertrophied MAT, using an animal model of colitis. In contrast, an earlier study found high levels of leptin being expressed in CD mesenteric fat when compared to control (Barbier et al., 2003). Leptin is a protein mainly secreted by adipocytes in relation to the size of AT; it has metabolic and endocrine functions as well as immune functions (Karmiris et al., 2008), and has both pro-inflammatory and anti-inflammatory properties. The role of leptin in CD is still not clearly understood. Nevertheless, the low expression of leptin in CD mesenteric fats observed in this study can be related to the increased number of small adipocytes rather than large adipocytes, which is a specific morphology of this type of fat tissue, previously reported (Zulian et al., 2012). The secretion and expression of leptin and other adipokines was found to be higher with increased adipocyte size. (Skurk et al., 2007). Yet, the suggested mechanism leading to the reduced expression of leptin in CD hypertrophied mesenteric fat needs further studies. The level of adiponectin mRNA in this study was not different between the two sets of patient samples. Adiponectin possesses many anti-inflammatory functions. Earlier studies have shown a significant upregulation in the level of secreted adiponectin from CD mesenteric fat versus

control, which could be related to an inflammatory response in the adjacent inflamed intestines (Yamamoto et al., 2005, Paul et al., 2006).

Consistent with our findings of low leptin expression, the gene expression of CEBPα (a key marker of adipogenesis) was significantly reduced in CD mesenteric fat tissues when compared to CRC fat tissues. Thus, defective regulation of adipogenesis could be a characteristic of CD hypertrophied mesenteric AT. However, no published study has yet investigated CEBPα expression in the context of CD MAT. Therefore, these findings should be carefully interpreted and further studies are needed for validation. In this study, the expression of other relevant markers like PPARγ (adipogenesis specific marker) and FABP4 (marker for fat metabolism) were not altered in CD mesenteric fat, whereas a previous study found an overall reduction in lipid metabolism genes in CD mesenteric fat (Zulian et al., 2012). Also, another study that used an animal model of colitis characterized the hypertrophied mesenteric fat by defective lipolysis and absence of lipid (triglycerides) storage (Olivier et al., 2011). The knowledge about lipid metabolism in CD mesenteric fat is still lacking and further investigations are required.

Inflammation of the MAT in CD is a complex process, where the upregulation of both pro-inflammatory and anti-inflammatory cytokines is a specific associated characteristic. However, the findings of this study indicated a reduction in the expression of the M2 macrophage-specific anti-inflammatory markers, CD163 and CD206, in CD hypertrophied mesenteric fat. Contrarily, no differences in gene expression of the pro-inflammatory markers IL-6 and CD68 were reported between CD and CRC MAT. These findings are inconsistent with earlier studies. Previous studies have shown an increased expression of anti-inflammatory genes associated with CD mesenteric fat, suggesting a protective role of this type of tissue (Zulian et al., 2012). M2 subtype macrophage infiltration is a characteristic feature of CD MAT, which is highly stimulated by adipokines (leptin and adiponectin), thus counteracting local inflammation in CD and possibly offering protection from further systematic inflammation (Kredel et al., 2013). Unfortunately, this study was not able to measure the gene expression levels of TNFα and CRP. TNFα is one of the main pro-inflammatory cytokines produced by fat tissues and has been a target for CD treatment for many years. Whereas CRP has only recently been investigated in the context of CD mesenteric fat, where its release was found to be triggered by bacterial

translocation and an inflammatory response (Peyrin-Biroulet et al., 2012). Therefore, further research is needed to elucidate the mechanism of action of mesenteric fat inflammation and its role in the pathogenesis of CD.

The contribution of adipose fatty acids to the immune functions of mesenteric fat tissue can modulate inflammation in CD. For instance, the fatty acid content in adipose tissue can stimulate the production of eicosanoids, molecules that have a direct involvement in the inflammatory process. Moreover, dietary fatty acids have an important role in the development of IBD. Epidemiological studies have shown an inverse association between dietary *n*-3 PUFA (DHA) intake and CD development (Chan et al., 2014a). This study has shown no difference in the fatty acid composition of CD mesenteric fat tissue and CRC. However, a previous study investigating the same type of tissue found notable differences in its fatty acid profile, where CD mesenteric AT and associated lymphoid tissues were higher in saturated fatty acids and lower in PUFA than control tissues (Westcott et al., 2006). The same study also showed that the ratio of *n*-6: *n*-3 PUFA was higher in CD AT and lower in lymphoid cells, when compared to control tissues, suggesting an interrupted physiological interaction between the adipocytes and the lymphoid cells, leading to defective fatty acid transport. The proposed mechanism still needs more validation as no other studies in the literature have yet investigated the role of fatty acid in CD MAT. Furthermore, our study was able to show few relationships between the level of fatty acids and the gene expression levels of selected markers. Surprisingly, gene expression of the pro-inflammatory marker CD68 was inversely associated with the SFA levels, while it was positively associated with the MUFA levels. This finding is in contradiction to the general statement that high levels of SFAs are associated with an increased pro-inflammatory response. *In-vitro* studies of adipocytes have shown that treatment with palmitic acid resulted in increased gene and protein expression of the pro-inflammatory cytokine IL-6 (Ajuwon and Spurlock, 2005), while MUFAs were characterized by anti-inflammatory properties. The findings of this study also show that expression of the anti-inflammatory marker MCP1 was positively correlated with the level of total PUFAs and total n-6 fatty acids. Linoleic acid (an *n*-6 PUFA) is considered a precursor in the biosynthesis of the pro-inflammatory arachidonic acid; however, the current evidence is still contradictory and not clear about the inflammatory potential of *n*-6 PUFA (Joffe et al., 2013). Lastly, the expression of

PPARγ in this study was negatively correlated with the level of total *n*-3 PUFAs. This result contradicts the previous finding that fatty acids, particularly PUFAs, are considered ligand (activators) for PPARγ (Wahli and Michalik, 2012).

So far, available studies trying to characterize the MAT in CD are limited. This study aimed to further characterize the molecular profile of this type of adipose tissue in relation to its fatty acid content. The use of human adipose tissue to characterize the fat-wrapping phenomenon associated with long-standing CD is considered more representative than employing an animal model. However, the restricted number of samples, especially for CD patients, presents itself as limitations. Furthermore, this study measured only the gene expression level of investigated markers and not the protein expression level, which may have omitted any interesting observations of post-translational regulations of the protein. Another limitation of this study is the statistically low number of paired CD samples, which limited the comparison between normal and inflamed mesenteric tissues in CD patients.

In conclusion, proper characterization of CD hypertrophied mesenteric fat is still lacking from the current literature. Inconsistent findings between published studies is a reason for this lack of knowledge, and so are inconsistencies between methodology and control group choices. This study has shown that MAT in CD patients is an abnormal type of AT. Unlike typical adipose tissue, this type of fat tissue was associated with defective adipogenesis via reduced expression of leptin and CEBPα and was associated with a low anti-inflammatory profile via decreased expression of M2 macrophages markers (CD163 and CD206). The fatty acid profile of CD MAT was observed to be similar to the control. However, the level of fatty acids impacted the expression of selected inflammatory and adipogenesis markers, which support the involvement of fatty acids in CD pathogenesis.

4 Chapter 4. *In-vitro* Study: Effect of Fatty Acids on Mesenteric Adipocyte Differentiation: Identifying Their Role in The Pathogenesis of Crohn's Disease

4.1 Introduction

4.1.1 Adipocyte hyperplasia controls mesenteric fat hypertrophy and inflammation

Generally, one-third of adipose tissue are composed of mature adipocytes, while the remaining consist of other types of cells, such as macrophages, fibroblasts, pericytes, red blood cells, endothelial cells, smooth muscle cells, mesenchymal stem cells (MSCs), and adipose precursor cells (Ruiz-Ojeda et al., 2016, Moreno-Navarrete and Fernández-Real, 2017). The type and location of the adipose tissue affect the composition and the functional characteristics of these cells (Ruiz-Ojeda et al., 2016). For example, a study found that MSCs from the subcutaneous fat depot tend to proliferate and differentiate into adipocytes more than MSCs from the visceral fat depot after adipogenesis induction in an *in-vitro* model (Ong et al., 2014).

The increased number of adipocytes (adipocyte hyperplasia) in the expanding adipose tissue observed in some types of inflammation results from the high recruitment rate of adipose tissue precursor cells. This is usually influenced by signalling factors that initiate the MSCs to convert into pre-adipocytes, which eventually differentiate into bigger and mature adipocytes (Tang and Lane, 2012). Nevertheless, it seems that the increased expansion of MAT in CD is dependent on the increased number of small adipocytes rather than on adipocyte size, which is a characteristic of visceral adipose tissue in obesity. Findings from a recent study found that adipose precursor cells isolated from MAT derived from a CD patient were more highly proliferative than MAT from control patients. It was therefore suggested that these cells could be responsible for the expansion of fat-wrapping (Serena et al., 2017). Moreover, histological findings from earlier studies found that morphological features of the MAT of CD patients differ from other patients. The size of MAT adipocytes from CD patients was found to be significantly smaller, and adipocyte numbers were,

furthermore, higher compared to MAT adipocytes from control cases (Peyrin-Biroulet et al., 2007).

Adipocytes are considered to be important factors in adipose tissue inflammation. Inflammatory mediators are synthesized by these cells. Alteration in the balance of pro-inflammatory and anti-inflammatory adipocytokines in mesenteric adipocytes has been previously reported (Paul et al., 2006). Smaller adipocytes tend to secret higher amounts of adiponectin than larger adipocytes (Yamamoto et al., 2005). Another inflammatory factor suggested to be involved in the fat-wrapping mechanism is the peroxisome proliferator activated receptor (PPAR-γ), which is specific to adipocytes and considered one of the key regulators in adipogeneis. An earlier study found high levels of PPAR-γ in MAT, which was suggested to be related to fat hypertrophy due to CD (Desreumaux et al., 1999). In addition, infiltration of the inflammatory cells is another significant characteristic of the inflamed MAT in CD. Compared to non-inflamed MAT, the expression of macrophages and T lymphocyte markers (i.e. CD68 and CD3 positive cells) were found to be significantly higher in hypertrophied CD MAT (Yamamoto et al., 2005). The evidence is still inconclusive on whether adipocyte hyperplasia is primarily responsible for activating inflammation in hypertrophied fat in CD, or whether it is a secondary outcome resulting from the transmural inflammation of the diseased intestine. According to Schäffler et al. (2005), a schematic hypothetical model (Figure 15) demonstrates how the interactions between intestinal inflammation, adipocyte hyperplasia, secreted inflammatory mediators, and macrophage infiltration could lead to the pathogenesis of fat-wrapping in CD.

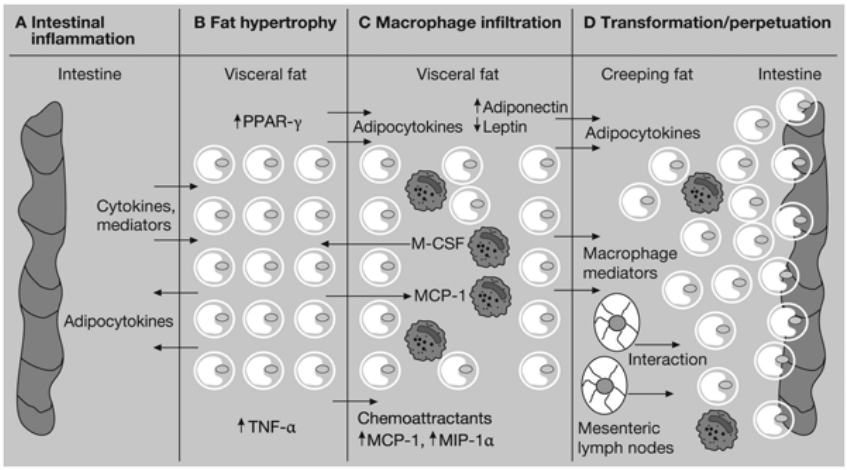


Figure 15: Hypothetical four-step concept of the pathogenesis of creeping fat in Crohn's disease.

Adopted with permission from "Mechanisms of disease: adipocytokines and visceral adipose tissue—emerging role in intestinal and mesenteric diseases", by Schäffler, A., Schölmerich, J. & Büchler, C. 2005, Nature Reviews Gastroenterology and Hepatology, 2, 103. (Schäffler et al., 2005).

4.1.2 Regulation of adipogenesis via fatty acids

Adipogenesis is a complex process regulated by a cascade of adipogenic transcription factors which eventually lead to the development of mature adipocytes. This process first starts with the cellular commitment of MSCs into an adipocyte specific lineage, which then terminally differentiates into fully mature adipocytes. Activation of the CCAAT/enhancer-binding protein alpha (C/EBPα) and PPAR-α are required for this process; both act in growth arrest, which is needed for adipocyte differentiation (Vanella et al., 2013). PPAR-γ is usually induced in the early stages of the adipocyte differentiation process, which also functions as a sensor for fatty acids (Warnke et al., 2011). When activated, PPAR-γ regulates the expression of other target genes that usually are found in the mature adipocyte, such as Fatty acid binding protein 4 (FABP4 or aP2), lipoprotein lipase (LPL), acyl-CoA synthase, CD36, and adiponectin (Moseti et al., 2016, Garin-Shkolnik et al., 2014). FABP4 is one of the master regulatory factors in adipogenesis, acting as a lipid transporter within cells. FABP4 helps in pairing intracellular lipids with their biological target molecules (Garin-Shkolnik et al., 2014).

There are several factors that could either induce or inhibit adipogenesis. For example, growth factors, hormones, cytokines, and specific drug compounds have the ability to regulate adipogenesis (Moreno-Navarrete and Fernández-Real, 2017). Also, dietary fatty acids are important regulators of adipose tissue function. Different fatty acids have varying functions in regulating adipogenesis and lipid metabolism. PUFAs are considered PPAR-γ ligands (activators) (Madsen et al., 2005). Linoleic acid has shown both pro-adipogenic and anti-adipogenic effects in pre-adipocyte cell-lines (Madsen et al., 2005, Petersen et al., 2003), while α-linolenic acids can decrease adipocyte differentiation and stimulate fatty acid biosynthesis and lipid turnover during adipocyte differentiation contributing to lower pro-inflammatory response (Polus et al., 2015). Studies investigating the differentiation of pre-adipocytes isolated from the MAT are very limited. Only a recent study by Serena 2017 explored the differentiation and immune properties of this type of pre-adipocyte. However, the fatty acid effect was not investigated in this study (Serena et al., 2017). The role of fatty acids in mesenteric pre-adipocyte differentiation hence requires further investigation.

4.1.3 Research gaps

Our understanding of how therapeutic lipids and fatty acids could possibly reduce intestinal inflammation in Crohn's disease is still limited. Mesenteric fat hypertrophy adjacent to inflamed intestines has been recognized as a specific pathological feature in CD patients for decades. However, knowledge of the aetiopathogenesis of fat-wrapping in CD is inconclusive about whether it has a leading role in regulating intestinal inflammation, or whether it's just a secondary outcome. The increased number of small adipocytes within the MAT results from the high rate of pre-adipocyte proliferation and differentiation. Different fatty acids can have differing functions in regulating adipogenesis in the adipose tissue. However, the impact of fatty acids on mesenteric pre-adipocyte differentiation has not yet investigated.

4.1.4 Hypothesis

Specific dietary fatty acids have a direct influence on adipocyte differentiation in hypertrophied mesenteric adipose tissue *in-vitro*, which could contribute to chronic intestinal inflammation in CD. This will be tested by collecting fresh mesenteric

adipose tissue for pre-adipocyte isolation, which will then be incubated with different fatty acids to study their differentiation potential.

4.1.5 Objectives

To study and compare the effect of fatty acid treatment during mesenteric pre-adipocyte differentiation, on the expression of key markers for adipocyte differentiation, inflammatory adipocytokines, and fatty acid metabolism.

4.2 Methods

4.2.1 Human tissue collection

For details regarding human mesenteric adipose tissue collection, please see the Methods section in Chapter 3.

4.2.2 Cell culture

4.2.2.1 Isolation and culture of human mesenteric pre-adipocytes

The protocol for primary pre-adipocyte isolation and culture was kindly provided by Seema Ali (School of Biological Sciences, UEA), which was described earlier, with minor modifications (Sideri et al., 2015, Serena et al., 2017, Dubois et al., 2008). Upon arrival, MAT specimens were kept in a sterile tube in a cold tissue culture room until ready for use. Tissue culture hoods were sterilized with 2% trigene prior to each procedure. 25 ml Digestion medium was prepared by weighing 12.5 mg of collagenase (Sigma C9891) and 2.5 mg of DNase (Roche 11284932001) and mixing them in a falcon tube containing 25 ml of pre-warmed serum free Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific 21969-035); the solution was then filter sterilized through a 0.22 μm sterile filter (Thermo Fisher Scientific 10268401). For the isolation process, two grams of fresh MAT were transferred into a sterile petri dish and were cut into smaller pieces using a sterile blunt-ended scissor. Any apparent blood vessels or white fibrous tissues were removed from the MAT sample, before it was minced to facilitate the following digestion step (see Figure 16). Just enough pre-warmed digestion medium media (2 ml) was added to the minced tissues, to help liquefy it so it can be taken up by the sterile 25 ml pipette and transferred in the tube containing the whole digestion medium. The tube was then sealed with parafilm and placed in a shaking water bath or oven at 37℃ (100 rpm) for 30 minutes to allow digestion, after which it was filtered through a 70 μm sterile cell

strainer. (Thermo Fisher Scientific 11597522). Basal medium was added to the filtered homogenate, up to the 50 ml mark in a sterile falcon tube (see Figure 17). The basal medium was composed of DMEM (with 4.5 g/L glucose and sodium pyruvate) (Thermo Fisher Scientific 21969-035), 10% fetal bovine serum (FBS) (Thermo Fisher Scientific 10500064), and 0.5% Penicillin-Streptomycin (Thermo Fisher Scientific 15140148). The tube was then centrifuged at 1500 rpm for 5 minutes to separate the stromal-vascular fraction (SVF) (in the form of a cell pellet) from the floating mature adipocytes (see Figure 17).

The pellet was then resuspended in 1 ml of red blood cell (RBC) lysing buffer (Sigma R7757) and incubated for 1 minute at room temperature to help remove any RBCs. Thereafter, the pellet was washed with 15 ml of phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 25 ml basal medium and plated onto a T-75 culture flask (Greiner Bio-One 658175) and incubated in a humidified culture incubator at 5% CO_2 and 37°C. After 24 hours, cells were washed with 10 ml of warm sterile PBS for allowing better cell growth and fed with new 25 ml growth basal medium. The cell culture basal medium was changed every 4 days.

Cells were sub-cultured till they reached 80% confluence, which took 8 to 10 days. To prepare the cells for sub-culture, the basal medium was removed before washing with 10 mL of warm, filter-sterilized PBS. The cells were then trypsinized with 2 ml of pre-warmed 0.25% trypsin (Thermo Fisher Scientific 25200056) and incubated for 5 minutes in a tissue culture incubator to allow cell dissociation. The cells were observed under a light microscope to check for cell detachment from the flask (the flask was gently tapped to help detach the cells). Once the cells were completely detached, the trypsin was inactivated by adding fresh basal medium with 10% FBS. Then, the basal medium containing the cell suspension was transferred from the flask into a sterile 15 ml falcon tube and centrifuged at 1500 rpm for 5 minutes to allow cell separation. The cell pellet was then re-suspended in pre-warmed basal medium and cultured at a suitable dilution to reach a sub-culturing ratio of 1:2. The cultures were expanded for up to 4 weeks (Figure 18A-C). Passages 2 and 3 where used for this study. Previous studies have shown that earlier passages of adipose tissue SVF have a heterogeneous cell population. Thus, additional *in-vitro* passages are

required to ensure a homogenous cellular population of adipose tissue MSCs (Zamperone et al., 2013).

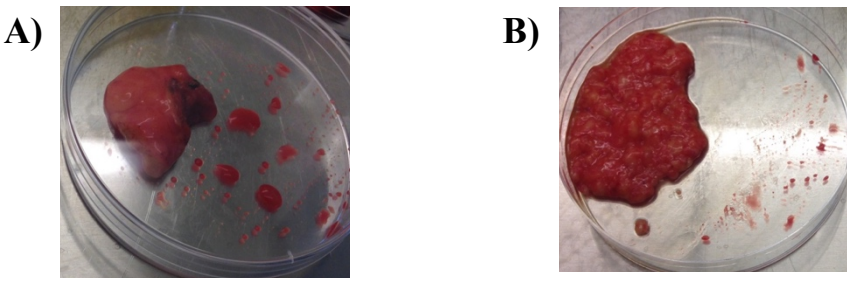


Figure 16: A diagram demonstrating the mesenteric fat tissue specimen collected for the pre-adipocyte isolation.

A) Before cutting the tissue sample and before removal of any apparent blood vessels or white fibrous tissues. B) After mincing the sample into a fine consistency to make it ready for the digestion step.

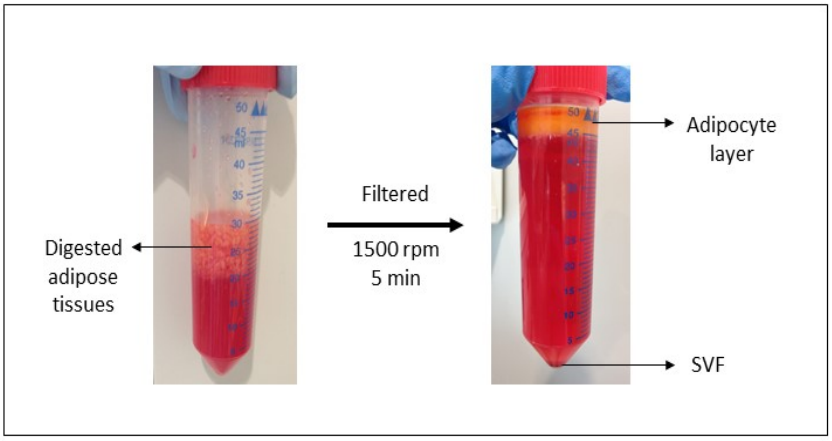


Figure 17: A schematic demonstrating the digestion and centrifugation steps is isolating mesenteric fat pre-adipocytes.

Picture on left-hand side show the tube containing the digested tissues after digestion step. The digested tissue homogenate was filtered through cell strainer into a new tube (picture on right-hand side) with basal medium added enough to fill the 50 ml falcon tube; this tube was then centrifuged at 1500 rpm for 5 minutes to separate the stromal-vascular fraction cells pallet from the floating mature adipocytes.

4.2.2.2 Pre-adipocyte differentiation and fatty acid treatment

For fatty acid differentiation experiments, cells at passage 2-3 were plated in 24-well tissue culture plates (Thermo Fisher Scientific 103809320) at a seeding density of 40,000 cells per well. The cell number was counted before plating using the haemocytometer counting method, where 10 µl of cell suspension (pre-mixed with trypan blue solution in a 1:1 ratio) was pipetted into a Neubauer Haemocytometer Counting Chamber. The area of the counting chamber was observed under light microscopy and non-blue dye cells were counted as viable cells. Cells in all four quadrants of the chamber were counted and averaged. This number was then used to calculate the total number of cells in the original cell suspension by multiplying it by 10,000, times a dilution factor of two. Once the plated cells reached 100% confluence, 48 hours were allowed to initiate growth arrest before inducing differentiation. The differentiation medium consisted of basal growth medium (DMEM with 4.5 g/L glucose, 10% FBS, and 0.5% Penicillin-Streptomycin) supplemented with pro-adipogenic factors that are required for activating the cAMP signalling pathway at the early phase of AT expansion; these factors include 100 nM insulin (Sigma I9278), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma I5879), 1 µM dexamethasone (Sigma D1756), and 0.2 mM indomethacin (Sigma I7378). The cells were incubated with the differentiation medium for up to 15 days (Figure 18D).

Oleic acid (Sigma O3008), linoleic acid (Sigma L9530), and α-linolenic acid (Sigma L2376) were chosen to study the effect of fatty acids on adipocyte differentiation. All the fatty acids were delivered to the cultured pre-adipocytes as fatty acid/bovine serum albumin (BSA) complexes, in a 2:1 molar ratio of fatty acid to BSA. A range of 50, 100, 200 mM α-linolenic acid stock solutions were prepared by dissolving the free fatty acid in dimethyl sulfoxide (DMSO) organic solvent (Sigma D2650), and kept frozen at -80°C until use. Oleic acid and linoleic acid in 10% BSA solution, and at a 2:1 fatty acid to BSA molar ratio was purchased. The concentrations of fatty acids used in this study are considered to be within optimal physiological levels to stimulate the adipocyte differentiation response in the cells, as shown in previous studies (Abdelmagid et al., 2015, Polus et al., 2015). When used in a given experiment, a desired volume of the stock solution was added to the adipocyte differentiation media to reach a final working concentration of 15 µM, 30 µM, or 60 µM fatty acid, as required by the experiment. α-linolenic acid treated media had to be

supplemented with 10% BSA solution (Sigma A1595) to obtain a molar ratio of 2:1 fatty acid to BSA. Differentiation medium with 10% BSA was used as the control. Also, the DMSO concentration in all fatty acid treatment groups and controls was normalised to 0.5% (v/v). Cells were treated with fatty acid enriched differentiation medium for 2, 10, and 15 days. Following the treatment, gene expression analyses were performed at day 2 and day 10 only, while lipid droplet staining was performed at day 15.

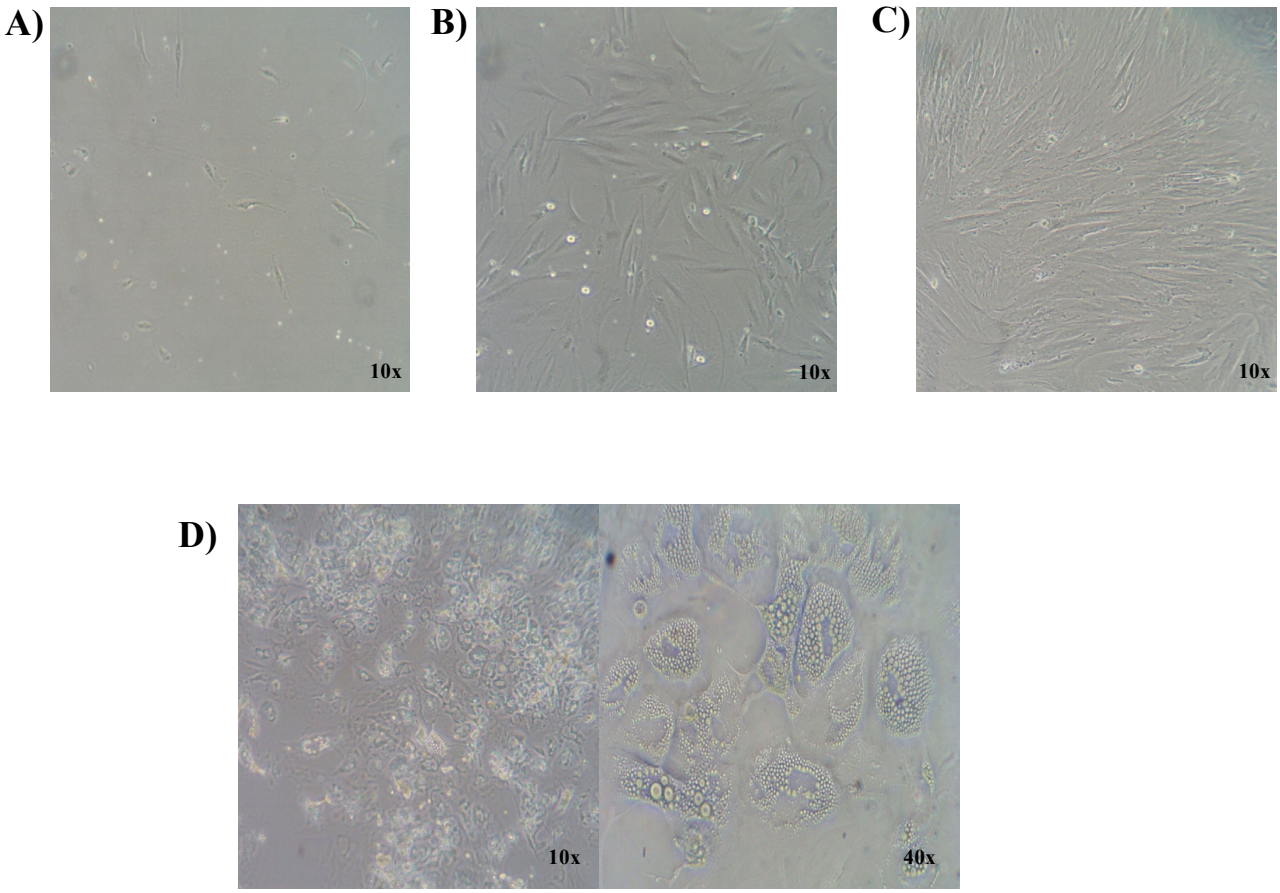


Figure 18: Morphology of isolated/cultured and differentiated mesenteric pre-adipocytes.

A, **B**, & **C)** Growth of mesenteric adipose tissue pre-adipocytes over a period of 4 weeks. **D)** Differentiated pre-adipocytes after 15 days of adipocyte differentiation. Images were acquired by light microscopy.

4.2.3 RNA extraction and Quantitative real-time PCR (qRT-PCR)

At day 2 and 10 of the above described differentiation experiment with fatty acids, RNA was extracted from the cells using the ReliaPrep RNA cell extraction kit (Promega Z6011), to measure the gene expression of adipogenic markers. The extraction was performed based on the manufacturer's description. Briefly, culture medium was removed and the cells were washed with 1 ml of ice-cold PBS. 250 µl of BL+TG lysis buffer was added and the cell suspension was gently mixed by aspiration seven times to help lyse the cells, after which the lysate was transferred to a sterile 1.5 mL eppendorf tube. The required volume of 100% isopropanol was added and the lysate was then transferred to an extraction kit minicolumn and centrifuged at 14000g for 30 seconds. 500 µl RNA Wash solution was added to the minicolumn and centrifuged at 14000g for 30 seconds. Next, DNase I incubation mix was prepared by mixing 24µl of Yellow Core Buffer, 3µl 0.09M $MgCl_2$ and 3µl of DNase I enzyme and added to each minicolumn and incubated at room temperature for 15 minutes. The minicolumn was then washed with 200µl of column wash solution, followed by 500µl of RNA wash solution. The minicolumn was placed into a new collection tube and washed with 300µl of RNA wash solution. Finally, the minicolumn was transferred to an elution tube, where the RNA was eluted with 15µl nuclease-free water. Extracted RNA samples were stored at -20°C till further analysis.

Please refer to the methods section in Chapter 3 for details about nucleic acid quantification (sub-section 3.2.2.1), cDNA synthesis (sub-section 3.2.2.2), qRT-PCR (sub-section 3.2.2.3), and the primers used (Table 4).

4.2.4 BODIPY lipid droplet staining and quantification

To measure lipid accumulation, fatty acid treated cells at day 15 of differentiation were stained with neutral lipid-specific BODIPY 493/503 dye (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Thermo Fisher Scientific D3922). Live cells were washed with filtered PBS and then stained with BODIPY (10 µg/ml) and kept in the tissue culture incubator for 10 minutes. Cells were then washed with filtered PBS and sixteen-bit images of the lipid droplets were taken with an AxioCam ICm1 monochrome charge-coupled device camera attached to the Apotome.2 Imaging System and a confocal microscope, (Zeiss LSM 800 with

Airyscan) using the Axiovision 4.8.2 software (CarlZeiss). Image staining intensities were analyzed with the ImageJ software. Images were background subtracted and thresholds were manually applied for regions of interest. For each treatment group, at least 7-8 images were selected for analysis and their averages were calculated.

4.2.5 Statistical analysis

GraphPad Prism software (Version 7.0, GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Results of at least 3 independent experiments (unless specified otherwise) are presented as mean ± standard error of the mean (SEM). The Mann-Whitney U test was used to compare the difference between test groups and $p < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Tissue donor characteristics for primary mesenteric pre-adipocytes

Live MAT was collected from 10 patients. However, the isolation of pre-adipocytes was successful (without any risk of contamination and infection) only for 6 cases, of which four were diagnosed with CRC and were admitted for right hemicolectomy, and two were diagnosed with CD and were admitted for ileal resection. The median age for the patients was 68 (37-86) years and the median BMI was 26.03 (24.84-33.39) kg/m^2.

4.3.2 Effect of unsaturated fatty acids on the gene expression of adipogenic markers during the differentiation of mesenteric pre-adipocytes at different time points

The effect of oleic acid, linoleic acid, and α-linolenic acid (at 30 μM concentrations) on the differentiation of human mesenteric pre-adipocytes was assessed by measuring the gene expression level of key markers for adipogenesis. Gene expression was assessed during early phase (day 2) and late phase (day 10) adipocyte differentiation (Figure 19). The level of PPARγ mRNA did not increase significantly from control after fatty acid treatments on day 2 of differentiation. This is illustrated in Figure 19A, which shows that PPARγ mRNA levels increased only by 1.6-fold, 2.1 fold and 1.4-fold when treated with oleic acid, linoleic acid, and α-linolenic acid, respectively. Significant changes in PPARγ mRNA expression levels also did not occur at day 10 of differentiation for all three fatty acid treatments.

Conversely, a marked increase in CEBPα mRNA levels was observed at day 2 of differentiation, after treatment with all the three fatty acids. Nevertheless; no changes to gene expression were observed at day 10. Figure 19B shows that CEBPα mRNA levels in mesenteric pre-adipocytes at day 2 increased 3-fold ($p = 0.08$) when treated with oleic acid, 3.1-fold ($p < 0.05$) when treated with linoleic acid, and 4.1-fold ($p < 0.05$) when treated with α-linolenic acid. No significant effect of the fatty acids on FABP4 mRNA expression was observed at day 2 and day 10 of differentiation (Figure 19C). Adiponectin mRNA expression was only measured at day 10 of differentiation because it was considered an adipocyte specific marker that only expressed in the late phases of adipogenesis. Figure 19D illustrates that adiponectin mRNA expression was highly increased (7.3-fold, $p > 0.05$) only upon linoleic acid treatment.

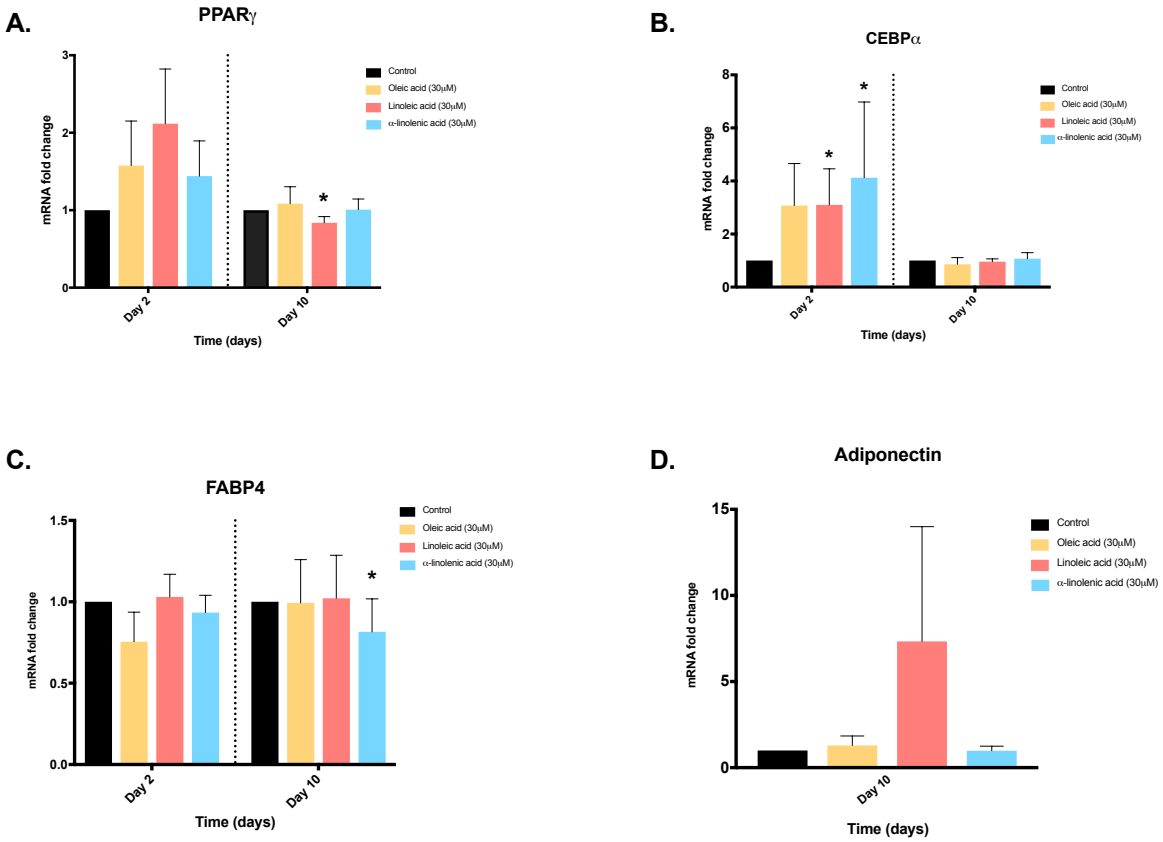


Figure 19: Fold change in adipogenic markers mRNA expression in human mesenteric pre-adipocytes treated with oleic, linoleic, and α-linolenic fatty acids (30 μM) during differentiation.

Data are presented as mean fold change over control group and standard error of mean (error bars). n=6 (all disease cohort combined). Statistical analysis was done by Mann-Whitney U test to test the difference between fatty acid treatment groups at a given time point of differentiation. *$p < 0.05$. All genes of interest have been normalised to the housekeeping gene RPL13A.

4.3.3 Effect of unsaturated fatty acids on the gene expression of adipogenic markers during mesenteric pre-adipocytes differentiation in Crohn's disease and colorectal cancer

To examine the effect of fatty acids on mesenteric pre-adipocyte differentiation in Crohn's diseases, primary pre-adipocytes isolated from CD-affected MAT samples were used. Healthy MAT samples from patients with colorectal cancer (CRC) were used as control. PPARγ mRNA expression levels following fatty acid treatments in both disease cohorts underwent a slight increase, which was nonetheless insignificant. Figure 20A shows that PPARγ mRNA from CRC and CD mesenteric pre-adipocytes at day 2 of differentiation increased 1.9-fold in CRC and 1.3-fold in CD when treated with oleic acid, and 2-fold in CRC and 2.3-fold in CD when treated with linoleic acid, and 1.6-fold in CRC and 1.04-fold in CD when treated with α-linolenic acid ($p < 0.05$). PPARγ mRNA levels at day 10 were unchanged in both CRC and CD mesenteric pre-adipocytes. Comparatively, expression levels of CEBPα mRNA were highly increased at day 2 in CRC pre-adipocytes (3.9-fold, 3.7-fold, and 5.6-fold when treated with oleic acid, linoleic acid, and α-linolenic acid respectively, $p < 0.05$) while in CD pre-adipocytes CEBPα mRNA levels underwent a moderate increased of 1.8-fold, 2-fold, and 1.2-fold, when treated with oleic acid, linoleic acid, and α-linolenic acid respectively (Figure 20B). However, CEBPα mRNA levels at day 10 of differentiation were unaltered in both CRC and CD mesenteric pre-adipocytes. For the FABP4, Fatty acid treatment did not alter FAB4 mRNA levels in CRC and CD mesenteric pre-adipocytes at the indicated time points (Figure 20C). For adiponectin mRNA, a 10.6-fold change ($p > 0.05$) was observed at day 10 in CRC pre-adipocytes, treated with linoleic acid, whereas CD pre-adipocytes showed no alteration in adiponectin mRNA levels in response to any of the fatty acids (Figure 20D).

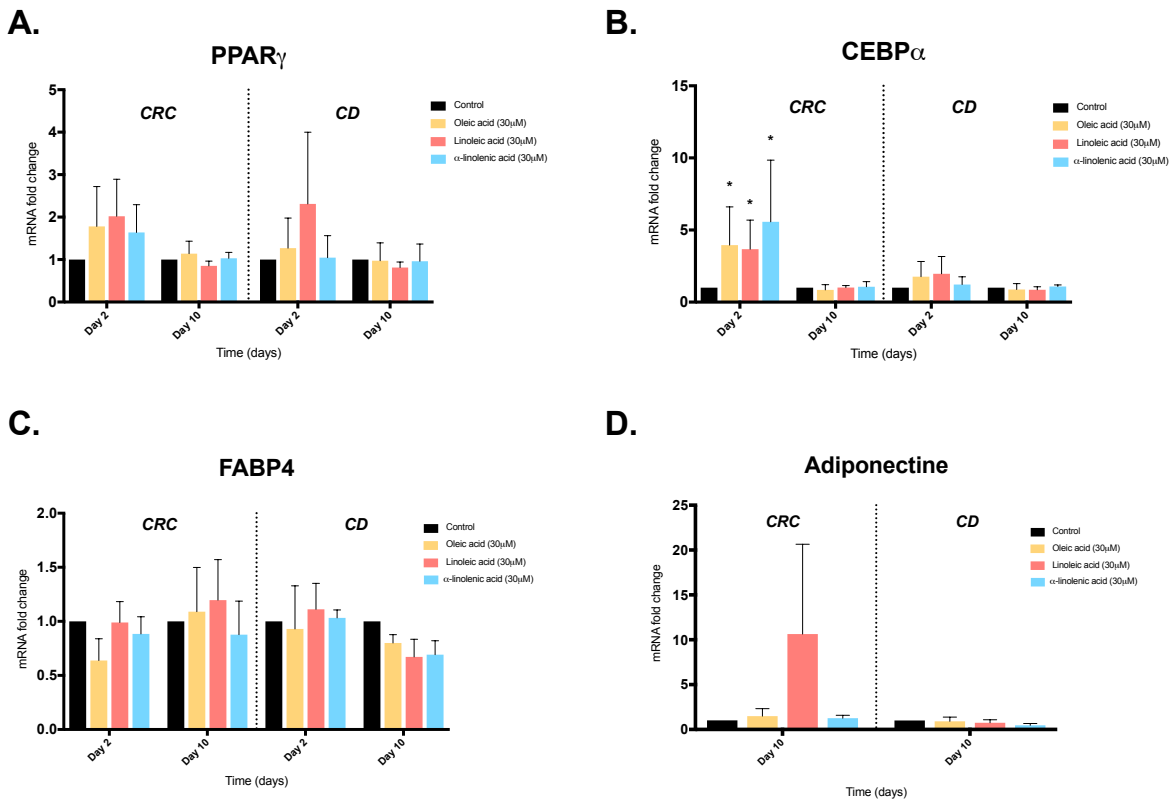


Figure 20: Fold change in adipogenic markers mRNA expression in Crohn's disease (CD) and colorectal cancer (CRC) isolated mesenteric pre-adipocytes treated with oleic, linoleic, and α-linolenic fatty acids (30 µM) during differentiation.

Data are presented as mean fold change over control group and standard error of mean (error bars). n=4 for CRC group and n=2 for CD group. Statistical analysis was done by Mann-Whitney U test to test the difference between fatty acid treatment groups at a given time point of differentiation. *$p < 0.05$. Due to the very limited number of mesenteric samples for Crohn's diseases no statistical analysis was applied to compare the differences in the gene expression level for the adipogeneic markers between the two disease cohorts. All genes of interest have been normalised to the housekeeping gene RPL13A.

4.3.4 Effect of different doses of unsaturated fatty acids on the gene expression of adipogenic markers during mesenteric pre-adipocytes differentiation

To further examine the role of oleic, linoleic, and α-linolenic acids on gene expression levels of adipogenic markers, mesenteric pre-adipocytes were treated with varying doses of the fatty acids (15, 30 and 60 μM), in a dose response experiment (Figure 21). Oleic acid stimulated an increase, albeit non-significant, in PPARγ mRNA and CEBPα mRNA levels with all doses tested on day 2 of differentiation (Figure 21A). While on day 10, oleic acid stimulated a non-significant increase in PPARγ, CEBPα, and adiponectin mRNA expression only at the lowest dose (15 μM) and highest dose (60 μM) (Figure 21B). For linoleic acid, a similar trend was observed, where there was a non-significant increase in PPARγ mRNA and CEBPα mRNA expression levels with all doses tested on day 2 of differentiation (Figure 21C). However, on day 10, linoleic acid treatment resulted in a small, but non-significant reduction in PPARγ, CEBPα, FABP4, and adiponectin mRNA levels with all doses tested (Figure 21D). For α-linolenic acid, a sharp increase in CEBPα mRNA levels at 60 μM concentration was observed at day 2 of differentiation, which was not statistically significant, while the other genes shown a modest increase (Figure 21E). α-linolenic acid treatment also did not show any noticeable and significant changes in adipogenic marker expression levels at day 10. (Figure 21F).

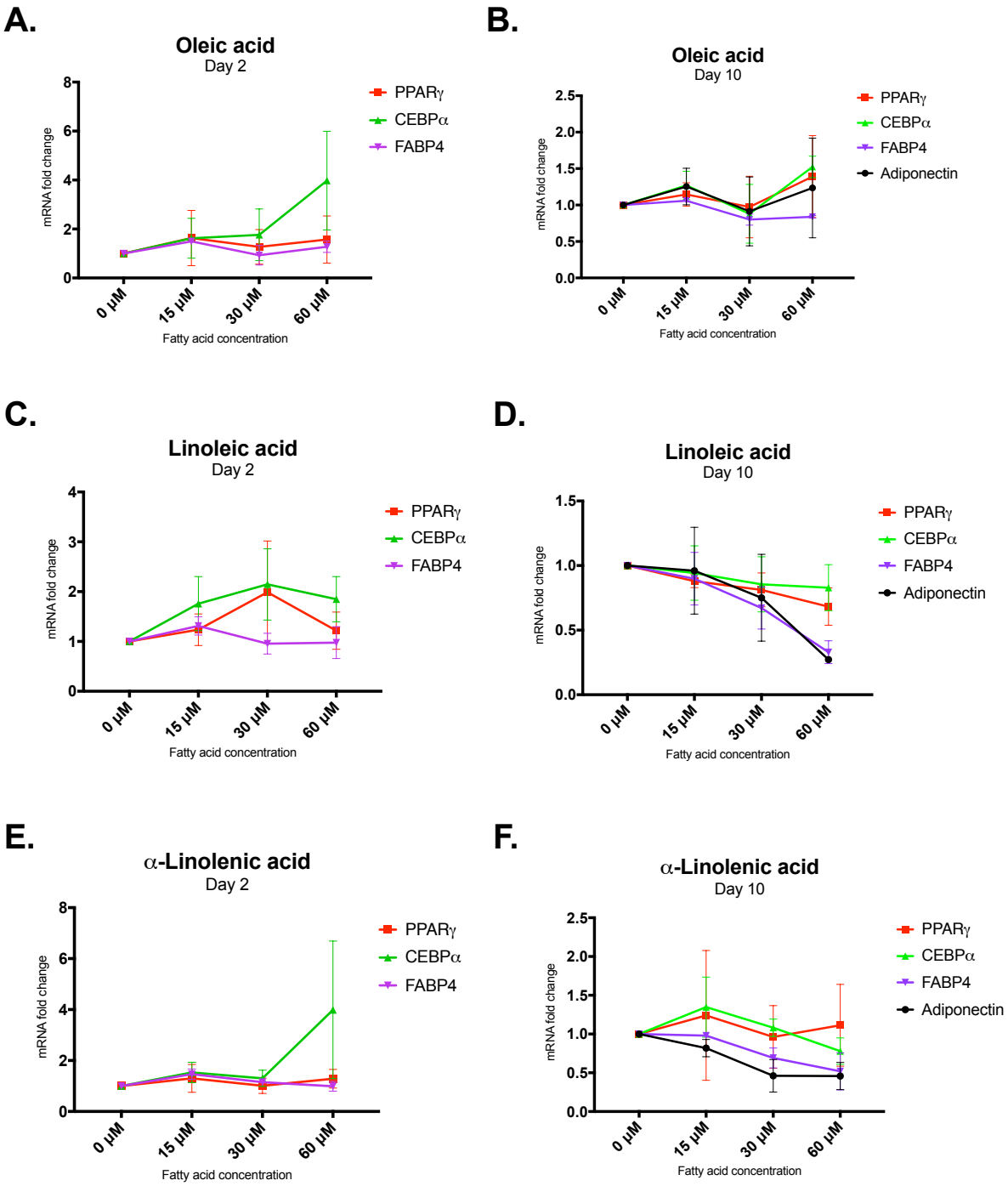


Figure 21: Effect of increasing concentrations of oleic, linoleic, and α-linolenic fatty acids on gene expression of adipogenic markers in mesenteric pre-adipocytes.

Data are presented as mean fold change over control group and standard error of mean (error bars). n=3. Statistical analysis was done by Mann-Whitney U test to test the difference between the effect of different concentrations for each fatty acid at a given time point of differentiation. All genes of interest have been normalized to the housekeeping gene RPL13A.

4.3.5 Effect of unsaturated fatty acids on lipid droplet formation during mesenteric pre-adipocyte differentiation

To examine the effect of fatty acids on lipid droplet formation during adipogenesis, BODIPY staining of differentiated mesenteric adipocytes was performed by measuring the fluorescence of BODIPY-stained lipid droplets after 15 days of differentiation with fatty acid treatment. As hypothesized, lipid accumulation was potentially increased in pre-adipocytes that were treated with adipocyte differentiation medium, with or without fatty acids (as in control, oleic acid, linoleic acid and α-linolenic acid groups), compared to cells that were treated with basal medium only (Figure 22A). A 7-fold increase in lipid accumulation was observed for cells treated with linoleic acid, while α-linolenic acid treated cells demonstrated a 34-fold increase, which was not statistically significant. Oleic acid has showed a non-significant inhibitory effect on lipid accumulation (Figure 22B).

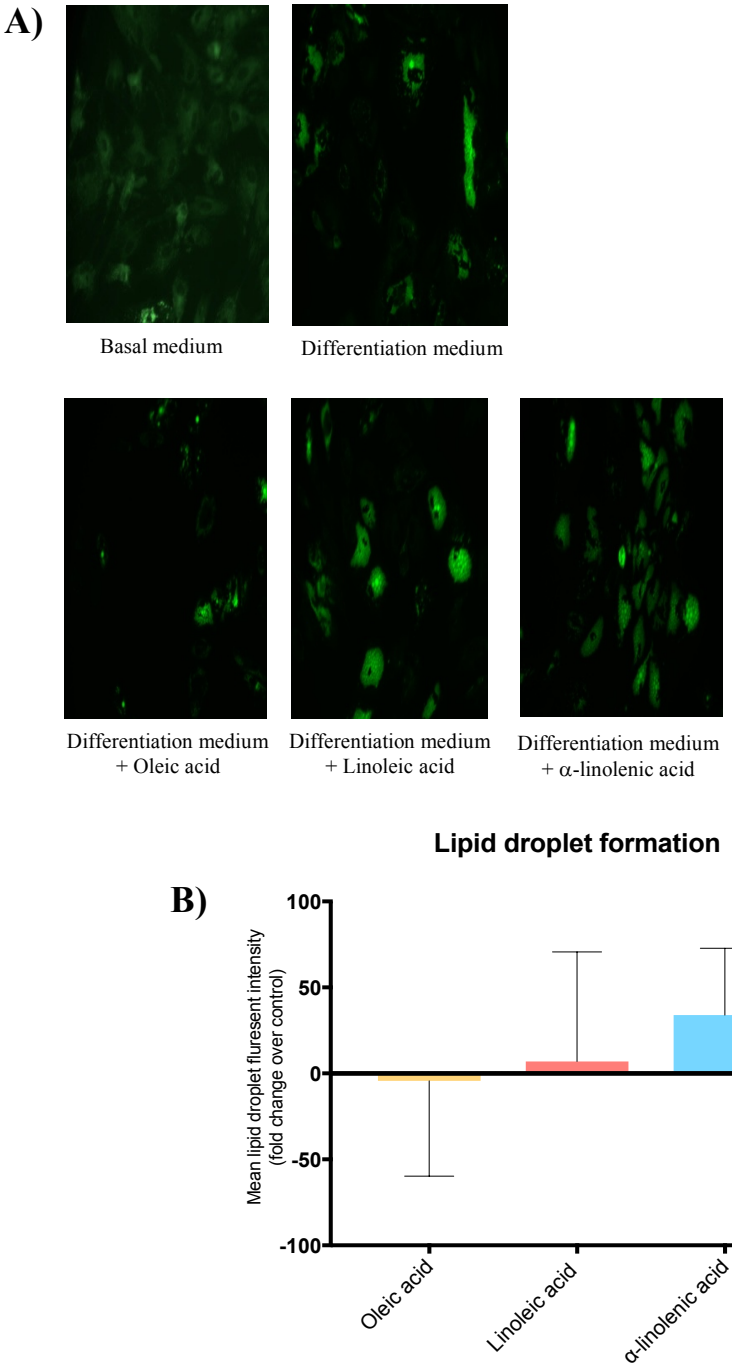


Figure 22: Effect of fatty acids on lipid droplet formation at day 15 of adipocyte differentiation.

A) Live cells at day 15 of fatty acid differentiation treatment were stained for lipid droplets by using BODIPY. **B)** Data are presented as fold change of the mean fluorescent intensity calculated over control group (differentiation medium only) and standard error of mean (error bars). n=3. Images were analysed with ImageJ. Statistical analysis was done by Mann-Whitney U test to test the difference between fatty acid treatment groups.

4.3.6 Results Summary

Oleic acid, linoleic acid and α-linolenic acid all seemed to cause an increase in adipogenesis in the early phase of differentiation, mainly via elevating CEBPα mRNA, while in late phase, only linoleic acid was observed to continue upregulation of adipogenesis via increasing adiponectin mRNA levels (non-significant). The effect of all three fatty acids on elevating CEBPα mRNA levels, was more potent in CRC mesenteric pre-adipocytes compared to CD pre-adipocytes, during the early phase of differentiation. A similar trend was also observed in the late phase of differentiation for linoleic acid, but only on adiponectin mRNA. The effect of fatty acids on CEBPα mRNA expression was observed to be relatively dose dependent, especially during the early phase of differentiation. Finally, linoleic acid and α-linolenic acid, both demonstrated increased lipid accumulation, which was dose-dependent, while oleic acid relatively downregulated lipid droplet formation. These results were, however, statistically non-significant.

4.4 Discussion

The results in this study have shown that unsaturated fatty acids generally induced a pro-adipogenic effect in mesenteric pre-adipocytes by varying degrees. This increasing effect on adipogenesis was mainly regulated by CEBPα, which is a key adipogenic transcription factor during the early stages of adipogenesis. This effect was relatively more potent in CRC derived mesenteric pre-adipocytes than those derived from CD patients. Furthermore, the observed pro-adipogenic effect was further confirmed by increasing lipid droplet formation in the fully differentiated mesenteric pre-adipocytes, an effect that was enhanced only for linoleic and α-linolenic acids but not for oleic acid.

Previous studies consistently reported an increased expression of PPARγ and CEBPα in differentiated pre-adipocytes that were treated with fatty acids (Regassa et al., 2017). In the present study, dietary fatty acids seemed only to significantly increase the expression of CEBPα in mesenteric pre-adipocytes. The level of PPARγ mRNA was insignificantly elevated, while no changes occurred in FABP4 expression. PPARγ and CEBPα are both induced during the early phases of adipogenesis, which subsequently regulate the expression of later adipogenesis target genes (Madsen et al.,

2005). While further research is required, it is proposed that the high expression of CEBPα reported here, in response to fatty acids, could be specific to mesenteric pre-adipocytes. The effect of oleic acid and linoleic acid on adipogenic gene expression reported in this study is consistent with previous findings, which demonstrated their upregulating effect on PPARγ, as well as CEBPα expression (Madsen et al., 2005). However, the pro-adipogenic effect of α-linolenic acid reported in this study is inconsistent with the current evidence, which reports a reduction in adipogenesis when pre-adipocytes were treated with α-linolenic acid (Siriwardhana et al., 2013). Moreover, the expression of the adipocyte specific marker (adiponectin) was relatively elevated only with linoleic acid, indicating that the pro-adipogenic effect at later stages of adipogenesis could be enhanced with linoleic acid.

Only very few studies have investigated the role of mesenteric adipose tissues in CD. However, the effect of dietary fatty acids on CD mesenteric pre-adipocyte differentiation is not yet investigated. This study is the first to look at the adipogenic potential of mesenteric pre-adipocytes in response to fatty acid exposure, where the findings have demonstrated an increase in adipogenesis only in CRC mesenteric pre-adipocytes treated with unsaturated fatty acids, whereas adipogenesis was very modestly upregulated in CD mesenteric pre-adipocytes. The pro-adipogenic effect was reflected by the significant increase in CEBPα mRNA expression. Nevertheless, these findings should be carefully interpreted as a small sample size due to very limited surgical samples, especially for CD patients, is one of the limitations of this study. Therefore, future studies are needed with larger sample sizes to elucidate the role of fatty acids on the adipogenesis of CD MAT.

Accumulation of intracellular lipids is an important characteristic during the process of adipogenesis. The degree of formation of lipid droplets will consequently determine the size of adipocytes and its adipocytokine profile; the bigger the adipocyte, the more pro-inflammatory mediators will be secreted, and so its anti-inflammatory potential will be lower (Skurk et al., 2007, Polus et al., 2015). Therefore, lipid droplet formation in differentiating mesenteric adipocytes was also investigated in later phases of differentiation in this study. Oleic acid was observed to relatively down-regulate lipid droplet formation, as indicated by the low lipid droplet fluorescent intensity. This result is relatively consistent with earlier animal studies, that reported an inhibitory effect of oleic acid on the degree of adiposity (Hsu and Huang, 2006,

García-Escobar et al., 2008). However, linoleic acid and α-linolenic acid have both shown an increasing effect on lipid droplet accumulation. Similar results for linoleic acid have been observed in a recent study using human subcutaneous pre-adipocytes, whereas linoleic acid precursor (arachidonic acid) significantly reduced lipid accumulation within treated cells by 25% compared to control cells (Polus et al., 2015). However, for α-linolenic acid our results are inconsistent with previous evidence that consistently reported decreased lipid droplet formation in pre-adipocyte cell-lines treated with α-linolenic acid (or its derivative EPA) (Warnke et al., 2011, Manickam et al., 2010). The disagreement in this data with the existing literature could be related to the fact that the types and origins of pre-adipocytes used in previous studies are different to what we have used for our investigation. In this study, primary human mesenteric pre-adipocytes were used, while only very few studies have started to investigate this type of pre-adipocyte. Therefore, these findings should be carefully interpreted as cells from different origins could behave differently to the influence of fatty acids. Moreover, the reported differences between the effect of oleic acid and the two PUFAs might suggest an opposing effect of unsaturated fatty acids on the inflammation of mesenteric fat. The anti-inflammatory effect of oleic acid on MAT has been previously investigated *in-vivo*; the level of anti-inflammatory markers specific for M2 macrophages was increased after supplementation of oleic acid in the animals' diet for three days, while no changes occurred after palmitic acid supplementation (Camell and Smith, 2013). Unfortunately, there is no available evidence for the suggested pro-inflammatory effect of PUFAs on mesenteric fat.

Unlike previous studies, this study has used a primary human mesenteric pre-adipocyte model to investigate the effect of dietary fatty acids, which is more representative than using cell-lines. One of the limitations of this study is the shortage of human mesenteric tissue samples: the number of CD patients undergoing intestinal surgical resection was very low, which limited the power of the study. Moreover, the risk of contamination and infection associated with the use of primary human cells further limited the number of successfully isolated mesenteric pre-adipocytes. Another limitation in this study is the absence of measuring the protein level of the investigated adipogenic markers, to account for any post-translation modifications of these markers in protein form.

Pre-adipocytes are a major cellular component of AT and are found to strongly control the secretion of inflammatory adipocytokines associated with the pathophysiology of chronic disorders (O'Hara et al., 2012). Hypertrophied MAT is a key phenomenon in CD as it is predominantly involved in the intestinal inflammation. Therefore, understanding the role of dietary fatty acids on MAT cellular function has an important clinical implication. Collectively, the findings of this study can guide future researches to identify the most optimal proportion of fatty acids in therapeutic diets prescribed for CD patients, that might beneficially modulate adipogenesis and MAT inflammation. Interestingly, other similar dietary constituents have also been identified as modulators of adipogenesis. Although not investigated in this study, curcumin is another potential dietary supplement that has been reported to have an anti-adipogenic effect in *in-vitro* studies, in addition to its reported anti-inflammatory effect in an IBD therapeutic diet (Siriwardhana et al., 2013, Alhagamhmad et al., 2017). More investigations are required to elucidate the modulatory effect of these nutrients in the context of CD adipogenesis.

In conclusion, this study has shown that mesenteric pre-adipocytes have an increased adipogenic response to unsaturated fatty acids. This effect was predominantly modulated via CEBPα, a key marker in the early stages of adipogenesis. Moreover, an increase in lipid droplet accumulation was observed in differentiated mesenteric pre-adipocytes treated with linoleic and α-linolenic acids, while oleic acid relatively reduced lipid accumulation. However, differences in the effect of the fatty acids on CD versus CRC mesenteric pre-adipocytes could not be concluded in this study as a larger sample size was required. Recent observations introduced mesenteric pre-adipocytes as a novel cellular population with immune functions that are likely to be involved in controlling intestinal inflammation in IBD (Sideri et al., 2015). Given the complexity of the MAT in CD, future studies targeting the potential interaction between dietary fats and the inflammatory responses produced by pre-adipocytes and immune cells present in hypertrophied MAT, and epithelial cells located in the adjacent inflamed intestinal mucosa are required.

5 Chapter 5. Preliminary *in-vitro* studies: impact of lipids on human intestinal crypt proliferation

5.1 Introduction

CD is characterised by transmural inflammation, that penetrates the intestinal wall, leading to serious complications. Transmural inflammation in CD is linked to mesenteric fat hypertrophy (Kruis et al., 2014). Impaired gut barrier function and increased gut permeability have been found to be associated with increased visceral fat deposition (Gummesson et al., 2011). Therefore, achieving transmural healing in CD patients is associated with better long-term outcomes, rather than achieving mucosal healing alone (Fernandes et al., 2017). Interestingly, diet has an important role in modulating the intestinal inflammatory response in CD. Enhancement of the gut mucosal barrier function is one of the mechanisms of action of nutritional therapy in CD (Ioannidis et al., 2011).

The alteration in tissue remodeling of intestinal epithelium by high fat intake is a newly suggested and plausible cellular mechanism for intestinal diseases. Intestinal epithelium is a key player in controlling inflammation, and is considered the fastest self-renewing tissue in the body. Intestinal stem cells (ISC) help maintain the rate of self-renewal for adequate tissue homeostasis in the gut. Digested nutrients in the gut can lead to an alteration in circulating factors that stimulate the biology of ISC, which could in turn affect the pattern of tissue remodeling and renewal in the mucosal epithelium (Luo and Puigserver, 2016). A recent study has shown that animals fed a high-fat diet have a significantly higher rate of ISC proliferation, which resulted in elongation of the crypts (the lower part of the epithelium in which ISCs are found), thus predisposing the animals to intestinal cancers (Beyaz et al., 2016). Interestingly, the resulting changes in the pattern of ISCs were produced by specific fatty acids in the interventional high-fat diet. Although the findings of this study focus on the effect that dietary fat has on tumorgenicity of the gut, rather than inflammatory response, it does suggest a new mechanism of action for the intestinal handling of dietary fats, which could be applicable to the understanding of CD aetiopathogenesis.

5.1.1 Intestinal epithelium in health and Crohn's disease

The intestinal epithelium is a highly renewable tissue in our body and has an important physiological function in regulating gut homeostasis. It consists of epithelial cells that stimulate interactions between the intestinal mucosa immune response and intestinal contents, which is an important mechanism in regulating intestinal homeostasis (Turner, 2009). Generally, the intestinal lining is comprised of proliferative crypts, consisting of ISCs that are responsible for epithelial tissue renewal, and villi, which have varying differentiated enterocytes. Both, the villi and the crypts aid in the absorption of nutrients, water, and electrolytes. Contrarily, the colon epithelium consists only of crypts, where water is primarily absorbed with ions and bile salts.

There are five types of epithelial cells located in intestinal crypts: enterocytes, which are the most abundant epithelial cells with major functions in nutrient and electrolyte absorption; enteroendocrine cells, which produce hormones; goblet cells, which secrete mucus that acts as a gut barrier against luminal bacteria; tuft cells, which are thought to have secretory functions, and ISCs which are located at the base of the crypt, and are constantly self-renewing. The communication between intestinal epithelial cells, luminal microbes and gut immune cells is considered to be an important characteristic of gut homeostasis; such interactions are crucial for maintaining normal homeostasis, as well as for enhancing the protective immune response against pathogens. (Maloy and Powrie, 2011).

The inner lining of the intestines plays an important role as a barrier against luminal bacteria, its metabolites and other toxic molecules. However, an abnormal immune response to gut antigens may occur in genetically predisposed individuals, which would consequently lead to intestinal inflammation, thereby causing symptoms characteristic of CD. It is proposed that an aspect of CD pathogenesis involves the impairment of gut barrier functions and the enabling of bacterial translocation into the inner lining of the gut. In addition, the disturbed immune response associated with the disease can result in an increased rate of immune cell recruitment and an increased expression of pro-inflammatory cytokines, with reduced levels of anti-inflammatory cytokines (Manuc et al., 2016).

5.1.2 Crypt proliferation and mucosal healing

Generally, achieving mucosal healing requires a dynamic balance between the three cellular mechanisms of restoration, proliferation, and differentiation of epithelial cells in the injured gut epithelium. Dysregulation in these cellular mechanisms that affect the rate of crypt proliferation will allow the development of mutation. Several signalling pathways are involved in regulating the function of intestinal crypts; the Wnt/β-catenin signalling pathway is mainly responsible for directing crypt proliferation. Thus, an active mutation in the Wnt pathway would lead to disturbances in intestinal homeostasis and, consequently, influence the development of CRC (Van Der Flier and Clevers, 2009). However, cellular mechanisms are less clearly understood for IBD. Restoring the barrier functions and immune functions of epithelial cells are essential for achieving mucosal healing in IBD, as crypt proliferation is required for mucosal tissue repair. Interactions between the intracellular signalling pathways, stimulated by pro-inflammatory cytokines, or by growth factors, or by intrinsic proliferative signals, can result in tissue repair in IBD (Okamoto and Watanabe, 2016). Other factors, such as diet, have also been suggested to play a role in regulating mucosal healing.

5.1.3 Role of dietary lipids in crypt proliferation

The association between obesity (related to high-fat diets) and intestinal tumours has been suggested for many years. A mechanism for the effect of dietary fat on intestinal tumour development, via excessive ISC proliferation in the crypt was recently proposed (Figure 23) (Beyaz et al., 2016). In this animal study, the long-term intake of a high-fat diet led to intestinal changes, such as intestinal villi shortening and increased proliferation of ISCs within intestinal crypts. Moreover, PPARδ (a nuclear receptor transcription factor which can bind to fatty acids) was suggested to act as a signalling component, which could link the role of a high fat-diet in intestinal tumour initiation. On the other hand, epidemiological studies have identified fat intake, independent of obesity, as a risk factor for IBD disease development. Gruber et al. found that a high-fat diet can modulate gut inflammation in an animal model of CD, via enhanced gut permeability and impaired gut barrier functions, resulting in a high rate of cellular recruitment of dendritic cells, and a high rate of immune cell infiltration into the lamina propria (Gruber et al., 2013). Nevertheless, further studies are still needed to validate the precise role of dietary lipids in intestinal disease development.

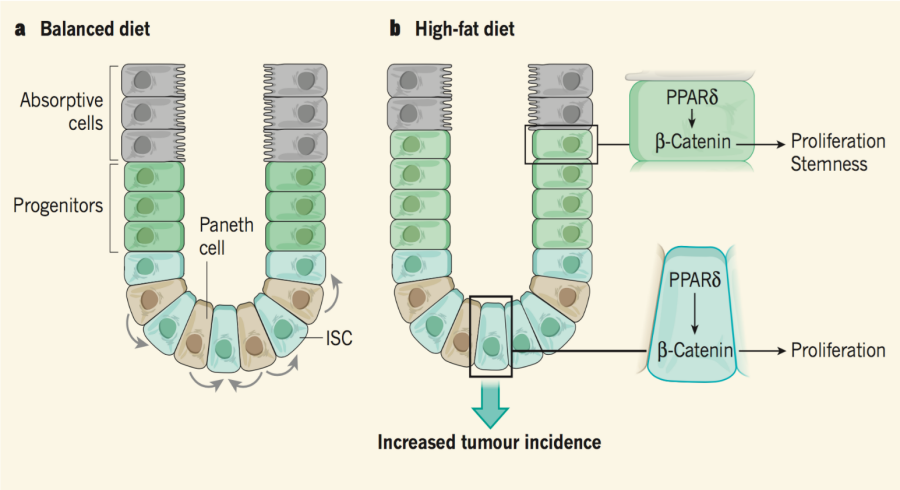


Figure 23: Dietary fats remodel the intestine.

Adopted with permission from LUO, C. & PUIGSERVER, P. 2016. Stem cells: Dietary fat promotes intestinal dysregulation. Nature, 531, 42-43 (Luo and Puigserver, 2016).

5.1.4 Research gap

The cellular mechanism behind mucosal healing in CD is not yet clear. The role of dietary factors in modulating gut epithelium function has been investigated, and a newly proposed mechanism of action suggests that dietary fat can remodel the intestinal epithelium in CRC via crypt proliferation (Beyaz et al., 2016). However, fat composition of the diet was found to regulate inflammatory cytokines in IBD studies using IBD organ culture, suggesting an anti-inflammatory effect of fish oil enriched-nutritional feeds (Meister and Ghosh, 2005). Another study found that an elemental diet administered to animals reduced the rate of crypt proliferation, while the addition of fat emulsion to the same elemental feed was found to increase absorption via the distal jejunum, suggesting that fat intake might enhance the mucosal integrity of the gut (Kawano et al., 2010). As dietary fat intake is linked to CD incidence rate, the role of fat on crypt proliferation and in controlling mucosal tissue repair in CD has been suggested to be a working model for future investigations.

5.1.5 Hypothesis

Dietary fat has an effect on the intestinal epithelial cellular function via regulating the rate of crypt proliferation, which could contribute to mucosal healing in CD. This effect will be investigated in this study using a human crypt culture model

isolated from colonic and small bowel mucosa, which will also be treated with different concentrations of lipid mixture to test their proliferative effect.

5.1.6 Objectives

The main objective of this study is to compare the effect of fatty acid treatment on the rate of colonic and small bowel epithelial crypt proliferation.

5.2 Methods

5.2.1 Human tissue collection

For details regarding ethical approval and patient eligibility for human tissue collection, please consult the Methods section in Chapter 3. For this study, mucosal tissue samples (normal colonic mucosa and normal small bowel mucosa) were collected from some of the recruited patients. For specimen collection, a 3 cm x 3 cm full thickness portion of fresh normal mucosa was collected. In CRC cases, mucosal samples were taken with more than a 10 cm tumor margin, while in IBD cases, mucosal samples were taken from non-ulcerated/non-inflamed regions as much as possible.

5.2.2 Isolation and culture of crypt from intestinal epithelium

Cultured crypts were kindly supplied by Dr. Mark Williams and Dr. Alyson Parris (School of Biological Sciences, UEA), who also performed crypt isolation.

The protocol for crypt isolation was described earlier by Williams and colleagues (Parris and Williams, 2015, Reynolds et al., 2014, Reynolds et al., 2007). During tissue collection, the mucosal sample was washed in Hepes-buffered saline (HBS; 140 mM NaCl, 5mM KCl, 10 mM HEPES, 5.5 mM D-glucose, 1 mM Na_2HPO_4 1, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$), and placed in a 25ml screw top plastic bottle (Universal) containing HBS without Ca^{+2} and Mg^{+2}, and supplemented with diaminoethanetetraacetic acid disodium salt (EDTA; 1mM), and incubated for 1 hour at room temperature. The crypts were then liberated by serial rounds of vigorous shaking, crypt sedimentation and collection. Sedimented crypts were collected and mixed in Matrigel (BD Bioscience), and a 20 µl droplet containing 50-100 crypts was placed onto a no. 0 glass coverslip (VWR), contained within a 12-well cell culture plate. After polymerization at 37^0C for 5-10 minutes, crypts were flooded with 0.5 mL of human crypt culture medium, which contained advanced F12/DMEM

supplemented with B27 and N2 (Invitrogen), n- acetylcysteine (1 mM), HEPES (10 mM), penicillin/ streptomycin (100 U/ml), L-Glutamine (2mM), Wnt-3A (100 ng/ml) (R&D systems), IGF-1 (50 ng/ml) (Sigma), Noggin (100 ng/ml) (Peprotech), RSPO-1 (500 ng/ml) (Sino Biological or R&D Systems), and the ALK 4/5/7 inhibitor, A83-01-01 (0.5 μM) (R&D systems). The human crypt culture medium was changed every two days and was modified further, depending on the stated experiments. The morphology of isolated and cultured human colonic crypt is shown in Figure 24.

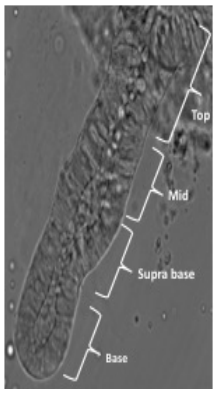


Figure 24: Morphology of isolated and cultured human colonic crypt.

5.2.3 Lipids and fatty acid treatment

On day 1-2 after isolation, cultured crypts were treated with a lipid mixture supplement (Sigma L0288), containing 10 μg/mL each of linoleic, linolenic, myristic, oleic, palmitic, and stearic acids, and only 2 μg/ml of arachidonic acid. The lipid mixture was added directly to the standard human crypt culture medium (with 100% growth factors). Only in one separate set of experiments on colonic crypts, the lipid mixture was added directly to a 20% growth factor supplemented human crypt culture medium, to help lower the high proliferation rates associated with high concentrations of growth factors. Lipid was added at 0.1% and 1% concentrations. Concentration choices were based on a previous study that used a similar lipid mixture at up to 2% concentration (Beyaz et al., 2016). The incubation time with lipids was up to 48 hours. Additionally, the effect of individual fatty acids (linoleic acid) was also tested. Pre-conjugated linoleic acid (Sigma L9530) was added to the medium, which was pre-dissolved in 10% bovine serum albumin (BSA) solution, giving a final fatty acid to

albumin molar ratio of 2:1. Linoleic acid supplement was added directly to the culture medium at 0.1% working concentration.

5.2.4 Proliferation (EdU) assay

The Click-iT 5-ethynyl-2′-deoxyuridine (EdU) Alexa Fluor cell proliferation assay kit (Invitrogen) was used in experiments assessing cell proliferation rates. Briefly, this assay uses a nucleoside analog of thymidine, called EdU, which efficiently incorporates itself into cells with newly synthesized DNA (cells in the S phase of the cell cycle), and then fluorescently labels these cells with the Alexa Fluor dye, which can be detected by using a fluorescence microscope.

After stimulating the crypt culture with selected concentrations of lipids, crypts where incubated with EdU (10 μM) for 2 hours in a cell culture incubator; this was done by replacing the spent culture medium with a fresh medium supplemented with EdU. Thereafter, crypts were immediately processed for fixation and permeabilization. Media was removed and 4% paraformaldehyde (PFA) solution was added (about 200 μl) to each cover slip containing the crypts, and incubated for 1 hour at room temperature. The fixative was then removed and the crypts were washed two times with phosphate buffered saline (PBS); this was done by aspirating away the PFA from the coverslips, and pipetting a sufficient amount of PBS to cover the Matrigel in each coverslip. To help permeabilize crypt cell membranes, ammonium chloride (NH_4Cl_2) was added for 13 minutes and 1% Sodium dodecyl sulphate (SDS) for 5 minutes, each step followed by 2-5 PBS washes. Then, a 1% Triton X-100 solution in PBS was added to the fixed crypts, and incubated for 30 minutes at room temperature, followed by two washes with 3% BSA solution in PBS.

The Click-iT reaction was subsequently induced by replacing the wash solution with a freshly prepared reaction mix, according to the kit manufacture's instructions. For a 1 ml reaction mixture, 860 μl reaction buffer, 40 μl $CuSO_4$ solution, 2.5 μl Alexa Fluor azide, and 100 μl reaction buffer additive were mixed. The Click-iT reaction cocktail was protected from light and used within 15 minutes. About 40 μl of the reaction cocktail was added to each coverslip and incubated for 35 minutes at room temperature (protected from light). The reaction mix was then removed with two 3% BSA solution washes. After this step, crypts were ready to be mounted on a glass microscope slide for florescence imaging. Crypts were mounted as follows: aspirating away all the wash solution from the previous step; lifting each coverslip from the

culture plate using a tweezer and putting it on a clean tissue paper to drain off any excess fluids; adding a drop of mounting media (Vectashield containing nuclear stain DAPI (marker for nucleus (DNA)); inverting the coverslip on a clean glass microscope slide, sealing the edges with a transparent nail varnish to help prevent mounting media leakage; finally, the slides were stored in a light-protected slide box in a fridge, before fluorescence imaging.

Depending on the experiment, immunolabeling with primary and secondary antibodies was additionally performed. This process simply involved extending the EdU proliferation assay by immunostaining the EdU-labeled crypts with primary antibodies and a suitable, species-specific Alexafluor-conjugated secondary antibody, which were raised in donkey. The primary antibodies mainly used were against ki67 and E-cadherin, which are considered to be markers for proliferation and the plasma membrane, respectively.

Briefly, after the final step in the EdU protocol (just before mounting the coverslips), serum block solution with 10% secondary antibody serum and 1% BSA was added to each coverslip for 2 hours at room temperature (protected from light) to help block non-specific binding. The blocking solution was then removed, followed by two PBS washes. The required combination of primary antibodies was diluted in PBS (1:100 dilution); these were rabbit anti-ki67 (R&D) and goat anti-E-cadherin (R&D). 50 µl of the primary antibody working solution was added to each coverslip and the culture plate was wrapped with wet tissue paper and cling film to minimize water evaporation, and stored overnight at 4ºC. The following day, the primary antibody solution was removed, followed by two washes with cold PBS. Fluorophore-conjugated secondary antibodies were prepared at half the dilution of primary antibodies (1:200); these were donkey anti-rabbit Alexa Fluor 488 (green) and donkey anti-goat Alexa Fluor 647 (red) (Thermo Fisher Scientific); 50 µl of secondary antibody working solution was added to each coverslip followed by 2 hours of incubation on ice (protected from light). The secondary antibodies solution was removed and washed once with PBS. Fresh PBS solution was added again and left for 1 hour (about 1 ml in each coverslip). The microscope slide was now prepared for fluorescence imaging, and mounted as described earlier.

Reagents used in this assay were either prepared fresh for immediate use, or were prepared earlier and aliquoted by Dr Mark Williams's team (School of Biological

Sciences, UEA). Reagents used include: PBS solution (1 tablet (Oxoid BR0014G) dissolved in 100 ml deionized water which was then autoclaved); 4% PFA solution, which was prepared weekly by dissolving 4 g of PFA powder (Sigma P6148) in 100 ml PBS solution with gentle warming for up to 4 hours till the solution was clear and stored in aliquots at -80°C); 1% TritonX (Roche) solution in PBS, which was prepared for immediate use; 100 mM Ammonium chloride NH_4Cl_2 solution (Sigma A9434) in PBS; 1% SDS solution (Melford B2008), in PBS; 10% donkey serum (sigma 9663); 1-3% BSA solution, which was prepared by dissolving 0.1-0.3 gm of BSA (Sigma A2153) in 10 ml PBS at room temperature; coverslip mounting media with nuclear counterstain Hoechst "VectashieldTM" (Vector labs H1000, H1200 or H1300); and coated microscope slides (PolysineTM VWR).

5.2.5 Epifluorescence/confocal imaging

Following EdU assay and immunolabeling treatment, slides of mounted crypts were imaged by epifluorescence microscopy (Nikon Ti) or confocal microscopy (Zeiss 510 META). Usage of the fluorescence microscopy facility was supervised by Dr. Paul Thomas (Henry Wellcome Laboratory for Cell Imaging, UEA), Dr. Alyson Parris, and Victoria Jeffery (School of Biological Sciences, UEA). For epifluorescence microscopy, a 40x objective lens was used to acquire images of crypts at the equatorial plane.

5.2.6 Data analysis

5.2.6.1 Image analysis

Acquired images of crypts were analysed using the Fiji ImageJ software. The total number of DAPI-positive nuclei was counted at the crypt equatorial plane. The number of EdU-positive cell nuclei was then counted. The percentage of EdU-positive cells was calculated from the number of DAPI-positive nuclei. Counting for colonic crypts was divided into four regions (base, supra base, mid, and top) along the crypt axis, each region containing an equal number of nuclei; the percentage of EdU-positive cells was calculated for each region. However, for small bowel crypt, counting was done for the whole crypt region. For each experiment, at least four representative crypts were analysed for each treatment group, to give the mean rate of proliferation in each case.

5.2.6.2 Statistical analysis

Prism software (Version 7.0, GraphPad Software, San Diego, CA, USA) was used to analyze the data. The statistical difference between the treatment groups was determined by using one-way analysis of variance (ANOVA) and to correct for multiple comparisons, Tukey's post-hoc test was performed. A p-value < 0.05 was considered as significant. Results were expressed as mean ± standard error of the mean (SEM) of three or more independent experiments.

5.3 Results

5.3.1 Characteristics of tissue donors

Between April 2016 and February 2017, mucosal tissue samples from small and large bowels were collected from a total of 21 patients (2 were diagnosed with Crohn's disease, 2 were diagnosed with ulcerative colitis, and 17 were colorectal cancer patients). However, crypt isolation was successful in only 12 cases, of which only 10 were investigated in this study (the other two were used for training purposes). These were all patients diagnosed with CRC, confirmed by histopathology. Two patients had right hemicolectomy, one had small bowel resection, one had panproctocolectomy, and six had anterior resections.

Successful isolation of intestinal crypts was limited due to several factors, including: 1) a limited amount of non-ulcerated mucosa in IBD cases; 2) the long durations of surgical operations, typically > 5 hours, which affected the viability of live tissues due a limited blood supply to intestinal tissues; 3) the duration of tissue transportation from the surgical theatre to the histopathology lab and to the cell culture lab for isolation, which was long enough to affect tissue viability; 4) and on three occasions, infected tissue.

5.3.2 Effect of different concentrations of lipids on colonic crypt proliferation

This sub-section reports the results of experiments investigating the effect of various lipid mixtures on colonic crypt proliferation (as measured by the percentage of EdU positive cells).

In the first set of experiments, stimulating the colonic crypts with different concentrations of lipids (in a standard human crypt culture medium with 100% growth

factors) resulted in no difference in proliferation rates in the base of the crypt; the mean rates of proliferation for the control, 0.1% lipid, and 1% lipid groups were 30.6 ± 2.35, 31.5 ± 1.94, and 28.33 ± 2.73, respectively. A similar pattern was observed in the supra base region as well. In the mid region of the crypt, lipid treatment resulted in a numerically increased proliferation rate that did not reach statistical significance. The mean proliferation rate was 20.49 ± 2.4 for the 0.1% lipid group, 18.23 ± 3.32 for 1% lipid group, and 12.52 ± 2.18 for the control ($p = 0.09$). In the uppermost region of the crypts, proliferation was only increased when crypts were treated with 1% lipid (13.42 ± 3.92) compared to control (6.15 ± 1.71), however, this was not statistically significant ($p = 0.11$) (Figure 25 A-B).

In the second set of experiments, stimulating the colonic crypts with 0.1% and 1% lipid solution (in a reduced-growth factor crypt culture medium with only 20% growth factors) resulted in a difference in proliferation rates across all crypt regions. At the base of the crypt, both concentrations of lipids resulted in a lowered percentage of proliferative cells compared to control, however this difference was not statistically significant: 21.67 ± 4.08 (0.1% lipids), 24.67 ± 4.36 (1% lipids), versus 33.57 ± 3.52 (control). In the supra base region, treatment with 0.1% lipid solution also reduced proliferation (9.44 ± 3.27) compared to control (17.25 ± 2.97) and the 1% lipid solution (22.54 ± 3.86); the difference was statistically significant after adjusting for multiple comparison (Tukey's test) between the 0.1% lipid group and 1% lipids group ($p < 0.05$). Similarly, in the mid region, 0.1% lipid treatment achieved the lowest percentage of proliferation (3.61 ± 2.2) compared to 1% lipid treatment (11.44 ± 3.82) and control (18.05 ± 3.26); the difference was statistically significant between the control and the 0.1% lipid treatment group ($p < 0.05$). In the uppermost region, treatment with 0.1% lipid only numerically reduced cell proliferation (2.68 ± 1.81) compared to the 1% lipid treatment group (5.87 ± 4.69) and control (6.48 ± 2.23) ($p = 0.65$) (Figure 26 A-B).

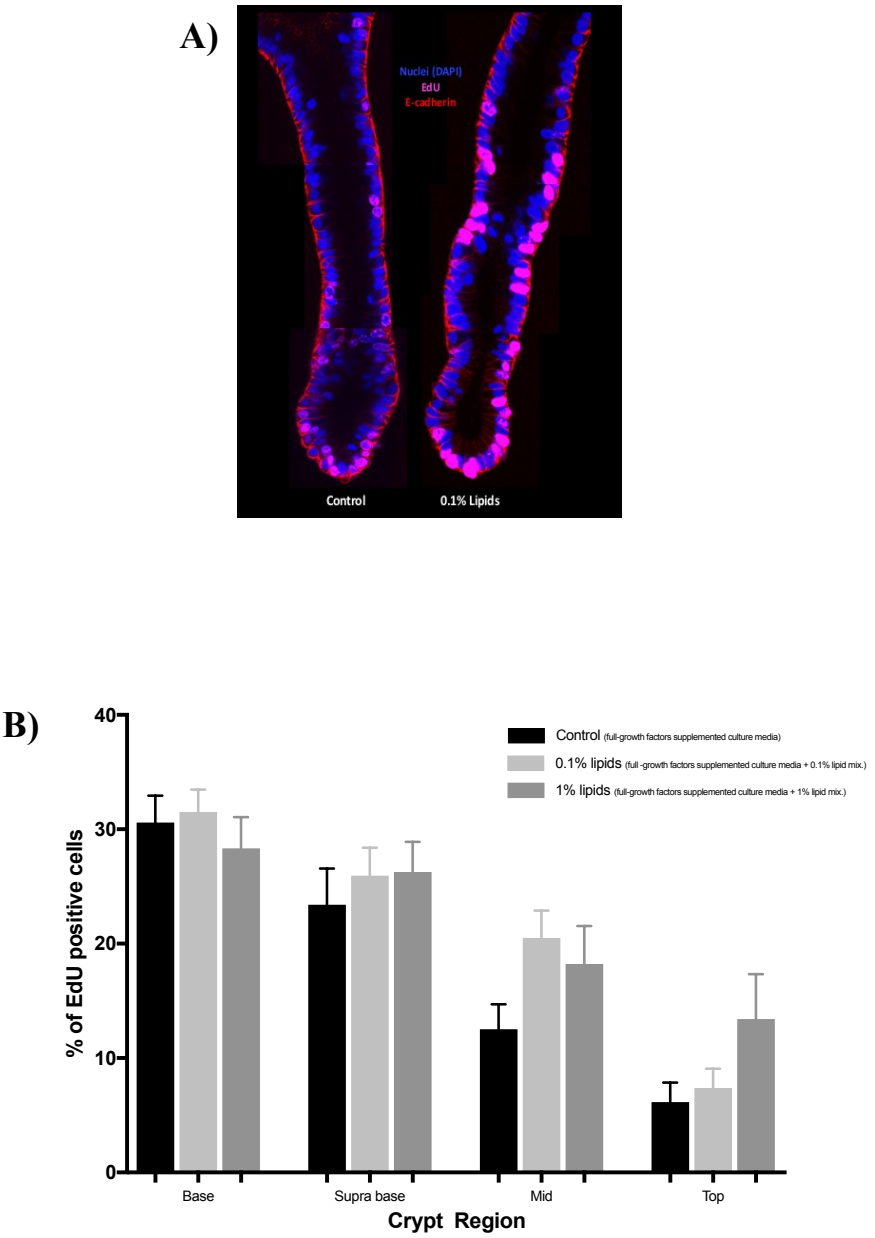


Figure 25: Effect of lipids on human colonic crypt proliferation.

A) Confocal image showing the immunolabeling for lipids stimulated colonic crypts for 48 hours (in this experiments lipids added to a standard human crypt culture medium with 100% growth factors), EdU (pink), E-cadherin (red), and blue (nuclei). **B)** Histogram showing the cell proliferation rate (expressed as mean percentage of EdU positive cells and standard error of mean (error bars)) in the different regions of colonic crypts that have been stimulated with 0.1% and 1% lipids in a standard human crypt culture medium (with 100% growth factors) for up to 48 hours (n=4 CRC). Statistical analysis was done by one-way ANOVA and to correct for multiple comparisons Tukey's post-hoc test was performed. *$p < 0.05$.

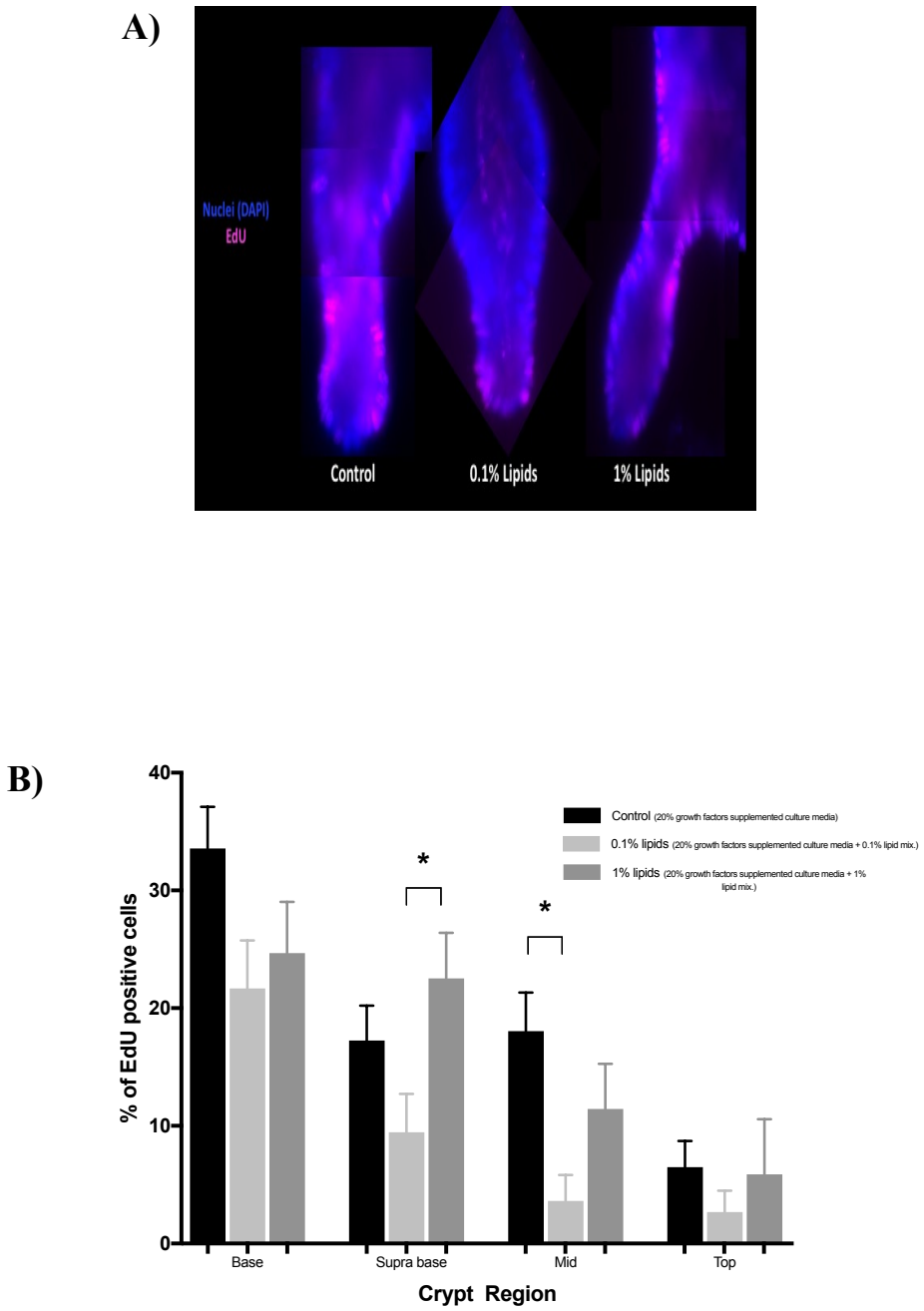


Figure 26: Effect of lipids on human colonic crypt proliferation.

A) Epifluorescence image showing EdU labeling for lipids stimulated colonic crypts for 48 hours (in this experiments lipids added to a reduced growth factors (20%) human crypt culture medium), EdU (pink) and blue (nuclei). **B)** Histogram showing the cell proliferation rate in the different regions of colonic crypts that have been stimulated with 0.1% and 1% lipids in a reduced growth factors (20%) human crypt culture medium for up to 48 hours (n=3 CRC). Data are presented as mean ± standard error of mean (error bars). Statistical analysis was done by one-way ANOVA to test the significance between the three treatment groups in each region across the crypt axis and Tukey's test was used to correct for multiple comparisons. *$p < 0.05$.

5.3.3 Effect of different lipid concentrations on small bowel crypt proliferation

The effect of lipid mixtures on small bowel crypt proliferation was also investigated in this study. The proliferation rate per crypt (quantified in the whole region of the small bowel crypt) was not significantly different between treatment groups. Compared to the proliferation rate achieved by the control group (20.37 ± 3.57), treatment with 0.1% lipid slightly reduced proliferation to 14.73 ± 1.86 compared to the control, while treatment with 1% lipid showed no difference in proliferation rate versus control (21.38 ± 3.8) (Figure 27 A-B).

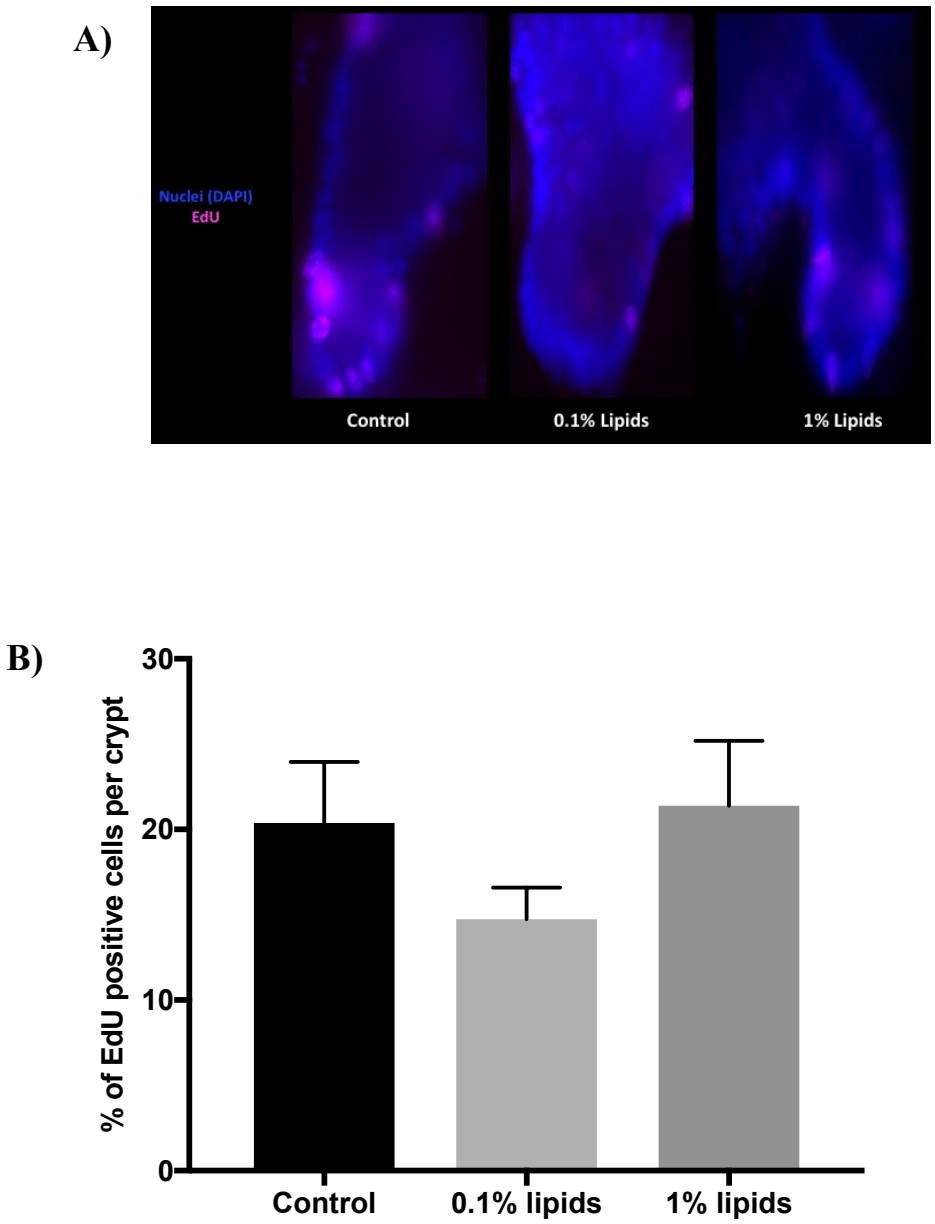


Figure 27: Effect of lipids on human small bowel crypt proliferation.

A) Epifluorescence image showing EdU labeling for lipids stimulated small bowel crypts for 48 hours (in this experiments lipids added to full growth factors human crypt culture medium), EdU (pink) and blue (nuclei). **B)** Histogram showing the cell proliferation rate (expressed as percentage of EdU positve cells) in the whole region of small bowel crypts that have been stimulated with 0.1% and 1% lipids in a standard human crypt culture medium (with 100% growth factors) for up to 48 hours (n=3 CRC). Data are represented as mean ± SD. Statistical analysis was done by one-way ANOVA to test the significance between the three treatment groups and Tukey's test was used to correct for multiple comparisons. *$p < 0.05$.

5.3.4 Effect of linoleic acid on colonic crypt proliferation

According to the results from only a single representative experiment, treatment with linoleic acid (0.1%) and a reduced concentration of growth factors (20%) for 48 hours did not show any significant difference in the number of EdU positive cells compared to the control (Figure 28).

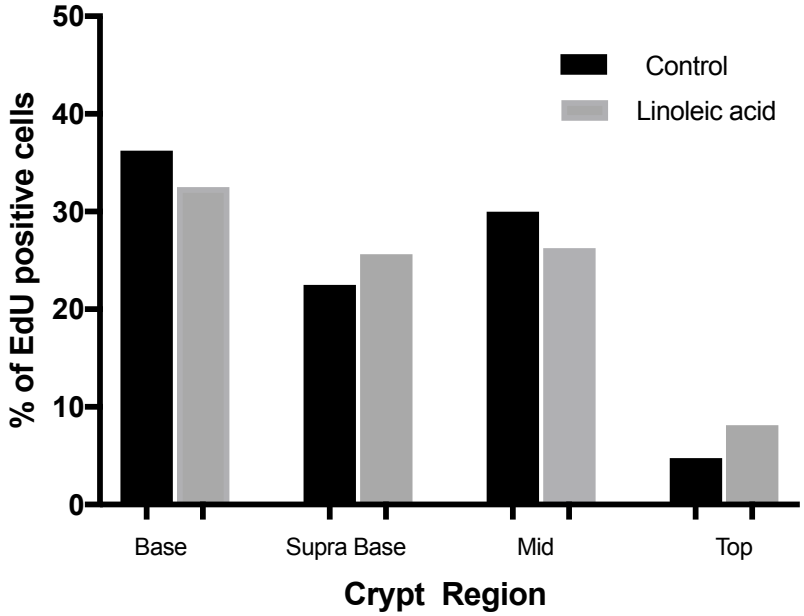


Figure 28: Effect of linoleic acid on human colonic crypt proliferation.

Histogram showing results from a single representative experiment (n=1 CRC). Bars represent the mean cell proliferation rate (expressed as percentage of EdU positve cells) in the different regions of colonic crypts that have been stimulated with linoleic acid (0.1%) in a reduced growth factors (20%) human crypt culture medium for 48 hours. Four crypts counted in each treatment group.

5.3.5 Effect of different concentration of lipids on the rate of Ki67-positive cells in colonic and small bowel crypts

A technical problem with Ki67 immunolabeling, related to high background staining, did not allow the quantification of Ki67-positive cells in culture crypts.

5.3.6 Summary of results

This study has shown that low concentrations of lipids reduced the rate of proliferation in mostly all regions within the colonic crypt, and was statistically significant only in supra base and mid regions; this is proposed to be attributed to the lower growth factor concentration used in the experiment. At higher lipid concentrations, a numerically increased rate of proliferation in the upper regions of the colonic crypt was observed. The different concentration of lipids did not significantly affect proliferation in small bowel crypts. Reduced proliferation in the small bowel crypts was only observed at low concentrations of lipid. This study was unable to find any differences in crypt proliferation rates between treatment groups after linoleic acid treatment.

5.4 Discussion

The dynamic crosstalk between intestinal epithelial cells is essential for maintaining gut homeostasis in health and disease, which balance between epithelial cell proliferation, differentiation, and apoptosis. The different subsets of intestinal epithelial cells have a potential role in controlling intestinal inflammation, which is a result of the loss of gut homeostasis between epithelial cells, the immune response, and gut microbes. In this study, we have investigated the effect of lipids on epithelial cell proliferation using the human cultured crypt model that helped in determining the epithelial cellular response to dietary fat. The main findings of this study have shown that a low concentration of lipids reduced the rate of proliferation in mostly all regions of the colonic crypt and was significant only in the supra base and mid regions; this was mainly achieved when lipids were added to a culture medium with a lower concentration of growth-factors.

The role of dietary fat in CD has long been recognized. Epidemiological studies on the western diet have identified high-fat diets as a risk factor in rising CD incidence rates (Hou et al., 2011). This indicates a potential role of low-fat diets in preventing CD. The findings of this study have shown that lower lipid concentrations significantly reduced intestinal crypt proliferation rates in CRC, but not in CD. Unfortunately, due to the very limited availability of CD mucosal tissue samples, the findings from this study were not able to identify the effect of lipid treatment on crypt proliferation in a CD context. Similar to chronic intestinal

inflammation, the development of intestinal tumors is induced by dysregulations in the cellular processes of the epithelium. CRC is induced by enhanced crypt proliferation, which forms into a cluster of irregular crypts resulting in a localized adenoma, that can consequently form into a carcinoma (Humphries and Wright, 2008). Moreover, as with CD, a high-fat western diet has also been identified as a risk factor for CRC. Our findings are relatively consistent with previous studies that indicate a negative effect of high-fat diets on increasing intestinal crypt proliferation and consequently, enhancing intestinal tumour formation (Tuominen et al., 2013, Dermadi et al., 2017, Beyaz et al., 2016).

While a low-fat diet is recommended for reducing the risk of CD development, a high-fat diet in established disease could offer a possible therapeutic potential in CD treatment. In active CD, destruction of crypts and reduced proliferation is a morphological characteristic of the disease leading to mucosal atrophy. The effect of enhanced crypt proliferation on disease pathogenesis and outcomes might differ between CRC and IBD. In CRC, increased crypt proliferation is characteristic of tumorigenesis. While increased cellular proliferation in the gut epithelium is considered a tissue renewing process, which could help in healing mucosa that were damaged due to inflammation (Shaw et al., 2012). Therefore, patients with active CD may benefit from using dietary factors that stimulate gut epithelial proliferation and turnover. The findings of this study indicate that at high concentration, lipids have numerically (but not statistically) increased the rate of proliferation in intestinal crypts; however, this was only observed in CRC and not CD samples. To our knowledge, the role of fat in enhancing mucosal integrity via cellular proliferation in CD is not yet investigated.

The importance of the type of fat in CD has also gained attention over the past years. Different types of fatty acids could modify the synthesis of inflammatory eicosanoids by altering the rate of conversion of fatty acid derivatives like EPA, DHA, and arachidonic acid. In this study, the difference in crypt proliferation rate in response to specific fatty acid treatment (i.e. *n-6* PUFA) was unable to be measured. However, the low proliferation rate observed in this study, after treatment with low concentrations of lipids in both, colonic and small bowel crypts, might suggest a protective effect of selected fatty acids at low ratios. There might be a threshold for certain fatty acids to induce their inhibitory effect on crypt proliferation. The effect of

specific fatty acids on intestinal epithelium remodelling has been investigated previously; LCT fats were found to enhance ISC function, resulting in higher rates of crypt proliferation (Beyaz et al., 2016).

A limitation of this study is the risk of contamination and infection accompanied with the use of primary human cell culture, which limited the number of successfully isolated crypt cultures, and consequently, limited the findings in this study. However, the study has used a primary human crypt culture as a model to investigate the effect of dietary fat, which is considered more representative of the human gut than using epithelial cell-lines.

Collectively, the results in the study show that reduced crypt proliferation after treatment with low concentration of lipids could be one mechanism of action of how a low-fat diet can be protective against CRC development, and possibly against CD. Nonetheless, the fatty acid(s) that promoted such protective cellular effects needs to be determined. Although, the findings of this study could not address the effect of fat on intestinal epithelial cell proliferation in CD, that might lead to inflammation, it does provide a working model for future research investigating the mechanism of action of dietary fat in CD. Further studies are required to determine the generalizability of these findings in a more diverse patient population.

6 Chapter 6: General discussion

6.1 Overview

The overall aim of this thesis was to investigate the effect of dietary fat composition on the treatment and aetiopathogenesis of CD via a systematic review and meta-analysis of RCTs, also via an observational study to characterize mesenteric adipose tissue (MAT) in human CD, and *in-vitro* studies to investigate the effect of fatty acids on the behaviour of mesenteric pre-adipocytes and on gut epithelial physiology. Novel observations have been made that show effect of dietary fat on the adipogenic potential of mesenteric pre-adipocytes and on the crypt proliferation of gut epithelium in CD.

The treatment of CD is complex and challenging. The use of enteral nutrition (EN) as primary therapy in adult patients with CD is not yet supported by high quality evidence. The current evidence is inconclusive regarding what, how, and why specific types of enteral feed perform better than others. The fat composition of EN feeds has been suggested to be a key factor in controlling inflammation in CD. As part of this research, a systematic review was initially conducted to reanalyse the findings of previous clinical trials, and to focus on evaluating the relationship between EN fat content and remission rate (RR) in CD patients. The quality of the included studies was highly variable. However, a few trends were identified between disease response rate and the percentage of long chain triglycerides (LCTs) and polyunsaturated fatty acids (PUFA) of total calories.

Moreover, a deep understanding of disease pathogenesis is also lacking. In CD, the mesentery attached to the inflamed intestines is usually thickened due to unknown reasons, a condition known as the “fat-wrapping” phenomenon. Additionally, the alteration in tissue remodeling of the intestinal epithelium due to high fat intake is a newly suggested, plausible cellular mechanism for intestinal diseases. Therefore, this research project aimed to determine the effects of lipids on the cellular and molecular environment of hypertrophied MAT and its adjacent intestinal epithelium. The results of this work form the basis of a human clinical trial investigating the effects that EN fat composition may induce in CD patients, with a focus on the alteration of the MAT. Lipids are, thus, an interesting and important dietary factor for future IBD research.

Altogether this thesis aims to contribute to our understanding of the potential mechanisms through which lipids could modulate disease treatment and pathogenesis.

6.2 Relevance of dietary fat in CD treatment

The relationship between lipids and CD has long been recognized. The current guidelines for CD state that an increased intake of *n*-3 PUFA and a reduced intake of saturated fats and *n*-6 PUFA should be recommended to reduce the risk of developing CD (Forbes et al., 2017). Fat could also modulate disease outcomes via their action on eicosanoid synthesis, which can alter the immune response. Therefore, the efficacy of nutritional therapy in CD has been considered to correlate with the content of dietary fat. The fat composition of EN feeds has gained interest over the years. In the systematic review presented in this thesis, a significant positive correlation was found between *n*-6 PUFA intake and remission rates in adult CD patients, while only a positive, non-significant trend was found between medium chain triglyceride (MCT) composition and remission rates, and a negative trend was reported for the intake of monounsaturated fatty acids (MUFA). The practice of recommending or prescribing a specific type of fat for patients with active CD remains a matter of debate and an existing challenge. The choices are many, and would consist of deciding between increasing MCT or MUFA proportions, or rebalancing the ratio of different PUFAs. The delivery of MCT in the diet is considered less inflammatory than LCT. Studies using animal models of colitis have demonstrated that a reduction in inflammatory cytokines levels occurred in animals that were fed with an enteral diet high in MCT (Papada et al., 2014). MCT can control intestinal inflammation and mucosal damage. Previous studies have suggested that the beneficial effect shown with MCT administration in CD is related to its efficient absorption in the small intestine and colon, and its reduced pro-inflammatory action (Andoh et al., 2000). For MUFA composition, although it is considered anti-inflammatory, one high quality RCT in this context found that EN feeds with a high MUFA content are less effective than a high-PUFA feed in controlling inflammation in CD patients (Gassull et al., 2002). However, a recent updated meta-analysis found no significant difference in remission rates of CD patients who were treated with enteral feeds with varying degrees of fat composition (Narula et al., 2018). Only the very low fat feeds and the very low LCT feeds showed a higher remission rate. It is remains challenging to elucidate the individual effect of each fatty acid within a given feed; it could be that the whole fatty

acid profile of the enteral feed is responsible for the immunomodulatory effect rather than a particular fatty acid itself (Gassull et al., 2002). Therefore, the inconsistency in current evidence can be better validated by future studies investigating the effect of different fatty acid profiles in the diet of CD patients.

6.3 Dietary fat has various effects on adipose tissues in CD

Hypertrophied MAT associated with CD has long been recognized as a manifestation of the disease. Over the past years, several studies were conducted to understand the pathophysiology of the fat-wrapping phenomenon and its relevance in CD. As part of my research, a study (detailed in chapter 3) was preformed to characterise MAT by investigating its inflammatory gene expression profile and fatty acid composition. An *in-vitro* study was also conducted (detailed in chapter 4) to examine the effect of fatty acid treatment on the differentiation of mesenteric pre-adipocytes.

Dietary fat can influence the pathogenesis of MAT in CD via three proposed mechanisms. First, certain fatty acids could modify the expression of inflammatory markers in adipose tissues. Secondly, dietary fat could have an impact on altering the fatty acid profile of the MAT, which is found to be abnormal in CD patients (Westcott et al., 2006). Thirdly, dietary fat can control the process of adipogenesis in MAT. The findings from chapter 3 have demonstrated a few associations between fatty acids levels and the mRNA levels of selected genes related to adipogenesis and the inflammatory response in the MAT of CD and colorectal cancer (CRC) patients, while the fatty acid profile for MAT was not significantly different between CD and control tissues. The findings reported in chapter 4 showed that unsaturated fatty acids induced a pro-adipogenic effect in mesenteric pre-adipocytes, which was mainly regulated by CEBPα. This effect was relatively more potent in CRC derived mesenteric pre-adipocytes than CD mesenteric pre-adipocytes. These findings were first to be reported in CD mesenteric pre-adipocytes. Previous studies in this field only reported the effect of fatty acids on cell-lines or on pre-adipocytes derived mainly from subcutaneous fats or from visceral fats of non-CD patients. Therefore, this study was able to use a more representative cell culture model. Overall, the combined results of this work have provided new insights for MAT characterisation, and for investigating the role of dietary fat in CD pathogenesis.

6.4 Dietary fat can modulate gut epithelium physiology: a relevant mechanism for CD

The gut epithelium forms an important platform for handling nutrients by the body. It is considered one of the most rapid, self-renewing tissue in the body, which comprises specialised intestinal stem cells (ISCs) that reside in the crypt structure of the epithelium. Yet, the role of dietary fat and other dietary factors in regulating the ISCs' function and crypt proliferation is not clear. In this thesis, it was hypothesised that intestinal epithelial cells can respond to dietary fat provision. The alteration in tissue remodelling of intestinal epithelium by dietary fat is a newly suggested plausible cellular mechanism for intestinal diseases (Beyaz et al., 2016). Our work in chapter 5 aimed primarily to study the role of dietary lipids on ISCs function in CD via assessing the rate of crypt proliferation. Earlier *in-vitro* studies in CD mainly focused on measuring pro-inflammatory markers as outcomes rather than intestinal epithelial cellular function which is an important factor contributing to CD aetiopathogenesis. In the methodology, we have used a highly sophisticated 3D human cell culture model to investigate the effect of lipids on epithelial cell proliferation (detailed in chapter 5), where an alteration in the rate of crypt proliferation after lipid treatment was observed. The model used in this study is unlike the model applied by previous studies which used cell-lines that only mimic the characteristics of intestinal cells. The crypt culture model used here is able to display all the different types of cells present in human intestine which make it a near-native culture system.

In this study, low concentration of lipids resulted in a statistically significant lower rate of colonic crypt proliferation compared to a higher concentration. Unfortunately, due to limited availability of CD mucosal tissue samples the findings could not be employed to elucidate differences in the level of crypt proliferation in response to lipid treatment in CRC versus CD tissues.

A mechanism whereby dietary fat induces intestinal tumour development via ISC proliferation in crypts was recently proposed. Recent studies using animal models have demonstrated a change in proliferation rate, where a high fat diet promoted a significantly higher rate of ISC proliferation, which resulted in elongation of the crypts, thus predisposing the animals to intestinal cancers (Beyaz et al., 2016). However, in the context of CD, although there is a well-established association

between high fat diet and increased disease risk, the mechanism of fat in controlling mucosal tissue repair in CD is yet to be identified. A high fat diet can modulate gut inflammation via enhanced gut permeability and impaired gut barrier functions, resulting in a higher rate of cellular recruitment of dendritic cells and immune cell infiltration into the lamina propria (Gruber et al., 2013). Additionally, the different types of fat in the diet can impact the physiology of gut epithelium in different ways. For example, supplementing the diet with *n*-3 PUFA, in addition to other nutrients like curcumin and pectin has been shown to reduce colonic tumorigenesis, via promotion of apoptosis in damaged ISCs, and by suppressing proliferation (Kim et al., 2016, Hong et al., 2015). Nevertheless, the current evidence still needs further validation to clarify the role of dietary nutrients in regulating gut epithelial physiology.

6.5 Other approaches to investigate EN efficacy in CD

6.5.1 A plausible effect of dietary emulsifiers on intestinal inflammation

It is also suggested that dietary agents related to fat formation in processed foods could be involved in the mechanism of action of disease pathogenesis. Emulsifiers, such as polysorbate 80, are suggested to be potential dietary agents in this context and are found in most commercial food products. Recent studies have found that dietary emulsifiers can physiologically impact the gut epithelium in mice by enhancing bacterial overgrowth and translocation and, consequently, promoting intestinal inflammation (Chassaing et al., 2015). Another study found a strong positive association between dietary emulsifiers intake and CD incidence rate in Japan (Roberts et al., 2013). Therefore, it is also possible that CD patients' response to EN therapy could be related to the emulsifier composition of the feed. The use of emulsifiers is important for the stabilization of feed lipid emulsion.

A few years ago, a group of researchers hypothesized that high intake of emulsifiers could be associated with increasing CD incidence, suggesting that patients' response to EN feeds might be affected by the emulsifiers' content, rather than the fat content of the feed (Roberts et al., 2013). Thereafter, the same group tested the *in-vitro* effect of emulsifiers on the translocation of *E coli* bacteria isolated from CD patients, and found that translocation increased significantly across Caco2-cl1 cell-line and M-cells (Roberts et al., 2010). These findings indicated a need to study the clinical effect of food emulsifiers in enteral feeds on CD outcomes.

In the systematic review presented in this thesis (detailed in Chapter 2), it was attempted to analyse the possible correlation between, both, emulsifier composition and fat composition in enteral feeds on CD outcomes. However, not all feed manufacturing companies reported the type and amount of emulsifiers, which limited the amount of data available. Where emulsifier contents were disclosed, lecithin and polysorbate 80 were found to be the main dietary emulsifiers present in the investigated enteral feeds. Future research is needed in this area to clarify the impact of emulsifiers in CD pathogenesis and treatment.

6.6 Clinical implications and future directions of research

The main objective of this research was to better understand the impact of dietary fat in CD treatment and pathogenesis. The evidence is currently inconclusive and this study helped to clarify the relevance of dietary fat composition in influencing CD clinical outcomes, pertaining to mesenteric inflammation regulation, and in modulating the gut epithelium repair mechanism. This study suggests that optimizing the fatty acid composition of enteral feeds for CD patients could promote better disease outcomes via their action on adipogenesis and MAT inflammation regulation, and on the gut epithelium repair mechanism.

In terms of understanding disease aetiopathogenesis, further research should work to better characterize MAT in CD. Characterization studies related to histological, immunological, and molecular analysis of MAT are still limited. In addition, investigating the association between disease behaviour, clinical manifestations, and mesenteric fat characteristics will help in clarifying the significance of the fat-wrapping phenomenon on CD pathogenesis. Future prospective studies based on investigating different depots of MAT from the same CD patient would help in better characterization. A key limitation of this study was the limited availability of paired inflammed and non-inflammed MAT samples from CD patients; also, the availability of surgically resected healthy mesenteric tissues was limited and varied across patients. Additionally, assessing nutritional status and food intake (via food frequency questionnaires) before MAT samples collection would have been helpful in determining the association between nutrient intake and fatty acid composition of MAT in CD patients.

The current evidence supporting the effect of individual dietary oils and fatty acids in enteral feeds for CD is still limited. Robust, randomized control trials (RCTs)

of exclusive enteral nutrition in CD are still needed. Future work in CD should focus on investigating the effect of enteral feeds with different fat composition on the physiological alteration of mesenteric fat (i.e. histological, biochemical, and inflammatory changes). Also, investigating the role of fat in modulating the gut epithelial cells in CD would help in elucidating its mechanism of action.

Based on the findings in this thesis, the next step in evaluation of future RCTs of enteral nutrition should aim to compare the effect of at least two feeds with relatively comparable amounts of total calories, carbohydrate, protein, and fat but with different fatty acid profiles. The effect of the feed will best be investigated among presurgical CD patients. The reason for this first choice of patient group is partially pragmatic because studying the mesenteric adipose tissue (MAT) directly is challenging as its collection is highly invasive and non-routine in clinical practice, surgery being the only way for researchers to gain access to it. Therefore, CD patients undergoing intestinal resection would be the only eligible candidates for such a clinical investigation. Nonetheless a pertinent RCT can be devised which would apply four arms of intervention so the intervention feed given exclusively would be compared against the standard feed (control) also given exclusively, and the same intervention feed but given as a part of a regular diet will be compared again against a control supplement to regular diet. The intervention would be given for a period of four to six weeks before surgery. Because compliance to an intervention like exclusive enteral nutrition is potentially a major issue in adult CD patients, minimizing the period of such an intervention should be considered: the duration of exclusive enteral nutrition in most of the previous trials was between 3 and 8 weeks. The primary outcome measure would be the degree of adipogenesis in MAT and its association with inflammation. This thesis suggests a couple of potential biomarkers that can be investigated in the future RCTs to study the role of fat in CD. For example, the level of adipogenic markers like CEBPα are important in showing the effect of intervention on the degree of adipogenesis in MAT in CD. Also, adipokines levels such as those of leptin in MAT and serum are considered important for studying MAT function in association with inflammation. Moreover, it will be interesting to obtain radiological quantification of mesenteric fat (acquired from CT and MRI scans) which will help in showing the degree of mesenteric fat hypertrophy before and after the intervention. Other biological markers include assessing the level of mucosal healing, transmural

healing, and mucosal tissue crypt proliferation in association with mesenteric tissue alterations, which would be useful in identifying a novel mechanism of action of enteral feeding in CD.

There are still many gaps in the knowledge on the relationships between CD phenotype, dietary fat, mesenteric adiposity, and inflammation, to determine disease risk and outcomes. Many clinical, scientific, and environmentally relevant questions need to be addressed in future research.

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Appendices

Appendix 1: PRISMA 2009 Checklist

1. Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4-5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5-6

Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6-7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6-7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	7

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8 &22
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8-7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	9
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	22-23
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	20-21
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	App.B(p.11)

Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	20-21
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12-15
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	14-15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	15

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Appendix 2: Study design and criteria for measuring remission rate

Study	Type of intervention	Trial design	Duration of intervention (weeks)	Remission rate criteria	Study geographical location
Bamba et al. (2003)[14]	Three elemental feeds with different fat levels were compared	Randomized controlled, not double-blinded (multicentre)	4	Organization of Inflammatory Bowel Disease (IOIBD) rating > 2 and at least one abnormal inflammatory marker	Japan
Gassull et al. (2002)[11]	Polymeric feed high in n-9 vs. polymeric feed high in n-6 vs. steroids	Randomized double-blinded (multi-centre)	4	Van Hees activity index (VHAI)< 120	Spain, UK, Germany
Giaffer et al. (1990)[21]	Elemental feed vs. polymeric feed	Randomized, not double-blinded	4	Control of symptoms, avoidance of complications, complete withdrawal of all medication including steroids, and CDAI<150	UK
Leiper et al. (2001)[16]	Polymeric feed low in LCT vs. polymeric feed high in LCT	Randomized controlled, double-blinded (two centres)	3	CDAI of <150	UK
Mansfield et al. (1995)[22]	Semi-elemental feed vs. elemental feed	Randomized controlled, not double-blinded	4	Reduction in CDAI of 100 points or 40% of the initial value, control of symptoms, and withdrawal of all treatment including corticosteroids.	UK
Middleton et al. (1995)[7]	Elemental feed vs. elemental feed with added MCT vs. elemental feed with added LCT vs. semi-elemental feed	Randomized, not double-blinded	3	Harvey & Bradshaw Index (HBI)<3 within 3 weeks of commencing enteral feed	UK
Park et al. (1991)[25]	Elemental feed vs. polymeric feed	Randomized controlled, double-blinded	4	Harvey-Bradshaw simple CDI<2 after 28 days of treatment	UK
Raouf et al. (1991)[30]	Elemental feed vs. polymeric feed	Randomized controlled, not double-blinded	3	Harvey-Bradshaw simple CDI<4 after 21 days of treatment	UK
Rigaud et al. (1991)[13]	Elemental feed vs. polymeric feed	Randomized controlled, not double-blinded (two centres)	4	CDAI<150 during the last 7 days	France
Royall et al. (1994)[23]	Elemental feed vs. semi-elemental feed	Randomized controlled, double-blinded	3	CDAI<150 after 21 days of treatment	Canada
Sakurai et al. (2002)[15]	Elemental low fat feed vs. semi-elemental high MCT feed	Randomized controlled, not double-blinded	6	Reduction of CDAI by at least 40% or by 100 or more after 42 days of treatment	Japan
Verma et al. (2000)[31]	Elemental feed vs. polymeric feed	Randomized, double-blinded	4	Amelioration of bowel symptoms, reduction in the CDAI to < 150 or by at least 100 points from baseline level, and normal CRP	UK
Gonzalez-Huix et al. (1993)[32]	Polymeric feed vs. steroids	Randomized controlled, not double-blinded	4	VHAI < 120 after 28 days of treatment	Spain
Lindor et al. (1992)[28]	Steroids vs. semi-elemental feed vs. both	Randomized, not double-blinded	4	Reduction in CDAI of 100 points or more of the initial value after 30 days	US
Lochs et al. (1991)[33]	Semi-elemental feed vs. drug therapy (sulfasalazine and 6-methyl-prednisolone)	Randomized, not double-blinded	6	Reduction in CDAI of 100 points or 40% of the initial value after 42 days	Austria & Germany
Malchow et al. (1990)[34]	Semi-elemental feed vs. drug therapy (sulfasalazine and 6-methyl-prednisolone)	Randomized, not double-blinded (multi-centre)	6	Reduction in CDAI of 100 points or 40% of the initial value after 42 days	Europe
Greenberg et al. (1988)[35]	TPN vs. polymeric feed vs. partial PN with oral diet	Randomized controlled, not double-blinded (multi-centre)	3	CDAI<150 after 21 days of treatment	Canada & US
Kobayashi et al. (1998)[36]	TPN vs. elemental feed vs. polymeric feed	Randomized controlled, not double blinded	4	IOIBD < 1 and normal inflammatory markers (CRP, ESR)	Japan
Mantzaris et al. (1996)[12]	Polymeric feed vs. steroids vs. both	Randomized, not double-blinded	4	Reduction in CDAI of 100 points or CDAI < 150	Greece
O'moráin et al. (1984)[37]	Elemental feed vs. steroids	Randomized controlled, not double blinded	4	Harvey-Bradshaw simple CDI	UK
Gorard et al. (1993)[38]	Elemental feed vs. steroids	Randomized, not double-blinded, multi-centre	4	Harvey-Bradshaw simple CDI	UK
Okada et al. (1990)[24]	Elemental feed vs. steroids	Controlled (not randomized), not double-blinded.	6	Harvey-Bradshaw simple CDI	Japan
Bodemar et al. (1991)[18]	Polymeric feed only	Uncontrolled trial	2	Harvey-Bradshaw simple CDI	Sweden
Coyle and Sladen (1989)[39]	Polymeric feed only	Uncontrolled trial	4	Harvey-Bradshaw simple CDI	UK
Riordan et al. (1993)[19]	Elemental feed only	Uncontrolled trial, multi-centre	2	HBI < 3	UK
Guo et al. (2013)[40]	Polymeric feed only	Uncontrolled trial	4	CDAI < 150 + CRP < 10 mg/L	China

Zoli et al. (1997)[20]	Semi-elemental feed vs. steroids	Randomized controlled, not double blinded	2	HBI	Italy
Hu et al. (2014)[17]	Semi-elemental feed only	Uncontrolled trial	12	More than 70 points reduction of CDAI score	China
Zhu et al. (2013)[41]	Polymeric feed vs. drug therapy (infliximab)	Randomized, Not double blinded, multi-centre	8	CDAI of <150	China

Appendix 3: Patients characteristics

Study	Type of enteral nutrition	Number of participants	Males%	Females%	Age (mean)	CD location			Mean of disease duration (years)
						Small bowel%	Large bowel%	Both%	
Bamba et al. (2003)[14]	Elemental, Low fat (6 packs of Elental + 6 packs of dextrin)	10	50%	50%	27	10%	10%	80%	-
	Elemental, Medium fat (6 packs of Elental + 3 packs of dextrin+ 3 packs of C-1 dextrin)	10	60%	40%	31	40%	0%	60%	-
	Elemental, High fat (6 packs of Elental + 6 packs of C-1 dextrin)	8	75%	25%	26	38%	38%	25%	-
Gassull et al. (2002)[11]	Polymeric, high in n-9 MUFA	20	45%	55%	31.1	35%	25%	40%	2.41
	Polymeric, high in n-6 PUFA	23	43%	57%	30.8	35%	17%	48%	2.05
Giaffer et al. (1990)[21]	Elemental (Vivonex)	16	25%	75%	34	50%	19%	31%	-
	Polymeric (Fortison)	14	29%	71%	42	29%	36%	36%	-
Leiper et al. (2001)[16]	Polymeric, 5% LCT	27	-	-	36.9	33%	22%	44%	-
	Polymeric, 30% LCT	27	-	-	35.6	22%	33%	44%	-
Mansfield et al. (1995)[22]	Elemental (E028)	22	50%	50%	-	32%	18%	50%	8.6
	Semi-elemental (Pepti-2000 LF liquid)	22	23%	77%	-	41%	14%	45%	8.7
Middleton et al. (1995)[7]	Elemental (E028)	17	41%	59%	34	24%	35%	41%	8.6
	Elemental (E028), High LCT	22	50%	50%	35	32%	36%	32%	4.6
	Elemental (E028), High MCT	19	26%	74%	30	37%	21%	42%	4.7
	Semi-elemental (Peptide 2+)	18	33%	67%	32	22%	22%	56%	9.1
Park et al. (1991)[25]	Elemental (E028)	7	14%	86%	33	14%	29%	57%	5.8
	Polymeric (Enteral 400)	7	0%	100%	38	14%	14%	71%	2.4
Raouf et al. (1991)[30]	Elemental (EO28)	13	-	-	-	-	-	-	-
	Polymeric (Triosorbon)	11	-	-	-	-	-	-	-
Rigaud et al. (1991)[13]	Elemental (Vivonex HN)	15	60%	40%	33	0%	67%	33%	5.9
Royall et al. (1994)[23]	Elemental (Vivonex-TEN)	19	53%	47%	31.5	26%	11%	63%	9.5
	Semi-elemental (Peptamen)	21	62%	38%	31.4	52%	10%	38%	6.8
Sakurai et al. (2002)[15]	Elemental, Low fat (Elental)	18	78%	22%	26.3	28%	17%	56%	-
	Semi-elemental, High MCT (Twinline)	18	89%	11%	25.3	28%	17%	56%	-
Verma et al. (2000)[31]	Elemental	11	36%	55%	38	18%	9%	64%	6.9
	Polymeric	10	40%	70%	33	30%	50%	30%	4.3
Gonzalez-Huix et al. (1993)[32]	Polymeric (Edanec HN)	15	47%	53%	31.1	20%	20%	60%	-
Lindor et al. (1992)[28]	Semi-elemental (Vital HN)	9	44%	56%	34.6	33%	33%	33%	3.2

Lochs et al. (1991)[33]	Semi-elemental (Peptisorb)	55	40%	60%	27.5	15%	16%	69%	-
Malchow et al. (1990)[34]	Semi-elemental (Survimed)	51	41%	59%	30.1	12%	27%	53%	4
Greenberg et al. (1988)[35]	Polymeric (Precision-Isotonic)	19	57.90%	42.10%	31.6	-	21%	-	5.5
Kobayashi et al. (1998)[36]	Elemental (Elental)	10	-	-	21.6	30%	20%	50%	2.2
	Polymeric (Clinimeal)	9	-	-	19.2	33%	22%	44%	1.8
Mantzaris et al. (1996)[12]	Polymeric (Nutrison HE)	10	-	-	-	-	-	-	-
O'moráin et al. (1984)[37]	Elemental (Vivonex)	11	82%	18%	31.9	18%	27%	55%	-
Gorard et al. (1993)[38]	Elemental (vivonex TEN)	22	55%	45%	31.6	32%	18%	50%	-
Okada et al. (1990)[24]	Elemental (elental)	10	50%	50%	21	40%	20%	40%	1.28
Bodemar et al. (1991)[18]	Polymeric (Semper lowfat)	10	70%	30%	46.6	20%	10%	70%	18
Coyle and Sladen (1989)[39]	Polymeric (Enteral 250)	12	42%	58%	38.5	33%	42%	25%	-
Riordan et al. (1993)[19]	Elemental (E028)	136	-	-	-	-	-	-	-
Guo et al. (2013)[40]	Polymeric (Nutrison Fibre)	13	69%	31%	26.1	15%	8%	77%	1.4
Zoli et al. (1997)[20]	Semi-elemental (Peptamen)	10	60%	40%	33.5	20%	0%	80%	-
Hu et al. (2014)[17]	Semi-elemental (Peptisorb liquid)	59	71%	29%	32.3	41%	17%	42%	-
Zhu et al. (2013)[41]	Polymeric (Nutrison Fibre)	52	-	-	31.4	8%	4%	69%	-

Appendix 4: Saturated fatty acids profile for enteral nutritional formulas

Study	Type of enteral nutrition	Total Saturated Fatty Acids									
		C6:0	C8:0	C10:0	C12:0 Lauric acid	C14:0	C16:0 Palmitic acid	C18:0 Stearic acids	C20:0	C22:0	C24:0
Bamba et al. (2003)[14]	Elemental, Low fat (6 packs of Elental + 6 packs of dextrin)	0	0	0	0	0	12.4	4.4	0	0	0
	Elemental, Medium fat (6 packs of Elental + 3 packs of dextrin+ 3 packs of C-1 dextrin)	0	0	0	0	0	12.4	4.4	0	0	0
	Elemental, High fat (6 packs of Elental + 6 packs of C-1 dextrin)	0	0	0	0	0	12.4	4.4	0	0	0
Gassull et al. (2002)[11]	Polymeric, high in n-9 MUFA	0	4.7	0.8	0.31	3.7	3.3	3.3	0	0	0
	Polymeric, high in n-6 PUFA	0	7	1	0	0.4	10.9	5.5	0	0	0
Giaffer et al. (1990)[21]	Elemental (Vivonex)	0	0	0	0	0	6.1	2.3	0.4	0.3	0.1
	Polymeric (Fortison)	0	0	0	0.25	0.1	5.7	3.1	0.45	0.55	0.2
Leiper et al. (2001)[16]	Polymeric, 5% LCT	0.1	51	35	0.1	0	1.5	0.5	0	0	0
	Polymeric, 30% LCT	0.1	9	6	0.2	0	34.5	4	0	0	0
Mansfield et al. (1995)[22]	Elemental (E028)	0	0	0	1	1	10	3	1	3	1
	Semi-elemental (Pepti-2000 LF liquid)	0.05	32.5	17.5	0.05	0	6.15	0.95	0.2	0.05	0.05
Middleton et al. (1995)[7]	Elemental (E028)	0	0	0	1	1	10	3	1	3	1
	Elemental (E028), High LCT	0	3	2	0	1	4	1	1	1	0
	Elemental (E028), High MCT	1	21	15	1	0	3	2	0	1	0
	Semi-elemental (Peptide 2+)	0	27.9	6.7	15	15	8.1	2.1	0	0	0
Park et al. (1991)[25]	Elemental (E028)	0	0	0	0	0.1	10.4	3	1.2	2.3	1.4
	Polymeric (Enteral 400)	0	16.3	8.75	0	0	7.9	2.3	0.9	1.7	1.1
Raouf et al. (1991)[30]	Elemental (EO28)	0	0	0	1	1	10	3	1	3	1
	Polymeric (Triosorbon)	0.1	50.7	27.3	0.2	0	1.4	1	0.1	0.2	0
Rigaud et al. (1991)[13]	Elemental (Vivonex HN)	0	0	0	0	0	6.1	2.3	0.4	0.3	0.1
Royall et al. (1994)[23]	Elemental (Vivonex-TEN)	0	0	0	0	0	6.1	2.3	0.4	0.3	0.1
	Semi-elemental (Peptamen)	0.07	45.5	24.5	0.22	0	1.92	1.35	0.09	0.24	0.06
Sakurai et al. (2002)[15]	Elemental, Low fat (Elental)	0	0	0	0	0.1	10.8	3.9	0.3	0.2	0.3
	Semi-elemental, High MCT (Twinline)	0.07	46.8	25.2	0.07	0	1.7	0.64	0.11	0.08	0
Verma et al. (2000)[31]	Elemental	-	-	-	-	-	-	-	-	-	-
	Polymeric	-	-	-	-	-	-	-	-	-	-
Gonzalez-Huix et al. (1993)[32]	Polymeric (Edanec HN)	-	-	-	13	-	28	-	-	-	-
Lindor et al. (1992)[28]	Semi-elemental (Vital HN)	0.05	29.25	15.75	0.35	0.05	3.52	2.48	0.17	0.5	0.1
Lochs et al. (1991)[33]	Semi-elemental (Peptisorb)	0.5	32.5	17.5	0.05	0.05	5.4	1.95	0.15	0.1	0.15
Malchow et al. (1990)[34]	Semi-elemental (Survimed)	0	0	0	0.5	0.1	6.4	4.5	0.3	0.8	0.2
Greenberg et al. (1988)[35]	Polymeric (Precision-Isotonic)	0	0	0	0	0.1	10.8	3.9	0.3	0.2	0.3
Kobayashi et al. (1998)[36]	Elemental (Elental)	0	0	0	0	0.1	10.8	3.9	0.3	0.2	0.3

	Polymeric (Clinimeal)	0	3.8	3.25	24.1	9.25	10.5	2.3	0.25	0.05	0.05
Mantzaris et al. (1996)[12]	Polymeric (Nutrison HE)	0	0.66	0.57	4.04	1.99	26.51	3.11	0.33	0.1	0.1
O'moráin et al. (1984)[37]	Elemental (Vivonex)	0	0	0	0	0	6.1	2.3	0.4	0.3	0.1
Gorard et al. (1993)[38]	Elemental (vivonex TEN)	0	0	0	0	0	6.1	2.3	0.4	0.3	0.1
Okada et al. (1990)[24]	Elemental (elental)	0	0	0	0	0.1	10.8	3.9	0.3	0.2	0.3
Bodemar et al. (1991)[18]	Polymeric (Semper lowfat)	0	0	0	0	0.1	10.8	3.9	0.3	0.2	0.3
Coyle and Sladen (1989)[39]	Polymeric (Enteral 250)	0	0	0	0	0	12.3	1.9	0.4	0.1	0.1
Riordan et al. (1993)[19]	Elemental (E028)	0	0	0	1	1	10	3	1	3	1
Guo et al. (2013)[40]	Polymeric (Nutrison Fibre)	-	-	-	-	-	-	-	-	-	-
Zoli et al. (1997)[20]	Semi-elemental (Peptamen)	0.07	45.5	24.5	0.22	0	1.92	1.35	0.09	0.24	0.06
Hu et al. (2014)[17]	Semi-elemental (Peptisorb liquid)	0.5	30.5	16.45	0.05	0.05	5.7	2.07	0.16	0.1	0.16
Zhu et al. (2013)[41]	Polymeric (Nutrison Fibre)	-	-	-	-	-	-	-	-	-	-

Appendix 5: Monounsaturated fatty acids profile for enteral nutritional formulas

Study	Type of enteral nutrition	Total Monounsaturated Fatty acids								
		C16:1 n-7	C16:1 n-9	C17:1 n-7	C18:1 n-9 Oleic acid	C18:1 n-7	C20:1 n-9	C20:1 n-7	C22:1 n-9	C24:1 n-9
Bamba et al. (2003)[14]	Elemental, Low fat (6 packs of Elental + 6 packs of dextrin)	0	0	0	23.9	0	0	0	0	0
	Elemental, Medium fat (6 packs of Elental + 3 packs of dextrin+ 3 packs of C-1 dextrin)	0	0	0	23.9	0	0	0	0	0
	Elemental, High fat (6 packs of Elental + 6 packs of C-1 dextrin)	0	0	0	23.9	0	0	0	0	0
Gassull et al. (2002)[11]	Polymeric, high in n-9 MUFA	0	0	0	79	0	0	0	0	0
	Polymeric, high in n-6 PUFA	0	0	0	28.2	0	0	0	0	0
Giaffer et al. (1990)[21]	Elemental (Vivonex)	0.1	0	0	13.4	0	0.2	0	0	0.2
	Polymeric (Fortison)	0.15	0	0	41.05	0	0.8	0	0.25	0.15
Leiper et al. (2001)[16]	Polymeric, 5% LCT	0	0	0	3.4	0	0	0	0	0
	Polymeric, 30% LCT	0	0	0	35.5	0	0	0	0	0
Mansfield et al. (1995)[22]	Elemental (E028)	0	0	0	58	0	1	0	0	0
	Semi-elemental (Pepti-2000 LF liquid)	0.05	0	0	13.85	0	0.15	0	0	0
Middleton et al. (1995)[7]	Elemental (E028)	0	0	0	58	0	1	0	0	0
	Elemental (E028), High LCT	0	0	0	66	0	1	0	1	0
	Elemental (E028), High MCT	0	0	0	42	0	0	0	0	0
	Semi-elemental (Peptide 2+)	0	0	0	11.7	0	0	0	0	0
Park et al. (1991)[25]	Elemental (E028)	0.2	0	0.1	47.9	0	1.3	0	0.1	0
	Polymeric (Enteral 400)	0.15	0	0.08	36.4	0	0.98	0	0.08	0
Raouf et al. (1991)[30]	Elemental (EO28)	0	0	0	58	0	1	0	0	0
	Polymeric (Triosorbon)	0	0	0	4.9	0	0	0	0	0
Rigaud et al. (1991)[13]	Elemental (Vivonex HN)	0.1	0	0	13.4	0	0.2	0	0	0.2
Royall et al. (1994)[23]	Elemental (Vivonex-TEN)	0.1	0	0	13.4	0	0.2	0	0	0.2
	Semi-elemental (Peptamen)	0	0	0	6.63	0	0.06	0	0	0
Sakurai et al. (2002)[15]	Elemental, Low fat (Elental)	0.2	0	0	23.9	0	0.1	0	0	0
	Semi-elemental, High MCT (Twinline)	0	0	0	3.75	0	0.05	0	0	0.05
Verma et al. (2000)[31]	Elemental	0	0	0	45	0	0	0	0	0
	Polymeric	0	0	0	45	0	0	0	0	0
Gonzalez-Huix et al. (1993)[32]	Polymeric (Edanec HN)	0	0	0	41	0	0	0	0	0
Lindor et al. (1992)[28]	Semi-elemental (Vital HN)	0.06	0	0	12.16	0	0.1	0	0.06	0
Lochs et al. (1991)[33]	Semi-elemental (Peptisorb)	0.1	0	0	11.95	0	0.05	0	0	0
Malchow et al. (1990)[34]	Semi-elemental (Survimed)	0.1	0	0	22.1	0	0.2	0	0.1	0
Greenberg et al. (1988)[35]	Polymeric (Precision-Isotonic)	0.2	0	0	23.9	0	0.1	0	0	0
Kobayashi et al. (1998)[36]	Elemental (Elental)	0.2	0	0	23.9	0	0.1	0	0	0

	Polymeric (Clinimeal)	0.05	0	0	16.85	0	0.2	0	0	0
Mantzaris et al. (1996)[12]	Polymeric (Nutrison HE)	0.14	0	0	31.2	0	0.2	0	0	0
O'moráin et al. (1984)[37]	Elemental (Vivonex)	0.1	0	0	13.4	0	0.2	0	0	0.2
Gorard et al. (1993)[38]	Elemental (vivonex TEN)	0.1	0	0	13.4	0	0.2	0	0	0.2
Okada et al. (1990)[24]	Elemental (elental)	0.2	0	0	23.9	0	0.1	0	0	0
Bodemar et al. (1991)[18]	Polymeric (Semper lowfat)	0.2	0	0	23.9	0	0.1	0	0	0
Coyle and Sladen (1989)[39]	Polymeric (Enteral 250)	0.1	0	0	27.7	0	0.3	0	0	0
Riordan et al. (1993)[19]	Elemental (E028)	0	0	0	58	0	1	0	0	0
Guo et al. (2013)[40]	Polymeric (Nutrison Fibre)	0	0	0	56.4	0	0	0	0	0
Zoli et al. (1997)[20]	Semi-elemental (Peptamen)	0	0	0	6.63	0	0.06	0	0	0
Hu et al. (2014)[17]	Semi-elemental (Peptisorb liquid)	0.1	0	0	12.7	0	0.05	0	0	0
Zhu et al. (2013)[41]	Polymeric (Nutrison Fibre)	0	0	0	56.4	0	0	0	0	0

Appendix 6: Polyunsaturated fatty acids for enteral nutritional formulas

Study	Type of enteral nutrition	Total Polyunsaturated Fatty acids									
		C18:2 n-6 Linoleic acid	C18:3 n-3 Linolenic acid	C18:3 n-6	C18:4 n-3	C20:2 n-6	C20:3 n-6	C20:4 n-6 Arachidonic acid	C20:5 n-3	C22:2 n-6	C22:4 n-6
Bamba et al. (2003)[14]	Elemental, Low fat (6 packs of Elental + 6 packs of dextrin)	51.6	7.7	0	0	0	0	0	0	0	0
	Elemental, Medium fat (6 packs of Elental + 3 packs of dextrin+ 3 packs of C-1 dextrin)	51.6	7.7	0	0	0	0	0	0	0	0
	Elemental, High fat (6 packs of Elental + 6 packs of C-1 dextrin)	51.6	7.7	0	0	0	0	0	0	0	0
Gassull et al. (2002)[11]	Polymeric, high in n-9 MUFA	6.5	1.5	0	0	0	0	0	0	0	0
	Polymeric, high in n-6 PUFA	45	1.6	0	0	0	0	0	0	0	0
Giaffer et al. (1990)[21]	Elemental (Vivonex)	76	0.3	0	0	0	0	0.5	0.5	0	0
	Polymeric (Fortison)	43.55	5.2	0	0	0.05	0	0	0	0	0
Leiper et al. (2001)[16]	Polymeric, 5% LCT	7.4	1	0	0	0	0	0	0	0	0
	Polymeric, 30% LCT	9.5	1.2	0	0	0	0	0	0	0	0
Mansfield et al. (1995)[22]	Elemental (E028)	20	1	0	0	0	0	0	0	0	0
	Semi-elemental (Pepti-2000 LF liquid)	28.05	0.5	0	0	0	0	0	0	0	0
Middleton et al. (1995)[7]	Elemental (E028)	20	1	0	0	0	0	0	0	0	0
	Elemental (E028), High LCT	17	4.5	0	0	0	0	0	0	0	0
	Elemental (E028), High MCT	11	3	0	0	0	0	0	0	0	0
	Semi-elemental (Peptide 2+)	22.5	0.4	0	0	0	0	0	0	0	0
Park et al. (1991)[25]	Elemental (E028)	30.2	0.4	0	0	0	0	0	0	0	0
	Polymeric (Enteral 400)	22.9	0.3	0	0	0	0	0	0	0	0
Raouf et al. (1991)[30]	Elemental (EO28)	20	1	0	0	0	0	0	0	0	0
	Polymeric (Triosorbon)	14.4	0.1	0	0	0	0	0	0	0	0
Rigaud et al. (1991)[13]	Elemental (Vivonex HN)	76	0.3	0	0	0	0	0.5	0.5	0	0

Royall et al. (1994)[23]	Elemental (Vivonex-TEN)	76	0.3	0	0	0	0	0.5	0.5	0	0
	Semi-elemental (Peptamen)	19.68	0.15	0	0	0	0	0	0	0	0
Sakurai et al. (2002)[15]	Elemental, Low fat (Elental)	52.1	7.8	0	0	0	0	0	0	0	0
	Semi-elemental, High MCT (Twinline)	21.28	0.08	0	0	0	0	0.14	0.14	0	0
Verma et al. (2000)[31]	Elemental	12	-	-	-	-	-	-	-	-	-
	Polymeric	12	-	-	-	-	-	-	-	-	-
Gonzalez-Huix et al. (1993)[32]	Polymeric (Edanec HN)	-	-	-	-	-	-	-	-	-	-
Lindor et al. (1992)[28]	Semi-elemental (Vital HN)	36.08	0.3	0	0	0	0	0	0	0	0
Lochs et al. (1991)[33]	Semi-elemental (Peptisorb)	26.05	3.9	0	0	0	0	0	0	0	0
Malchow et al. (1990)[34]	Semi-elemental (Survimed)	65.6	0.5	0	0	0	0	0	0	0	0
Greenberg et al. (1988)[35]	Polymeric (Precision-Isotonic)	52.1	7.8	0	0	0	0	0	0	0	0
Kobayashi et al. (1998)[36]	Elemental (Elental)	52.1	7.8	0	0	0	0	0	0	0	0
	Polymeric (Clinimeal)	28.95	0.55	0	0	0	0	0	0	0	0
Mantzaris et al. (1996)[12]	Polymeric (Nutrison HE)	30.63	0.61	0	0	0	0	0	0	0	0
O'moráin et al. (1984)[37]	Elemental (Vivonex)	76	0.3	0	0	0	0	0.5	0.5	0	0
Gorard et al. (1993)[38]	Elemental (vivonex TEN)	76	0.3	0	0	0	0	0.5	0.5	0	0
Okada et al. (1990)[24]	Elemental (elental)	52.1	7.8	0	0	0	0	0	0	0	0
Bodemar et al. (1991)[18]	Polymeric (Semper lowfat)	52.1	7.8	0	0	0	0	0	0	0	0
Coyle and Sladen (1989)[39]	Polymeric (Enteral 250)	56.1	1	0	0	0	0	0	0	0	0
Riordan et al. (1993)[19]	Elemental (E028)	20	1	0	0	0	0	0	0	0	0
Guo et al. (2013)[40]	Polymeric (Nutrison Fibre)	-	-	-	-	-	-	-	-	-	-
Zoli et al. (1997)[20]	Semi-elemental (Peptamen)	19.68	0.15	0	0	0	0	0	0	0	0
Hu et al. (2014)[17]	Semi-elemental (Peptisorb liquid)	27.6	4.1	0	0	0	0	0	0	0	0
Zhu et al. (2013)[41]	Polymeric (Nutrison Fibre)	-	-	-	-	-	-	-	-	-	-

Appendix 7: Downs and Black quality assessment checklist for included trials

Reference	Reporting (11)	External validity (3)	Internal validity (bias) (7)	Internal validity (confounding-selection bias) (6)	Power (1)*	Overall score (28)
Bamba et al. (2003)[14]	10	1	5	4	0	20
Gassull et al. (2002)[11]	11	2	7	5	1	26
Giaffer et al. (1990)[21]	9	2	5	5	0	21
Leiper et al. (2001)[16]	10	1	5	4	1	21
Mansfield et al. (1995)[22]	9	1	5	5	1	21
Middleton et al. (1995)[7]	9	2	3	3	0	17
Park et al. (1991)[25]	9	1	6	3	0	19
Raouf et al. (1991)[30]	10	1	3	2	0	16
Rigaud et al. (1991)[13]	9	1	5	3	0	18
Royall et al. (1994)[23]	11	1	6	3	0	21
Sakurai et al. (2002)[15]	9	1	5	3	0	18
Verma et al. (2000)[31]	10	2	7	5	0	24
Gonzalez-Huix et al. (1993)[32]	11	2	5	4	1	23
Lindor et al. (1992)[28]	10	1	4	4	0	19
Lochs et al. (1991)[33]	10	2	4	2	1	19
Malchow et al. (1990)[34]	10	1	4	3	1	19
Greenberg et al. (1988)[35]	10	1	5	5	0	21
Kobayashi et al. (1998)[36]	9	1	4	4	0	18
Mantzaris et al. (1996)[12]	4	1	3	2	0	10
O'moráin et al. (1984)[37]	9	1	4	2	0	16

Gorard et al. (1993)[38]	9	1	4	3	0	17
Okada et al. (1990)[24]	9	1	4	3	0	17
Bodemar et al. (1991)[18]	9	1	5	2	0	17
Coyle and Sladen (1989)[39]	9	1	4	2	0	16
Riordan et al. (1993)[19]	9	2	4	3	0	18
Guo et al. (2013)[40]	10	2	5	4	0	21
Zoli et al. (1997)[20]	8	2	4	4	0	18
Hu et al. (2014)[17]	10	1	5	3	0	19
Zhu et al. (2013)[41]	10	2	5	4	0	21

Appendix 8: The Norwich Biorepository information sheet and consent form for patients

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

The Norwich Biorepository

The donation, collection, storage and use of samples of tissue and/or fluids and/or other material from an adult for research

Information sheet for patients - Version 15 (21 February 2014)

Thank you for considering giving a sample for biomedical research. This information sheet provides a brief summary to help you to understand what this means and involves.

There is a consent form after the information sheet. It is important that you complete and sign it, if you decide to give us a sample. Please complete all parts of the consent form.

Doctors and other health professionals often take samples (which may be blood, small biopsies, or something else) from patients to help tell us what is wrong and how best to treat it. Larger pieces of tissue or whole organs may be removed by surgeons from some patients as part of their treatment.

Quite often, some of the test sample or surgical tissue is left over at the end. The left-over sample can be used by other doctors or researchers in special experiments to learn more about illness, how it happens and how to treat it, and sometimes to help make new medicines.

We would like to do some experiments on any left-over sample or surgical tissue, once your tests are finished. To do that, we need your permission and signed consent.

Samples donated (given) to the Norwich Biorepository are not:

- Normally used in animal research. It will be made clear to you if animal research is an integral part of the project for which we are seeking a donation.
- Used in cloning experiments. However, the Biorepository would consider the use of donations in non-reproductive cloning experiments based on their scientific value and in the context of prevailing law and ethical standards. It will be made clear to you if cloning experiments are part of the research project for which we are seeking a donation.

If you want to help us, please sign the form at the end of this document.

Continued.....

If you give permission for a sample to be taken –

- The Hospital will own the sample.
- The sample may be stored, usually in a deep freezer, until it is used. The freezer is referred to as a tissue bank in the consent form.
- Nobody involved in the research will know where the sample has come from.
- The sample will be used only in experiments that are ethical and to help other people. Please see the section entitled 'Scientific and ethical approval' below to understand what we mean by *ethical*.
- Your donated sample(s) and any genetic material derived from it (them) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing. *Whole genome sequencing* means reading your total DNA code (your genetic blueprint) in a single assessment.
- We might give some of or all the sample to other doctors or researchers for their experiments, if they are ethical and to help other people. Some of these people might work in companies in this country or abroad.
- Data derived from your sample(s) may be placed anonymously in an international database to be used in future research. While we will take all possible steps to maintain your anonymity and protect your privacy, there is a very small risk that genetic information produced in the research and stored on databases could lead to your identification by being linked to other stored information.
- We will keep some facts about you on our Biorepository database.
- Although these facts might be given to the research doctors or scientists to help their experiments, we will NOT tell them your name or other details that would let them know who you are.
- Doctors in the Hospital might also read your hospital records to help them understand what the doctors or scientists find out in the experiments. This is possible because your hospital records can be linked to the anonymous research sample without loss of confidentiality as far as the researchers are concerned. If the research results are important for you, it might be possible using this linkage to feed back the information to your doctor, so that any appropriate action can be considered.

Your treatment will not be affected in any way, if you do not feel able to say yes to our request for a research sample.

The next sections give you some more detailed information. If you have any questions, please do not hesitate to ask the person who is seeking your permission.

WHAT WILL HAPPEN

Tissue, blood or other samples taken from you for diagnosis and/or treatment of your condition will be sent to the Pathology Laboratories, where they will be tested to decide exactly what they are and whether any further treatment will be necessary. This is a standard part of treatment. **Only as much tissue or fluid as is needed will be removed.**

Continued……

MEDICAL RESEARCH AND WHY THIS PROGRAMME IS IMPORTANT

When all the routine tests have been done, if any samples are left over, with your consent, they could be donated (given) for use in medical research. As part of a research programme which now includes the Norfolk and Norwich University Hospitals NHS Foundation Trust, the James Paget University Hospitals NHS Foundation Trust, the University of East Anglia (UEA), and the Institute of Food Research (IFR), some of the sample or material extracted from it will be stored in the Norwich Biorepository (usually in a special deep freezer) for use by ourselves or by researchers from other centres at a later date. Some of this research may involve an assessment of genetic material (DNA and/or RNA) to help us understand the genetic basis of health and disease.

The purpose of this research is to understand more about human health and disease, and to develop new methods of prevention or new treatments for the benefit of future patient care. Some of these research programmes could lead to the development of new products and processes, which may be developed commercially for the improvement of patient care, in which case there would be no financial benefit to you.

Medically qualified doctors or other suitably qualified staff at the hospital may need to review your hospital records, including case notes, as part of this research in order to relate the research findings to the clinical outcome. It is important to be able see how you progress after the tissue or other samples you have donated (given) have been used in the research project(s). They will not give your name to those doing the research.

The research may also involve training doctors and researchers in scientific medicine, and may lead to higher qualifications for them (e.g., PhD or MD degrees). This is important for future research into diseases and for looking for new, more effective, treatments for them.

LINKS WITH OTHER ORGANISATIONS

If you agree, we may send stored material or products derived from it to other approved tissue banks or companies in this country or abroad to support their research programmes or the research programmes of those companies' clients. Such outside organisations will provide financial support for the Norwich Biorepository (our tissue bank), to help it recover its operating costs. We are not, however, allowed to sell tissue or other samples in order to make any financial profit from these commercial links.

SCIENTIFIC AND ETHICAL APPROVAL

The Norwich Biorepository acts as a custodian of the samples it holds. It releases them only to individuals or organisations that have an acceptable scientific background and work to high ethical standards. We require that all such medical research has been approved by a properly constituted Research Governance Committee before it starts. It must also be approved by a Research Ethics Committee or on behalf of the Research Ethics Committee that oversees the work of the Norwich Biorepository under the terms of the Biorepository's own Research Ethics Committee approval. That committee is the Cambridge East Research Ethics Committee. These committees look particularly at the purpose and validity of the research proposal, the welfare of any participants and issues of consent and confidentiality. We will release samples to commercial companies only if they work to appropriate ethical and scientific standards.

Continued.....

DONATING EXTRA SAMPLES FOR RESEARCH

In certain circumstances you may be asked by the doctor treating you (or by a doctor, research nurse or nurse practitioner working with him/her) to consider donating (giving) tissue or other samples in addition to those to be removed as part of your diagnostic investigation or treatment. **Such extra samples will be taken only if you give your consent and if their removal does not cause you any harmful effects now or in the future.**

YOUR RIGHTS

If your samples are stored, information about your case will be kept on a computer in the Norwich Biorepository. This will help us understand what your illness was like and relate what we find in experiments to what happens to patients. Under the Data Protection Act, you are entitled to ask to see what is recorded about you by applying to the Chairman of the Norwich Biorepository Committee, Norfolk & Norwich University Hospital, c/o Dept. of Cellular Pathology, The Cotman Centre, Colney Lane, Norwich, NR4 7UB. No one other than you has the right to see these records and any information needed for research purposes will be made anonymous before it is given to the researcher.

The researchers will not be able to find out your name or any personal details about you from the information that they receive.

You will have the opportunity to discuss with a doctor issues relating to the possible use of your samples for research purposes. He or she will answer any questions you may have.

MAKING A DONATION (GIFT) OF TISSUE AND/OR OTHER MATERIAL FOR RESEARCH

If you decide that you want your tissue, etc., to be stored in the Norwich Biorepository and used for research purposes, you will be asked to sign a special Consent Form confirming your decision and stating that you have read and understood this sheet. When you sign the form you will give the ownership of the tissue or other samples to the Norfolk & Norwich University Hospitals NHS Foundation Trust. The tissue or other samples will then belong to the Trust, which will store it for an indefinite period of time and will able to decide how it should be used for research. It will also have the right to dispose of unused stored material in an appropriate legal and ethical manner following normal procedures.

If you do not want your tissue to be stored in the Norwich Biorepository, please tell us and do NOT sign the special Consent Form.

If you do not sign this form, the tissue or other samples will still be sent to the laboratory to undergo those tests that are necessary for your care but they will not be used for research purposes. All unused tissue from your procedure will be disposed of using normal hospital methods. We will respect your decision and it will not affect in any way the treatment you receive.

Continued……

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I agree (Please initial small box) that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List samples for research:	☐

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust") ☐

The Trust may store these samples in a tissue bank / biorepository ☐

The Trust may use these samples at its discretion in properly approved research programmes ☐

The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes

Yes ☐
No ☐

My genetic material and donated sample(s) may be stored for an indefinite amount of time for future research projects, which may include whole genome sequencing ☐

Information about my case may be kept on the Norwich Biorepository database ☐

Anonymous data derived from my sample(s) may be placed in an international database for future research ☐

Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings ☐

I agree that appropriately qualified staff employed by the Trust may review my hospital Medical records, including case notes, as appropriate, for the purposes of research using the donated samples ☐

These samples may be used in ethically approved animal research

Yes ☐
No ☐

These samples may be used in ethically approved cloning research

Yes ☐
No ☐

Continued.....

Affix an addressograph label here or complete the following details:

Patient's name.....

Date of birth.....

Hospital no. .....

The Norwich Biorepository

Consent for the collection, storage and release of human samples for research

I confirm that:

1) **I have read and understand the Information Sheet for Patients, Version 15, dated 21 February 2014**
2) **The issues have been explained to me, and that I have had the opportunity to ask questions.**

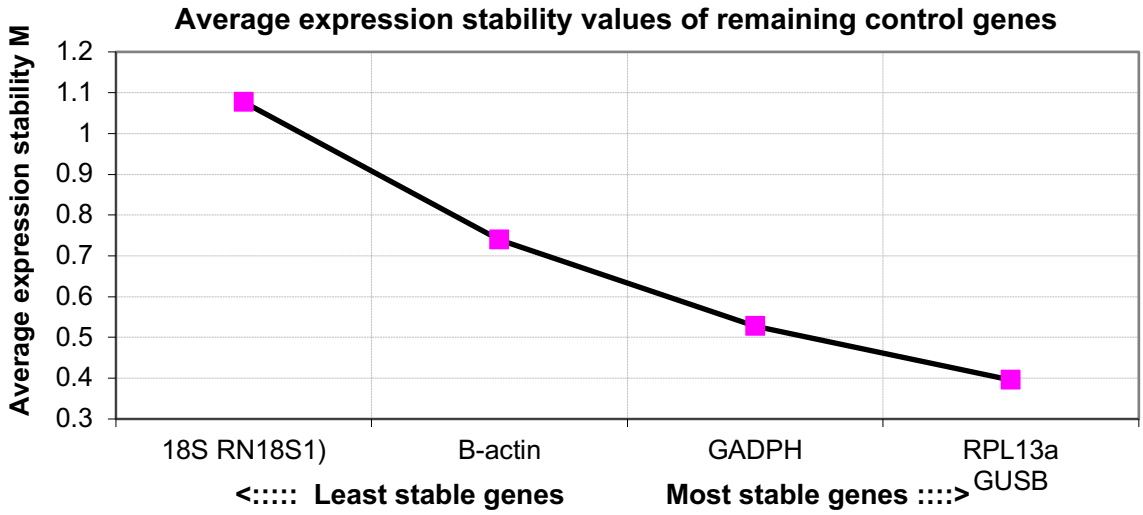
Signed _____ (Patient) Date _____

I have explained the request for tissue for research purposes and have answered such questions as the patient has asked.

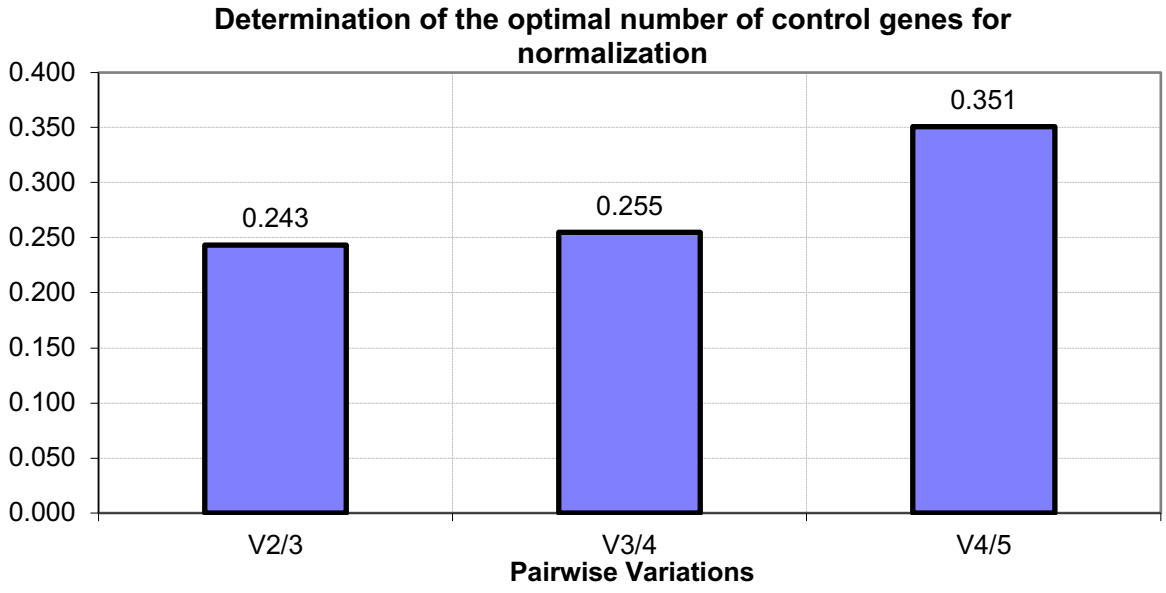
Signed_____ Print name _____
Medical / Nursing Practitioner

Date _____

Appendix 9: qRT-PCR data normalization



GeNorm M graph presenting the average expression stability of the housekeeping candidate genes. The geNorm M graph shows the ranking of the reference candidate genes according to their stability, expressed in geNorm M values, from most unstable genes at the left (high M value) to the best reference genes at the right (low M value). The threshold value for M is 0.5, with values ≤ 0.5 indicating a high expression stability. Three from the five screened housekeeping genes (18S rRNA gene (18S), B-actin, and glyceraldehyde-3phosphate dehydrogenase (GAPDH)) had M values higher than 0.5, indicating poor expression stability. Whereas RPL13a and GUSB had M values lower than 0.5 indicating high expression stability.



GeNorm V graph indicating the optimal number of housekeeping genes.