Gut Microbial Taxonomy and Metabolism in Paediatric Crohn's Disease during Exclusive and Maintenance Enteral Nutrition using OMICS Technologies.

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Author's declaration

I declare that the original work presented in this thesis is the work of the author, Clare Martha Clark. I have been responsible for the organisation, recruitment, sample collection, laboratory work, statistical analysis and data processing of the whole research, unless otherwise stated.

Clare M. Clark

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Publications

Published papers

- Logan, M., Clark, C.M., Ijaz, U.Z., Gervais, L., Duncan, H., Garrick, V., Curtis, L., Buchanan, E., Cardigan, T., Armstrong, L. and Delahunty, C., 2019. The reduction of faecal calprotectin during exclusive enteral nutrition is lost rapidly after food re-introduction. *Alimentary pharmacology & therapeutics*. 50(6), pp. 644-674 DOI: 10.1111/apt.15425
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- Clark, C.M., Kountouri, A., MacKinder, M., Hansen, R., Russell, R.K. and Gerasimidis, K., 2016, March. Intestinal fatty acid binding protein as a biomarker of intestinal damage in children with coeliac and Crohn's disease. *In Journal of Crohn's & Colitis* (Vol. 10, pp. S129-S129). Great Clarendon St, Oxford OX2 6DP, England: Oxford Univ Press.

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Glossary

25OHD	25-hydroxyvitamin D
C2	acetate
C3	propionate
C4	butyrate
C5	valerate
C6	hexanoate
C7	heptanoate
C8	octanoate
AIEC	adherent-invasive E. coli
ANOVA	analysis of variance
ALT	alanine transaminase
AST	aspartate transaminase
AUC	area under the curve
AZA	Azathioprine- immunosuppressant
BCFA	branched chain fatty acids
BMI	body mass index
CLR	Centered Log Ratio normalisation
CI	confidence interval
CRP	C-reactive protein
DADA2	divisive amplicon de-noising algorithm2
EAR	estimated average requirement
ECCO	European Crohn's & Colitis Organisation
ELISA	enzyme-linked immunosorbant assay
EEN	exclusive enteral nutrition
ESR	erythrocyte sedimentation rate
FFQ	food frequency questionnaire
GWAS	genome-wide association study
GPCR	G-protein coupled receptors
GC-FID	gas chromatography-flame ionisation detector
GC-MS	gas chromatography-mass spectrometry
HIF-1	hypoxia-inducible factor 1
Ht	height
HPLC	high performance liquid chromatography
IL	interleukin
IBD	inflammatory bowel disease
IU	International Units
IQR	interquartile range
LPS	lipopolysaccharide
MCH	mean corpuscular haemoglobin
MCV	mean corpuscular volume
MEN	maintenance enteral nutrition
NFkB	nuclear factor kappa B
NOD2	nucleotide-binding oligomerization domain-containing protein 2
PBMC	peripheral blood mononuclear cells
PUFA	polyunsaturated fatty acids
pKa	-log10 Ka (acid dissociation constant)
qPCR	quantitative polymerase chain reaction
rpm	revolutions per minute
rRNA	ribosomal RNA
SCFA	short chain fatty acid
SDS	standard deviation score
SIMD	Scottish index of multiple deprivation
SNV	single-nucleotide variant
Sp1	transcription factor Sp1
STAT3	signal transducer and activator of transcription 3
TGGE	temperature gradient gel electrophoresis
Th17	T-helper 17 cells
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cell
TSS	Total Sum Scaling normalisation
UC	ulcerative colitis
Wt	weight
wPCDAI	weighted paediatric Crohn's disease activity index

Note for pdf users: Anchor link connections allow the user to click on contents, as well as references to figures, tables, citations and appendices as a direct route to these sections. The methods references in chapters have anchor links which take the reader to the relevant sections in Methods chapter 2.

Abstract

Objectives Treatment with exclusive enteral nutrition (EEN) is an effective therapy, successfully helping children with Crohn's disease achieve remission without the need for corticosteroids. The use of maintenance enteral nutrition (MEN) following induction of remission using EEN, is thought to help maintain clinical remission. Although the efficacy of EEN is well established, the mechanism remains unknown. There is now strong evidence to support an aetiopathogenesis in Crohn's disease which implicates an interaction between environmental factors and the indigenous gut microbiota in genetically susceptible individuals.

The aim of this prospective observational study was to test the hypothesis that clinical response to EEN and reduction of colonic inflammatory markers are associated with a characteristic bacterial taxonomy (composition) and short/medium chain fatty acids (C2-C8) (functionality); and that maintenance of these profiles with MEN, while returning to habitual diet, reduces the risk of relapse. The idea that gut bacteria can be altered using diet, is an important area of research in paediatric Crohn's disease, hence this study also aimed to link the gut microbiota with dietary intake.

Methods Thirty-four children with Crohn's disease; 10 with ulcerative colitis (UC); 11 patients without inflammatory bowel disease (non-IBD); and 25 healthy controls were asked to provide faecal samples. Children with Crohn's disease also provided samples at 4-weeks, and 8-weeks of treatment with EEN; and then at 2-weeks and 8-weeks of MEN. Dietary intake was estimated using a food frequency questionnaire (FFQ), at week-0 and at 2-weeks and 8-weeks post-EEN. Post-EEN, children were treated with either 20% MEN, an immunosuppressant, or both. Bacterial DNA was extracted using the chaotropic method followed by amplification of the 16s rRNA gene (V4) for Illumina MiSeq sequencing. SCFA extraction was carried out with diethyl ether followed by gas chromatography.

Results ***Baseline:*** Children with Crohn's disease had retarded growth and weight gain compared with healthy children ($p<0.01$). BMI z-scores correlated with inflammatory markers: albumin (rho=0.611; $p<0.001$), CRP (rho=-0.536; $p<0.001$) and ESR (rho=-0.407; $p<0.03$), showing disease severity led to an increased risk of poor growth outcomes. Faecal calprotectin was higher in children with Crohn's disease and UC than non-IBD patients and healthy children ($p<0.001$). Multi-dimensional scaling using euclidean distance of white blood cell counts; ALT/AST; CRP; ESR and albumin, shows children with Crohn's disease have a blood marker profile which has 20% variance from children with UC and non-IBD conditions (R2=0.197; $p<0.001$).

Patients with Crohn's disease and UC had reduced microbiota diversity compared with healthy children ($p<0.001$). Many gram-positive commensal bacteria, includ-

ing butyrate producing species of Firmicutes (particularly Clostridiales) were decreased; while gram-negative potential pathobionts including Gamma-proteobacteria; *Fusobacterium* and *Veillonella* increased in children with Crohn's disease compared with healthy children. *Akkermansia*, a genus associated with healthy gut mucosa, was also reduced in Crohn's disease patients (mean $\log_2$ difference $p<0.05$).

The faecal short chain fatty acid valerate ($p=0.02$) and medium chain fatty acids, hexanoate and octanoate ($p<0.001$), were reduced in children with Crohn's disease compared with healthy children. The profile of short/medium chain fatty acids in children with Crohn's disease differed from healthy children ($p=0.01$). Reduced hexanoate was associated with reductions in a number of Firmicutes in children with Crohn's disease ($p<0.05$). Estimated dietary intake suggested children with Crohn's disease also had reduced fibre intake, particularly fruit, along with reduced intake of vitamins (A, E, B_7 and C) ($p<0.05$).

EEN: Of 32 children who took EEN, 23 (72%) went into remission (wPCDAI <12.5). Nine (28%) failed to respond and went on to corticosteroids. No differences were seen for baseline faecal calprotectin or blood markers, between children who responded to EEN and those who failed EEN. In children who responded to 8-weeks of EEN calprotectin, ESR and CRP were reduced ($p<0.001$, $p=0.002$ and $p=0.02$ respectively); mirrored by an increase in albumin ($p<0.001$). Blood inflammatory marker profiles after treatment with EEN were similar to non-IBD controls ($R2=0.225$; $p<0.001$).

Although 25 bacterial species discriminated responders from non-responders at baseline, there was no pattern of taxonomic relatedness between these. Successful treatment with EEN changed the microbiota community structure further from that of healthy children ($R2=0.070$; $p<0.001$). During EEN gram-negative bacteria including Pasteurellaceae, Bacteroidales S24-7, Fusobacteriaceae and Veillonellaceae (Negativicutes) were reduced, while increases mostly came from gram-positive Clostridiales ($p<0.05$). During EEN short/medium chain fatty acid profiles moved towards that of healthy children ($R2=108$; $p=0.002$), while the concentration of short/medium chain fatty acids decreased, particularly butyrate ($p=0.001$). Neither energy intake nor composition of diet at baseline predicted response to EEN.

MEN: Of the 23 children who responded to EEN, 17 (74%) were treated with MEN post-EEN. Although children responding to treatment had age appropriate growth during EEN ($p=0.056$), growth velocity was not maintained once children went back onto normal diet ($p=0.96$). No differences in growth were seen between types of maintenance therapy. The use of MEN was not associated with an increase in length of remission, however the number of children in the non-MEN group was too small ($n=6$) to reject the null hypothesis.

Faecal calprotectin returned to near pre-treatment levels, having a significant increase after only 8-weeks post-EEN ($p=0.004$), showing MEN failed to maintain the reduc-

tion of inflammation achieved during EEN. Bacterial changes during EEN, were also not maintained post-EEN. Patients using MEN, had increases in gram-negative bacteria (Veillonellaceae, Enterobacteriaceae, Bacteroidaceae, Prevotellaceae and Verrucomicrobiaceae). Within 2-weeks post-EEN, short chain fatty acids including butyrate returned towards pre-treatment levels ($p<0.001$). The use of MEN did not appear to prevent the ratio of short/medium chain fatty acids returning to pre-treatment levels. One year after the start of treatment with EEN, only 8/23 (35%) original respondents had maintained remission.

Conclusions Reduced fibre intake in children with Crohn's disease appears to lead to reduced diversity of Firmicutes, particularly Clostridiales. This reduction in commensal bacteria opens a niche for gram-negative bacteria like the Enterobacteriaceae. These changes could lead to decreased short/medium chain fatty acids, valerate, hexanoate and octanoate, which provide antimicrobial protection against gram-negative bacteria; again opening a niche for Enterobacteriaceae and other gram-negative pathobionts. Decreased intake of fibre may trigger some bacteria to switch from fermenting fibre to feeding on mucin glycans, consequently damaging the protective layer of the gut mucosa. It is thus possible that low fibre intake, under certain conditions, drives increased gut inflammation. Malabsorption, of vitamins A, C, D and E, which have immunoregulatory roles, could further exacerbate inflammation. As faecal calprotectin levels rise this limits bacterial access to zinc, manganese and iron, thus further depleting Clostridiales and increasing dysbiosis. Therefore a chronic escalation of calprotectin, could contribute in part to increased inflammation.

This study does not support the hypothesis that 20% MEN can extend remission times in children with Crohn's disease. The fact that 74% of children went onto MEN post-EEN, but only 35% remained in disease remission after 1-year, suggests that either MEN is dose dependent or that exclusion of the normal diet leads to reduced inflammation. Altering normal diet in order to induce and maintain remission in children with Crohn's disease is an attractive option, for both patients and treatment centres. Further studies are needed to explore whether increased fibre or vitamin intake can protect against inflammation and relapse in Crohn's disease. Studies also need to examine the role calprotectin plays in modifying the gut microbiota.

1 Introduction

The purpose of this introductory chapter is to provide a framework for the original research presented in following chapters. Included are practical background descriptions of the topics being researched. Detailed descriptions of selected methods can be found in chapter 2 and the references cited herein, or in appendices where appropriate. The means by which the material presented in chapters 3-7 are interlinked is discussed in chapter 8, and how these relate to our ability to address and answer questions about the role the gut microbiota, bacterial metabolites and diet might play in terms of risk and disease activity in inflammatory bowel disease (IBD) and specifically Crohn's disease.

As in all animals the human gut is home to millions of symbiotic micro-organisms including bacteria, called the microbiota. The genomic information of all the microbiota that live in a particular organ or tissue such as the gut is collectively known as the microbiome. The gut microbiota are not silent, they interact and communicate with each other and the host to provide crucial and beneficial functions for the human host, including stimulating immune development, digestion of food, provision of essential vitamins and nutrients as well as protection against infectious agents like pathogenic bacteria and viruses.

However, the homoeostasis of this mutualistic symbiosis between the microbiota and its human host can become out of balance, potentially leading to disease. This 'dysbiosis' is associated with a number of inflammatory diseases including IBD. There are many factors that are potential modulators of the microbiota including our own genome, diet, medication, environmental exposure to pathogens and other bacteria, hence research into the microbiome is complex. To understand it, we must explore interactions between diet, the microbiome and other functional systems in the body. By understanding the relationship between the microbiota as a whole and how microbial changes impact on the health of the host, it might become possible to create new personalised and targeted strategies to treat conditions like IBD.

1.1 Overview of inflammatory bowel disease

IBD is an inflammatory condition affecting the human gut and refers to two related but distinct conditions, ulcerative colitis (UC), and Crohn's disease (Fig. 1.1). The key differences are firstly the location of inflammation and secondly the extent of the inflammation. UC is restricted to the colon while Crohn's disease can affect any part of the digestive system from the mouth to the anus. In UC the inflammation only affects the inner lining of the gut (mucosa) while in Crohn's disease the inflammation can extend through the entire thickness of the intestinal wall to the outer most wall of the digestive tract. These differences lead to different outcomes and treatments. A

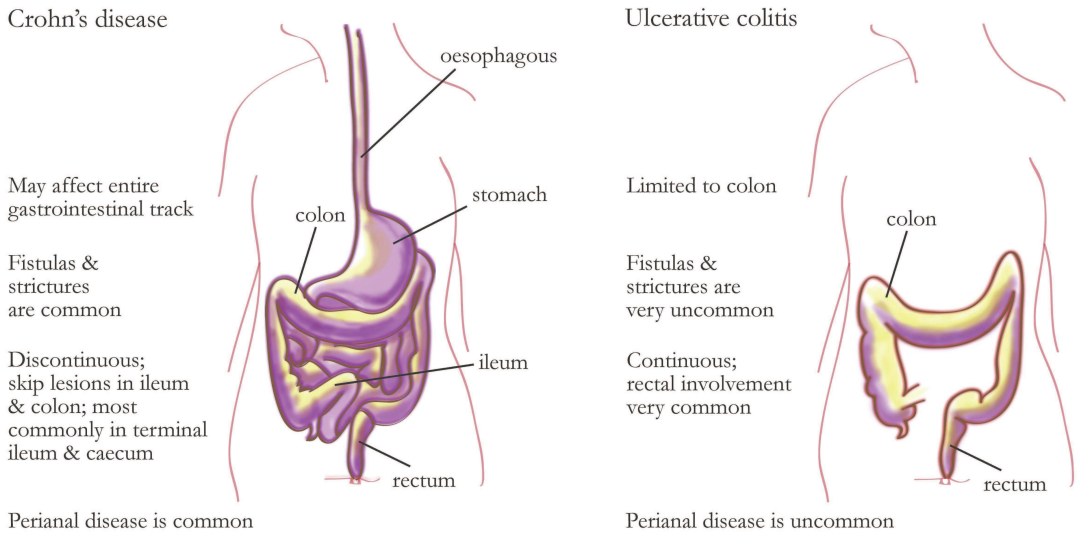


Figure 1.1: Differences between Crohn's disease and UC

small number of children with chronic inflammatory colitis cannot be classified into either of the two sub-types of IBD after endoscopic and histological assessment and are characterised as IBD unspecified (IBD-U).[2]

The major symptoms of UC and Crohn's disease overlap (Table 1.1). These include abdominal pain, changes in bowel habits, with additional symptoms such as weight loss, decreased appetite, fever and fatigue. Symptoms can vary a great deal between people and over periods of time, with patients having times when symptoms are severe (flare-ups) and times with few or no symptoms (remission). Inflammation can also affect the eyes (uvitis), skin, liver and joints.[3] Both UC and Crohn's disease can also lead to growth delay in children. IBD is more commonly diagnosed in young people aged 15-25 and there is an increased risk of IBD in those who have a family member with the condition. The disease tends to be more severe in children with IBD but there is no notable difference in the distribution of disease types between children and adults.[4] Defining the exact epidemiology of IBD can be challenging due to the insidious nature of the condition, often marked by delays in disease diagnosis as well as occasional incorrect diagnosis of patients with early stage disease. The small number of children presenting with IBD can also hinder the speed at which the correct diagnosis is reached.

1.2 Crohn's disease

Crohn's disease is a clinical classification of IBD, often presenting in childhood. It is a chronic condition which relapses and remits throughout the patient's life, having a substantial impact on the child and their families quality of life, as well as a significant cost to NHS resources.[5]

Table 1.1: Typical symptoms in children with IBD

Diarrhoea, with or without blood and mucus
Severe cramping abdominal pain, often before passing a stool
Weight loss due to loss of appetite and malabsorption
Delayed growth and development
Tiredness and fatigue
Anaemia, due to poor dietary intake and blood loss
Mouth ulcers
Potential complications, especially in Crohn's disease:
- strictures (scarring of tissue create narrowing of bowel)
- fistulas (channel connecting internal organs or to epidermis)

Because of variability in the severity and location of Crohn's disease, as well as its transmural nature, patients can have a wide range of presentations. In addition to diarrhoea, children usually present with abdominal pain, particularly before a bowel movement; and around 40% of patients can show a range of extra-intestinal manifestations affecting the function of a wide range of body systems.[6] Systemic symptoms including fatigue, anorexia, and weight loss can also be present in more severe cases. Although less common, children with Crohn's disease may initially present with stricturing or perforating complications.[7] Around 10% of children have perianal disease,[8] possibly a distinct phenotype of Crohn's disease linked with a susceptibility locus on chromosome 5.[9] A delay in puberty often complicates the clinical course of young people with IBD, particularly in those with Crohn's disease and more often in boys.[10] A 1994 study of children with Crohn's disease showed that in 73% (n=11) of girls menarche occurred 2-years later than girls with UC and healthy girls (NS).[11] Another study of fifty-five children with IBD found a mean delay of puberty of 0.70 years (SD 1.14), while a more robust US study found that in 104 children with Crohn's disease puberty was delayed by 1.5-years ($p<0.01$) compared with 233 healthy children.[12]

Timely diagnosis in children is very important, since missing school due to symptoms like pain, diarrhoea and fatigue as well as possible isolation from peers, exacerbated by a prolonged diagnostic work-up, can have a long-term psychosocial and physical impact on children.[13]

1.3 Classification of disease location and phenotypes of Crohn's disease

Crohn's disease is typically characterised by inflammation of the gastrointestinal tract which is non-continuous unlike inflammation in UC which is continuous and restricted to the colon and rectum. Treatment strategies for paediatric Crohn's disease are dependent on disease location and disease extent, however there is discordance between endoscopic (macroscopic) and histological (microscopic) disease extent, which can

make classification of disease location challenging, and inaccurate in terms of research where microscopic evaluation might be unavailable.[14] In order to properly identify disease location it is normally recommended that the whole gastrointestinal tract is examined by upper endoscopy, ileocolonoscopy, and ileal imaging.[15] A number of studies, have suggested that disease location can be an indicator of treatment outcomes in Crohn's disease.

Disease classification as well as being a tool for clinical practice, also has a role to play in research. In IBD research studies an increasing number of bio-banks are being created to allow the sharing of molecular data linked with phenotypic clinical data. Therefore high quality disease classification systems will become an important feature of clinical data used in multi-centre prospective research studies. Previous classification methods such as the "Rome classification"[16] used disease location (stomach/duodenum, jejunum, ileum, colon, rectum, anal-perianal); disease behaviour (primarily inflammatory, primarily fistulising, primarily fibrostenotic) as well as extent of disease (localised, diffuse) and surgical history (primary, recurrent). This method could theoretically result in as many as 756-subgroups of Crohn's disease. It had also been shown that for some aspects of the classification, particularly disease location and behaviour, there was inter-observer bias creating the potential for non-comparable results between patients and over-time.[17] Mucosal healing based on histology is often referred to as the 'true' measure of disease remission, where earlier clinical classification systems were based exclusively on endoscopic and radiological disease extent. In 2000, the Vienna classification (Table 1.2) was created to try and simplify the classification of the clinical phenotypes of Crohn's disease.[18] Following on from this in 2003 a group of investigators sought to resolve some of the issues around creating a Crohn's disease sub-classification that took into account the clinical, molecular, and serological markers of IBD. The results were presented at the Montreal World Congress of Gastroenterology in 2005 where they tried to address issues that affect both the needs of clinicians and the needs of scientific researchers in the field.[1] In 2011 Levine *et al.*[19] devised the Paris classification, containing further sub-categories based on histology which also took into account growth abnormalities in children. However, the small number of patients with Crohn's disease limit the use of the Paris disease classification sub-sets for research, as it can reduce the power of data sets in small scale studies such as the current study.

Isolated upper disease Using upper gastrointestinal endoscopy in paediatric patients as well as the growing use of magnetic resonance imaging enterography and computed tomography enterography have increased awareness of how common upper disease is among children with Crohn's disease. Upper involvement has been reported as anywhere between 30%-80% in children and adults.[20]

Table 1.2: Vienna, Montreal and Paris classification for Crohn's disease

	Vienna	**Montreal**	**Paris**
Age at diagnosis	A1 <40 yrs	A1 <17 yrs	A1a: 0–<10 yrs A1b: 10–<17 yrs
	A2 >40 yrs	A2 17-40 yrs	A2 17-40 yrs
		A3 >40 yrs	A3 >40 yrs
Location	L1 ileal	L1 terminal ileal ±limited caecal disease	L1 terminal ileal ±limited caecal disease
	L2 colonic	L2 colonic	L2 colonic
	L3 ileocolonic	L3 ileocolonic	L3 ileocolonic
	L4 upper	L4 isolated upper disease*	L4a upper disease proximal to ligament of Treitz* L4b upper disease proximal to ligament of Treitz and proximal to distal 1/3 ileum*
Behaviour	B1 non-stricturing, non-penetrating	B1 non-stricturing, non-penetrating	B1 non-stricturing, non-penetrating
	B2 stricturing	B2 stricturing	B2 stricturing
	B3 penetrating	B3 penetrating	B3 penetrating B2B3 both penetrating and stricturing disease, either at same or different times
		p perianal disease modifier†	p perianal disease modifier†
Growth	n/a	n/a	G_0 no growth delay G_1 growth delay

*In the Montreal and Paris classification L4 and L4a/L4b can coexist with L1-L3.
† is added to B1-B3 when concomitant perianal disease is present. Taken from Satsangi *et al.* 2006 [1] and Levine *et al.* 2011. [19]

The L4 category of the Montreal classification does not distinguish between ileal disease, and oesophageal or gastric involvement. A large 2011 study concluded that patients with jejunal disease were at significantly greater risk of stricturing disease and surgeries than either oesophageal, gastric, duodenal or ileal (without proximal) disease, thus they suggested this category be further split in the new Paris classification (Table 1.2). The study also found that adult patients with upper (L4) disease were less likely to have concurrent colonic (L2) disease (12% vs. 21%; p<0.001). [21] Very few patients have isolated upper (L4) disease without concurrent ileal/colonic disease (L1-L3) making it difficult to include this group of patients in studies.

Ileal, ileocolonic and colonic disease The terminal ileum, ileocaecal valve, and caecum are the most common sites involved at disease presentation. Disease location is restricted to the ileum (ileal) in approximately 40%-50% of patients, while 30%-40% involve both the ileum and colon (ileocolonic). The remaining cases are isolated to the colon (colonic). [2] The age of disease onset has been recorded as a factor in disease location, with very early age onset usually being isolated colitis, while ileal disease is found more often in children who are diagnosed after age nine. [22;23]

A retrospective study which evaluated pathology of Crohn's patients that underwent

surgery for either stricturing or fistulas, found no specific pathology differentiated patients with stricturing from fistulising disease. However, in terms of histology they did find that ileocolonic (L3) localisation was significantly more common in non-perineal fistulising disease than in patients with strictures.[24] Another 2016 study recorded from 120 adults patients found that Crohn's disease evolved, progressing to more aggressive stricturing and penetrating phenotypes over a 5-year period. Interestingly disease location remained relatively stable over time, with 93% of patients showing no change in disease location. The study reported that ileal involvement, stricturing, presence of fistulas and perineal lesions were predictive of surgery and immunosuppressant or immunomodulatory treatment.[25] With respect to treatment with exclusive enteral nutrition (EEN), a meta-analysis of ten trials was unable to link disease location and phenotype with efficacy of treatment, or time to relapse,[26] despite limited evidence suggesting that patients with ileal involvement respond better to EEN.[27;28]

Extra-intestinal features Extra-intestinal features in Crohn's disease can include the eyes, mouth, skin, joints, liver, bile ducts and anus. Oral lesions, anal fissures, abscesses, ulceration around stomas and anal skin tags being common features of Crohn's disease.[29] In a 2015 European inception cohort, patients with Crohn's disease were found to be twice as likely as patients with UC to experience extra-intestinal symptoms.[30]

Orofacial granulomatosis (OFG) is seen as swelling of the tissues around the mouth due to granulomatous inflammation.[31] It is often referred to as cheilitis (inflammation of the lip). The swelling can cause midline fissuring of the lip called median cheilitis or produce sores in the corners of the mouth called angular cheilitis. OFG is associated with Crohn's disease sometimes preceding gastrointestinal symptoms by a number of years;[32] however OFG might also be the only obvious symptom of underlying Crohn's disease. OFG can also be associated with oral ulcers, gingival inflammation and cobblestone appearance of the buccal mucosa, the histological features of which are indistinguishable from Crohn's disease and systemic sarcoidosis.[33] In 2007 Freysdottir *et al.*[34] showed in oral biopsies from ten patients with OFG that the Th1 immune response resembled that seen in gut biopsies from patients with Crohn's disease. Thus there is no clear way to determine when OFG is a stand-alone condition and when it is an oral manifestation of Crohn's disease.

Studies of perforating perianal disease report an incidence of around 8-15% in children with a diagnosis of Crohn's disease.[4] Perianal disease can also be a strong predictor of developing a more complicated pathology in Crohn's disease and in children can be debilitating, either preceding or following intestinal inflammation.[35] Perianal abnormalities can include lesions on perianal skin or the anal canal, as well as abscesses and fistulas. This perianal component of Crohn's disease can be asymptomatic in

some children but for others it can be a serious source of disability and distress.[36;37]

Paediatric Crohn's disease The extent of disease in adult Crohn's tends to remain more stable, unlike that found among children, where a significant and rapid change in both disease location and behaviour was recorded over time in a large cohort of 276 Scottish paediatric patients.[38] The same study found that disease location was more dynamic in childhood disease onset, such that within 2-years of diagnosis, disease progressed to involve additional sites in 39% of patients that did not have maximal ileocolonic (L3) and upper (L4) involvement. It was also noted that disease behaviour decreased in non-stricturing, non-penetrating disease (B1) from 91% to 83% by the second year and 76% by the fourth year after diagnosis. Another study involving 404 children with childhood-onset Crohn's disease found that complicated disease behaviour (stricturing B2, and penetrating B3) doubled during a 7-year follow-up from 29% at diagnosis to 59% plateauing after ∼9-years.[39]

1.4 Understanding chronic inflammation in Crohn's disease

It is hypothesised that IBD occurs in genetically susceptible people after exposure to unknown environmental factors which leads to aberrant immune responses. Although a number of environmental factors have been suggested, the causal roles of these potential disease triggers have yet to be determined.[40] A number of tissue defects have been described in both Crohn's disease and UC, including gut tight junction barrier function, defective trans-epithelial cell transport, extracellular matrix barrier proteins, and Paneth cell antibacterial peptides.[41] Also the polarisation of T-cells to a Th1 cytokine profile has been a recognised feature of Crohn's disease. More recent studies also point to a role for Th17 cells, hence taken together, both Th1 and Th17 cells are important mediators of inflammation in Crohn's disease.[42]

It has also been suggested that neutrophil dysfunction could be playing a role in altered innate immunity in the early stage of Crohn's disease development.[43] There are known genetic disorders with neutrophil function deficiency which give rise to clinical symptoms and pathology that is indistinguishable from Crohn's disease.[44] The exact mechanisms underlying disease pathogenesis are yet to be elucidated, however the current hypothesis for the aetiology of Crohn's disease suggests a T-cell driven inappropriate mucosal immune response in the gut, leading to an overproduction of inflammatory cytokines, most likely against endogenous bacteria.[45] Unlike acute inflammation, chronic inflammation continues for long periods (months or years), leading to tissue damage and long term associated disease.[46] Until recently, chronic and acute inflammation were thought to be separate processes activated by different mediators and outcomes, but it is now accepted that the processes are interlinked in such a way that defects in the processes that drive acute inflammation play a role

in chronic inflammation,[47] with tissue damage being the net result of the activated inflammatory process. Standard therapy for Crohn's disease is therefore directed towards suppression of this inappropriate immune response.

1.5 Growth failure in children with Crohn's disease

Malnutrition is common in children with IBD, particularly in active Crohn's disease where weight loss at diagnosis is noted in up to 90% of cases,[48;49] but there are no standards or screening tools to specifically identify malnutrition in IBD. In 2016 Jansen *et al.*[50] proposed a new screening tool which uses BMI, weight loss over 3-months and plasma CRP levels to approximate a malnutrition score specifically for IBD. The tool is limited in that BMI can be a poor predictor of malnutrition and growth over the short term.[50]

As a result of weight loss and malnutrition, children with IBD, especially those with Crohn's disease, tend to present with lower BMI and lower body weight when compared to the national reference range of healthy children.[51;52] It is not always easy to identify the cause of growth failure in individual children due to factors including: reduced food and nutrient intake; malabsorption due to inflammation; and possibly treatment with corticosteroids.[48]

More recent data suggests the risk of IBD patients being underweight is decreasing, with some studies showing a large number of patients being overweight at diagnosis which is most likely linked with increases in obesity in the general population. Although there is limited evidence showing the prevalence of obesity among newly diagnosed patients with IBD, a number of paediatric and adult studies suggest that the current number of overweight (BMI 25.0-29.9kg/m_2) and obese (BMI 30.0kg/m_2 or more) IBD patients matches that of the general population (∼20%-30%). One US study found that in a population of 1598 children with IBD, around one in five children with Crohn's disease and one in three with UC were overweight or obese.[53] A similar US adult study in 2015 found the rate of obesity to be 30.3% in Crohn's disease and 35.2% in UC patients.[54] Another study of 489 Scottish IBD adults from Tayside found that 18% were obese in comparison to ∼ 23% for the total Scottish population. Another 38% were over-weight which was equivalent to the general Scottish population. Overall, obesity was found to be more prevalent in Crohn's disease than in UC patients (p=0.05).[55]

A retrospective study in Philadelphia (1997-2002) looking at 126 patients with Crohn's disease found that 32.4% were overweight or obese at diagnosis. They also found that time to first surgery for patients with a BMI of under 18.5kg/m_2 was considerably longer than patients with a BMI of 25kg/m_2 or higher (p=0.04).[56] Although two of these US studies found that an increase in BMI was associated with an increased need for surgery, the other studies found that obesity in IBD (using BMI) was associated

with a less severe disease course.[56]

A large Canadian time-trend analysis of body weight and disease activity between 1991-2008; including forty randomised controlled trials involving a total of 10,282 patients with Crohn's disease, saw a significant increase in weight (r=0.36; 95% CI) and BMI (r=0.14; 95% CI) as well as a significant increase in clinical disease activity (r=0.109; 95% CI) and disease duration (r=0.063; 95% CI) in Crohn's patients over the time period.[57] It is therefore possible that there is a link between adiposity and inflammation in Crohn's disease which needs to be studied further.

1.6 Risk factors for Crohn's disease

The aetiology of Crohn's disease is complex, with the most widely acknowledged hypothesis being that Crohn's disease is a dysregulation of the normal immune response in genetically susceptible individuals, where the onset of disease is triggered by one or more environmental factors that lead to gut inflammation and abnormal gut immune responses to the gut microbiota.

Genetics The link with genetic risk factors is supported by ethnic and racial variation in the prevalence of IBD, with the highest rates being found among Caucasians, particularly Ashkenazi Jews who have a two to ninefold greater prevalence of IBD.[58] However, there is a trend towards growing prevalence in populations previous thought not to be at risk, thus the balance between genetic risk and environmental factors are complex. The idea that IBD has a heritable component is well established through family studies which show that 5-30% of these patients, particularly those with Crohn's disease, report a family member with IBD.[58] A 2003 study suggested IBD patients who have a family history of IBD are at risk of developing disease at a younger age.[59] They reported that in a group of eighty-two children with Crohn's disease, 42% of those under 11-years of age had a positive family history of IBD, significantly greater than older children (19%). However this type of split age analysis can be scientifically biased and misleading.

The genetic component of IBD risk is supported by the higher concordance rate found among monozygotic twins, that is not seen in same sex dizygotic twins; as well as the increased incidence among first degree relatives of those affected.[60] It is likely that some genetic variants affect the host relationship with the gut microbiota via immune expression, which put some individuals at higher risk of developing IBD. As well as understanding risk factors genetic studies are beginning to highlight the importance of host–microbe interactions in the pathogenesis of IBD (see section 1.9.3).

Genome-wide association studies (GWAS) created a framework for new insights into the aetiology of Crohn's disease. Identification of 163 IBD loci with 30 being Crohn's specific, is more than any other complex disease.[61] The strongest links are with genes

involved with intracellular killing of bacteria and innate immunity (CARD15/NOD2, IRGM, IL23R, LRRK2, and ATG16L1) as well as adaptive immune responses (IL-23 and Th17).[62] Current GWAS are based on samples from North America and Europe, hence wider studies including non-Caucasian populations are needed to properly understand how these genes might put individuals at risk of IBD. Although there is good evidence that NOD2, autophagy, and Th17 immune responses have strong links in Crohn's disease pathogenesis, susceptible loci only contribute around 14% of total disease variance.[61]

North-south divide There is a higher incidence of both Crohn's disease and UC reported in northern latitudes, compared to southern latitudes in the northern hemisphere.[63;40;64] There is growing evidence that a relationship exists between IBD and a north-south gradient in a number of countries, while some studies have shown a relationship between latitude and incidence of Crohn's disease linked with low exposure to sunlight and vitamin D insufficiency.[65;66] Higher dietary vitamin D levels among fish eating coastal populations might explain why not all studies observe a higher incidence of IBD at higher latitudes (*Prof C. Edwards pers. comm.*).

A 2010 clinical trial[67] found that supplementing vitamin D (1,200IU) to the normal diet of adults with Crohn's disease resulted in the mean serum 25OHD going from 69nmol/L to 96nmol/L after 3-months ($p<0.001$) and a reduction in relapse rate at 12-months (13% v 29%, $p=0.06$); although it should be noted, the group which received the vitamin supplement had greater azathioprine use than the control group.

Nutrition Diet is known to have a role in the expression of IBD, however most studies are retrospective case-controlled, meaning dietary habits are either asked to be recalled from a time before the onset of the disease, or as the current diet, which may not represent normal habitual diet prior to developing symptoms. In 2011 Hou *et al.* carried out a systematic review, concluding that not only were total fats, polyunsaturated fatty acids (PUFAs), omega-6 fatty acids, and meat associated with an increased risk of IBD but that higher fibre and fruit intake reduced the risk of Crohn's disease.[68]

Gut microbiota The importance of the microbiota in the pathogenesis of Crohn's disease has been demonstrated since diverting the faecal stream results in healing and prevention of Crohn's disease relapse in humans and animal colitis models.[69] Identifying a 'high risk' microbiota profile in IBD and Crohn's disease has therefore become a priority. A reduction in bacterial species abundance and diversity within the commensal gut microbiota community of patients with IBD have become key features however a link with causality has yet to be shown (Section: 1.9).[70;71;72]

Antibiotics Lewis *et al.* 2015 using a multi-variable model which included use of antibiotics within the last 6-months found that antibiotic use was independently associated with dysbiosis in IBD.[73] They also reported that genera associated with Crohn's disease in the absence of antibiotic use were different from those associated with antibiotic use. This evidence was based on creating two bacterial composition clusters of Crohn's patients which may have introduced some bias into results.

1.7 Epidemiology of Crohn's disease

The incidence of Crohn's disease is increasing worldwide with prevalence being particularly high in the northern countries of Europe and America. A Canadian study recorded the incidence rate of paediatric IBD rising from 9.5 per 100,000/yr in 1994 to 11.4 per 100,000/yr in 2005; including an incidence in Crohn's disease of 6.2-7.0 per 100,000/yr.[74] Crohn's disease had previously been classed as a western disease because it was more common in industrialised nations, due to low prevalence rates in places such as South America, Eastern Europe and Asia.[75;76] However, recently it has become clear that Crohn's disease is an emerging disease in countries with previously low rates, such as Japan where the adult prevalence rate rose to 21.2 per 100,000 by 2005.[77] The speed at which incidence is increasing in areas where Crohn's disease was previously absent suggests that environment, including diet, is pivotal in triggering disease.[76] It is possible that the introduction of a more westernised diet in these countries is playing a role in the elevation of patient numbers. This is supported by the fact that there is an increase in occurrence of IBD among families when they emigrate from a country where incidence is low to one where incidence is high, highlighting the importance of identifying environmental triggers.[76]

Some limitations in describing the worldwide epidemiology of Crohn's disease are that increases in IBD have not been described in a uniform way, with many countries lacking accurate estimates of incidence and prevalence particularly for paediatric IBD. There is also a particular lack of data about rates of paediatric IBD from developing countries in Asia, Africa, and South America,[78] hence most of the data regarding epidemiology is taken from European and North American cohorts. Additionally most reports do not include the category of IBD-U thus the overall incidence of IBD is higher than that inferred from available data.[79] In Europe the highest incidence and prevalence rates have been found in Scandinavian countries[80] and the UK, with the highest in Scotland.[81] Scotland has one of the highest IBD rates in the world. Since the mid-1990s the incidence of IBD in Scottish children has increased by 76% including a 66% rise in Crohn's disease (Table 1.3). There has also been a marked increase over the past 10-years in the incidence of children with Crohn's disease in Ireland.[82] These increases in the Scottish paediatric IBD population, which have lead to an overall IBD incidence rate of 7.82/100,000/yr (derived from the 2003-2008 figures) may have continued to rise towards levels of incidence described in Canada

(2005)[74] and Scandinavia (2005-2007),[83] which have reported rates of 11.4/100,000/yr and 10.6/100,000/yr, respectively.

Table 1.3: Paediatric IBD incidence in Scotland per 100,000/yr

Year	1990-1995 (n=260)	2003-2008 (n=436)	difference sig. at
IBD	4.45	7.82	p<0.0001
Crohn's	2.86	4.75	p<0.0001
UC	1.59	2.06	p=0.023

Taken from Henderson *et al.* 2010[84]

Age of disease onset IBD is now being diagnosed at a younger age in Scotland, particularly Crohn's disease where the age at diagnosis has fallen from 13.2 in the early 1990s to 12.1 years by 2008.[84] Around a quarter of patients with Crohn's disease are diagnosed before the age of twenty, often presenting with severe nutritional deficiencies.[85] About 85% of these children present with weight loss, with up to 15–40% of them failing to reach their growth potential.[86;87] This risk of malnourishment can impair growth and affect pubertal development,[3] therefore nutritional therapy is an integral part in the management of children with Crohn's disease. The onset of IBD in childhood tends to be characterised by a more extensive disease location as well as more aggressive and severe disease condition than found in adult onset IBD.[88;89] Overall the impact on the health of children with Crohn's disease can be more serious, leading to children requiring increased NHS resources.

Gender differences In Asian studies a male predominance has been recorded in both, paediatric and adult populations.[90;91;92;93] For European and North-American studies the female/male ratio has varied from an even distribution to as much as 2.5:1.[94] Many cultures have particular gender roles, potentially resulting in different exposure to environmental risk factors, which could in part account for geographical differences in IBD gender ratios.

Henderson *et al.* 2011[84] reported that among Scottish children with IBD that more boys were affected than girls especially for Crohn's disease. The adjusted male/female ratio for the 2003-2008 figures was 1.29, rising from 1.07 in 1990-1995; and mainly due to an increase in male/female incidence ratio in children with Crohn's disease (1.22 to 1.38). It will be important for future studies in Scotland to look at the reasons behind gender differences in Crohn's disease which could be down to difference in exposure to environmental risk factors; dietary choices; or hormonal differences. The possibility that girls, especially in their teen years, are less willing to come forward with bowel problems, to discuss diarrhoea and other gastrointestinal symptoms with parents and clinicians, leading to a possible gender bias in mild cases, or greater time to diagnosis in more severe cases, needs to be considered. It has also been noted in

some clinical gender studies, that general practitioners can be more prone to interpret male symptoms as physiological, while attributing a more psychological element to the symptoms of female patients, which affects referral and diagnosis.[95] Increased side-effects from bowel preparation have been recorded in female IBD patients[96;96] which could in turn affect the completeness and accuracy of an endoscopic result. Another study has recorded a lower rate of follow-up colonoscopies in female IBD patients.[97] Hence there are a number of potential biases which could account for gender ratio differences. However this type of bias could also be due to genuine biological gender differences in the severity of symptoms, which need to be identified by future research.

1.8 Current treatment strategies for paediatric Crohn's disease

Crohn's disease is currently incurable and thus therapeutic approaches aimed at achieving clinical remission of the condition only provide short term relief from symptoms.[98] The management of Crohn's disease firstly involves inducing remission and is then followed by medical treatment to maintain remission.[99] The use of corticosteroids to induce remission in patients with active Crohn's disease is effective.[100] However the side effects over the long-term, particularly in children, make their use less than ideal and they are ineffective at inducing mucosal healing. The side effect of weight gain and acne as well as poor bone health and development can also be a significant problem for adolescents with Crohn's disease being treated with corticosteroids.[101] Choice of treatment is not only determined by whether a child with IBD has UC or Crohn's disease but also the location, severity and extent of disease. Due to the large range of disease phenotypes treatment is tailored according to the individual needs of each child.

1.8.1 Medications used to treat Crohn's disease

A recent NHS review in England has estimated that to treat adult IBD costs around £720 million per year,[102] with approximately 1/4 of costs taken up by medications used to treat IBD.[103] Medications used to treat Crohn's disease and IBD are aimed at inducing and maintaining disease remission through modification of immunomodulatory processes (Table 1.4).[104]

Although the 2014 second European consensus on prevention, diagnosis and management of infections in IBD, concluded that IBD patients should not be routinely considered to have altered immunocompetence *per se*, there are a number of immunomodulators used in the treatment of IBD which are associated with increased risk of infections.[105] These include corticosteroids, thiopurines, methotrexate, calcineurin inhibitors, anti-TNF agents and other biologics. Unfortunately there is no sound biological means to measure immunosuppression in IBD patients.

Table 1.4: Medication used to treat children with Crohn's disease in the UK.

Type	Drug name	Role	Action
Antibiotics	Metronidazole Ciprofloxacin	induction of remission (esp. perianal disease)	reduction of gut bacteria
Corticosteroids	Prednisolone Hydrocortisone	induction of remission	down-regulation of pro-inflammatory cytokines; interference of NFκB inflammatory signalling
Immuno-suppressant	Azathioprine 6-Mercaptopurine	maintain remission	immune suppression and cytotoxicity; induce T cell apoptosis
Biologics	Infliximab	induction of remission	antibody against TNFα

Observational studies like the current one cannot control for the confounding effect that medications might have on the composition and functional behaviour of the gut microbiota, hence it is important to examine the potential impact drugs might have on results. A 2007 study reported from mucosal biopsies that the suppressed migration of leukocytes in UC patients treated with azathioprine was associated with a 28-fold higher concentration of mucosal bacteria when compared with patients treated with 5-ASA, and 1000-fold when compared with healthy controls.[106] Therefore it is important to know when measuring bacteria that changes are due to dietary treatment like maintenance enteral nutrition (MEN) rather than the immunosuppressant Azathioprine, which many children take along with MEN therapy.

Antibiotics Although medical therapy has targeted suppression of the immune system, antibiotics are frequently used as an ancillary treatment in Crohn's disease.[107] There is conflicting evidence regarding the efficacy antibiotics to help induce remission on Crohn's disease. A 2011 review paper concluded that in active Crohn's disease using data from ten RCTs involving 1,160 patients, that treatment with antibiotics was superior to placebo (RR=0.85; 95% CI=0.73–0.99, p=0.03);[107] however interpretation is challenging due to the diversity of antibiotics used. It is unclear whether antibiotics work due to reduction of a single species, or via changes to overall bacterial composition.[108] It is possible, gut bacteria are not driving IBD, but rather cause secondary infection due to underlying inflammation, exacerbating disease activity.[109]

Immunosuppressants Immunosuppressants on their own cannot induce remission and are therefore used to help maintain remission in IBD patients. As such azathioprine or 6-mercaptopurine are the primary treatment for maintenance of remission of moderate to severe Crohn's disease.

Biologics Tumour necrosis factor-α (TNF-α) inhibitors are biological agents introduced in the 1990s for the treatment of IBD. It is recommended for treatment of children aged 6–17 with severe active disease who have not responded to immunosup-

pressants, corticosteroids and nutritional therapy.[110] The introduction of anti-TNF therapy has improved the quality of life as well as reducing time in hospital and surgery for Crohn's disease patients.[111] The most common TNF-α antagonist used for Crohn's disease is infliximab. Although it is effective in lowering inflammation, treatment-induced immunosuppression is suspected to increase infection risk.[112]

Corticosteroids Corticosteroids were first used to treat IBD in the 1950s with the successful use of cortisone acetate in patients with UC.[113] Since this time corticosteroids have become the cornerstone in treating IBD patients with moderate to severe active inflammation.[114] Despite the widespread use of corticosteroids, studies concerning optimal therapeutic strategies have mostly been done in adults with IBD, with as many as 80-90% of adults treated with corticosteroids reporting at least one adverse event during their therapy.[115] Although corticosteroids are clinically efficacious and induce remission, their use in children can have unwanted side effects such as weight gain, growth impairment, increased acne as well as low mood,[116] thus children with IBD can be more sensitive to the side effects of corticosteroids than adults, particularly with respect to impaired growth.[117]

A number of studies have shown that systemic use of corticosteroids work via a number of routes including: inhibiting epithelial cell gene expression of cytokines (IL-1β, IL-6, & TNF-α), growth factor and receptors (GM-CSF & TGF-β), as well as various inflammatory chemokines of the CXC and CC families.[118] They can also reduce diarrhoea by increasing sodium and water absorption.[119]

1.8.2 Treatment with exclusive enteral nutrition (EEN)

As a chronic inflammatory condition of the gut, Crohn's disease causes severe nutritional complications. The use of exclusive enteral nutrition (EEN) to treat the dietary and growth needs of children with Crohn's disease led to EEN becoming an effective therapy which could successfully help children achieve disease remission without the need for corticosteroids.[120]

EEN is a liquid nutrition diet which exclusively replaces the child's normal diet.[121] Being a liquid preparation with no fibre, it is easily digested in the small intestine. Once the individual calorific requirement for a child about to start EEN has been calculated, the formula is normally introduced slowly over 2-4 days and continued for around 6-12 weeks.[122] Normal food is then reintroduced, although some centres slowly reintroduce specific types of food first.[122]

Only one study has attempted to answer the question as to whether it is necessary to have full exclusion of the normal diet in order to achieve remission in paediatric Crohn's disease, and they found that EEN increased the remission rate 3-fold when compared to the energy requirement being met from 50% enteral nutrition and 50%

normal diet.[123] However paediatric centres often permit a variety of additional foods in addition to EEN,[124] including sugarless chewing gum,[121] clear soup or carbonated beverages such as lemonade. More than 40% of studies allow tea and coffee, and almost half allow the introduction of additional sugar in the form of hard boiled sweets.[124] Further studies are needed to explore the efficacy of these additional food items, before being added to EEN protocols.

It would be unethical not to treat patients with active disease, hence no placebo controlled trial has ever been carried out to test the efficacy of EEN in IBD. The efficacy of EEN to induce remission in active Crohn's disease has therefore been measured against medications, in particular corticosteroids.[110] EEN has been shown to induce remission in 80-85% of children with active Crohn's disease,[120] with recent guidelines advising the use of EEN as the first line induction treatment for children with inflammatory luminal disease.[125] A 2008 Cochrane review looked at the effectiveness of EEN as primary therapy to induce remission in children with Crohn's disease from 1966-2006.[26] From the fifteen studies included they concluded corticosteroid therapy was more effective than EEN for inducing remission of active Crohn's disease, but that EEN had the added benefit of mucosal healing. Many of these studies were of low quality, and the two highest quality studies in this review had opposing findings, with one in favour of steroid therapy[126] and the other EEN;[127] although neither study reached statistical difference. However, recent reviews have shown EEN is just as effective as corticosteroids in inducing remission (OR=1.26 [95% CI 0.77, 2.05]) in children with Crohn's disease; and mucosal healing was significantly higher in children receiving EEN compared to corticosteroids (OR=4.5 [95% CI 1.64, 12.32]).[128] Enough studies showing EEN to be a beneficial primary treatment for active Crohn's disease triggered the European Crohn's and Colitis Organization (ECCO) in 2014 to revise their consensus guidelines and recommend EEN as the first line therapy to induce remission in children with luminal Crohn's disease (Table 1.5).[98;125]

There are factors which could influence the efficacy of EEN, including variation in type and severity of Crohn's disease, the way disease activity and remission is defined; composition of EEN; duration of EEN, as well as the timing of clinical assessments.[125] In some studies, length of treatment is effectively too short to properly evaluate efficacy of EEN against corticosteroids. For example Tjellstrom *et al.* 2012 concluded that children with active perianal Crohn's disease, saw no improvement in clinical status, or inflammatory parameters after treatment with EEN.[129]

There are trade-offs to using EEN as a therapy to induce remission over corticosteroids including: palatability; distress caused by use of nasogastric tubes; and stress or isolation from the social aspects of normal family/school meal times.[110] These issues are important considerations since the 2012 NICE guidelines[110] reported that all studies included in the Cochrane review[26] of EEN are of low to very low quality. Hence it might be premature to assume EEN is always the better option.

Table 1.5: Studies since 2012 on exclusive enteral nutrition (EEN) showing remission rates, length of treatment and type of feed.

Study	No. of children	Type	Feed	Duration	Remission rate	Notes
Connors *et al.* 2017[130]	111	Retrospective	unknown	6-16 weeks	86.6%	Remission with EEN was associated with reduced risk of CS treatment over 2-years
Luo *et al.* 2015[131]	28	Retrospective	Not given	8 weeks	90%	Longer time to relapse in those treated EEN vs CS (significant)
Lee *et al.* 2015[132]	90	Prospective cohort	Not given	8 weeks	88%	Partial enteral nutrition induced remission in 64% of children
Levine *et al.* 2014[133]	201	Prospective cohort	unknown	6-8 weeks	79%	No difference seen between EEN and CS
Hojsak *et al.* 2014[134]	74	Retrospective	Polymeric	6-8 weeks	84.2%	Longer time to relapse in those treated EEN vs CS (significant)
Soo *et al.* 2013[135]	105	Retrospective	33 polymeric; 3 elemental	6 weeks	88.9%	No difference seen between EEN and CS
Lambert *et al.* 2012[136]	57	Retrospective	Polymeric	4-8 weeks	84%	compared with corticosteroids
Saadah *et al.* 2012[137]	50	Retrospective	Polymeric	6 weeks	32%	No comparison with corticosteroids

CS -corticosteroid therapy; EEN -exclusive enteral nutrition. Studies prior to 2012 are given in the '*2012 NICE guidelines*'.[110] Similar results have not been reproduced in adult studies, with EEN resulting in lower response rates than using steroids (perhaps due to lack of compliance in adults).[138]

Effect on inflammation and mucosal healing Although the effect of EEN on mucosal inflammation is not fully understood, it has been shown that clinical response to treatment with EEN is associated with mucosal healing and down-regulation of mucosal pro-inflammatory cytokine mRNA in the terminal ileum and colon.[139] A 2005 study in patients with adult Crohn's disease observed that EEN reduced mucosal cytokine production, correcting an imbalance between pro- and anti-inflammatory cytokines.[140] Two 2006 paediatric studies also showed that EEN is more effective than corticosteroids at healing inflammatory associated lesions in the gut.[100;141] This beneficial effect of enteral nutrition on mucosal healing has been shown to a lesser degree during maintenance therapy for quiescent Crohn's disease.[45]

Potential mechanisms How EEN works to induce remission is unclear. There are a number of theories including removal of dietary antigens and reduction of microbial antigens. In 2004, Bannerjee *et al.*[142] conducted a study on twelve children with active Crohn's disease treated with EEN for 6-weeks. They saw changes in ESR and IL-6 within 3-days; and in disease activity scores, CRP, and insulin-like growth factor-1 within 7-days. They did not see nutritional improvements until 14-21 days; hence suggest an early anti-inflammatory effect rather than nutritional improvement is driving remission in Crohn's patients. Recent studies have provided insight into possible mechanisms linking changes in the gut microbiota and bacterial metabolites with gut inflammation.[143] These show EEN causes a reduction in bacterial diversity, leading to changes in bacterial metabolites and functionality.[144;145] However causality

has yet to be established.

1.8.3 Treatment with maintenance enteral nutrition (MEN)

Following the success of EEN, studies have gone on to supplement the normal diet with enteral nutrition (MEN), to try and maintain disease remission (Table 1.6), thus reducing the need for corticosteroid therapy.[101] Several approaches have been tested, including overnight nasogastric feeds combined with normal eating during the day; short periods of nasogastric feeds several times a year; and a daily supplement drink used alongside normal eating. In one study overnight nasogastric feeding significantly reduced relapse to 43% at 12-months compared with 79% for children who chose not to take MEN.[146] Another study using nasogastric enteral nutrition for one out of every four months over a year, also saw a significant increase in remission rates.[147] A study on adults with Crohn's disease saw an interim remission rate using MEN, which was double that of the non-MEN treatment group, and hence felt it was inappropriate to continue the study as they decided the benefit had been clearly demonstrated;[148] a similar success rate to a paediatric study which reported 66% of Crohn's disease children in remission after 12-months on MEN.[149] MEN has also been shown to reduce relapse after surgery in Crohn's disease adults, particularly in those with penetrating disease and non-active lesions.[150] A later Japanese study also found MEN reduced recurrence rates after bowel resection.[45]

In a recent review, although ten studies found remission rates were significantly higher in Crohn's disease patients who received MEN compared with patients who did not, only one of these was a randomised controlled trial.[151] It could be argued that although the sample size in these studies was small ($\sim$20 MEN patients), combined they represent a reasonable sample population. Eight studies took place in Japan, thus these results may not translate to Western populations. Also, the studies as a whole found that the ability of enteral nutrition to induce remission was dose-dependent with higher doses resulting in higher remission rates.

A recent adult study compared MEN with the use of the immunosuppressive drug 6-mercaptopurine (6-MP) and non-treatment controls,[152] reported a remission rate of 46.9% for MEN which was comparable with 60% remission in the 6-MP group; remission in controls were 27.2%. Two patients in the 6-MP group developed liver injury and one alopecia, thus highlighting the need to develop safer forms of long term treatment such a nutritional therapy. If MEN is an effective treatment for the maintenance of remission in Crohn's disease, as some studies suggest, more work needs to be done to understand the mechanisms behind why EEN and MEN work, so that treatment to induce and maintain remission can be optimised for Crohn's disease patients.

Table 1.6: Evidence on whether maintenance enteral nutrition (MEN) affects time to disease relapse

Study	Number of patients	Liquid feed	Time on treatment	Notes
Gavin *et al.* 2018[153]	102 CD: 58 MEN, 44 non-MEN	30-50% polymeric or elemental formula (300-1200kcal)	median 4-months (upto 12-months)	Relapse rate did not differ between MEN and non-MEN group at either 6, or 12-months 78% MEN vs 77% non-MEN
Duncan *et al.* 2014[154]	15/48 (31%) CD patients 14/15 patients oral MEN and 1/15 via NGT	polymeric formula	12-months	Remission at 12-months: MEN 60% (9/15) vs 15% (2/13) non-MEN no meds (p=0.001) and 65% (13/20) non-MEN taking azathioprine (p=0.14).
Pham *et al.* 2014[155] Abstract	10 CD children (2 did not comply)	Polymeric; 6 via NGT: 75% formula 25% solid food	6-12 months	6/8 children maintained remission using nutritional therapy alone.
Hanai *et al.* 2012[152]	95 adult CD: ∼1/3 in each group	3 groups: 6-mercaptoppurine (6-MP); MEN 900kcal per day & non-treatment control	24-months	Log-rank test showed better efficacy for 6-MP (p=0.004) and MEN (p=0.035) vs control. No difference between 6-MP and MEN. In 6-MP group 2 had liver injury; 1 had hair loss.
Grogan *et al.*, 2012[156]	24 CD children:	19 polymeric 15 elemental	6-weeks	In both groups, ∼1/3 of children still in remission at 2-yrs. Mean days until relapse not significantly different (elemental feed: 183 days; polymeric feed: 162 days)
Takagi *et al.* 2006[148]	51 CD adults: 26 MEN; 25 Non-MEN	50% elemental formula vs normal diet	2-years	Study finished early (11 months) as intermediate analysis showed relapse rate in MEN group significantly lower [34.6% vs. 64.0%; multivariate hazard ratio 0.40 (95% CI: 0.16–0.98)] than free diet group.
Day *et al.* 2006[121]	CD children 4 male MEN; 8 non-MEN	Polymeric formula 300-1800kcal per day (oral)	Mean 15.2 (10-21) months	All 4 maintained remission during average follow-up period of 15.2 months (no other therapy was given).
Verma *et al.* 2001[157]	33 CD adults	19 elemental; 14 polymeric (35-50% calorie intake) with normal diet	12-months	MEN maintained remission in 14 (43%) patients. Response to elemental diet (42%) similar to polymeric diet (43%). MEN failed in 13 (39%). No non-treatment control was included.
Verma *et al.* 2000[149]	CD children 21 MEN vs 18 non-MEN	Elemental (35-50% calorie intake) with normal diet	Up to 12-months	Response to MEN at 12-months was 48% vs 22% in non-MEN group (p<0.0003).
Wilschanski *et al.* 1996[146] Canada	CD children: 28 MEN; 19 non-MEN	Elemental or semi-elemental	Overnight NG tube/normal daytime diet for 12-months	Children who continued nasogastric MEN remained in remission longer than non-MEN group (p<0.02). 1/3 of children experienced problems with night-time waking.
Belli *et al.* 1988[147]	8 CD vs same children over previous 12-months	Elemental by NGT 25% of daily caloric intake	1 out of 4-months	Increased height and weight in MEN vs controls (p<0.01). Disease activity decreased in MEN group compared with themselves and with controls on medical therapy (p<0.05).
Harries *et al.* 1983[158]	20 adult CD patients	Polymeric formula (Ensure plus)	2-months normal diet then 2-months normal diet plus formula.	Not a maintenance study, all patients were malnourished. Crossover study

CD - Crohn's disease; **HC** - healthy controls; **NGT** - nasogastric tube; **Modulen** - Polymeric formula
All CD patients were in remission at start of MEN therapy.

1.9 Role of the gut microbiota and microbiome in Crohn's disease

Involvement of the gut microbiota in Crohn's disease pathogenesis was first shown in studies where faecal stream diversion prevented recurrence of ileal disease[159] and that subsequent post-operative exposure to the gut microbiota resulted in relapse.[69] Treatment with antibiotics is also associated with clinical improvement in IBD patients.[160;161] Accumulating evidence now suggests, that IBD is linked to an inappropriate inflammatory response to the intestinal microbiota in genetically susceptible hosts.[162] If children with Crohn's disease have a gut microbiota profile, both distinct from healthy children and other inflammatory diseases including UC, it could have potential as a clinical marker for Crohn's disease. Understanding why the gut microbiota is altered in these children could also lead to improving treatment strategies.

The term microbiome refers to ¨the collective genome of the human indigenous microbes (microflora or microbiota), the idea being that a comprehensive genetic view of *Homo sapiens* as a lifeform should include the genes from our microbiome˝.[163] The term microbiota refers to the actual microbes, but in the literature is often interchangeable with the term microbiome.[164] The human gut contains a surprisingly complex ecosystem with an abundance of over a thousand different bacteria and other micro-organisms. This structural composition in most people include the dominant bacterial phyla, Bacteroidetes and Firmicutes, as well as the less abundant Proteobacteria and Actinobacteria.

As well as their role in digestion and absorption, epithelial cells that line the luminal surface of the gut form a physiochemical barrier to bacteria. In healthy individuals intestinal goblet cells produce mucus which contain antimicrobial peptides all of which keep the majority of bacteria to the more fluid outer layer of the mucus and the lumen of the gut, keeping bacteria away from the surface of epithelial cells. However the depth of the mucus layer varies significantly along the length of the gut. In the colon where bacterial load is greater, goblet cells are very dense where they secrete an additional sterile layer of mucus which acts as a barrier at the cell surface. It has been shown in mice that lack the MUC2 gene that the mucus layer is unable to prevent bacteria coming into contact with epithelial cells which can in turn result in inflammation.[165;166]

Pattern recognition receptors (PRRs) towards bacteria have been shown to control the production of microbicidal peptides produced by enterocytes and Paneth cells in the gut.[167] PRRs produced by epithelial cells include membrane associated toll like receptors (TLR) and intracellular nucleotide binding oligomerisation domain containing protein (NOD) like receptors (NLR) as well as retinoic acid inducible gene related receptors (RIG) which are able to recognise microbial molecular patterns. TLR1-5 and TLR9 can recognise bacterial motifs as can a number of intracellular PRRs (NOD1/CARD4 NOD2/CARD15). The NOD2 gene encodes for a protein which

is mostly expressed in peripheral blood leukocytes, recognising bacterial molecules that contain a muramyl dipeptide moiety as well as activating NF-κB.[168] Mutations in this and other genes have been associated with Crohn's disease,[169] suggesting that the way epithelial PRRs interact with commensal bacteria underpin host protection against both resident and pathogenic bacteria.[167]

From altering gut composition as a treatment via probiotics; dietary changes or faecal transplant, to searching for causative agents or risk factors amongst disease groups like IBD, IBS and arthritis, crosstalk between bacteria and host cells, as well as modulation of the immune system, are all important areas of current research. Bacteria also produce bacteriocins, which play a role in competitive dynamics between strains of bacteria, possibly helping to maintain microbial homoeostasis.[170]

Culture based plating was the primary method of investigating the gut microbiota for many years, and was subsequently replaced by conventional DNA analysis using either sequencing or fluorescent detection. These focused approaches did not account for the vast array of bacteria that interact to make up the gut microbiota, resulting in an incomplete picture.[171] In the last decade culture independent detection methods have now progressed to include 16S rRNA sequencing and high throughput DNA sequencing[172] as well as third generation sequencing analysis.[173] This has allowed highly detailed bacterial classification as well as information about the functional genes that make up the gut microbiome.[174;175] These methods now allow us to characterise phylogenetic disease-associated gut microbiota changes and highlight changes in individual microbial species, as well as telling us something about the functional capability of the gut bacterial community. As 'omics' technologies and analysis have become more widely available and affordable, this has resulted in the exploration of global gut microbial taxonomy and function, allowing a more comprehensive investigation of the complex relationships between the gut microbiota and host tissues.

While a number of bacterial species have been linked with Crohn's disease, such as species from *Mycobacterium, Campylobacter, Escherichia* and *Helicobacter*, present evidence does not support the idea that Crohn's disease is caused by a single species or strain of bacteria.[176] Evidence from both stool and mucosal biopsy samples, suggests that Crohn's disease is associated with significant differences in gut microbiota at a community-level often referred to as dysbiosis.[176]

1.9.1 Types of dysbiosis associated with Crohn's disease

Loss of beneficial commensal bacteria In infancy, development of the core commensal microbiota is vital for the maturation and development of the normal intestinal immune response.[177;178;179] The mucus layer is composed of mucin glycoproteins which are secreted into the lumen by epithelial goblet cells. It consists of two layers: an inner layer of stratified mucus which is adherent to gut epithelial cells ($\sim$50μm

thick); and a thicker outer non-attached layer which is home to the commensal flora. Recent research into the identification and development of commensal bacteria are beginning to shed light on some of the mechanisms responsible for driving the maturation of host immunity in the gut. In order to establish homoeostasis the immune response needs to prevent undesirable immune response towards self and beneficial commensal bacteria while at the same time be primed to deal with potentially harmful pathogens. Establishing this level of homoeostasis initially involves production of mucus and antimicrobial peptides which create this protective, but dynamic barrier, between the host gut wall and the commensal bacteria. Vaishnava *et al.* 2011 showed in a mouse model that a secreted antibacterial lectin (Reg3γ), was necessary for maintaining a 50μm layer that acts as a physical barrier to the luminal microbiota.[180] Loss of this barrier in Reg3γ-/- mice resulted in bacterial colonisation of the gut epithelial surface along with increased adaptive immune responses to bacteria. These findings suggest that antimicrobial peptides play a crucial role in maintaining host-bacteria mutualism by regulating the spatial relationships between bacteria and the host. It has also been shown that members of the commensal gut microbiota can inhabit the intestinal mucus without activating the host inflammatory response.[166] Hence the mucus layer is not a simple physical barrier but is a dynamic layer that controls the interaction between nutrients, antigens, commensal bacteria and the immune system.

Immunotolerance to self tissue and beneficial commensals is controlled in large part by a subset of T-lymphocytes known as T_{reg}.[181] Studies in germ-free mice have shown that T_{reg} function is compromised but can be restored by introducing the human commensal bacterium, *Bacteroides fragilis* or with a combination of *Clostridium* strains IV and XIVa.[182;183] Although mouse models do not fully represent the human gut, it is likely the molecular basis of the evolution of commensal tolerance is similar across all mammals. There are other human strains of *Bacteroides* which have also been shown to modulate the amount of colonic T_{reg} in mouse models including *B. caccae, B. thetaiotaomicron*, and *B. vulgaris*;[184;182] as well as a number of *Clostridia* spp. isolated from humans;[185;183] and a number of *Lactobacillus* strains as well as some strains of *Bifidobacterium* in a cell culture model, suggesting the modulation of T_{reg} is a common mechanism to induce tolerance in the gut. However these experiments do not test a comprehensive selection of bacteria therefore cannot represent the overall make-up or complexity of the developing human microbiota which modulate T_{reg} and other immune responses in the gut.

Reduced diversity Members of the gut microbiota have various functions that contribute to host well-being, such as being involved in anti-inflammatory pathways or the induction of inflammatory responses. Some members of the microbiota promote the growth of anti-inflammatory networks within the host, as well as protective inflammatory responses. In a germ-free mouse model it has been shown that some

bacterial/host interactions such as changes in metabolite concentrations or accumulation of gut T_{reg} cells, are initiated only when a number of bacterial species are present.[184] This demonstrates a role for bacterial diversity in altering host immunological phenotype,[184] and the loss of total microbial diversity is an important aspect of dysbiosis in Crohn's disease.

T_{reg} function has also been shown to be compromised in germ-free mice, however when human faecal sample enriched for chloroform-resistant bacteria (selecting for thirty strains Clostridiales) were used to recolonise mice, it induced a 3-fold expansion of T_{reg} compared with controls.[186;183] If only one strain of *Clostridia* was used, T_{reg} induction was diminished.[186;183] This demonstrates that increased diversity, even within the same family, maximises cellular development in the host gut. It is possible that early exposure to a diverse microbiota is crucial to the development of healthy host/microbial interactions.[178] For example, a study looking at microbial diversity in newborn babies at 1-week and 1-month has shown children who developed asthma at 7-years of age had lower microbial diversity in early infancy, but not at 12-months, compared with healthy children in the study ($p<0.05$).[187]

Although studies of patients with IBD, regardless of disease activity, show they have a marked loss of symbiont bacteria within the gut, specifically Clostridium groups XIVa and IV,[188;189] it is not yet clear whether lack of beneficial bacteria play a role in driving inflammation, or conversely, inflammatory processes play a role in reducing commensals.

Expansion of pathobionts The gut microbiota also contains bacteria that have potential to cause harm. The term pathobiont was coined to describe members of the 'normal' commensal community that can, under certain circumstances, cause damage to the host.[190] The most commonly recorded incidence of pathobiont expansion is that of the phylum of gram-negative bacteria, Proteobacteria, and particular the family Enterobacteriaceae, including *Escherichia coli, Shigella* and *Klebsiella.*[191] This increase in Enterobacteriaceae has also been recorded in mouse models of colitis,[192] especially those with the TLR5 mutation.[193] It is not likely that pathobiont expansion on its own could cause IBD, since recent years of study have failed to identify a single organism specific to IBD patients.

Although three distinct forms of dysbiosis are described here, it is likely that gut microbial dysbiosis, in any one individual, will include a mixture of all three types.

1.9.2 Evidence for microbial difference in Crohn's disease

The idea of gut bacteria being altered as a collective, as opposed to a single species or strain resulting in IBD is a crucial step forward in IBD research. The development of 'omics' technologies,[172] now permit analysis of microbiota in a way which allows

researchers to see global changes in the gut which are being linked with the functionality of the microbiome, and its impact on the host. Both human and animal studies are starting to provide clues as to how the microbiome could be altering mucosal immune function, potentially leading to chronic inflammation. It is therefore vital that research, not only aims to identify which groups of bacteria are changing in the gut, but also tries to determine what impact these changes have on gut immunoregulation.

In 2014 Gevers *et al.*[194] carried out a large study on 425 ileal and 300 rectal tissue biopsies, and 199 faecal samples collected from children with Crohn's disease prior to treatment. They recorded an increased abundance in families Enterobacteriaceae, Pasteurellacaea, Veillonellaceae, and Fusobacteriaceae, as well as decreased abundance of Bifidobacteriaceae, Erysipelotrichales, Clostridiales and Bacteroidales compared to a control group of non-IBD children, which correlated well with disease status. The differences were less pronounced in faecal samples than was seen in both ileal and rectal biopsies, but did include an increase in *Streptococcus* and a loss in a taxa belonging to the order of Clostridiales, including *Dorea*, *Blautia*, and *Ruminococcus*. They also found that antibiotic use amplified bacterial dysbiosis in Crohn's disease. Previous studies had mostly included patients with ongoing disease and were small in scale hence lacked statistical power, and thus could not easily take into account confounding factors such as medication use.[195] It should also be noted that although this study did a comparison with faecal samples only 12% (n=26) of non-IBD controls gave a stool sample.

The study by Quince *et al.* 2015[144] also highlighted that differences in gut bacteria between children with Crohn's disease are not uniform across genera with species like *Ruminococcus obeum* being lower in children with Crohn's while *R. gnavus* was higher. It is therefore important to see the whole picture to understand how patterns of gut microbiota might play a role in aetiology or patient symptoms.

Metagenomic studies have suggested Crohn's disease can be identified from distinctive microbiota compositions compared to healthy controls,[196] supported by Davenport *et al.* 2014[197] who used 16S ribosomal sequencing of mucosal biopsies from twenty-one Crohn's disease patients and twenty-four healthy controls, to show an overall alteration in the ratio between Firmicutes and Bacteroidetes, with a relative abundance being significantly greater than healthy controls (p=0.05). The controls were not age-matched (20-year mean difference) and it is therefore not just possible, but quite probable that at least some of the observed difference, especially in Bacteroidetes, are attributable to age related changes.[198] Other meta-analysis studies have also identified a dysbiosis characterised by a reduction in Firmicutes and an increase in Proteobacteria.[195] A study looking at the metagenomic profile of monozygotic twin pairs discordant and concordant for Crohn's disease also found that the faecal microbiota profile was more similar between healthy twins, than between twins with Crohn's disease, particularly if they were discordant for the disease.[199]

A 2010 study using metagenomic sequencing has shown that not only did twenty-five patients with IBD have lower bacterial diversity than ninety-nine non-IBD individuals, but also an average of 25% fewer genes.[200] A loss in microbial functional genes in IBD patients could affect crosstalk between host cells and the mucosal bacteria. An IBD study looking at monozygotic twins went on to show bacterial abundance at the genus level, when linked with transcriptomic profiles, demonstrates a loss of host bacterial interaction in patients with UC.[201] A linked study looking at six monozygotic twins and their mothers, showed community level metabolic network changes in the microbiome of Crohn's disease patients, mostly at the periphery of these networks, highlighting the importance of the host/microbiome interface in the aetiology of Crohn's disease.[202] Demonstrating links between the bacterial taxa, environmental factors, and a host genome is complicated by the various disease states in Crohn's disease, as well as the large intra-and inter patient variability in microbial diversity.

A study has reported that mucosal biopsy samples in Crohn's disease patients show little difference in gut bacterial composition between inflamed and neighbouring non-inflamed tissue, whereas those taken from UC patients appear to demonstrate clear bacterial clustering between inflamed and non-inflamed tissue,[197] suggesting that any dysfunctional aspect of the mucosal bacteria in Crohn's patients could be more systemic than that of UC. However differences could be present, especially among species of low abundance, which were not detected using these methods.

1.9.3 Interaction between host genome and the gut microbiota

It is an over simplification to suggest that a specific bacteria or group of bacteria cause Crohn's disease and hence these studies looking at changes in the gut microbiota need to be interpreted in the context of the host genome as well as other environmental factors.

Although studies have shown that some bacterial species such as *Akkermansia muciniphila* (human) and *Enterorhabdus mucosicola* (mouse) have the ability to damage the intestinal mucosa,[203] alterations in host genes such as the gel forming mucin MUC19 might make patients more susceptible to this type of microbial interaction allowing some bacterial species that degrade mucus to thrive in the mucus layer.[204] The mucosal barrier which protects the lamina propria from bacteria in the lumen, has been shown to be reduced in IBD patients.[205] It has been suggested that inherited mutations (NOD2 and IL23R) affecting mucosal composition in turn alter the composition of gut bacteria in contact with the mucosa. This in turn impairs the ability of the mucosa to protect the gut, allowing opportunistic bacteria to colonise the mucosa leading to a further increase in the immune response and chronic inflammation. Defects in the genes SLC22A5A, GPR35 or GPR65 which are involved in sensing bacterial metabolites could also be responsible for triggering an inappropriate immune

response. Genetic susceptibility to bacterial dysbiosis has been demonstrated in colitis mouse models deficient in the NOD like receptor and pyrin domain 6 (NLRP6) genes.[61] However, to put the role of genetic variants into context, Jostins *et al.* 2012 have identified 163 susceptibility loci for IBD,[61] which have been calculated to contribute to only 33% of genetic susceptibility in Crohn's disease and 16% in UC.[206] Although there is good evidence that NOD2, autophagy, and Th17 immune responses have strong links to Crohn's disease pathogenesis, it is important to note that these susceptible loci only contribute around 14% of total disease variance.[61] However, much of this genetic evidence still points towards a key role between the host immune response and the gut microbiota.

Genetic variants do not explain why some people within a family develop IBD while others do not. When exploring the aetiology of Crohn's disease it is essential to consider the context of a complex microbial milieu, rather than individual species, and how changes in the environment might drive changes which activate an inflammatory state. Bacteria associated with compromised gut mucosal barriers often consist of mixed communities,[207] and future research studies need to try and build study designs and models of analysis which take these multiple factors into account.

1.9.4 The microbiome and innate immunity

The gut microbiome is a hub of signalling connecting input from the diet, with genetically controlled immune signals to control metabolic processes, as well as innate immune regulation of pathogens. Both haematopoietic and non-haematopoietic innate immune cells are located at the host/microbiome interface, where they can sense micro-organisms and metabolites, which can in turn be translated into an appropriate host physiological response to regulate the microbial milieu. Dysfunction in this communication network between the innate immune system and the gut microbiota might play a role in the aetiology of a number of complex diseases including Crohn's disease.

1.9.5 The role of diet in shaping the gut microbiota

One of the most important factors which impact the human gut microbiota composition are dietary choices, a factor which has been shown to drive the evolution of microbiome composition in mammals.[208] Colonisation of the gut microbiota is established very quickly in the newborn gut, reaching adult concentrations within only a few days. Subsequent microbiota development is then usually characterised by an increase in bacterial diversity until around three years of age.[209] The process from colonisation to a stable microbiota in homoeostasis is not well understood; however it appears control mechanisms, particularly related to innate immunity, control the establishment of the normal commensal microbiota which in turn acts to preclude

further colonisation by exogenous bacteria. This might suggest that the primary inoculum after birth and initial microbial exposure are very important to development of a healthy gut.

For the first six months of life, human milk is regarded as the optimal diet for infant health, growth, and development, however for a number of reasons around 80% of babies have been either fed fully or partially with formula milk.[210] A study has shown that the microbiota of six breastfed babies was predominately made up from *Bifidobacterium*, while six babies fed formula milk generally had a more diverse microbiota with lower abundance of *Bifidobacterium* and higher levels of bacteria such as *Escherichia coli, Clostridium difficile, Bacteroides* and *Lactobacillus* that have the potential to act as pathogens.[210] Another study looking at the gut bacteria of 3-month old babies found higher levels of *Clostridium cluster XVIII, Lachnospiracea incertae sedis, Streptococcus, Enterococcus*, and *Veillonella* in the formula fed group.[211]

A number of studies have also shown how changing the diet of healthy people can alter the gut microbiota. In a controlled feeding study of ten adults, microbiota composition changed within 24-hours of taking a high-fat/low-fibre or low-fat/high-fibre diet, affecting ratios of *Bacteroides, Prevotella,* and Firmicutes. Interestingly the enterotype remained stable for the 10-days of dietary intervention, suggesting that enterotypes are more stable in the longer-term, and that short-term changes may have less impact.[212] A strict vegan or vegetarian diet has been shown to have significantly lower counts of *Bacteroides* spp. (p=0.001), *Bifidobacterium* spp.(p=0.002), *Escherichia coli* (p=0.006) and *Enterobacteriaceae* spp.(p=0.008) compared with an omnivorous diet.[213] Although the relationship between diet and the gut microbiota is complex it has become a key target for therapeutic intervention in IBD.

In the last 20-years, interest in using nutritional therapy to induce remission of active Crohn's disease has increased.[214] Recent studies investigating the suggested mechanisms of action of enteral nutrition in Crohn's disease, have highlighted the importance of interactions between nutritional therapy and the host gut microbiota.

1.9.6 Changes in gut bacteria during exclusive enteral nutrition

The idea that gut bacteria could be altered using diet, is a particularly important area of research in paediatric Crohn's disease. Treatment with corticosteroids can reduce the speed of growth in children, making dietary alternatives attractive as a strategy to induce disease remission, while also achieving optimum growth.

Although no specific diet has yet been identified which could directly induce, prevent, or cure IBD, it is becoming more evident that the interaction between nutrition, nutrients, and the gut microbiota have a role to play in disease aetiology. The therapeutic effect of EEN in treating Crohn's disease suggests that diet plays a crucial role in this disease. However, to set up large scale dietary studies in humans is very challenging

and hence the amount of data linking diet with IBD is limited. Most gut microbiota studies into the effects of EEN have been carried out on children and usually with small sample sizes. Comparing these studies is also difficult due to variation in methods such as type of formula; inclusion of non-EEN foods; percentage of EEN used and method of delivery (oral/NGT). Also variation in sample type (stool/mucosal); gut site of sample collection; inter-patient variation; geographical and genetic variation; as well as differences in how samples were handled and processed for analysis, all contribute to making comparison and interpretation of data challenging.

However as the role of gut bacteria in IBD has become more established, some interesting patterns have emerged from gut microbiota studies looking into the effect of EEN. In the past decade a number of studies, both human and animal, have looked at changes in the gut microbiota before, during and after EEN (p197, Table 5.10).

In 2005, Lionetti *et al.*[215] was the first to report on the modulating effect EEN had on the gut bacteria of children with Crohn's disease. They used temperature gradient gel electrophoresis (TGGE) to visualise 16S rRNA bands from faecal samples taken from nine children with active Crohn's disease undergoing treatment with 8-weeks EEN and five healthy controls. They reported that EEN could be characterised by changes in banding patterns in all patients whereas healthy children were stable over time; although they only show evidence for four of their patients and one healthy child. They also failed to provide any data or formal analysis of bands and hence their results were somewhat subjective. In a similar study, Leach *et al.* 2008[216] using 16s rRNA and denaturing gradient gel electrophoresis (DGGE) on stool samples taken from six children with Crohn's disease, showed that the percentage similarity was significantly lower ($p<0.05$) before and after 8-weeks of EEN, than an 8-week duration in healthy children for Eubacteria; *Bifidobacteria*; *Bacteroides* and two *Clostridium* species. The study did find a strong positive correlation ($r^2=0.738$, $p=0.028$) between the percentage of *Bacteroides/Prevotella* group during EEN with a change in paediatric Crohn's disease activity index (ΔPCDAI). They also recorded a correlation between faecal calprotectin levels and PCDAI ($R=0.529$, $p=0.002$), but the strength of the correlation might suggest the (PCDAI) scores were not entirely representative of gut inflammation. It was also interesting to note that healthy children had only between 59% to 84% similarity in these bacteria groups, suggesting the gut microbiota of healthy children is variable over time. However, as DGGE bands can only give an estimate of diversity, some of this variance might be due to the method used.

Research had begun to identify differences in specific groups of bacteria in children with Crohn's disease, thus studies started to ask questions about whether specific bacteria changed during an elemental diet of EEN (028 Extra), and if these changes could be linked with disease remission after EEN. Particular emphasis was given to the Firmicutes phylum, especially *Faecalibacterium prausnitzii* which had been shown to be reduced in active Crohn's disease.[217] Interestingly, one study showed

that in twenty adults with Crohn's disease levels of one strain of *F. prausnitzii* A2-165, decreased significantly after treatment on 2-weeks of EEN compared to baseline, while levels of another strain *F. prausnitzii* M21/2 decreased without significance (p=0.61).[218] Their analysis showed the levels of both *F. prausnitzii* strains in Crohn's patients were significantly lower than those in other patients groups both before and after successful treatment with 2-weeks EEN; hence EEN did not increase levels of *F. prausnitzii* towards that seen in healthy controls.

Using terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16s rRNA, Shiga *et al.* 2012 went on to show a reduction of bacterial diversity in the gut of eight adult patients treated with an elemental formula (Elental®),[219] however results were not statistically significant. They also treated nine patients with total parental nutrition (TPN), a method of feeding fluids into a vein thus bypassing the gut. In these patients there was a significant reduction in bacterial diversity after 8-weeks treatment. Although when applied to complex microbial communities such as the gut microbiota, T-RFLP compresses the output data to around only 20-50 peaks creating both a bias and oversimplification of actual bacterial diversity; the group carried out further qPCR analysis which showed a reduction in *Bacteroides fragilis* after treatment with EEN. It should be noted the reduction in *Bacteroides* after EEN was matched by a non-significant reduction of *Bacteroides* in healthy controls. This was inconsistent with the difference over time seen in other bacterial groups, hence this result could be an experimental anomaly due to storage conditions, or *Bacteroides* numbers could be more variable in the gut of healthy people than other bacteria groups measured.

In 2014, Gerasimidis *et al.*[145] using TTGE and qPCR went on to show that the global bacterial diversity and abundance in fifteen children with Crohn's disease decreased ($p<0.05$) after treatment with EEN. This was accompanied by a reduction in the stability of bacterial composition in the gut in comparison to healthy children ($p<0.01$). *F. prausnitzii* spp. was shown to decrease after 30-days on treatment with EEN ($p<0.05$), a finding supported by an earlier adult study into EEN,[218] and might counter the previously held assumption that *F. prausnitzii* plays a protective anti-inflammatory role in the gut.[217] This paradoxical decrease in *F. prausnitzii* is not yet understood, and a recent mucosal biopsy study on thirty-seven children with Crohn's disease has reported that *F. prausnitzii* is significantly more abundant in the gut mucosa of Crohn's patients than controls (p=0.02).[220] The same study also reported a non-significant trend for decreasing concentrations of *Bifidobacteria* after 4-weeks (p=0.108) and 8-weeks (p=0.120) on EEN which went back to pre-treatment levels once children went back to their normal habitual diet. Gerasimidis *et al.*[145] also found that in children with Crohn's disease who went into remission after treatment with EEN, the bacterial changes were greater and the amount of *Bacteroides/Prevotella* was significantly reduced ($p<0.05$). All these recorded changes along with bacterial

diversity, returned to pre-treatment levels once children returned to their normal habitual diet. In 2015 this group went on to carry out next generation sequencing of these samples.[144] During EEN, bacterial diversity in children with Crohn's disease decreased and the community profile moved further away form healthy children than at baseline. They calculated that for every 10-days on EEN, there was a loss of 0.6 genus diversity equivalents. They also recorded that thirty-four genera had decreased including *F. prausnitzii* while *Lactococcus* increased during EEN.

Lewis *et al.* 2015 evaluated changes in gut microbiota from faecal samples taken at 1-week, 4-weeks and 8-weeks EEN in a Canadian cohort of children with Crohn's disease. The treatment regime was approximately 90% EEN (formula not given) hence some foods were consumed during the diet. They also included a group of twelve children taking partial EEN, around 50% (PEN). Rather than using a disease activity index they chose to use a cut-off in faecal calprotectin below 250mg/g which is less subjective to gauge levels of inflammation. They found that the gut bacteria profile in children treated with EEN changed significantly within 1-week of treatment, moving further away from gut microbiota profile of healthy children (relative to baseline $p=0.005$). The abundance of six out of forty genera were nominally different but this did not reach significance. A similar pattern was not seen among the PEN patients ($p=0.83$) or anti-TNF patients ($p=0.02$). Interestingly they found that children treated with anti-TNF moved closer to healthy gut microbiota profile within 1-week, the opposite of those on EEN.

Although only a single case study, D'argenio *et al.* 2013[221] is the only study to have looked at the mucosal microbiota (ileal) rather than faecal samples. Consistent with faecal studies, when compared to a single control, dysbiosis was characterised by reduced diversity, less Bacteroidetes and increased Proteobacteria. After treatment with EEN the diversity and relative abundance of Bacteroidetes increased and Proteobacteria decreased in-line with control sample levels. In a small study of only four children with active Crohn's taking EEN, Guinet-Charpentier *et al.* 2016[222], reported an increase in abundance of *Alistipes* along with a decrease in the genera *Escherichia-Shigella* ($p<0.01$) and *Sutterella* ($p<0.05$) of the phylum Proteobacteria.

In another small study of five children with Crohn's disease and five controls, Kaakoush *et al.* 2015,[223] also recorded decreased microbial diversity, and that dysbiosis was highly variable in Crohn's disease. In-line with previous studies, after treatment with EEN a further decrease in the number of OTUs and bacterial diversity was seen. They also recorded a correlation between relative abundance of six Firmicutes families (Erysipelotrichaceae, Ruminococcaceae, Lachnospiraceae, Streptococcaceae, Veillonellaceae and Peptostreptococcaceae) with clinical improvements. However there was a lot of variation in changes across these families between children.

Schwerd *et al.* in a small study of eight Crohn's disease patients reported the microbiota was characterised by a reduction in Firmicutes complexity at baseline, but

contrary to other studies, treatment with EEN increased their relative abundance, particularly in the Christensenellaceae.[224] They did however find that EEN decreased the relative abundance of gram negative Bacteroidetes. The number of species (Shannon effective diversity) ranged between 8-50 per faecal sample and did not change significantly during EEN. This conflicts with Kaakoush *et al.*[223] who reported that EEN induced remission was linked with reduced numbers of OTUs. Although Schwerd claimed to have used a more robust method for filtering OTUs, the small sample size of these studies makes variability a serious problem. This study also saw no significant difference in relative abundance of any single species (OTU) associated with treatment on EEN.[224]

It has been suggested from previous studies that the microbiota profile pre-EEN could identify which children will, or will not, respond to treatment with EEN. In 2016 Dunn *et al.* took faecal samples from ten children with Crohn's disease undergoing EEN and five healthy controls. They investigated this idea by comparing gut microbiota profiles between those children who maintained remission for at least 6-months after EEN, and those who either failed EEN, or failed to sustain remission. Interestingly in those who maintained remission after EEN, the pre-EEN relative abundance of Verrucomicrobia, Firmicutes and Bacteroidetes were similar to healthy controls. However those that failed EEN, or failed to stay in remission for 6-months, had higher abundance of Proteobacteria, a commonly reported aspect of dysbiosis in Crohn's disease.[225;221;226] This study also reported that in patients who failed to respond after treatment with EEN, Proteobacteria saw a further increase. However, studies which look at non-responders to EEN, need to keep in mind that failure to adhere to dietary treatment could influence results. Dunn *et al.* also found that during treatment bacterial diversity decreased in responders while increasing in non-responders. This group went on to use a new Bayesian analytical framework to deal with inter-individual variation, to show that the most common OTUs associated with sustained remission were *Akkermansia muciniphila, Bacteroides (B. fragilis & B. ovatus)*, Lachnospiraceae and Ruminococceae. The OTUs associated with non-response to EEN were from *Bacteroides (including B. plebeius)*, Enterobacteriaceae (*Klebsiella*), and *Prevotella*.[227]

A study using Denaturing Gradient Gel Electrophoresis (DGGE) examined mucosal biopsies before treatment with EEN in six patients with Crohn's disease and again at post-treatment (8-weeks).[228] 16S rRNA was used to generate cluster diagrams of similarity of the bacterial community, which showed not only was the mucosal bacteria different between healthy individuals, but also that in these individuals there was variation at different sections of the gut including between the ileum and colon. In patients with active Crohn's disease a similar pattern of variation was seen between individuals, but without the same normal variation along the length of the colon found in healthy individuals. What was interesting in the six Crohn's disease children was,

after treatment with EEN, this loss of variation across different sites in the gut, returned back to normal colonic variability.[228]

Given that some of these studies record a swing in ratio from Firmicutes towards Bacteroides, it will be interesting to see if future studies which look at changes in gut bacteria during EEN, identify a reversal of this ratio associated with induction of remission in Crohn's disease patients; although the reduction in bacteria such as *F. prausnitzii* seen in the Gerasimidis *et al.* 2014 study[145] suggests that this is not the case. Overall these studies show that EEN induces a rapid and complex change in the gut microbiota profile which can be linked with reduction in clinical markers of inflammation and clinical remission in children with Crohn's disease.

Once remission has been induced using EEN, enteral nutrition given as a supplement to normal diet has been trailed with the aim of keeping children in remission and preventing disease relapse (p23, Table 1.6). However, the role of maintenance enteral nutrition (MEN) has not been well studied in children with Crohn's disease. If induction of remission is driven by gut bacterial changes due to EEN, then the key question would be, can these microbial changes be maintained using MEN to sustain remission?

1.9.7 Changes in gut bacteria during maintenance enteral nutrition

Few studies have gone on to look at the gut microbiota composition in patients with Crohn's disease, who after treatment with EEN, have taken a course of maintenance enteral nutrition (MEN) to try and maintain disease remission. The current study aim is to fill major gaps in our understanding about how diet, and specifically MEN, might alter microbial composition in children with Crohn's disease.

In 2005 Lionetti *et al.*[215] collected faecal samples from nine children with active Crohn's disease who were undergoing EEN using a polymeric formula (Modulen); and five healthy children. After 8-weeks of EEN, children with Crohn's disease returned to their normal habitual diet but continued to supplement 40% of their daily energy intake with the Modulen. The faecal bacteria were analysed by 16S-rRNA PCR and temperature gradient gel electrophoresis (TGGE) with direct visual comparison of band profiles of PCR products. Changes in bacterial composition were seen in the gut of all nine children with Crohn's disease after treatment on EEN, and these changes continued once the child went back onto normal diet, but did stabilise after several months. In healthy children the TGGE results showed a stable bacterial profile over 3-months. A major drawback was that this study did not include a non-MEN control group, making it impossible to tell if MEN had an effect which would also been seen in children returning to normal diet without MEN supplementation. The change in composition is consistent however, with that recorded in an earlier study using TGGE which showed a similar difference in gut bacterial composition in

eight adult Crohn's disease patients with active disease, compared to eight patients in remission.[229]

In 2008 Leach *et al.*[216] carried out a small study to look at faecal samples from six children with Crohn's disease collected at diagnosis, during treatment with EEN and then on MEN for 4-months after treatment; and compared these with seven healthy children, where samples were collected 8-weeks apart. Although using 16S-rRNA DGGE, they showed a significant change in the gut bacteria on EEN, the study was completely underpowered to draw any conclusions about the use of MEN, with a maximum of three children being followed up at 26-weeks for each bacterial group. It was also interesting that in the five children who continued on MEN, although there was a partial return to the bacterial profile found at the start of treatment (40% similarity), the gut bacteria composition was still to some degree altered at 4-months post treatment with EEN. For example a negative correlation between changes in the *C. leptum* group and both changes in PCDAI and faecal calprotectin, suggested that *C. leptum* stability after treatment with EEN, was associated with a reduction in gut inflammation and disease activity.[216] It is worth noting that although five of the children went onto MEN, two of the six began medication on aminosalicylates while two started azathioprine, hence we cannot be sure whether medication contributed to these results.

MEN, if shown to be effective, is an appealing option for maintenance of remission in children with Crohn's disease, since it avoids the negative events associated with drugs such as immunosuppressants and biologics. It is therefore vital to gain an understanding of gut bacterial changes during MEN, in order to show whether MEN is linked with an anti-inflammatory gut composition, and hence had real clinical benefit in reducing inflammation in children with Crohn's disease.

Partial enteral nutrition Although Lewis *et al.* 2015 evaluated changes in gut microbiota from faecal samples taken at 1-week, 4-weeks and 8-weeks EEN they also gave a group of children partial enteral nutrition which made up 53% of the child's daily intake. When they compared the gut bacteria profile in children treated with EEN with those children treated with partial enteral nutrition the same pattern of change was not seen (p=0.83) hence they concluded that either changes to bacterial profile seen during EEN are dose dependant, or that only total removal of normal diet is influencing the microbiota composition during this week of treatment.

1.10 Bacterial fermentation in the gut

As discussed in the previous section, the type of bacteria which ferment fibre have been reported to be reduced in the gut mucosa and faeces of IBD patients. Therefore the products of colonic fermentation in relation to their impact on immunoregulation

in the gut, is a key area of interest, particularly because these metabolites could open a potential new source of therapy for patients.

The key function of bacteria that reside in the colon is fermentation of food that has escaped digestion or absorption in the upper gut. It is a complex process which involves a large number of metabolic processes, products of which can be used by host tissues and cells.[230] The end products of colonic carbohydrate fermentation include organic acids, lactate and short chain fatty acids (SCFA), along with hydrogen, carbon dioxide and methane. The branched-chain fatty acids, iso-butyrate and iso-valerate as well as ammonia, amines and phenols are all products of protein fermentation.[230] The main fatty acids associated with colonic fermentation are given in Table 1.7. Fermentation activity varies throughout the large intestine, with highest production of SCFA and reduced luminal pH in the caecum and ascending colon.[230;231] As the amount of available substrate diminishes in the distal colon, this is where protein fermentation tends to occur, leading to increased branched-chain fatty acids, ammonia and phenols. Here the pH increases towards neutral.

Table 1.7: Short and medium chain fatty acids produced and utilised by gut bacteria

Carbon chain length	Common name	Systematic name	Ester/Salt
Short chain			
C_2	Acetic acid	Ethanoic acid	Acetate
C_3	Propionic acid	Propionic acid	Propionate
C_4	Butyric acid	Butanoic acid	Butyrate
C_5	Valeric acid	Pentanoic acid	Valerate
Branched chain			
i-C_4	Iso-butyric acid	Iso-butanoic acid	Iso-butyrate
i-C_5	Iso-valeric acid	Iso-pentanoic acid	Iso-valerate
Medium chain			
C_6	Caproic acid	Hexanoic acid	Hexanoate
C_7	Enanthic acid	Heptanoic acid	Heptanoate
C_8	Caprylic acid	Octanoic acid	Octanoate

1.10.1 Introduction to short chain fatty acids (SCFA)

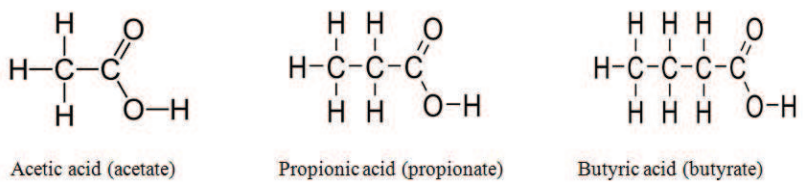


Figure 1.2: Short chain fatty acids (SCFA) are carboxylic acids with aliphatic tails of between 1-6 carbons, with acetate (C2), propionate (C3), and butyrate (C4) being the most abundant in the gut where they are an important source of fuel for colonocytes.

SCFA (Fig. 1.2) are produced via anaerobic fermentation of dietary fibres in the gut,[232] with dietary fibre being defined as 'carbohydrate polymers with three or more

monomeric units, which are neither digested nor absorbed in the small intestine of humans'.[233] A recent metabolic reconstruction, based on data from the Human Microbiome Consortium show that, although there is a great deal of variation in gut microbiota profiles between individuals, the metabolic profile which includes SCFA is more constant.[234] This is because many biochemical pathways are consistent across species and groups of bacteria. For gut bacteria, SCFA are a necessary waste product which are needed to balance reduction-oxidation reactions in the anaerobic environment of the gut lumen.[235] However, the absorption of SCFA as a product of bacterial metabolism provides an additional source of energy to host cells from dietary fibre which the host is unable to digest directly in the small intestine. Around 90% of SCFA which are produced from the digestion of foods, are absorbed in colon, while the remaining are excreted in faeces.[236]

The main products, acetate, propionate and butyrate, are usually found in an approximate ratio of 60:20:20, in the colon and stool of healthy individuals.[230] The production of SCFA depends on abundance and species of gut bacteria present, as well as nutritional availability in the gut for fermentation.[237] Also, depending on dietary composition, the total concentration of SCFA reduce from 70-140mM in the proximal colon to 20-70mM in the distal colon.[238] SCFA are mostly absorbed in the caecum and colon hence only small amounts can be detected in the faeces.[239] Once absorbed into the body via the gut, SCFA are metabolised in tissues; the colonocytes use butyrate as well as some propionate and acetate for energy production and cell maintenance; in the liver hepatocytes metabolise propionate (gluconeogenesis) and acetate (lipogenesis); while muscle cells can oxidise energy from residual acetate.[239] The metabolic effects of SCFA appear to be tissue specific, and their role at particular sites requires further research in humans. SCFA also have a role to play in controlling tight junction proteins, which control permeability of the gut epithelial barrier, thus regulating molecular transport across the gut lumen and hepatic portal.[240]

Acetate (C2) Acetate is the most abundant SCFA found in the gut, being produced by a large range of gut bacteria, mostly from the phylum Firmicutes.[241] The citric acid cycle uses acetate in the form of acetyl co-enzyme A, along with water to reduce NAD+ to NADH in cells. This energy is then utilised in the process of glycolysis to generate pyruvate (Fig. 1.3). According to stable isotope studies acetate contributes around 6-8% of energy expenditure.[242] Acetate is consumed by butyrate and propionate producing bacteria including *Faecalibacterium prausnitzii, Roseburia spp.*, and *Eubacterium spp.*[243;244] *Anaerostipes caccae* has also been shown to utilise acetate to produce butyrate in conjunction with the class of bacteria, Clostridia.[245] The ratio of acetate in faeces is therefore dependant on the ratio of butyrate producing bacteria and fibre availability in the gut.[246]

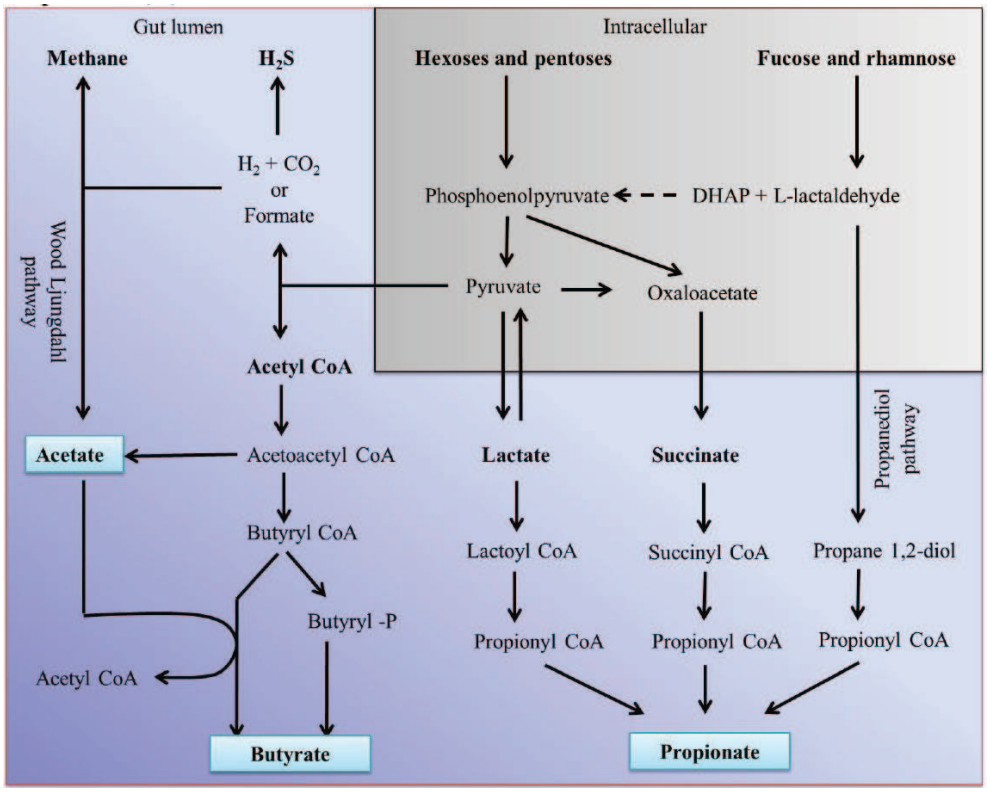


Figure 1.3: Most SCFA are produced via more than one pathway. The pathways represented are the major routes of production. Adapted from Louis *et al.* 2014[247]

Propionate (C3) In non-ruminants, including humans, propionate arises from the β-oxidation of odd chain and branched chain fatty acids. In humans it is relatively minor substrate for gluconeogenesis in the liver compared with ruminants where propionate is the principle gluconeogenic substrate.[248] Propionate can be used in the human gut as a source of energy by colonocytes, but is less efficient than butyrate.

Commensal gut bacteria which produce propionate are less diverse than butyrate producers, with their distribution being dominated by only a few genera. Three pathways (Fig. 1.3) have been described for the production of propionate.[249] The succinate pathway is the main route for hexose conversion to propionate by gram-negative *Bacteroides* as well the class of bacteria Negativicutes (Firmicutes). The acrylate pathway is restricted to members of the Lachnospiraceae family including *Coprococcus catus* and Negativicutes, where lactate is converted to propionate. However, butyrate is the main source of lactate utilisation among gut bacteria.[250] In the propanediol pathway, propionealdehyde dehydrogenase has been shown to convert deoxysugars such as fucose and rhamnose in members of the Lachnospiraceae family including *Ruminococcus obeum* and *Roseburia inulinivorans*.[249]

Butyrate (C4) Butyrate in particular has been identified as being important for maintaining the colonic epithelium, and being the preferred fuel utilised by colonocytes, it is the primary site of butyrate sequestration in the body.[251;252] In 2012 Wang *et al.* carried out a study using a cellular model of the colon, to show that butyrate

was an important regulator of tight junction assembly proteins, increasing intestinal barrier function via increased production of claudin-1, while inducing tight junction proteins, ZO-1 and occludin, redistribution in the cell membrane.[240]

These SCFA, not only provide a source of energy for the host, but also have anti-inflammatory and anti-apoptotic effects that could potentially protect the gut against colitis.[253;254;255;256] A recent 2014 study showed that butyrate could reverse the aberrant expression of ZO-1 as well as decreasing lipopolysaccharide translocation which lead to inhibition of macrophage activation, pro-inflammatory cytokine production and neutrophil infiltration in the liver of rats.[257] Increasing permeability is also linked with translocation of pathogens and antigens potentially triggering the inflammatory cascade. More recently studies have shown that SCFA have a role to play in regulating human immune responses and inflammation. It has been known for some time that butyrate is involved in regulation of the immune system via inhibition of NF-κB activation in macrophages in the lamina propria of patients with UC.[258] In 2010 Manco *et al.*[259] showed that an increase in bacterial lipopolysaccharide triggers a TLR4 mediated pro-inflammatory cascade in monocytes and macrophages. This in turn activates downstream pathways including NF-κB and mitogen-activated protein kinase, leading to cytokine (TNF-α and IL-6) driven inflammation.

The main butyrate producing bacteria in the gut belong to the Firmicutes phylum, with species from the families Ruminococcaceae and Lachnospiraceae being the most abundant.[244;260] The lactate utilizing bacteria such as *Eubacterium* and *Anaerostipes* species also contribute to butyrate production.[244] The Ruminococcaceae species *F. prausnitzii* is reduced in IBD patients, especially those with Crohn's disease, which accompanied by the evidence that *F. prausnitzii* has anti-inflammatory properties has made it of particular research interest. Recently a 15kDa anti-inflammatory protein (MAM) produced by *F. prausnitzii* has been shown to inhibit the NF-κB pathway in intestinal epithelial cells lines, leading to the possible development of new anti-inflammatory drugs for IBD in future.[261]

A few studies have suggested that administration of SCFA could have a positive impact on the treatment of UC[262;263;264;265] and Crohn's disease patients with mild to moderate ileocolonic disease.[266] In 2013, two studies went on to show that butyrate, in a dose dependant manner, promotes regulatory T-cell (T_{reg}) generation through inhibition of histone deacetylase.[267;268] These studies also found that *de novo* T_{reg} cell generation in the periphery, was enhanced by propionate via histone deacetylase inhibition. However acetate lacked this inhibitory functionality.

An *in vitro* study has shown that when T-cells are given butyrate under T_{reg} inducing conditions, acetylation of the Foxp3 promoter region increases along with enhancer elements thus allowing Foxp3 to be expressed. A number of host receptors including G-protein coupled receptors GPCR43 and GPCR109a can sense SCFA.[269;270] GPCR43 expression is specifically increased on T_{reg} within the gut epithelium,[269]

hence loss of GPCR43 or similar receptors could lead to host susceptibility to colonic inflammation. This highlights a possible route for gut bacteria via bacterial metabolites to alter the status of host immunity, theoretically affecting the balance between pro-inflammatory and anti-inflammatory mechanisms.

Valerate (C5) A recent paper looking at the gut bacteria as potent class I histone deacetylase inhibitor, which is primarily involved in cell survival, proliferation and differentiation, suggest that valerate (C5) plays a role in its expression.[271] After screening seventy-nine diverse commensal bacteria for their histone deacetylase inhibitory properties, the three most potent strains were evaluated for specific class I and class II histone deacetylase inhibition. All three were butyrate producing strains, but one also produced substantial levels of valerate (C5) and hexanoic acid (C6). This study identified valerate as a potential contributor to the histone deacetylase inhibitory effect. This *Megasphaera* bacterial strain, was then assessed in a mixed community where it was shown to increase the capacity of the community to produce butyrate and valerate, showing that bacteria via SCFA production have the ability to affect gene expression.[271] The structure of valerate is similar to the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), and a recent study has shown that plant extracted valerate could act as a GABA-agonist in rats.[272]

SCFA in the form mono-valerin and tri-valerin which are esters of valerate, when given as feed additives to chickens have been shown to reduce the colonisation of *Salmonella enteritidis*,[273] as well as reducing the incidence of necrotic enteritis caused by *Clostridium perfringens.*[274]

Branched-chain fatty acids (iC5 and iC4) The branched chain fatty acids, isobutyrate (iC4) and iso-valerate (iC5), are products of protein fermentation, specifically from the amino acids valine and leucine respectively.[275] Ammonia, amino acids, phenolic compounds, and branched chain fatty acid concentrations are higher in the distal colon where substrate sources are more limited and conditions including increased pH, allow protein fermentation to take place.[230;231] The production of isobutyrate (iC4) and iso-valerate (iC5) are correlated, even under changes in diet, a feature that has been replicated across animal models as well as humans.[275] Although products of protein fermentation such as ammonia, have been suggested as possible causes of inflammation in IBD,[276] studies measuring SCFA have not reported an increase in iso-butyrate and iso-valerate (Chapter 6, Table 6.1) suggesting that protein fermentation is not increased in IBD.

Medium chain fatty acids Hexanoate (C6) and Octanoate (C8) The medium chain fatty acid (MCFA) octanoate (caprylic acid) is commercially used as an antimicrobial agent on surfaces in food handling businesses and drinks processing plants,

as well as a disinfectant in health care facilities and schools. A 2010 study using a rabbit model, showed a supplement of octanoate reduced the number of coliform bacteria isolated from the stomach and caecum of rabbits, as well as a reduction in the number of anaerobes isolated from the caecum.[277]

Animal husbandry studies have shown that sub-minimal inhibitory concentrations of the fatty acids propionate (C3), butyrate (C4), octanoate (C8) and hexanoate (C6), significantly alter the invasive capability of *Salmonella typhimurium* in piglets.[278] This suggests that SCFA/MCFA, in optimum concentrations, have a role to play in maintaining bacterial homoeostasis in the gut. Animal husbandry studies have also shown that the MCFA hexanoate and octanoate affect the composition of the intestinal microbiota as well as having inhibitory effects on bacterial concentrations (*Salmonella* and coliform bacteria) in the digestive tract of weaned piglets.[279] Interestingly the inhibitory concentration of MCFA has been shown to be much lower than SCFA such as propionate (C3) and butyrate (C4).[279] It is therefore possible that within the gut microbiota, individual bacteria secrete particular profiles of SCFA/MCFA which work to inhibit the growth of competing species.

In summary SCFA/MCFA have the potential to alter gut barrier integrity, by regulating tight junctions;[240] alter cytokine expression;[280] promote T_{reg} generation;[267;268]; and directly inhibit the growth of potential pathogenic species.[278;279] These properties of fatty acids suggest they might play a key role in driving or maintaining inflammation in patients with IBD. Understanding the ways in which colonic fatty acid profiles differ between patients with IBD and Crohn's disease is therefore a vital area of research.

1.10.2 SCFA levels in IBD and Crohn's disease

The type of bacterial species which ferment dietary fibre to produce SCFA, are typically found to be reduced in the mucosa and faeces of patients with IBD when compared to healthy individuals. Studies have indeed gone on to show that faecal SCFA levels are reduced in patients with active IBD (Chapter 6, Table 6.1).

However, interpreting SCFA from samples has some limitations as the concentrations found in faeces are also affected by a number of confounding factors within the colon; including gut motility; transit time; SCFA absorption and host metabolism. Thus using SCFA as an indicator of bacterial activity in the colon where the majority are quickly absorbed or metabolised in the gut, means faecal levels cannot give an accurate picture of fermentation processes in the proximal colon;[281] such that two individuals with the same level of butyrate in faeces may have different bacterial fermentation and butyrate absorption capacities. Given this limitation, the potential functional differences between IBD patients and healthy individuals make the study of bacterial metabolites, an essential part of future research.

Decreasing levels of butyrate have been linked with lower levels of microbiota diversity in both UC and Crohn's patients.[195] Of SCFA producing bacteria, *Phascolarctobacterium* (Ruminococcaceae) and *Roseburia* have been shown to be reduced in Crohn's disease.[195] Given that Ruminococcaceae metabolise hydrogen to produce acetate, which can in turn be converted by *Roseburia* to butyrate, it makes sense that these interdependent bacterial groups might decrease together in Crohn's disease.[195]

If patients with IBD have impaired SCFA production, this would not only result in reduced energy to colonocytes but also a loss of normal immunoregulation. Understanding the role of SCFA in Crohn's disease could help us to understand why bacterial dysbiosis drives inflammation in these patients, and how modulation of the diet can alter SCFA levels in the gut, creating a pro-inflammatory state. The mechanisms of how SCFA, especially butyrate, regulate inflammation in IBD is still not understood. Hence it is important to try and link SCFA production, the microbiota and diet with inflammatory markers in individual patients in order to understand how these factors affect each other.

1.11 Key concepts of this thesis

It has already been shown that dysbiosis in Crohn's disease includes an increase in the numbers of bacteria with reduced bacterial diversity, a decrease in abundance of Firmicutes and an increase in Proteobacteria and Bacteroidetes. Although it is not the main aim, the current study will test this assumption against healthy children, but also a group of children with non-IBD conditions as well as children with UC.

In addition to exploring these differences in the gut microbiota between healthy individuals and those with Crohn's disease, microbial studies looking at the use of EEN as a treatment for children with Crohn's disease, have given an interesting insight into bacterial changes involved in remission and flare up in Crohn's disease. As well as being underpowered, these earlier studies were limited to using older technologies which limit the number of bacterial groups/species that could be included. The current study aims to fill this gap by using 16s rRNA and Illumina technology to build a wider picture of which bacterial groups, down to species level, are changing during treatment with EEN. This level of detail could give insight into why 1:5 children fail to respond to EEN. It will also provide a better picture of whether EEN works by altering the levels of specific pathogens or rather modifies the global balance of bacteria in the gut. Understanding changes in the gut microbiota composition and bacterial metabolic activity during the use of EEN, will help to increase our understanding of the mechanisms that control inflammation in Crohn's disease.

No previous studies had looked at gut bacterial or metabolite changes in children with Crohn's disease post-EEN who were being treated with MEN. The current study design was set up to explore whether MEN could maintain gut bacterial and metabolite

changes achieved during EEN. This work is of particular importance since MEN is currently being used as a treatment strategy, given as a supplement in addition to normal diet (usually 20% of EAR), with little understanding of how it might work.

Although previous studies have looked at dietary intake in children with Crohn's disease, no study has tried to link diet with the microbiota in the same patients. Thus the aim of the current study is to collect dietary data from children pre- and post-EEN to see if dietary composition can be linked with bacterial and metabolite composition.

Understanding what drives inflammation in Crohn's disease will not only improve delivery of treatment, but allow clinicians to predict which patients are most likely to benefit from enteral nutrition in the form of EEN and MEN, increasing its success rate. Although the successful use of EEN has been well demonstrated it is not used universally across the globe, hence understanding the mechanisms that lead to these distinct changes in gut microbiota composition which improve barrier function, could help to widen its use, as well as develop consistent protocols across centres.

1.11.1 Hypotheses summary

- Children with Crohn's disease have a gut microbiota and bacterial metabolite profile, which is distinct from that of children with UC, non-IBD conditions and healthy children.
- Gut microbiota profiles at baseline can predict which children with Crohn's disease will respond to treatment with EEN.
- Treatment with EEN is associated with changes in gut microbiota and metabolite composition, which is linked with changes in inflammatory status.
- For children in disease remission post-EEN, supplementing return to normal diet with (20%) maintenance enteral nutrition (MEN) can increase remission times; and that MEN helps to maintain gut microbiota and metabolite profiles associated with remission achieved during EEN.
- The profile of dietary intake at baseline for children with Crohn's disease is distinct from children with UC, non-IBD conditions and healthy children. Also that dietary intake in children with Crohn's disease post-EEN is associated with time to disease relapse.

To test these hypotheses, bacteria and metabolites in the form of short/medium chain fatty acids will be extracted from stool samples. Faecal markers of inflammation (calprotectin) and blood markers of inflammation (CRP, ESR, albumin, FBC, LFT) will also be collected. Dietary intake from food frequency questionnaires along with patient characteristics (disease activity, disease location, height, weight, BMI) will also be used (Chapter 2).

2 Recruitment, Sample collection and methods

2.1 Study design

This chapter will describe the method of child recruitment as well as ethical considerations and ethics approval including participant eligibility. It will also set forth how samples were collected; detailed lab methods and the statistical design used to answer the questions proposed in the hypotheses.

This prospective observational study was carried out in collaboration between University of Glasgow and the West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition (WoSPGHaN) network, with a core subspecialty service and endoscopy at Royal Hospital for Children, Glasgow and outreach services with named paediatricians at Forth Valley Royal Hospital, Wishaw General Hospital, University Hospital Crosshouse and the Royal Alexandra Hospital allowing some children to be seen within their own local centre. Within the University of Glasgow the study combined expertise from the Department of Human Nutrition and Glasgow Polyomics Facility, University of Glasgow. The study was approved by the West of Scotland Research Ethics committee and the NHS Greater Glasgow & Clyde Research and Development department to run from 24/6/14 for 4-years (study reference number 14/WS/1004) under the title, Gut Microbial Taxonomy and Metabolism in Paediatric Crohn's Disease during Exclusive and Supplementary Enteral Nutrition using OMICS Technologies (appendix 1). This study is publicly registered with ClinicalTrials.gov under the reference NCT02341248.

Observation, replication, and prediction are fundamental to all sciences. One of the key qualities of a scientist is to be a good observer, recording and reporting observations in a sufficient amount of detail to allow other scientists to replicate the study. Over recent years there has developed a pressure in publication to create shorter articles. It is true that papers can be reduced in size without any fundamental loss of meaning, however this can be more problematic when describing detailed methodology. Sadly, it has become often impossible to replicate studies because of insufficient detail in methods, and contacting authors to request these details can be difficult, particularly where the author is no longer working in academia or has died. Methodologic issues section of journals have to some extent helped to overcome this problem, as well as encouraging discussion around methodologies and facilitating the practice of experimental replication. As such this chapter will aim to provide sufficient detail and discussion around methods used, which should allow replication.

Another problem with biomedical research is that due to improvements in technology; company updates; or current understanding of processes, methodologies are constantly being updated or superseded by newer methods. Thus researchers need to keep themselves up to date with current improvements and make decisions based on

cost and the degree of improvement, about whether the questions they are trying to answer would be better served by moving to newer methods. Although using newer methods can make it difficult to compare across similar studies that have used different methods, hence decisions are sometimes made to use an older method if linking in with other studies is of key importance. Also within existing methodologies it is important that the researcher undertaking lab work understands, and designs experiments that account for the role that experimental variables play in producing data that help to power studies and answer research questions. In this chapter I will try to discuss where possible some of the issues around experiment variables and identify where improvements could be made.

This study is a prospective observational study and as such there is no placebo and no correction for any type of treatment bias. Observational studies lack the aspect of randomised allocation of a treatment as it would be unethical to withhold treatment that is known to be effective. Hence the current study is limited to discussing any bias which is observed and trying to understand results in light of these limitations.

2.2 Subjects

2.2.1 Identification and recruitment of participants

Patients were identified from the West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition (WoSPGHaN) network which covers a wide range of areas in the west of Scotland. Children from distant rural or island communities however were not recruited on to the study, as it was impracticable to collect a series of six stool samples over these distances. Three groups of children aged between 3 and 18-years were included in the study (Fig. 2.1).

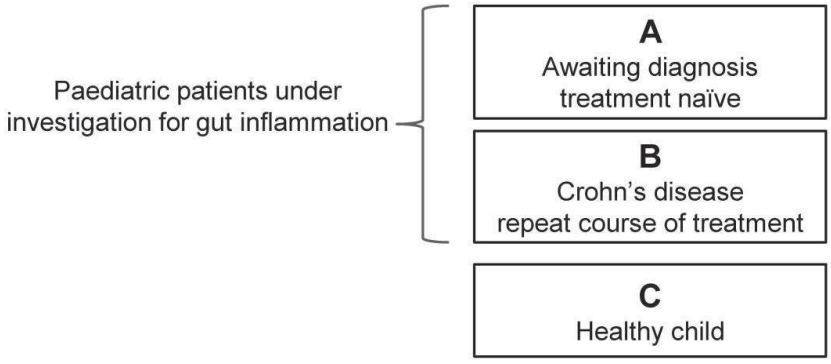


Figure 2.1: Groups of children recruited on to the study.

The first group invited to join the study were children undergoing endoscopic investigation (colonoscopy) to rule out colonic inflammation, including both ulcerative colitis (UC) and Crohn's disease. Only children who received a diagnosis of Crohn's disease then went on to participate in the follow up aspect of this study i.e. those

undergoing treatment with exclusive enteral nutrition (EEN). The second group invited to join the study were children with a previous diagnosis of Crohn's disease who were about to start on an 8-week standard course of treatment with EEN due to disease flare up. The final group invited to join the study were a cohort of age and sex matched healthy children, unrelated to Crohn's disease patients, who were recruited as a control group.

Group A. Children undergoing endoscopic investigation for IBD

Prior to diagnosis This study group were recruited from all children age three to eighteen years old, booked in to have a colonoscopy to investigate possible gut inflammation by a paediatric gastroenterologists at the Royal Hospital for Children, Glasgow. Eligible participants were identified through endoscopy appointment lists with assistance from the paediatric gastroenterologists who were co-investigators of this study. The clinician informed potential participants about the study during their routine appointment and asked them if they would be happy to speak with a researcher after their appointment. If the child and their parent/guardian were agreeable, the researcher met with them after their appointment to explain the study in full, and answer any questions they had. The family were given an information pack which contained a leaflet for the parents and an age appropriate leaflet for the child. If both parent/guardian and the child were willing to take part in the study the researcher requested to collect urine, a faecal sample, bloods if these were already being taken for clinical diagnostic reasons, and eight additional tissue biopsy samples from the gut lining if the patient was having a colonoscopy. We also requested permission to collect relevant medical information, such as information on medication and disease status from their patient notes.

After obtaining informed consent, the parent/guardian and child were asked to complete a food frequency questionnaire (FFQ). They were given a faecal and urine sample kit along with detailed instructions (appendix 2.3) and requested, if possible, to provide a faecal and urine sample prior to their colonoscopy. If unable to provide a sample before colonoscopy, a sample was requested before they started any treatment.

After diagnosis Once children had given the first set of samples and undergone their colonoscopy, only children who had a confirmed diagnosis of Crohn's disease continued on the study. The samples collected from children who were not diagnosed with Crohn's disease, were retained and used as patient control groups made up from those who had UC; and a second non-IBD group who had a range of other conditions. These two groups of children were not requested to give any further samples.

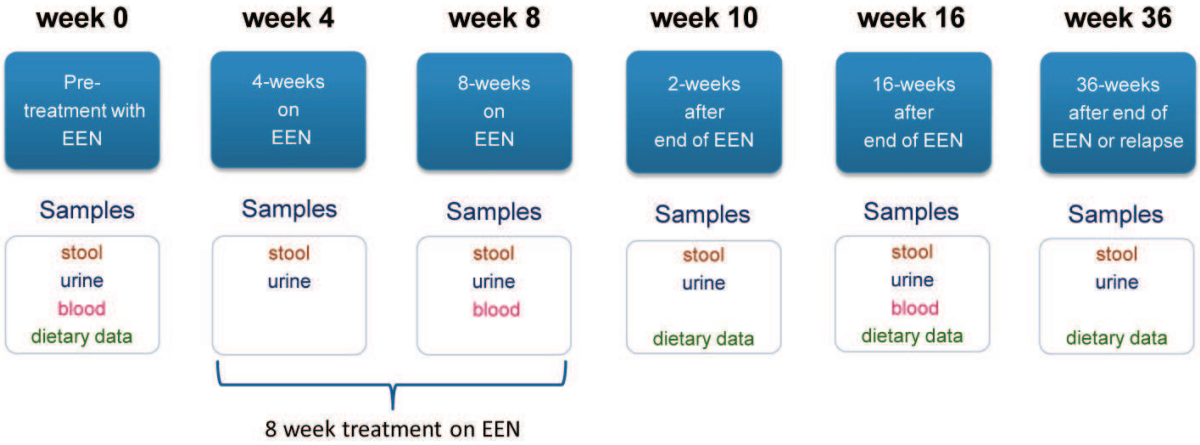


Figure 2.2: The target time points and type of sample collected from children with Crohn's disease undergoing treatment with exclusive enteral nutrition (EEN).

Group B. Children with previous diagnosis of Crohn's disease

Children with a previous diagnosis of Crohn's disease about to start on an 8 week course of treatment with EEN, were recruited from across the West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition (WoSPGHaN) network, including centres at Glasgow, Wishaw, Larbert (Forth Valley) and Crosshouse (Ayrshire). Eligible participants were identified through hospital appointment lists with the assistance of medical staff, including IBD nurses and dietitians. The process of recruitment was as described for group A, except this group of children were not asked to provide any mucosal biopsy samples, since they were not about to undergo a colonoscopy.

Children from patient groups A and B, diagnosed with Crohn's disease and undergoing 8-weeks of treatment with EEN, were asked to provide a total of six faecal and urine samples: at baseline; 2-samples during treatment with EEN and 3-samples post-EEN (Fig. 2.2). A sample of blood was requested during routine clinical blood collection. A food frequency questionnaire (FFQ) was requested at baseline then at three collection points post-EEN. An estimated 3-day food diary, to record detailed dietary intake, was requested at 2-weeks and 8-weeks post-EEN (Fig. 2.2).

Once children in group A and B had completed their treatment with EEN some children opted to take a maintenance form of enteral nutrition (MEN) along with their normal habitual diet. However some chose not to continue with MEN, due to the supplement becoming unpalatable during their exclusive diet or because they saw no benefit from taking it, if they had failed to respond to EEN. Both children who took MEN and those who did not, were retained on the study for the full 36-weeks.

Group C. Healthy children (control group) Healthy children unrelated to Crohn's disease patients, aged between 2 and 17-years were identified and invited to take

part in the study via word of mouth or advertising through posters and leaflets. Where possible, they were recruited from the same geographical areas as our patient cohort. After introducing the study to children and their parent/guardian and giving them age appropriate detailed information leaflets (appendix 2.2), healthy children who consented were requested to give a single faecal and urine sample and complete dietary questionnaires in the form of a 3-day food record and an FFQ.

2.2.2 Consent and ethical considerations

The recruitment strategy we chose followed the tried and tested procedure used for children at Yorkhill in the 'BISCUIT study' (IRAS Ref:09/S0802/24). A clinician informed potential participants about the study during their routine appointment and ask them if they would be happy to speak with a researcher after their appointment. If the child and their parent/guardian were agreeable, the researcher met with them to explain the study in full and answer any questions they might have at this stage.

The parents/guardians were given a detailed information leaflet (appendix 2.1) which was specific for each of the three groups: (A) children prior to diagnosis; (B) children with Crohn's disease; (C) healthy children. Children who were age seven or under were provided with a pictorial form of the information leaflet to help them to understand what the study involved and the researcher or parent/guardian read the simplified age appropriate information leaflet to them (appendix 2.2). The language used in each age appropriate information leaflet was carefully chosen, with the advice of primary school teachers, to be easy to understand yet detailed enough so that children were fully informed about the study. Participants willing to take part in the study were invited to sign a consent form in duplicate: one for the participant and one for the research site file. A third copy of the consent form was placed in the hospital medical notes of all hospital patients who took part. Parents were asked to sign an assent form to confirm they agreed with their child taking part. For children of primary school age it was the parent who signed consent and the child an assent form. If the child was unable to write their own name the parent completed a consent form on their behalf. It was also made clear that they could withdraw from the study at any time without giving a reason. In the interest of participant welfare, an external contact name and phone number was provided to all participants, whom they could contact if they had concerns or wished to raise a complaint.

To ensure the well being and safety of participants the researcher undertook NHS Greater Glasgow & Clyde Good Clinical Practice training courses. Access to patient medical records and storage of sensitive data by the researcher followed the rules governed by the NHS Code of Practice (Scotland) Version 1.0 and current data protection laws.[282] Only the researchers named in the study ethics had access to this information.

2.2.3 Inclusion and exclusion criteria

A health check questionnaire was used to screen patients. Patients were excluded if they had treatment with antibiotics or surgery to remove part of the gut, in the previous 6-weeks, since this would likely alter gut microbiota composition. Healthy children were also excluded if the had any gastrointestinal symptoms in the last 6-weeks.

Medication A note of all medications taken in the last 6-weeks was recorded at baseline as part of the health check questionnaire. Any additional medications as well as those taken as part of standard treatment during the term of the study were recorded from patient notes.

2.3 Treatment with exclusive and maintenance enteral nutrition

This was a prospective observational study and as such children with Crohn's disease who took part in the study underwent a standard course of treatment with EEN to induce disease remission under the care of the West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition team. Normal treatment requires them to complete 8-weeks of exclusive feeding with the polymeric enteral nutritional formula Modulen IBD (Nestle, Switzerland) or in children with cow's milk protein intolerance, Elemental O28 extra (SHS international, Liverpool, UK). The amount given was calculated for each child to meet their estimated average requirement (EAR) for age. This was usually increased by up to 10% for children who were underweight at the time of starting treatment.[283] As per normal treatment, at the end of the 8-weeks children either continued taking enteral nutrition (about 25% of EAR) as a supplement to their normal diet (MEN) or stopped enteral nutrition altogether and went back to their normal habitual diet. The study made no alteration or addition to the child's normal treatment for Crohn's disease, and only collected samples and monitored their progress under normal treatment conditions.

Children who were unable to tolerate EEN orally were given Modulen IBD via nasogastric intubation which involves the insertion of a plastic tube (NG tube) through the nose and throat to reach down into the stomach. The duration from the start of remission to subsequent disease relapse over the course of 28-weeks after treatment was documented for each child with Crohn's disease. Disease relapse was defined as an increase in disease activity resulting in a repeat course of treatment with either EEN or corticosteroids; or a step-up in medical treatment such as biologics or surgery.

2.4 Measurements and samples collected from participants

2.4.1 Anthropometric measures

The height of children was measured using a Seca® Leicester stadiometer (Seca 213, Birmingham, United Kingdom) to the nearest centimetre by the researcher or by nursing staff in clinics. Body weight and body composition were measured where possible with a TANITA® (TBF300, TANITA, Japan) body composition weighing scales; however as this method was not used by nursing staff in clinics, only height, weight and BMI was used to assess anthropometric measures in this study. All children had their height and weight measured at baseline. For children with Crohn's disease measurements were taken at time points: 0-weeks (pre-treatment with EEN); 8-weeks EEN; then 2-weeks and 8-weeks post-EEN.

LMS (lambda, mu, sigma) method software, based on the UK 1990 reference growth standards data, was used to calculate percentiles which were converted to standard deviation scores (SDS), commonly referred to as z-scores.[284] Theses growth standards provide sex and age specific LMS parameters that allow the calculation of z-scores that indicate how close each individual is to the population median. An example of typical conversions for comparison is shown in Table 2.1.

Table 2.1: List of usually used percentile and SDS z-score conversion values

percentile	Z-scores (standard deviation scores)
0.2nd	-3.00
2.3rd	-2.00
2.5th	-1.96
5th	-1.64
15th	-1.04
16th	-1.00
50th (median)	0
84th	+1.00
85th	+1.04
95th	+1.64
97.5th	+1.96
97.7th	+2.00
99.8th	+3.00

In statistics the z-score or standard deviation score (SDS) is the signed number of standard deviations above the mean. See equation (1).

$$\text{z-score or SDS} = \frac{\text{observed value - median value of reference population}}{\text{standard deviation value of reference population}} \quad (1)$$

Interpreting the results for height and weight as z-scores has advantages. Firstly the z-score scale is linear, and thus has a fixed height or weight difference for all children of the same age in the population. In terms of analysis, z-scores make comparison across different age groups possible. Secondly z-scores are produced independently for girls and boys, which means children's growth status reflects differences in both sex and age.

The BMI z-score categories were used to work out whether children were under or over weight as per the NHS National Obesity Observatory (NOO) guidelines:[285] A simple guide to classifying body mass index in children (Table 2.2).

Table 2.2: BMI z-score categories

Catergory	BMI z-score	Centiles
very underweight	$\leq$ -2.67 BMI z-score	($\leq$ 0.4th rounded centile)
low weight	$\leq$ -2 BMI z-score	($\leq$ 2nd rounded centile)
healthy weight	$>$ -2 to $<$ 1.34 BMI z-score	($>$ 2 to $<$ 91st rounded centile)
overweight	$\geq$ 1.34 BMI z-score	($\geq$ 91st rounded centile)
obese	$\geq$ 2 BMI z-score	($\geq$ 98th rounded centile)
extremely obese	$\geq$ 2.67 BMI z-score	($\geq$ 99.6th rounded centile)

NHS NOO guidelines 2011: A simple guide to classifying body mass index in children

2.4.2 Socioeconomic status

The Scottish Index of Multiple Deprivation (SIMD) was used to estimate socioeconomic status.[286] In all, seven indicator 'domains' are used: income, employment, education, health, access to services, crime and housing. These seven domains are then used to create a rank from 1 (most deprived) to 9,976 (least deprived). The resulting 9,976 individual zones are divided into areas of equal population size.[286] To visualise the data, the 6,976 SIMD data zones were subdivided into quintiles (5-bands) using the SIMD rank scores. Each band contains 20% of the data zones, with rank-1 containing the 20% most deprived and rank-5 containing the least deprived zones in Scotland.

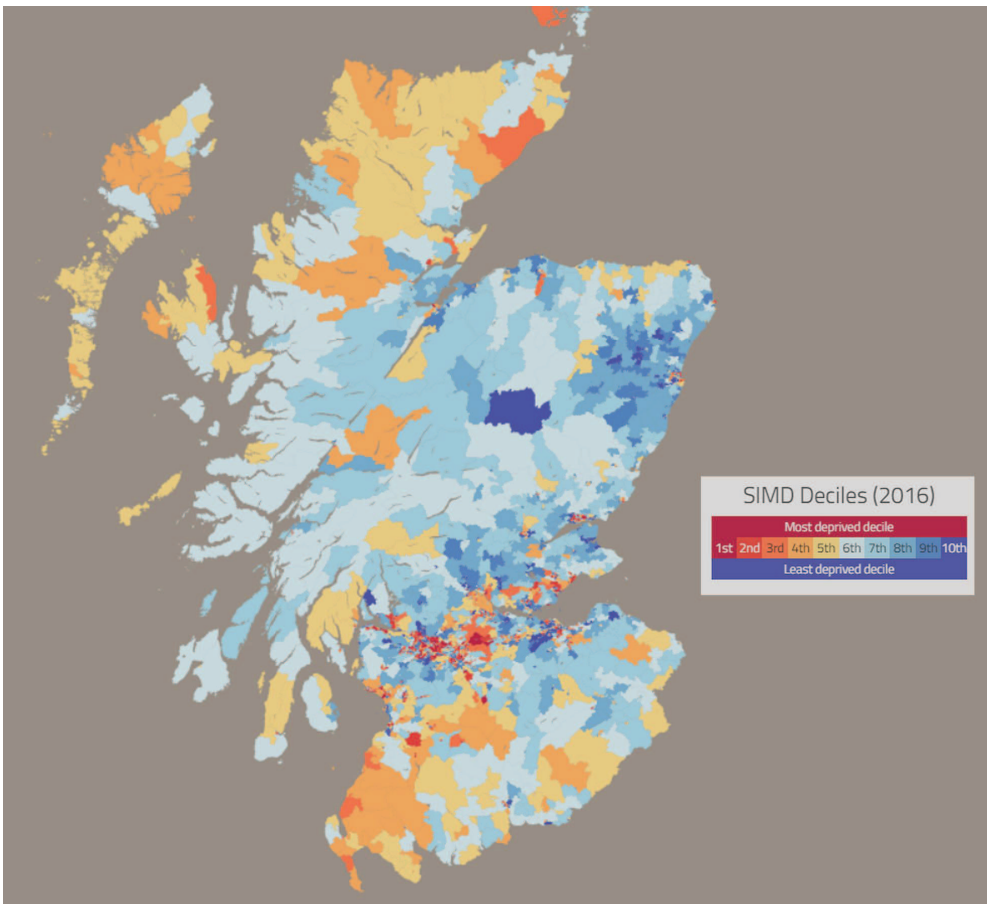


Figure 2.3: The Scottish Index of Multiple Deprivation 2016 by area distribution[286]

The SIMD scores need to be used with some caution. They are not always a clear indicator of deprivation because the difference between two ranks could be very small or quite large. The emphasis of different domains might also vary with some areas having similar scores for different reasons hence making it difficult or inaccurate to associate with health outcomes. Also not everyone who is deprived lives in a deprived area and vice versa, hence in a small study like this one there is some limitation in using the SIMD score to assume the socioeconomic status of any individual child in the study. It is however useful to look at any potential differences between groups of patients and outcomes, because it can help to target resources towards where they are needed, where socioeconomic status plays a role in risk or treatment outcomes.

When the national 2016 SIMD figures are split into the different health boards, they show that Greater Glasgow & Clyde followed by Ayrshire & Arran, then Lanarkshire, have the highest income deprivation and health deprivation in Scotland as well as the highest overall deprivation shown by SIMD scores[286] (Fig. 2.3). As these are the areas this study recruited from our cohort of patients may not be representative of Scotland or the UK as a whole, in terms of socioeconomic status.

2.4.3 Measurement of disease activity and location

Disease activity Although it is by no means a perfect tool, the Paediatric Crohn's Disease Activity Index (PCDAI) is the standard outcome measure in paediatric Crohn's disease research. The inclusion of blood results, a perianal examination, and measuring height velocity can make constructing the PCDAI score difficult, particularly if doing this retrospectively from patient notes. In our study a new weighted version (wPCDAI), now being used by clinicians in West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition network, was used because it has been shown to perform better than the previous PCDAI being more feasible to complete and easier to use in practice. In 2011 Turner *et al.* tested the wPCDAI against the PCDAI for 437 children with Crohn's disease, and concluded that the wPCDAI performed better in construct validity and responsiveness, as well as discriminating better between disease activity categories (ROC: 0.97; 95% CI: 0.95–0.99);[287] with a recent 2017 study confirming these findings.[288] In the current study children with Crohn's disease were assessed for wPCDAI scores at baseline, after 8-weeks of EEN, and 8-weeks post-EEN on normal diet (wPCDAI shown in appendix 3).

Table 2.3: Montreal classification for Crohn's disease (Satsangi *et al.* 2006).[1]

	Montreal
Age at diagnosis	A1 below 17 yrs A2 between 17 and 40 yrs A3 above 40 yrs
Location	L1 ileal L2 colonic L3 ileocolonic L4 isolated upper disease *
Behaviour	B1 non-stricturing, non-penetrating B2 stricturing B3 penetrating p perianal disease modifier †

* L4 is a modifier that can be added to L1-L3.
† is added to B1-B3 when concomitant perianal disease is present.

Disease location Crohn's disease is typically characterised by non-continuous inflammation which can effect the entire gastrointestinal system. The Montreal classification[1] was used to categorise disease location in children with Crohn's disease. Although not the most recent classification system, the small number of patients with Crohn's disease in our study limit the research use of the newer Paris disease classification sub-sets,[19] as these would reduce the power of our data sets. The problem with using disease extent in the classification system is that due to the dynamic nature of Crohn's disease, there is instability of disease extent over time, and children were not given a follow-up colonoscopy unless their condition deteriorated (Table 2.3).

2.4.4 Sample collection and processing

Faecal and urine samples: After giving consent, each participant was given a faecal/urine sample collection kit, which contained a pre-weighed stool collection pot, a plastic bag which could be sealed with the pot inside, an anaerobic sachet (Anaerocult® A Merck KGaA 62471 Darmstadt, Germany) and a sterile universal container for urine. The aim was to enclose the pot and limit expose to oxygen to protect anaerobic bacteria. The pack also contained an ice-pack and instruction sheet which included contact details. A potential sample collection day was agreed between the family and the researcher; courtesy calls and reminders were made every few days to provide support and answer any questions. As soon as the sample was ready, the family would call or text the researcher for collection with the aim to collect and have it in the lab within 4-hours. Samples where collected in person by the researcher form a wide range of areas in the west of Scotland (Fig. 2.4).

Figure 2.4: Map shows the distribution of children recruited onto the study. Children with Crohn's disease (green); UC (yellow); Non-IBD (red); healthy (blue).

Mucosal tissue biopsies: In children who were due to have a colonoscopy for diagnostic purposes, and with the consent of participants and their parent/guardian, an additional six mucosal pinch biopsy samples; two from the terminal ileum when possible; two from the ascending colon and two from the descending colon were taken by the gastroenterologist (14-16 are collected at normal colonoscopy for diagnostic purposes). An additional two biopsy samples were also collected from the duodenum during upper endoscopy. These samples were taken during routine biopsy sampling

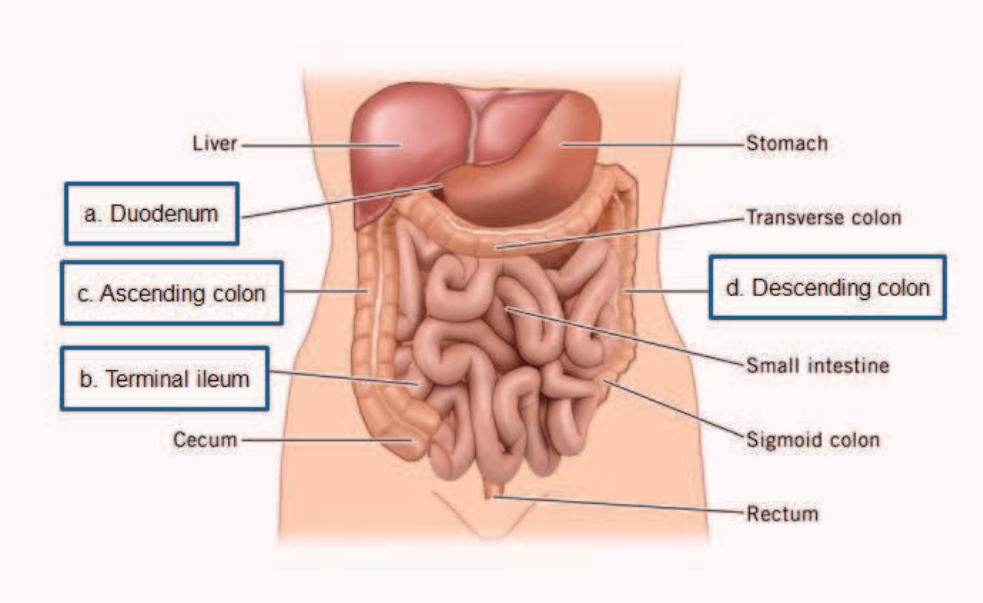


Figure 2.5: Two pinch biopsies were collected from sites highlighted with blue boxes, a) duodenum; b) terminal ileum; c) ascending colon; d) descending colon.

carried out as part of disease diagnosis at the Royal Hospital for Children, Glasgow. Biopsy samples were taken using standard endoscopic forceps by the gastroenterologist and transferred by the researcher from the forceps using a sterile blunt ended needle to a 2mL screw top tube. These were kept on ice for between 1-3 hours before being stored at -80°C. Biopsies were held in a biobank by the department of Human Nutrition to be included in a another study.

Bloods: Bloods were only collected from patient groups (5-8ml) and were only taken if the child was having routine bloods taken as part of their normal treatment. Hence no additional needle insertion was required. If the child had any anxieties about giving routine blood and the nurse felt the additional blood might add anxiety we did not request bloods.

Baseline bloods were normally taken from patients along with diagnostic bloods while the child was under general anaesthetic during their colonoscopy (week-0). The child was fasted and had taken an age appropriate dose of Picolax (sodium picosulfate combined with magnesium citrate) as a bowel emptying preparation for colonoscopy. Subsequent blood samples were not fasted. A blood sample was collected around 8-weeks of EEN during the child's end of treatment review; and after 8-weeks of normal diet post-EEN (Fig. 2.2). Bloods were held in a biobank by the department of Human Nutrition to be included in a another study.

Sample processing

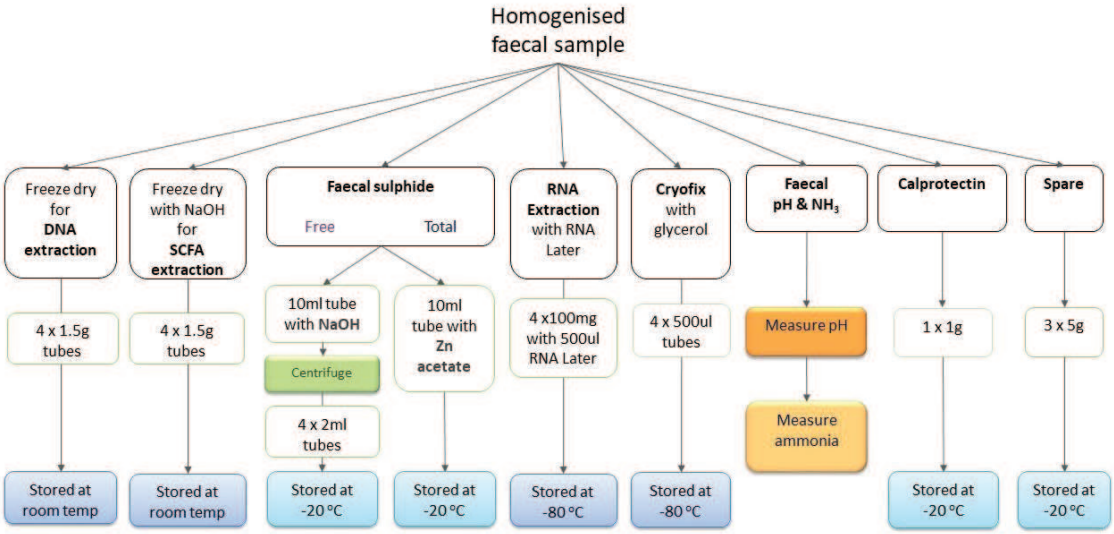


Figure 2.6: Immediately after collection faecal samples were homogenised and processed as shown, before being stored for further analysis.

Faecal and urine samples: To minimise any possible changes in bacterial composition, the aim was to start processing faecal samples within 4-hours after collection. This was not always possible due to travel distances and the collection of multiple samples. The mean elapsed time between production (telephone call) and arrival of sample for laboratory processing is shown in (Table 2.4).

Table 2.4: Time between stool sample production and lab processing

	group			
	CD	UC	non-IBD	HC
mean number of hours	05:31	03:09	03:28	02:39
standard deviation	02:15	02:13	03:24	01:16
<4-hours (%)	70%	83%	60%	78%

On arrival in the lab the sample was weighed and then homogenised using a sterile blender in a class-2 laminar flow cabinet. A summary of sample processing is given in Figure 2.6. 1.5g of homogenised faecal sample was weighed into a 5mL bijoux in quadruplicate and immediately stored at -80°C. 1g of faecal sample was weighed into 5mL bijoux containing five glass beads in quadruplicate. 1M NaOH (1:1 w/v) was added to samples to stabilise volatile short chain fatty acids such as acetate and stored at -80°C. These samples were then freeze dried within 4-weeks as described in section 2.7.1.

Bloods: After bloods were taken by the nurse they were decanted in two 4mL blood tubes, one containing K_2EDTA and one with lithium heparin. These were stored in a cool bag on ice and immediately transferred to the lab, where they were centrifuged at 3 000g for 15-minutes (4°C). The plasma from each tube was split into three eppendorf tubes and the remaining red blood cells transferred into a another eppendorf tube. These were stored at -80°C.

Data on clinical markers C-reactive protein (CRP); erythrocyte sedimentation rate (ESR); serum albumin; as well as full blood count (FBC); liver function test (LFT) and urea and electrolytes (U/Es) were obtained from routine clinical lab results.

Although blood, urine, and mucosal biopsies were collected, these were not used in the current project. These will be analysed as part of an ongoing wider research extension to this study by the department of Human Nutrition, University of Glasgow.

2.5 Collection of dietary information

This study is based on the relationship between the effect of diet in the form of enteral nutrition (EEN and MEN) and the outcome it has on gut inflammation and whether a positive outcome has a direct effect on changes in the gut bacteria profile. It was therefore necessary to gather additional information about the child's dietary intake at different time points over the course of the study, both before and after treatment with EEN to determine if differences in diet could be correlated with changes in the gut microbiota and inflammatory status of children with Crohn's Disease (Fig. 2.7). An ideal record of food intake should measure the true intake during the period of study to create a valid dietary record which is a comprehensive and precise record of all food consumption on recorded days. Due to the complexity of human behaviour this is actually quite difficult to achieve.

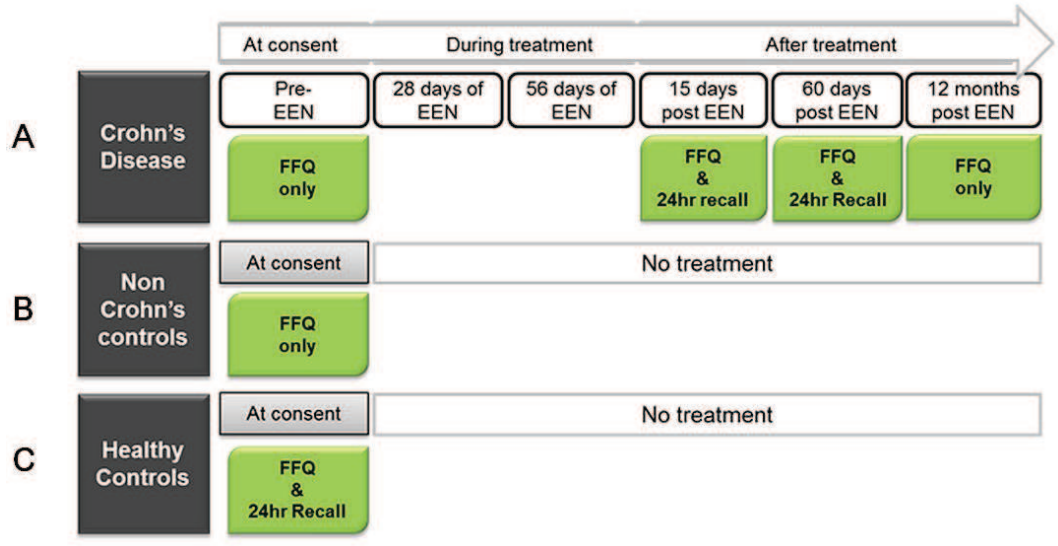


Figure 2.7: Dietary questionnaire time points. 24-hour recalls were replaced with 3-day food diaries.

After reviewing a number of methods we decided to use the five-step multiple pass 24-hour dietary recall questionnaire to collect data on the actual diet at the time faecal and urine samples were collected. A dietary recall is a retrospective method of dietary assessment whereby an individual is interviewed about their food and beverage consumption during a defined period of time, typically the previous day or the preceding 24-hours. Recall of intake over a longer time period is problematic due to the limitations of memory. Several national surveys use the 24-hour recall method because of its high response rate and its ability to obtain detailed information. The interview can be carried out in person, by telephone or increasingly via the Internet. The Norwegian arm of the EPIC software study showed no significant differences in dietary data between face to face (n=102) and telephone 24-hour recalls (n=103).[289] We planned to take this record on three separate days over the period of one week (two week days and one weekend day).

However after 2-weeks of starting to use this method it became apparent that the burden on patients of completing this questionnaire was greater than had been anticipated. Firstly many children had after school activities and it was difficult to find 3-evenings they were free to complete it. Secondly, on testing this method on adults, the questionnaire took between 15-25 minutes; however in practice telephone interviews often did not work well because parents/guardians found it difficult to answer questions unless their child was sitting with them, being unsure what children had eaten at school or away from home. The children found it difficult to report their own eating habits, because they often did not have the skills to describe food or portion sizes without parental help. The discussion between parents and children resulted in the average time to conduct the interview being 45-minutes, much longer than anticipated. Also a number a parents had very young children at home making demands on them and interviews were thus interrupted or ended before completion. It was not possible to visit the homes of children three times per week to carry out these interviews and after listening to feedback from patients, and a problem with non-compliance, it was decided to switch to a 3-day food record (Appendix 7.1.1) which could be filled out at the families convenience. Hence although we had chosen the best scientific method, in the context of young children, busy adolescents and parents coming to terms with chronic IBD, the time burden was too much for most families.

2.5.1 Food frequency questionnaire (FFQ)

The FFQ is a constructed list of foods and drinks with a frequency response tick-box section for participants to report how often each item was consumed over a specific period of time. Participants were shown pictures of portion sizes and asked to count larger portions as two incidences rather than one. Calculations for nutrient intake were then estimated by the diet survey team at the University of Aberdeen. These

calculations multiply the reported frequency of each food eaten by the amount of nutrient in that serving of food.[290]

FFQs are good when measuring habitual intake which can be variable from day-to-day and are relatively easy for participants to complete, as well as being low cost. Craig et al. 2010 compared the child FFQ with a 4 day food diary in 150 children. Children age 12-16 filled out their own questionnaire while parents completed it for those age 3-11 years. The study found that Spearman rank correlation coefficients were significant ($p<0.05$) for all nutrients, with the exception of energy, total fat and vitamins C and E, in the FFQ for children aged 12-16. Although the ranking agreement was best in the 3-11 age group, absolute intakes agreed best between the two methods for children in the 12-16 age group. Therefore the FFQ is a useful tool to look at generalised differences between groups.[290]

It does however have some drawbacks. It is a retrospective method that relies on the participant having a reasonably good memory and it is not very sensitive to measures of absolute intake for specific nutrients. Also the way foods are grouped might not make sense for some participants or may not contain foods popular with families originating from other countries or who choose to shop outside of mainstream UK stores. These factors might lead to inaccurate data if not taken into account.[290]

Paper copies of the Scottish Collaborative Group validated FFQ (Appendix 7.1.2) provided by the University of Aberdeen were used. These include around 150 commonly-eaten types of UK food or drink which are grouped into 19 sections.[291] Two versions were used: the children's FFQ which is designed for children aged 3-11 and is normally completed by the parent. The second version of the children's FFQ used is designed for children aged 12-17. It has additional foods such as coffee and alcoholic drinks, and is completed by the young person themselves. An FFQ was given to each participant both patients and healthy children at the start of the study. The researcher explained the FFQ in detail paying particular attention to the portion sizes shown on the front of the questionnaire (Appendix 7.1.2) and explaining how to record this in the tick boxes. They were also prompted to think about hidden foods in composite foods such as milk and sugar in tea; or butter in sandwiches. They were asked to record the foods and drinks they had consumed in the previous 2-weeks. An FFQ was also collected 2-weeks, 8-weeks and 28-weeks post-EEN (Fig. 2.7).

2.5.2 3-Day dietary records

It was decided in the current study to avoid using weighed food diaries, because it is known that asking participants to weigh food can alter the behaviour of the participant.[292] Weighing food takes extra time thus, subconsciously or consciously, participants may choose a meal that is easier to weigh to save time. There is also the problem that asking participants to weigh food makes them consider food types and

portion sizes that might be considered to be socially desirable.[292] This can lead to an inaccurate record of the actual 'normal' habitual diet. After researching different methods the current study chose to use the 5-step multi-pass 24-recall which has been shown to be an excellent method to help the participant recall all food consumed by using prompts which remind the participant to recall drinks and snacks out-with normal meal times, or forgotten items such as sugar added to hot drinks.[293] Participants were asked if the researcher could call them on the telephone on two typical weekdays and one weekend day to conduct the interview. For the first five recruited patients three refused to give dietary information over the phone as they felt it was either too inconvenient, or could not find time due to other activities. Another had difficulty completing the interviews due to the needs of young children in the home. The other interview went very well. Given the low level of participation in 3-day food records over the phone, and after listening to feedback from participants, it was decided to change the method to a 3-day written diary, which had the effect of increasing compliance, as participants could complete it at times more suitable to their own individual needs. An introductory instruction sheet based on the 5-day multi-pass interview was given to participants along with the dietary record, to try and prompt participants to remember snacks (Appendix 7.1.1).

Three-day food records were requested from participants once they had completed treatment with EEN and had returned to habitual diet for 2-weeks; then 8-weeks; and at disease relapse or seven months after completing treatment with EEN, whichever came first. Three day food records were not requested prior to the start of treatment, firstly because patients were being asked to fast as part of their preparation for colonoscopy, and secondly because a large number of patients started treatment within a few days of recruitment and hence would not have sufficient time to record three separate days before starting enteral nutrition either as the eight week course of EEN treatment or as 'tasters'in preparation for EEN.

Many people after filling out an FFQ failed to complete and return the 3-day food record. Compliance was so poor it was decided not to include these in the study.

2.6 Detailed lab methods

2.6.1 Stool sample consistency as a measure of disease activity

Bristol Stool Chart The Bristol stool chart was used to record the consistency of faecal samples before they were processed for analysis. It is a system of seven categories often used by health professionals to classify the form of human faecal samples. It was developed in 1997 at the University of Bristol[294] as proxy measure of colonic transit time. Although it has been shown that it has limited use in terms of measuring transit time,[295] it is a useful research tool to evaluate the effectiveness of treatments used to reduce inflammation in the gut.[296] Type 1 and 2 represent

constipation while 3 and 4 are ideal stools which are easy to pass. 5 & 6 contain excess liquid tending towards severe diarrhoea at type 7 (Fig. 2.8).

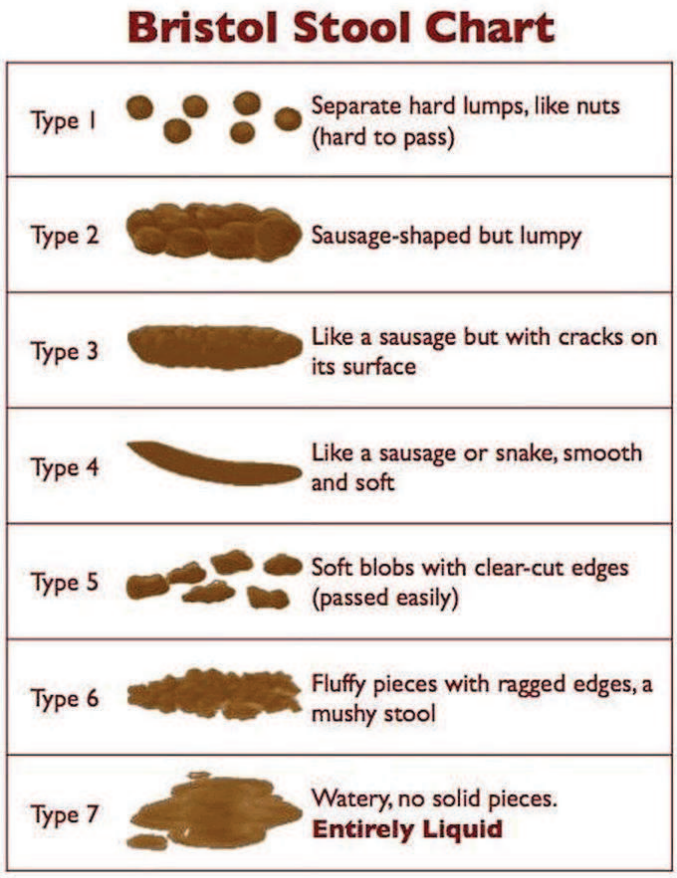


Figure 2.8: Bristol Stool Chart: Types 1 and 2 show constipation; 3 and 4 are ideal stools with 5, 6 and 7 tending towards diarrhoea.

2.6.2 Faecal calprotectin as a marker of disease activity

Faecal calprotectin was measured using the commercial ELISA kit CALP-0170 (CalproLab^{TM}, CALPRO, Lysaker, Norway) according to the manufacturer's instructions.

Sample extraction: Previously homogenised frozen (-20°C) faecal samples where thawed at room temperature and ~100mg weighed into 15mL corning tubes using an inoculation loop. The loop was left inside the tube as an agitator. The manufacturer's extract buffer was added (w/v 1:50) and the samples vortexed before being shaken for 45-minutes on an orbital shaker (IKA VIBRAX VXR BASIC) at 1000rpm. Samples were then centrifuged at 3,000g for 5-minutes at 4°C before 1.8mL of the supernatant transferred to 2mL eppendorfs. These extracts were stored at -20°C until used.

ELISA: On the day of analysis, extracts were thawed at room temperature and centrifuged at 3,000g for 5-minutes at 4°C. The extracts were then diluted 1:100 in the manufacturer's dilution buffer, vortexed and then 100μL added to wells in

duplicate. A set of six standard dilutions (0, 7.8, 31.3, 62.5, 125, 500) along with a high and low control sample were set up as per the manufacturer instructions and also added to the plate along with a negative control. The plate was incubated on a plate shaker (500rpm) in the dark for 45-minutes at room temperature then washed five times with 300μL of manufacturer's wash buffer. 100μL of manufacturer's ALP enzyme conjugate was then added to wells using a multi-channel pipette (reverse pipetting) and again incubated on a plate shaker (500rpm) in the dark for 45-minutes at room temperature. Solution was removed by inverting the plate, tapping dry and then washed five times with 300μL of manufacturer's wash buffer. Finally 100μL of pNPP enzyme substrate solution was added to wells and incubated in the dark for 30-minutes at room temperature before adding 100μL of 1M NaOH to stop the reaction. The OD values at 405nm were then read on an ELISA reader (ThermoLab Systems Multiskan Spectrum). The concentration of calprotectin in faecal samples was calculated with reference to the standard curve (4-parameter logistic non-linear regression model) and expressed as mg/kg of dry faecal material.

2.6.3 Faecal pH

Faecal pH was measured from a 1:3 w/v faecal slurry: 1g of homogenised faecal sample was suspended in 3mL of distilled water with four glass beads. The slurry was vortexed until completely dispersed. Measurement of pH was performed using an auto-calibrated portable digital pH meter (Hanna HI98140, Portugal).

2.6.4 Faecal ammonia (NH3)

Faecal ammonia was measured from the 1:3 w/v faecal slurry used for measurement of pH. This slurry was further diluted with water to 1:500. 10mL of slurry was then passed through a 0.22μL filter (Millex®GP, Millipore, USA cat. SLGP033RB) into a glass vial. This was used to obtain a blank measurement on the ammonia analyser (Hannah Electrical HI93715; Hannah manual.pdf) before adding 4-drops each of the proprietary reagents (Hannah HI-93715-01: Nessler method). The vial was inverted to mix reagents and the measure of ammonia (NH3) recorded as mg/L (ppm).

2.7 Measuring short chain fatty acids (SCFA)

2.7.1 Sample preparation and storage

Short chain fatty acids (SCFA) are the major end product of bacterial metabolism in the colon, and are associated with nutritional benefit for colonocytes and the host in general. Due to the importance of SCFA it was important to use an efficient, economical, and sensitive method to measure the range of short and medium chain

fatty acids in a large number of faecal samples. SCFA (C2-C8) and branched chain fatty acids (BCFA) iso-butyrate, iso-valerate, and iso-hexanoate (Table 2.5) were extracted and then measured using gas chromatography.[297]

Principle of extraction The pKa of acetic acid is 4.8 (other SCFA are similar) which means at 4.8 the ratio of the acid or anion (salt) form is equal. In their natural acidic form SCFA are volatile. However the base sodium hydroxide (NaOH) can be added to faecal samples to lock SCFA in their ionic form (salt) to prevent loss from the sample. To then unlock the SCFA the addition of a strong acid such as orthophosphoric acid decreases the pH to less than 1; and at this pH more than 99% of SCFA return to their acidic form. SCFA can then be extracted using the organic solvent diethyl ether which forms two phases when mixed with faecal slurry. The acidic form which is miscible in ether can then be extracted for analysis using Gas chromatography. Although this method recovers close to 100% of SCFA an internal standard (2-ethyl-butyrate) is added to faecal samples prior to extraction to account for any possible loss.[297]

Stabilisation of SCFA for storage Approximately 1g of faecal sample was added to a bijou tube containing 4 glass beads along with an equal volume of 1M NaOH w/v. The tube was vortexed until the sample was fully homogenised. Samples were then stored at -80°C until freeze dried and analysed.

Freeze drying faecal samples Faecal samples for short chain fatty acid analysis were removed from -80°C and holes made in the lids of sample tubes using syringe needles. The samples were returned to -80°C for one hour and then freeze dried (Edwards apparatus Micro Modulyo, Thermo Scientific®) for 36-hours. Fresh lids were placed on freeze dried samples after they had been homogenised with a sterile wooden spatula. Sealed tubes were stored at room temperature with a silica gel desiccant until further analysis.

Determination of water content Water content was determined by weighing samples before and after freeze drying. Once faecal sample dry weight was known, water content was then expressed as percentage of water per mass of stool sample less the weight of added NaOH.

SCFA extraction 100mg of freeze dried faecal sample was weighed out into 15mL corning tubes (Corning®, Mexico, USA). The sample was homoginised with 300µL of distilled water by vortexing. 100µL of orthophosphoric acid and 100µL of 2-ethyl butyric acid (as internal standard) was added to samples. 1.5mL di-ethyl ether was added to the tube and vortexed on an orbital shaker (IKA® Vibrax Orbital Shaker)

at 1200rpm for one minute. The upper ether phase was removed to a separate 15mL corning tube and the process repeated 3 times. The extracted supernatant was then transferred to 1.5mL glass vials (Agilent technologies®, USA, cat. 5181-3375) and sealed with silicone rubber seal crimp tops to avoid evaporation(Fisher scientific®, UK, cat. 11588150) in preparation for gas-chromatography.

SCFA standards Gas chromatograph does not give an equimolar response to samples being measured so an external standard to quantify the amount of SCFA in samples was used. An external set of 11-standards (Table 2.5) was extracted with 6 dilutions (10, 25, 50,100, 200, and 300μL) using the same protocol as for the unknown samples to obtain the retention times and draw quantification calibration curves. The concentration of individual acids used to construct standard curves were previously optimised by Laurentin and Edwards (2004).[297]

Table 2.5: Concentration of external standards used in SCFA extraction

no. of carbons	acid name	concentration (mmol/L)
2	Acetic acid	183.50
3	Propionic acid	134.52
4	Butyric acid	111.74
5	Valeric acid	89.92
6	Hexanoic acid	80.12
7	Heptanoic acid	68.53
8	Octanoic acid	57.59
4	Iso-butyric acid	104.22
5	Iso-valeric acid	85.51
6	Iso-hexanoic acid	52.41

Concentrations were based on those previously optimised by Laurentin & Edwards.[297]

2.7.2 Gas chromatography

A TRACE 2000 gas chromatograph (GC) (ThermoQuest Ltd, Manchester, UK) using a flame ionisation detector (250°C) and a Zebron ZB-Wax capillary column (15m x 0.53mm idx1μm film thickness), (Cat. 7EK-G007 Phenomenex, Cheshire, UK). Nitrogen set at a flow rate of 1.89cc/min was used as the carrier gas. 1μL of sample was auto injected at 230°C onto the column. The column temperature was held at 80°C for one minute ramping 15°C/min to a final oven temp of 210°C (Fig 2.9).

Samples were analysed on the GC using 32 bit Chrom-card (version 2.2 2003 Thermo-Scientific®, Milan Italy). Individual peaks were identified based on the retention times calculated from the analysis of known external standards (Table 2.5). To check the column prior to sample analysis 100% ether containing only the internal standard (sample blank) was run and the chromatogram checked for any potential contamination in the column or ether. The needle was washed with ether and absolute

methanol between each sample.

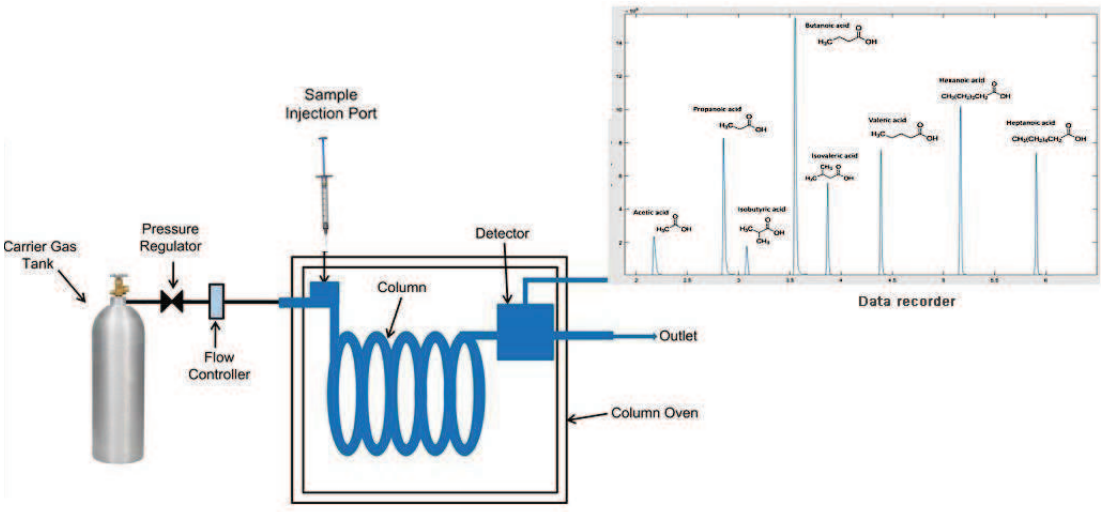


Figure 2.9: In gas chromatography (GC) sample is injected in and vaporised (210°C under pressure); it then flows through a column with a carrier gas (nitrogen). Via a mobile and static phase the sample separates into components. The detector then generates peaks on a chromatogram to identify and quantify SCFA.

Each sample was extracted in duplicate but as separate extractions and in reverse order to the previous extraction to account for any evaporation over time of volatile SCFA. If the variance between duplicates was low the mean between these was used. Samples with high variance were re-extracted. A standard control sample was included at the beginning and the end of the run to test repeatability of the extraction over time. The 100μL dilution of external standard was run after every 12-samples to test the coefficient of covariance. Measurement of SCFA concentration were based on the area under the curve calculated from chromatogram peaks. The concentration was expressed as μg/g of freeze dried faecal material. The area ratio for each individual SCFA was calculated by taking the area under the curve (AUC) for individual acid and dividing by the AUC for the internal standard.

$$Area\ Ratio = \frac{AUC\ for sample\ SCFA}{AUC\ Internal\ Std}$$

The relative response factor accounts for difference in the area ratios between SCFA in the external and internal standards.

$$Relative\ Response\ Factor = \frac{Area\ Ratio\ of\ SCFA in 100\mu L\ ExtStd}{Conc.\ Each\ SCFA\ in\ sample / Conc.\ Int\ Std\ in\ sample}$$

Finally the concentration of SCFA was calculated using the formula:

$$SCFA\ Conc. = \frac{Area\ Ratio\ of\ Individual\ SCFA}{Relative\ Response\ Factor} \times Conc.\ of\ Internal\ Std$$

2.7.3 Potential limitations of SCFA extraction

Although the extraction of SCFA using diethyl ether measures SCFA concentrations with a good level of accuracy it does have some limitations. Some faecal samples may hive a higher proportion of undigested food remnants. We removed any obvious undigested food items like seeds and thoroughly homogenised the sample with a blender. Diethyl ether is volatile and thus can facilitate the loss of SCFA such as acetate during sample handling and analysis after they are extracted from the sample. We checked and corrected for this by using an internal standard.

2.8 DNA extraction methods

The development of culture independent techniques has been followed by a need for the methods used to determine the microbial composition to be robust. This begins with obtaining good quality and yield of genomic bacterial DNA. Faecal samples contain a mixture of undigested food, mucosa, dead cells, bile, enzymes, and other substances, which can degrade bacterial DNA and inhibit PCR amplification. Thus optimal DNA recovery should take these factors into account and choice of a good DNA extraction method very important. There are a number of easy to use commercial kits available which can be used specifically for extracting DNA from faecal samples (Table 2.6) Although these kits are known to be efficient in extracting bacterial DNA, a number of studies have recorded variation in the quantity and purity of DNA between these kits,[298] which could to some extent explain differences in relative abundance between different studies.[299] There is also controversy about whether the temperature of storage and freezing has a significant[300;301] or minor[302;303] impact on the population of gut bacteria recorded after sequencing. There is also evidence that bacterial profiles change while in storage after as little as 2-months and can alter the amount of specific groups of bacteria such as *Bifidobacteria*.[304] This is a major issue when comparing the findings of different studies, and may play a role in why studies have contradictory findings.[299]

Table 2.6: Commonly used DNA extraction kits for stool samples

Kit	Manufacturer
PowerSoil® DNA Isolation kit	MoBio Laboratories Inc. Carlsbad, USA
Qiagen® DNeasy kit	Qiagen, Hilden Germany
QIAamp® DNA Stool Mini kit	Qiagen, Valencia CA USA
ZR Faecal DNA MiniPrep	Zymo Research Corp. Irvine USA

Faecal samples The chaotropic DNA extraction method[305], combined with bead-beating was chosen as it has a higher yield compared to commercial kits, and when

tested against other methods is better at capturing sub-dominant groups of bacteria.[145] This method had already been used by previous researchers in the same lab[145] allowing us to cross reference and potentially pool data. A number of components in samples cause PCR inhibition including: heme; bilirubins; bile salts and complex carbohydrates. The chaotropic method uses guanidine thiocyanate in extraction process to effectively remove these potential inhibitors. This method was chosen not only due to extensive cleaning and purification steps but because it is good at maintaining the gut microbiota composition because it keeps extracted DNA intact. Previous work has also shown that this method gives the highest yield and good purity of genomic bacterial DNA (on spectrophotometry and agarose gel electrophoresis) when compared to methods such as the QIAamp® DNA Stool Mini Kit, and phenol chloroform method.[298] A summary of reagents used in bacterial DNA extraction along with their concentrations and functions are given in Table 2.7.

Faecal samples were collected and homogenised as previously described. Approximately 200mg faecal sample was stored in 1.5mL screw cap tubes in quadruplicate and immediately stored in -80°C. Before starting DNA extraction, each sample was thawed at room temperature. To avoid variability of extraction between the samples and to maintain efficiency of the researcher, DNA was extracted from a set of twelve samples each time with all samples from the same patient included in the same run. Each set of DNA extractions needed 2-days to complete.

To lyse cells and virus particles in the sample and to prevent activity of RNase and DNase enzymes by denaturing them, 250µL of 4M Guanidine thiocyanate 0.1M Tris-Cl (pH7.5) (Sigma Aldrich,® UK) and 40µL of 10% N-Lauroylsarcosine (Sigma Aldrich,® UK) was added to each sample before being vortexed then centrifuged for 3-seconds at 15,000g. The sample was then incubated at room temperature for 10-minutes. Anionic surfactant, N-Lauroylsarcosine (5%) and 500µL, already prepared in 0.1M Phosphate buffer (pH8.0), was added to the sample. The sample was vortexed and centrifuged for 3-seconds before being incubated for 1-hour at 70°C in a dry bath (Dri-Block Teche, UK). The sample was vortexed at 20-minute intervals, then centrifuged for 3-seconds before adding 750mg of sterile 0.1 mm zirconia glass beads (Biospec Products. USA). Samples were vortexed before being placed on an MP FastPrep®-24 benchtop homogeniser for 2x30 seconds at 6m/s, resting between each burst for 15-seconds to allow cells to cool down. Samples were then placed on ice for 5-minutes before homogenising again for 2x30 sec at 6m/s. The samples were cooled on ice for 5-minutes and centrifuged for 3-seconds before adding 15mg of PVPP powder (Sigma Aldrich® Co) and vortexed upside down to mix the pellet. Samples were then placed on a shaker for 5-minutes (1,000rpm) and centrifuged for 3-minutes at 15,000g (4°C) before recovering the supernatant in sterile 2mL safe-lock tubes.

200mg of TENP buffer was used to wash the pellet by vortexing and then centrifuging for 3-min at 15000g (4°C). The supernatant was recovered in the same tube. This was

repeated 3 times. The supernatant was then centrifuged for 10-minutes at 15,000g (4°C) before being split into two 2mL tubes. An equal volume of isopropanol (v:v) was added to each sample to precipitate DNA and gently mixed by hand before being incubated at room temperature for 10-minutes. Samples were then centrifuged for 5-minutes at 15000g (4°C) and the supernatant discarded. Tubes containing the pellet were inverted and tapped onto absorbent paper until dry and then left to air dry in the hood for 30-minutes. 225μL of 0.1M phosphate buffer (pH 8) and 25μL 5M potassium acetate were added to each sample and tubes placed on an orbital shaker for 5-minutes to dissolve pellet. Samples were then stored overnight at 4°C.

The next day samples were shaken on an orbital shaker for 10-minutes before duplicate samples were combined in to one 2mL tube. Samples were centrifuged for 30-minutes at 15,000 g (4°C) and the supernatant recovered in a new 2mL tube. To remove RNA 5μL of RNAase (RNAse ONE, M426A, Promega®) 10mg/mL was added to each sample. Samples were vortexed, pulsed for 3-seconds to bring contents down, before being incubated for 45-minutes on a shaking dry bath at 37°C. 25μL of 10% SDS (Sigma Aldrich® Co), along with 12.5μL of proteinase K 800units/mL (Sigma Aldrich® Co) were added to the sample to digest proteins which have the potential to inhibit PCR reactions. The samples were vortexed and incubated at 45°C for 2-hours on a shaking dry bath. Samples were then pulsed for 3-seconds before adding 54μL of 3M sodium acetate buffer (S2889, Sigma Aldrich®) and 1mL 100% ethanol (-20°C) which was mixed by inverting. The sample was then kept at -20°C for 1-hour. Samples were then placed on an orbital shaker for 10-minutes and centrifuged for 10-minutes at 15,000g (4°C).

The supernatant was discarded and 240μL DNase/RNase free water added to the pellet and vortexed to dissolve it. 560μL 100% ethanol (stored at -20°C) was added to the pellet and transferred to an orbital shaker for 10-minutes at 1,000rpm. The sample was centrifuged for 10-minutes at 15,000g (4°C) and the supernatant discarded; these washing steps were repeated 3 times. The pellet was then tapped dry on lint-free paper and air dried under a class-2 biological cabinet for 1-hour. Samples were re-suspended in 300μL of TE buffer and after 10-minutes on an orbital shaker, aliquoted to 0.2mL PCR-tubes and stored at -20°C for further analysis. Negative extraction controls were performed using the same method protocols and then evaluated by gel analysis after both PCR amplification and sequencing. The DNA was then measured using Qubit dsDNA broad range assay reagents. See section 2.14.4.

Table 2.7: Reagents used in chaotropic DNA extraction method

Reagent	**Conc.**	**preparation**	**Function**
guanidine thiocyanate	4M	12.37g; ddH_2O 13.5ml; Tris-Cl 1M (pH7.5) 2.6ml	Lyse cells; denature RNase and DNase
N-Lauroylsarcosine	5%	1g; Phosphate buffer 0.1M (pH8) fill to 20ml	anionic detergent
N-Lauroylsarcosine	10%	NLS 1.1g + 19ml ddH_2O	anionic detergent
phosphate buffer	0.1M	Na_2HPO_4 1M with NaH_2PO_4 1M pH8	buffer
Tris-Cl pH7.5 and pH8	1M	Trizma base 12.11g; ddH_2O fill to 100ml	dispersion agent to clean DNA
Sodium Acetate	3M	2.461g; ddH_2O fill to 10ml	buffer (source of monovalent cations)
Polyvinylpyrrolidone		PVPP 15mg per sample	dispersion agent; removes polyphenol (can inhibit PCR)
TE buffer	10mM	Tris-CL (pH8) add 1ml	
TENP buffer	1M 0.5M 1%	Tris-Cl (pH8) 1ml 1mM EDTA (pH8) add 0.8ml 5M NaCl (pH8) add 0.4ml ddH_2O fill to 20ml PVPP add 200mg	dispersion agent to clean DNA
ethanol	70%		precipitate DNA
isopropanol	v/v		precipitate DNA
potassium acetate	5M	salt for isopropanol precipitation of DNA	salt aids precipitation of DNA in ethanol
RNAase	10mg/ml	1mM EDTA (pH8) add 0.8ml	denatures RNA
Sodium Dodecyl Sulphate (SDS)	10%	SDS 10mg in 90ml ddH_2O	strong anionic detergent; denature proteins
Proteinase K	800units/ml	12.5μl per sample	digests proteins

2.9 16S ribosomal RNA gene amplicons

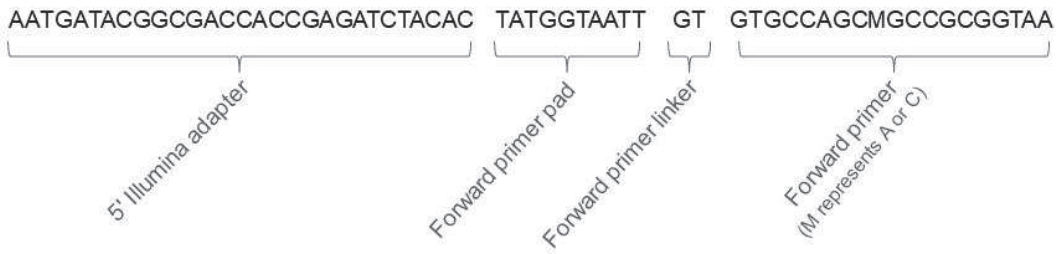
Profiling the gut microbiota can be undertaken by analysing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500bp long. It contains nine variable regions which are inter-spaced between highly conserved regions of DNA. These variable regions can be used in phylogenetic classification down to genus or species level allowing us to profile diverse microbial populations such as those found in the gut microbiota. The choice of 16S rRNA region to sequence is a current area of debate and is influenced by experimental objective; methods used and type of sample being analysed. The current study used 16S rRNA primers covering the variable V4 region (Fig 2.10), which is the most commonly used in microbiota research. V4 not only captures an optimum range of sequence variants, but also results can be reliably compared with similar studies.[306] Di-methyl sulfo-oxide (DMSO) was added to PCR reactions to prevent non-specific primer binding.

A point of note is that the copy number of the 16S rRNA gene per individual species or strain of bacterium is variable. Therefore a sequence of high frequency could either represent, a high copy number per bacterium of lower abundance, or a low copy number per bacterium of higher abundance. Therefore when we report relative abundance of 16S rRNA genes this may not fully represent true community structure.[307]

Although this has the potential to be a significant source of systematic bias in 16S rRNA studies, and there are ways to estimate copy number and correct this bias, doing so creates another potentially more serious bias, since different strains of bacteria have known to have widely varying copy number per bacterial strain. Therefore the correction for copy number was not used in this study as it would simply replace one bias with another.

2.9.1 16S rRNA amplicon sequencing library preparation

To create the amplicon library for paired-end community sequencing on the Illumina MiSeq platform, the V4 region of 16S rRNA was amplified using Golay fusion adaptors with the barcode added to the reverse strand (806R), in combination with the forward degenerate 515F primer (shown below).[306]



Using degenerate primers widens the scope of bacterial species represented by 16S amplicons giving a better representation of the gut bacterial profile.[304] Around 10ng/μL of template was added to each 25μL reaction. PCRs were run in triplicate along with a non template control. PCR components were added as shown in Table 2.8. The HiFi HotStart Kit (KAPABIOSYSTEMS®, USA, KK2502) was used for PCR, and DMSO (SIGMA-Aldrich D1970-5VL) added to improve reaction efficiency.

The reagents for four reactions were mixed in one tube, vortexed, and then 23μL transferred to each of the remaining PCR tubes. 2μL of DNA template was added to three of the PCR tubes the and same volume of water added to the non template control. Ten samples were amplified in each run (i.e. 40 tubes). Samples from disease and control groups were included in each set of ten to account for any environmental variation. All PCR tubes were vortexed and spun down before being placed in the thermal-cycler The samples were amplified as follows: 95°C for 5min; (98°C for 20sec; 60°C for 15sec; 72°C for 1min; for 25 cycles); 4°C ∞.

2.9.2 16S rRNA gel electrophoresis

2g agarose in 200mL 1X TAE buffer was melted in a microwave until clear. Once cooled to 55°C 20μL SYBR® safe DNA gel stain 10,000X in DMSO (Invitrogen®,

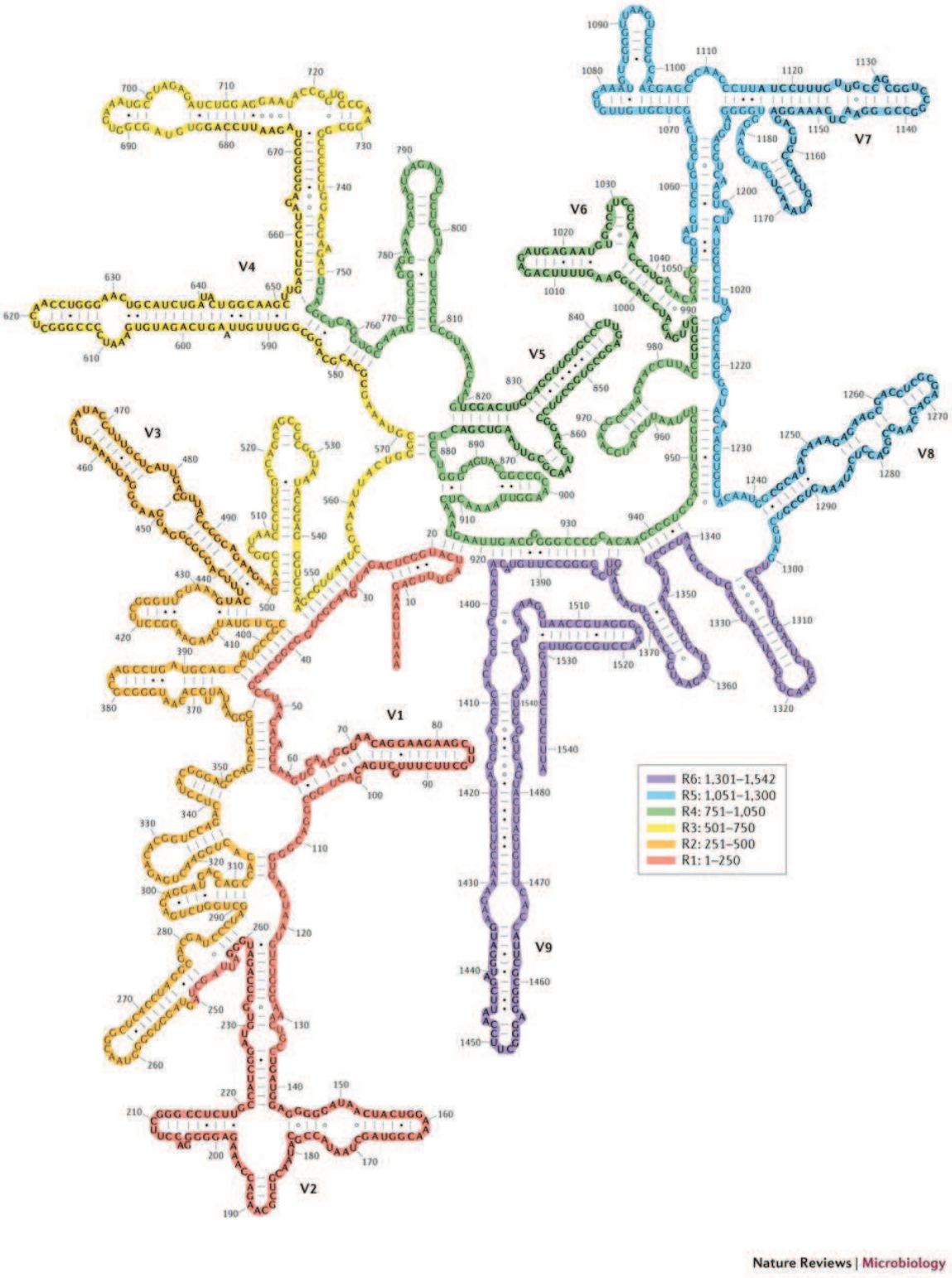


Figure 2.10: Diagram showing the structure and regions of 16s rRNA

Table 2.8: 16S rRNA PCR reagents

reagent	volume per reaction
Nuclease Free water	13.76μL
KAPA HiFi Fidelity buffer (5x)	5.00μL
KAPA dNTP 10mM mix	0.75μL
KAPA HiFi hotstart polymerase	0.50μL
forward Primer (same for all samples)	0.87μL
Reverse Primer (different for each sample)	0.87μL
DMSO(Di-Methyl Sulfo-Oxide)	1.25μL
DNA template (5ng/μL)	2.00μL
Total volume	25.00μL

USA) was added (1μL/10mL). The 1% gel was cast and allowed to cool for 50-minutes in the dark (covered by foil), before being transferred to the electrophoresis tank filled with 1X TAE buffer.

One of the triplicate sample tubes was distributed between the other two sample tubes, and 5μL blue/orange loading dye 6x (Promega Corporation, USA) was added. The dye contains xylene cyanol, bromophenol blue, and orange G to help optimise gel running time by tracking the smaller fragments. A 100bp DNA ladder was used to quantify the base-pair size of DNA template. Three wells were loaded for each sample (non-template control and replicate template). The gel was run for 60-minutes at 100 volts before being visualised under UV light using Gel Doc 2000 (Bio Rad). Each sample resulted in two bands: proximal band containing the amplified sample and a distal band containing residual primers, primer dimers, and any degraded DNA. The non-template control shows only a distal band indicating that only the target template was amplified. If smearing, indistinct bands, absent bands, or multiple bands were seen, these were re-amplified (Fig. 2.11).

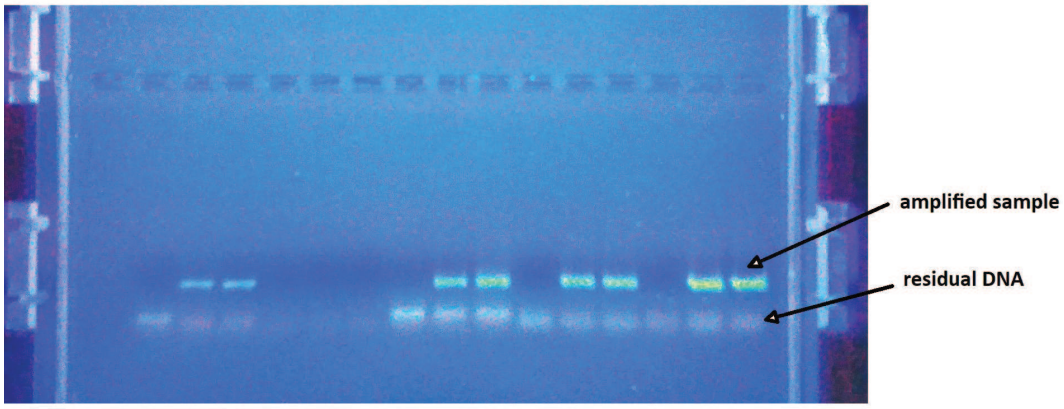


Figure 2.11: Electrophoresis gel showing amplified 16S rRNA bands done in duplicate with a negative control between. The PCR was not always successful due to poor quality samples containing mucus or blood.

Extraction of bands using Zymo-Spin extraction kit Amplicon bands were viewed under UV light (Fig. 2.11) and excised from the gel by cutting bands and placing in 2mL DNA low-bind eppendorf tubes. The Zymoclean Gel DNA recovery kit (ZYMO Research™cat. no. D4002) was used to recovery the target DNA. Gel fragments were weighed and the correct amount of agarose dissolving buffer added to tubes (3:1 w/v). The mixture was then incubated at 55°C for 10 mins in a shaking dry bath (Thriller Peqlab) to dissolve the gel fragment. The melted agarose solution was added to a Zymo-Spin™Column in a collection tube and centrifuged at 10,000g for 1min. The flow-through was then discarded. 200μL of DNA wash buffer containing ethanol was added to the column and centrifuged at 10,000g for 1min. This wash step was repeated and the flow-through again discarded. 12μL of elution buffer was added directly to the column and centrifuged at 10,000g for 1min to recover DNA, which was then stored at -20°C.

Qubit fluorometric quantitation Unlike Nano-drop™, Qubit™is based on the detection of target-specific fluorescence, making it more reliable than UV absorbance for DNA quantification. Hence Qubit™is a better tool for measuring double stranded DNA concentration in preparation for running on the Illumina HiSeq platform. Qubit has the additional advantage of having a range of high sensitivity reagents which measure very small amounts of DNA reliably and accurately.

The concentration of amplicons was measured using a working solution prepared by adding 1μL dsDNA high sensitivity assay reagent with 199μL buffer (Life technologies, USA). Standards were used to calibrate the unit before 1μL of amplicon was mixed with 199μL of the working solution in specialised 500μL PCR tubes supplied with the kit. Any sample with a concentration of less than 2.5ng/μL was re-amplified and extracted until this minimum concentration was reached.

2.9.3 Pooling the amplicons for running on MiSeq

The final DNA concentration of each sample was standardised to 5ng/μL. 3μL from each sample was transferred to a single 0.5mL DNA low-bind eppendorf tube to make an amplicon pool which was then stored at -20°C. DNA concentration of five samples measured by Qubit, selected in random, showed a median concentration of 2.45ng/μL. From this 300μL pool, 100μL was aliquoted into another 0.5 ml DNA lowbind tube and transported to the laboratory for sequencing, in dry ice.

2.9.4 16S rRNA sequencing on MiSeq Illumina

Defining microbial communities by sequencing 16S rRNA genes has grown dramatically due to an increase in lower cost high-throughput instruments. Illumina MiSeq

was chosen for these reasons, along with it's higher accuracy rate.[308] When compared with 454-pyrosequencing, Illumina MiSeq has a lower error rate and is not as prone to insertion/deletion errors in homopolymer stretches. The brightness of light indicates the length of the homopolymer in 454-pyrosequencing hence distinguishing the brightness of light in a sequence AAAAA and AAAAAA can cause errors.[309] Sequencing of the V4 region of the 16S rRNA gene was performed on the MiSeq platform (Illumina, Essex, UK) using our pooled 2x250bp paired-end reads. The TruSeq LT assay was used on FASTQ only to generate FASTQ files.

2.10 Bioinformatics and statistical methods

2.10.1 Creation of 16S rRNA abundance tables

Operational Taxonomic Units (OTU) based approach: *Sickle* v1.200[310] was used to trim and filter Illumina paired-end reads using a sliding window approach. Reads were trimmed when the average base quality fell below twenty. A 10bp length threshold was set below which reads were discarded. *BayesHammer*[311] from *Spades*V2.5.0 assembler was then used as a read error correction tool. *Pandaseq* V(2.4) was used to assemble overlapping pair-end read while correcting mismatches and uncalled bases using a minimum overlap of 50bp to assemble the reads. This workflow has been shown to be effective with substitution errors being reduced in MiSeq datasets by between 77% and 98% (mean 93.2%).[312]

To generate clusters in the form of OTUs the *UPARSE* (v7.0.1001) pipeline was used (url:UPARSE.link) According to research analysis on mock communities carried out by Edgar *et al.* 2013,[313] *UPARSE* generates OTUs that are superior to other frequently used methods, making these OTU sequences accurate predictions of actual biological sequences to represent species.

Briefly reads were pooled together from different samples (barcodes used to track sample origin). Reads were dereplicated and sorted by decreasing abundance while discarding singletons. Reads were then clustered by 97% similarity, discarding those shorter than 32bp. Although the cluster_otu command had removed chimeric reads, a few chimeras may remain, especially if parents are absent from reads or have very low abundance. Hence, a reference-based chimera filter was used from the 'gold database' (ChimeraSlayer reference database in the Broad Microbiome Utilities (url:ChimeraSlayer.link).The original sample barcode reads were then matched with OTUs with 97% similarity and an OTU table generated containing a total of 1,205 unique sequences for all samples.

OTU free approach: As a comparison a more recent method, divisive amplicon denoising algorithm, (DADA2)[314] was used to infer species from the sample sequences

taken from our amplicon sequencing reads. These will be referred to in chapters as single-nucleotide variants (SNVs). DADA2 is a reference free method which does not create OTUs. Rather it infers sample sequences exactly, being able to resolve differences of as little as one nucleotide and thus giving output data of a much higher resolution. The method is built on a model of the errors in Illumina-sequenced reads, and assigning an 'abundance p-value' to unique sequences that have associated abundance, as a 'goodness of fit' criteria to the Poisson density function. Starting by placing all sequences in a single partition, the algorithm iterates by forming new partitions (with their associated Poisson density function) whenever the criteria is not met, and allowing unique sequences to join the partition most likely to have produced them. When the algorithm has converged, all the partitions represent unique variants the central most abundance sequence of each partition as representative sequence. The DADA2 workflow (DADA2.link)`http://benjjneb.github.io/dada2/tutorial.html` learns the error model from the data first, dereplicates the reads and then runs the DADA2 algorithm separately on both forward and reverse reads. The SNVs are then reduced by merging the overlapping reads from both forward and reverse reads. For our data set this generated a SEQ table containing a total of 2,751 unique sequences for samples, more than double that of the OTU table.

The accuracy of the DADA2 approach has been tested on two mock communities and was found to be closer to the expected number of strains than any other methods.[315] At genus level DADA2 was able to identify 25/26 expected taxonomies (Bokulich mock community 1688), with the one missed taxonomy likely being absent from the sequencing data and 18/18 in a second dataset (Bokulich mock community 1685). DADA2 also detected some unexpected genera, all of extremely low abundance (<0.01%) likely to be correctly identified contaminants. DADA2's F-score at the genus level was 0.7659574, which was higher than any other methods tested (0.39-0.54).[315]

The OTUs and SNVs used in the current study were taxonomically classified against a custom database using the package *Qiime* 'assign_taxonomy.py' script workflow.[316] To calculate the phylogenetic distances between sequences, multi-sequences were aligned using multiple sequence alignment program *MAFFT* v7.04.[317] *FastTree* v2.1.7[318] was then used to infer 'approximately-maximum-likelihood' phylogenetic trees from alignments of sequences. Finally 'make_otu_table.py' was used to combine the abundance table with taxonomic information to create a Biological Observation Matrix (BIOM) file for both OTU and SNVs.

2.10.2 Modelling limitations

Describing microbial communities using mathematical models, in which the bacterial population are represented by global empirical functions does not fully describe the

complex functional workings of the human gut microbiota. Sequencing technologies now allow us to record the diversity of the gut microbiota in unprecedented detail. These new data sets can represent a relatively unbiased compositional snapshot, of not only the bacteria present, but also the genes and metabolites that might give insight into the functional activities present in both diseased and healthy gut environments. However, the challenge to translate this empirical data into testable predictions which lead to meaningful research used to improve the quality of life for patients, is challenging.[319]

Because of the way sequence variation differs in terms of defining bacterial species the following issues need to be kept in mind. Firstly the exact same genome can have difference in their sequences, due to multiple copies of the 16S target gene. The DADA2 method can detect these. Secondly two different strains of the same species might have the same sequence in the specific 16S region amplified. It is also possible on some occasions that two different species might have the same sequence in the region we amplified and no method can tell the difference between strains when this is the case. Also two different strains from the same bacterial species can have different sequences in the 16S region amplified and in this case DADA2 can frequently tell these apart, even when they differ by only one nucleotide. However these are usually classed under the same species by the taxonomic classification step.

2.10.3 Statistical methods and graphics

The statistical package **R**[320] was used to carry out statistical analysis and functions. The community ecology package *vegan*[321] and high-throughput microbiome handling package *phloseq*[322] was used for alpha and beta diversity analyses. *phloseq*[322] was used to calculate phylogenetic distances, including unweighted UniFrac and weighted UniFrac distance. More traditional plotting methods such as linear and scatter plots have been replaced by non-metric multidimensional scaling (NMDS) plots where large data sets are compressed into a readily viewable format. This provides an overview of data trends which would be impossible to obtain from data tables. NMDS plots of OTUs and SNVs were generated using the following packages: *phyloseq; vegan; ggplot2; ape* and *phangorn. Phyloseq* is used to organise, link, store, and analyse the phylogenetic sequencing data.

(*Vegan adonis*) was used to carry out permutational multivariate analysis of variance using distance matrices (PERMANOVA) to calculate statistical significance.[323] *Adonis* function fits linear models to distance matrices (Bray-Curtis, Unweighted UniFrac, Weighted UniFrac) and uses a permutation test with pseudo-F ratios to give sources of variation for metadata (it returns R2 values for a predictor, and if significant, $p<0.05$, is the percentage variability in community structure contingent upon the possible observable values of that predictor).

Alpha diversity (within sample) Alpha diversity is the species richness, or number of taxa, present within a single microbial environment or sample; i.e. how many different bacterial species can be detected in a specific patient sample? This seems like a simple question, however it is not always easy to define a bacterial species. Therefore in microbiota analysis operational taxonomic units (OTUs) are often used, where this proxy for 'species' is any bacteria with 97% genetic similarity.

Although an OTU count (richness) gives useful information about diversity it can be misleading because it fails to capture information about species relatedness. For example, sample A may contain three species from the same genus: sample B may contain two species from the same genus and one from another genus; sample C may contain bacteria from three different genera. A simple count would conclude that sample A, B and C had a species count of three, suggesting these three groups were equally diverse which is not really true. Hence using a phylogenetic tree and adding up the branch lengths between different species (or OTUs) in a sample to give a measure of phylogenetic diversity can be more useful. Using a phylogenetic approach would show sample A had the least diversity and sample C had the greatest diversity.

Another drawback with species richness is that it does not take into account the ratio of each species present in a given sample. A bacterial community which is dominated by one or two species is considered less diverse than one where several different species have a similar abundance. The Shannon diversity index[324] can account for both species richness and the evenness of bacterial species present, where species evenness is a measure of the relative abundance of bacterial species.

$$H = -\sum_{i=1}^{S} p_i * \ln p_i$$

H= Shannon diversity index; S= the number of genera; p_i= where p_i is the fraction of total species comprised by species i; [Richness=exp(H); giving estimated number of taxa observed in the rarefied samples]

Beta diversity (between samples) The term β-diversity was first introduced in a 1960 paper by Whittaker[325] where he defined it as "the extent of change in community composition, or degree of community differentiation, in relation to a complex-gradient of environment, or a pattern of environments".[325] He proposed several ways to quantify β-diversity. In its simplest form, β-diversity is defined as the ratio between regional and local diversities and as such for the gut microbiota, β-diversity represents the diversity in microbial community between different groups.

Non-metric (ordinal) distance scaling (NMDS) was used to visualise cluster differences in microbiota data. Bray-Curtis dissimilarity was used to incorporate species abundance counts.[326] Bray-Curtis is used to quantify the compositional dissimilarity between two different groups based on counts within each group. It is simple in that

unlike *unifrac*, it does not make assumptions about the genetic relationship between bacteria.[326] Samples were grouped around the mean ordination value using *vegan ordiellipse*, with the ellipse showing the 95% CI for each grouping. Bacterial community analysis including alpha and beta diversity analyses was done in **R** using *Vegan.*[321] Phylogenetic distances were calculated using *phyloseq.*[322] Pair-wise ANOVA was used to calculate p-values.

Metric distance scaling (MDS) plots were used to analyse metadata such as short chain fatty acids, using the *capscale* function. Data was normalised using autoscaling, before using *vegdist* 'euclidean' to calculate the distance between samples and *adonis* function (PERMANOVA) to test for significance. The *ordiplot* function (ordihull & ordispider) were used to visualise the data.[327]

Permutational Multivariate Analysis of Variance Using Distance Matrices To calculate multivariate homogeneity of groups dispersions between multiple conditions, *Vegan betadisper* function was used. *Betadisper* is a multivariate analogue of Levene's test[328] for homogeneity of variances. *Vegan adonis* was used to carry out permutational multivariate analysis of variance using dissimilarity matrices Bray-Curtis. Adonis analyses and partitions sums of squares using semi-metric and metric dissimilarity matrices of multivariate data sets and hence is directly analogous to the multivariate analysis of variance (MANOVA). Although previously know as a 'non-parametric MANOVA', McArdle and Anderson 2001 coined the term permutational MANOVA (PERMANOVA).[323;329] Because its inputs are linear predictors and it has a response matrix of arbitrary number of columns ranging from two to millions it is a robust choice over both parametric MANOVA and other ordination methods for describing how variation is linked to different treatments or covariates.

Temporal and space–time analysis of community composition data Local contribution to beta diversity (LCBD)[330] was calculated by using the Hellinger transformation to work out total sum of squares of species composition for all samples, from which sample-wise local contributions to β-diversity could be shown as a proportion of total beta diversity. These were plotted as bubbles under stacked bar plots (TAXAplots) to represent marked differences in species composition. ANOVA of LCBD values between different treatment groups was carried out to show significance.

Estimation of microbiota fold change between groups using DESeq2 To test if there was a significant difference in abundance between selected groups or conditions, differential expression analysis *DESeq2* was used in **R**. It is based on the 'Negative Binomial' (Gamma-Poisson) distribution. This method enables a more quantitative analysis which is focused on the strength rather than the presence of differential ex-

pression.[331] Results are expressed as the $\log_2$ fold change between patient/treatment groups and controls. **R** script was adapted from Albertsen.[332]

sPLS-DA Discriminant Analysis Sparse Projection to Latent Structure- Discriminant Analysis (sPLS-DA) was used within **R**'s *mixOmics* package.[333] This procedure constructs artificial latent components of the predicted dataset (genera table denoted as $X(N \times P)$ collated at genus level) and the response variable (denoted as Y with categorical sample data) by factorising these matrices into scores and loading vectors in a new space such that the covariance between the scores of these two matricescov $(X_h a_h, Y_h b_h)$ in this space is maximized under two constraints: $\|a_h\|_2 = 1$; and $\|a_h\|_1 \leq \lambda$, where a_h and b_h are the corresponding loading vectors for X and Y, and h represents the number of components (similar to PCA analysis). The first constraint ensures the loading vector to have unit magnitude (requirement of the procedure) and the second constraint (also called l_1 penalty) to ensure that in features that do not vary between the categories, the corresponding loading vector coefficients go to zero. This is achieved by using the sparsity control parameter λ in the above equation, enforcing shrinkage of loading vector coefficients. The recommendations in *mixOmics* package (http://www.mixomics.org), are to pre-filter 1% of the lowest abundant genera and then perform Total Sum Scaling followed by Centralised Log Ratio (TSS+CLR) normalisation, before applying *splsda*. To predict the number of latent components (associated loading vectors) and the number of discriminants, the *perf.plsda* and *tune.splsda* functions were used, respectively. The model was fine tuned by using leave-one-out cross-validation, by splitting the data into training and testing sets and then finding the classification error rates employing two metrics, overall error rates and balanced error rates (BER), between the predicted latent variables with the centroid of the class labels using the centroid distance. BER accounts for differences in number of samples between different categories.

The BVSTEP routine The *BVSTEP* routine, a method for linking multivariate community structure to environmental variables,[334] was used to search for the highest correlation, in a Mantel test, between dissimilarities of fixed and multivariate datasets. The *BVSTEP* algorithm is part of the *sinkr* package version 0.6.[335] It was used to best correlate dissimilarities (Bray-Curtis distance) of samples using subsets against dissimilarities of the samples given using all the genera. This analysis is complementary to the sPLS-DA which was used to identify which genera are causing major shifts in β-diversity but without considering their grouping (e.g. time points) allowing the identification of the genera that change the most in the sample space.

Kendall rank correlation coefficient Kendall tau test is a non-parametric test for statistical dependence based on the tau coefficient.[336] This was used to examine

correlations between gut microbiota and meta data in the form of a facet-grid using *ggplot2* in **R**. P-values were adjusted for multiple comparison using Benjamini & Hochberg (1995).[337]

Kernel density estimation Kernel density estimation[338] is a non-parametric method used to estimate the probability density function of a random variable i.e. inferences about a population are made, based on smoothing our finite data set.

The grouping of variables was created using pairwise Pearson correlation coefficients. The threshold correlation value for this grouping was set between 0.99 and 0.60 depending on the number of features or variables in different data sets. Under the chosen threshold for each data set, features were grouped into feature-sets. Then for each feature-set a distance based kernel score test and a stratified kernel score test were applied.[338]

A probabilistic PCA (ppca) was used which combines an expectation maximisation (EM) method for PCA with a probabilistic model. The EM method is based on an assumption that the latent variables as well as the noise have normal distribution. By using ppca the function is defined such that the likelihood for data to be far from the training set is lower, even when they are close to the principal subspace, allowing improved estimation accuracy.

Pearson's chi-squared test The Pearson's chi-squared test (χ^2) was used to assess the likelihood that an observed difference between sets of categorical data were due to chance. The Yates' correction[339] which is designed to prevent the overestimation of statistical significance in small data set was not used at it has been shown that the Yates formula has a tendency to over correct the P value, resulting in a type II error. Sokal *et al.* suggest that the Yates' correction can therefore be unhelpful where sample sizes are smaller.[340]

Kruskal Wallis/Dunn's test To look at differences between different groups of patients the Kruskal Wallis test followed by the Dunn's Test of multiple comparisons using rank sums was used. The Dunn's test[341] reports results among multiple pairwise comparisons after a Kruskal-Wallis test for stochastic dominance among groups.[342] The null hypothesis for each pairwise comparison is that the probability of observing a randomly selected value from the 1st group that is larger than a randomly selected value from the 2nd group equals one half. The Dunn's test can be understood as a test for median difference.

3 Participant characteristics

This chapter will provide more detailed information on the disease condition of children, as well as participant demographics including: socioeconomic background, gender and age. For children with Crohn's disease the medications taken before, during and after treatment with exclusive enteral nutrition (EEN) and maintenance enteral nutrition (MEN), and the implications of these will be discussed. The chapter will go on to describe details about disease activity and location of disease within the gut. It will then go on to explore body composition data as an objective measure of how well children were achieving growth targets, as well as assessing if children and study groups as a whole were achieving optimal height and weight for their age. In this section the question of whether EEN as a treatment for children with Crohn's disease has an impact on both short and long term growth outcomes will be studied; as well as asking if low weight or BMI at the start of EEN predicts how successful EEN is in inducing remission in children with Crohn's disease. The study subgroups are examined to judge whether they are a good representation of the Scottish population of children as a whole and identify any bias or limitations, as well as exploring what lessons could be learned from the recruitment process of this study which could benefit the planning of similar studies in future. Finally any limitations in the data set will be explored along with a discussion about the robustness of the data which will be used to help answer questions about how diet, bacterial composition and microbial metabolic status might play a role in disease activity in subsequent chapters.

3.1 Summary of hypotheses

- Children with IBD will have reduced height/weight/BMI at baseline compared to those with non-IBD conditions and healthy controls.
- Increased disease activity in children with Crohn's disease is associated with poorer growth outcomes.
- BMI at baseline in children with Crohn's disease can predict response to treatment with exclusive enteral nutrition (EEN).
- Disease location in children with Crohn's disease can predict response to treatment with EEN.
- For children in disease remission post-EEN, supplementing return to normal diet with (20%) maintenance enteral nutrition (MEN) will maintain the growth velocity associated with growth outcomes achieved during EEN.

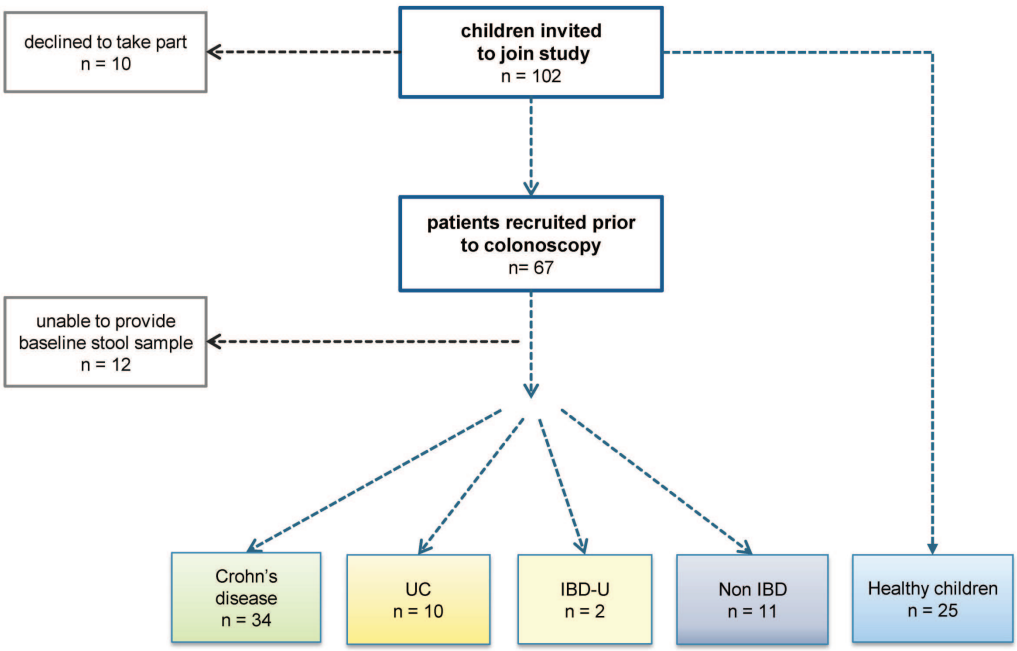


Figure 3.1: Numbers and groups of children recruited onto the study

3.2 Research participants

Of 102 children invited to join the study ten declined to take part (Fig. 3.1). Twenty-five, all of whom gave a stool sample, were a healthy cohort of children with no gastrointestinal symptoms or family history of inflammatory bowel disease (IBD), recruited from the West of Scotland general population. The remaining sixty-seven were children referred to hospital because of suspected IBD. Of these sixty-seven children twelve were unable to provide a baseline stool sample (ten dropped out of the study). After diagnosis thirty-four children were found to have Crohn's disease, ten had ulcerative colitis (UC), two were classified as IBD undetermined (IBD-U) and eleven had other conditions such as irritable bowel syndrome or colonic polyps which were not related to IBD (non-IBD). This non-IBD group were included in the study as a patient control group with the caveat that five of these had a family history of IBD and hence it cannot be ruled out that some of these children could have subclinical IBD. Of the thirty-four children recruited with Crohn's disease two were subsequently excluded from the study: one because they had additional treatment with antibiotics during treatment with EEN which would affect the composition of gut bacteria and the other because they stopped treatment with EEN via nasogastric tube after only 3-days, once the results of their colonoscopy showed very mild disease. Of the twenty-three children who went into remission on EEN, seventeen (74%) children took MEN once they returned to their normal diet, while only six (26%) children opted not to take MEN (Fig. 3.2).

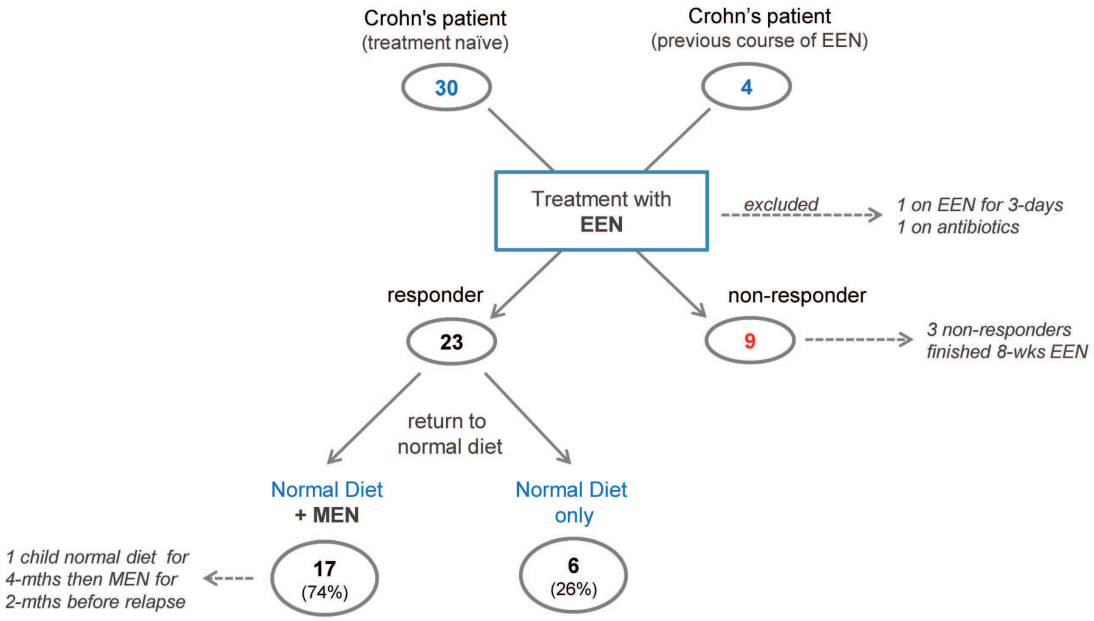


Figure 3.2: Flow chart showing numbers of children on each dietary treatment. EEN- Exclusive enteral nutrition; MEN- maintenance enteral nutrition

Sample collection Samples were collected over a period of 22-months (July 2014 to May 2016). Detailed methods and a summary of faecal, urine and stool sample collection as well as any issues, are described in Methods, section 2.4.4. The number of faecal samples collected for each group and time-point are shown in Table 3.1.

Table 3.1: Number of samples collected for each group

Group	HC	non-IBD	UC	Crohn's disease time-points					
				wk-0	wk-4	wk-8	wk-10	wk-16	wk36
faecal samples	25	11	10	34	24	21	21	23	13
FFQs	21	5	6	22	-	-	12	9	7

HC- healthy controls; UC- ulcerative colitis
FFQ- food frequency questionnaire

3.3 Demographics

Patients who were successfully recruited on to the study all fell within the West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition (WoSPGHaN) network which covers a wide range of areas in the West of Scotland (Methods, section 2.4.4; Fig. 2.4). The healthy control cohort were successfully recruited from the same geographical areas as our patient cohort.

Socioeconomic characteristics The postcode of participants was used to generate Scottish Index of Multiple Deprivation (SIMD) scores. The 6 976 SIMD data zones were subdivided into quintiles (5-ranks) with each rank containing 20% of total

zones (Methods, section 2.4.2). Of the thirty-two children with Crohn's disease who provided samples around 18% came from each deprivation rank giving a very even SIMD distribution, with the exception of Rank-2 containing 28% of children with Crohn's disease (Fig. 3.3). Although 4/10 children with UC (40%) came from the most deprived group (Rank-1), four children with UC (40%) also came from the least deprived areas (Ranks-4 & 5). The control group of eleven non-IBD patients came from all socio-economic backgrounds with a slightly higher proportion (4, 36%) from Rank-3. Of the twenty-five children who were recruited as healthy controls ten (40%) came from the least deprived areas of Scotland (Rank-5) and only three (12%) came from the most deprived areas (Rank-1).

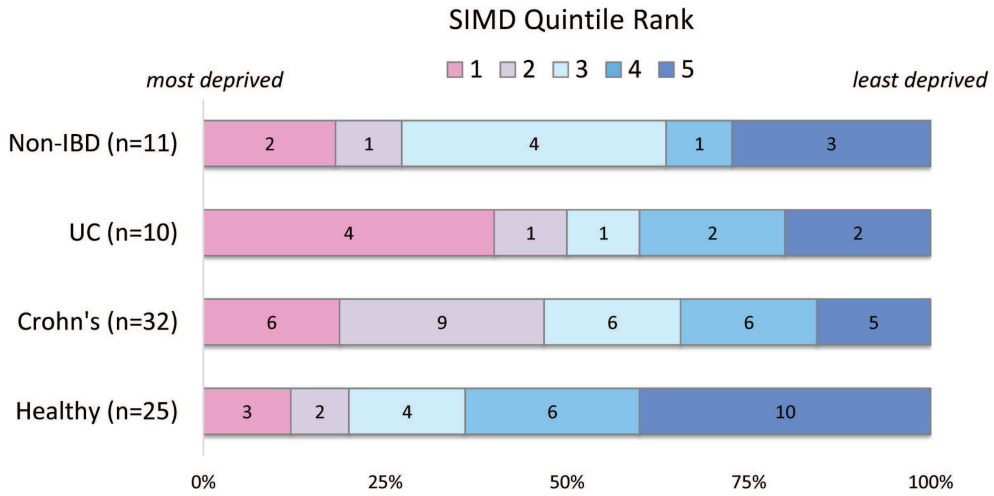


Figure 3.3: Scottish Index of Multiple Deprivation (SIMD) data zones were divided into 5-bands with each band containing 20% of total data zones. Rank-1 (pink) contains the 20% most deprived; rank-5 (blue) contain the 20% least deprived zones in Scotland. The number of children in the 20% band is shown on each band of the bar.

The difference between the median SIMD score for children with Crohn's disease (3454; IQR 3507) and healthy children (5034; IQR 2611) did not reach statistical significance (p=0.12). The group of children with non-IBD conditions (3477; IQR 3308) also came from an even spread of deprivation and were just as likely to come from the least or most deprived areas. The median SIMD score for children with UC (2879; IQR 4404) was not substantially different from other groups (p>0.12).

Gender Of thirty-two children with Crohn's disease nine were female (28%) and twenty-three were male (72%). The gender difference for the twenty-five healthy children was eleven female (44%) and fourteen male (56%). Of the nine children with UC four were female (44%) and five were male (56%). The patient control group of eleven children with non-IBD conditions also had a gender difference of two female (18%) to nine males (82%).

Age The median age for children with UC were, as a group, slightly older than children with Crohn's disease (p=0.24); Non-IBD (p=0.07) and healthy children (p=0.02) (Fig. 3.4).

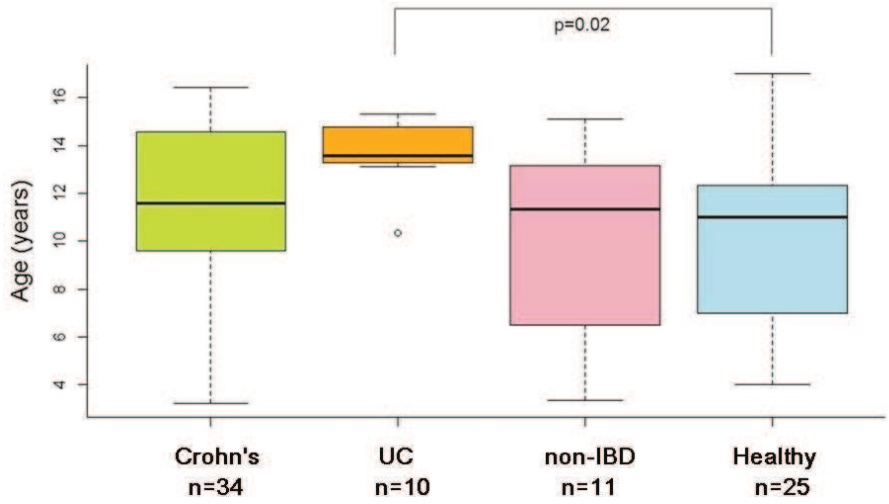


Figure 3.4: The age distribution of each group of children at the start of the study. Median age for children with UC was higher than the three other groups. Difference significant for healthy children. (Dunn's test of multiple comparisons was used following a significant Kruskal-Wallis test).

3.4 Disease characteristics at baseline

3.4.1 Non-IBD participants

The group of children who were found not to have IBD (non-IBD), had a number of different conditions including: no known medical condition; intestinal polyps; irritable bowel syndrome (IBS) and gastritis (Table 3.2). The five children who were not diagnosed with a medical condition all had a first or second degree relative with IBD.

Table 3.2: Diagnosis of patients with non-IBD conditions

diagnosis	no. of children	family history of IBD
no medical condition diagnosed	5	5
intestinal polyps	1	0
irritable bowel syndrome (IBS)	1	0
gastritis/enteritis	2	0
anal fissure	1	0
umbilical hernia	1	0

3.4.2 Disease location in Crohn's disease patients

The Montreal classification was used to subdivide children with Crohn's disease into groups based on disease location (Methods, section 2.4.3). Of the thirty-two children

with Crohn's disease four (13%) had isolated ileal disease (L1); nine (28%) had colonic disease (L2) and nineteen (59%) had both involvement of ileum and colon (L3) (Table 3.3). The overlapping relationship between disease locations in each patient is shown in figure 3.5. Twenty-three (72%) children had upper involvement (L4): three (9%) with orofacial granulomatosis (OFG); nineteen (59%) had gastric involvement and eleven (34%) children had duodenal involvement. Additionally two (6%) children with L3 had perianal disease.

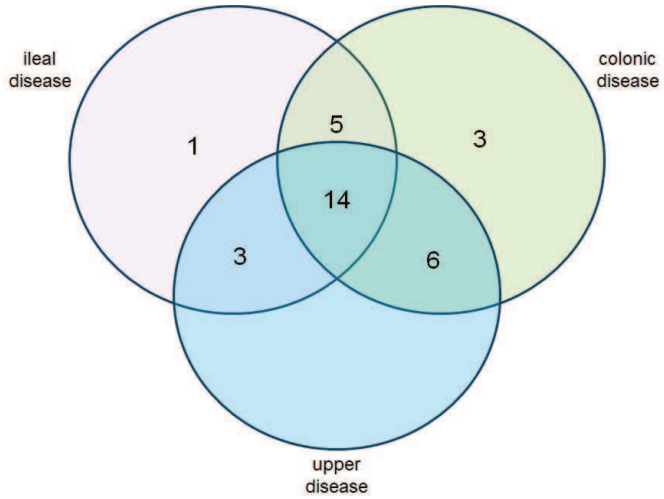


Figure 3.5: Venn diagram showing number of children with ileal, colonic and isolated upper disease. Overlap shows number of children who fall into two or all three disease location categories.

Table 3.3: Montreal classification of disease location of the thirty-two children with Crohn's disease.

	classification of disease distribution	n (%)
L1	terminal ileal +/- limited caecal disease	4 (13)
L2	colonic	9 (28)
L3	ileocolonic	19 (59)
L4*	isolated upper disease*	23 (72)

*may coexist with L1, L2 and L3

3.5 Changes in disease activity during EEN

Disease activity was assessed by clinicians using the weighted paediatric Crohn's disease activity index (wPCDAI)(Methods, section 2.4.3).[287] Scores were calculated for each patient at pre-treatment (week-0); after treatment with EEN (week-8); and 8-weeks normal diet (week-16) (Table 3.4). Disease remission was defined as a wPCDAI score of <12.5. Mild disease between 12.5-40; moderate disease >40-57.5 and severe disease as >57.5.[287]

Table 3.4: Number of children with Crohn's disease in each disease activity category (wPCDAI) before and after treatment with EEN

	Treatment Timepoint					
	week-0 baseline		week-8 end of EEN		week-16 normal diet	
in remission	1	(3%)	23	(72%)	18	(56%)
mild disease	14	(44%)	9	(28%)*	12	(38%)
moderate disease	7	(22%)	0	(0%)	2	(6%)
severe disease	10	(31%)	0	(0%)	0	(0%)

* 6 of these children did not complete the full 8-weeks of EEN. wPCDAI scores: remission <12.5; mild 12.5-40; moderate 40-57.5; severe >57.5

Of the thirty-two children diagnosed with Crohn's disease at baseline, one was in disease remission (wPCDAI score 7.5); fourteen had mild disease; seven had moderate disease and ten had severe disease. Although macroscopically the child in disease remission had mild to moderate disease and histologically mild disease, their calprotectin (inflammatory marker) was high (>1800 mg/kg); hence this child chose to undertake a course of EEN and was included in the study group.

After 8-weeks EEN, 72% of children were in disease remission (wPCDAI <12.5). The remaining 28% had mild disease. Five of these children who failed EEN were in the mild category at the start and end of treatment, however three had reduced their wPCDAI score by the end of EEN. Two children who failed treatment with EEN had stopped treatment after only 1-week. Another four of the children who did not respond well to EEN stopped treatment early; two after 4-weeks and two after 6-weeks. The remaining three children who failed EEN completed the full 8-weeks of treatment before going onto corticosteroids. In total nine children failed to achieve remission on EEN and went onto treatment with corticosteroids.

Of the seventeen children with Crohn's disease who had either moderate or severe disease at the start of treatment, all had reduced disease activity after the 8-weeks of EEN. Of twenty-three children who responded well to treatment with EEN one relapsed 6-weeks after returning to normal diet. Another child relapsed after 10-weeks of normal diet. Two children relapsed within 16-weeks and another two children within 24-weeks (∼6 months). Another two children relapsed within 36-weeks. At the 36-week timepoint (28-weeks of normal diet), fifteen children were still in disease remission. One year after the start of treatment with EEN, only eight of the original twenty-three respondents were still in remission. The number of days to relapse for each patient is shown in figure 3.6.

3.6 Medications taken by children

Of the thirty-two children diagnosed with Crohn's disease twenty-three were medication free at the start of the study (Table 3.5). The child taking an immunosuppressant

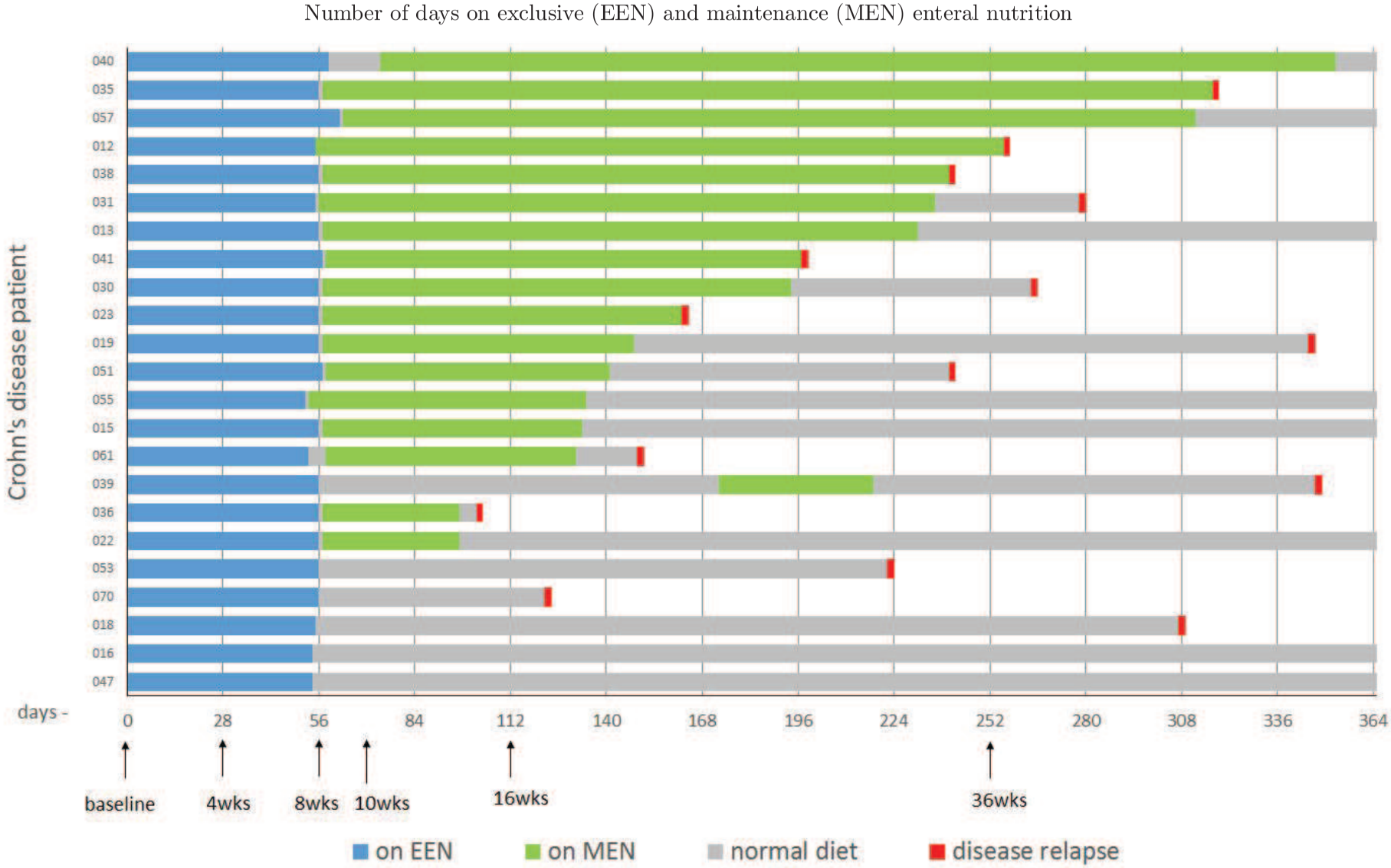


Figure 3.6: Shows the number of days each child with Crohn's disease was on EEN and MEN. Disease relapse (child was given corticosteroids) is marked in red. The six time points when faecal samples were collected are indicated (↑).

had a previous diagnosis of Crohn's disease at the time of recruitment. Of the nine children with UC, seven were medication free. For the group of eleven patients with non-IBD conditions ten were medication free; the child using a bronchodilator had asthma. Of the twenty-five healthy children all were medication free except two children using paracetamol occasionally for headaches and a one child using a bronchodilator for asthma. Children with IBD, especially Crohn's disease were more likely to be taking some form of dietary supplement (Table 3.5).

Table 3.5: Numbers of children in each study group taking medication prior to treatment

medication	Crohn's n=32	UC n=9	non-IBD n=11	healthy n=25
prescribed medication				
none	23	7	10	22
corticosteroids	0	0	0	0
immunosuppressant	1	0	0	0
antibiotic	0	0	0	0
biological	0	0	0	0
anti-acid (omeprazole/ranitidine)	2	0	0	0
laxative (movicol)	3	1	0	0
antispasmodic (buscopan)	2	0	0	0
anti-diarrhoeal (imodium)	1	0	0	0
pain killer (paracetamol)	3	1	0	2
bronchodilator	0	0	1	1
dietary supplement*				
multivitamin	7	2	1	1
probiotic	4	1	3	0
iron	6	0	0	0
omega 3	2	1	0	0
vit C	1	0	0	0

*nutritional supplements are discussed in chapter on dietary intake

Prior to 8-weeks treatment with EEN no children with Crohn's disease were taking regular medications with the exception of two children: one who was taking a proton pump inhibitor to control gastric acid (omeprazole); and another who was already on immunosuppressants due to pre-existing diagnosis of Crohn's disease (Table 3.6). This child was included in the study as one of four children with existing Crohn's disease who were undertaking a second course of treatment with EEN.

Table 3.6: Types of medication given to Crohn's disease patients during treatment with EEN and MEN

number of children	week-0 pre-treatment	week-8 end of EEN	week-16 normal diet
corticosteroids	0	5	7
immunosuppressant	1	15	22
antibiotic	0	1	0
biologic	0	0	1
omeprazole	1	7	6
no medication	30	10	4

By the end of 8-weeks treatment with EEN: five children were on steroids; fifteen were on an immunosuppressant; one was taking an antibiotic and seven were taking omeprazole. Ten children at this point were still taking no medication. At week-16 (2-weeks post-EEN) seven children were on steroids; twenty-two were taking an immunosuppressant; one was taking the chimeric monoclonal antibody biologic, infliximab, and six were taking omeprazole. Only four children were taking no medications at week-16 (Table 3.6).

3.7 Treatment with maintenance enteral nutrition (MEN)

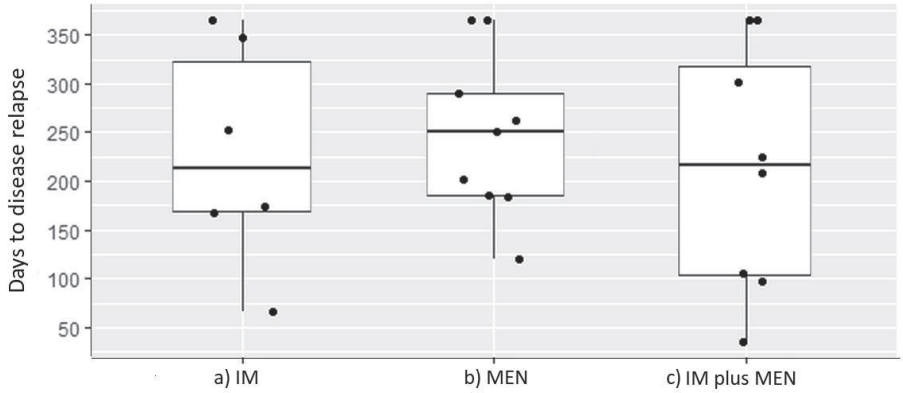


Figure 3.7: Number of days to disease relapse after end of EEN for children taking a) an immunosuppressant (IM); b) maintenance enteral nutrition (MEN) or an immunosuppressant plus maintenance enteral nutrition (IM plus MEN). KW-test p=0.82

It was predicted using clinical data from the previous 2-years,[283] that approximately 55% of children who had undergone a course of 8-weeks EEN would refuse MEN as a course of treatment to maintain disease remission. However, out of twenty-three children who went into remission on EEN only six (26%) children opted not to take MEN (Table 3.7). Another issue was treatment with immunosuppressants: all six children who declined MEN were on an immunosuppressant; whereas only 8/17 children taking MEN were on immunosuppressants. The number of days to disease relapse for each treatment group is shown in figure 3.7. All children were given some form of maintenance therapy post-EEN.

Table 3.7: Number of children with Crohn's disease taking maintenance enteral nutrition (MEN)

	declined MEN n=6	taking MEN n=17
immunosuppressants	6 (26%)	8 (35%)
no immunosuppressant	0 (0%)	9 (39%)

∗ All children on immunosuppressants were taking azathioprine, except for 4 children taking mercaptopurine (2 on MEN and 2 not).

3.8 Anthropometric characteristics and growth

Body composition data was collected from both patients and healthy children as an objective measure of how well they were achieving growth targets, as well as assessing if children and study groups, as a whole, were achieving optimal height and weight for their age. To correct for age, the standard deviation scores (z-scores)[284] for height, weight and BMI are used to describe differences between groups. The actual height, weight and BMI are also shown for reference but are not used for statistical analysis since z-scores better represent whether a child is reaching expected growth targets for their age (Methods section 2.4.1).

Table 3.8: Body composition in patient groups and healthy children (median; IQR)

Category	Crohn's n=32	UC n=9	non-IBD n=11	Healthy n=23	Kruskal-Wallis Test
Height (m)	1.47 (0.27)	1.58 (0.21)	1.48 (0.44)	1.50 (0.35)	-
Weight (kg)	35.6 (18.6)	49.9 (39.4)	34.9 (23.0)	40.0 (25.9)	-
BMI (kgm^2)	16.0 (3.33)	18.8 (12.15)	17.9 (2.78)	18.8 (4.73)	-
Height z-score	**-0.110**** (1.45)	0.061 (2.20)	**-0.670**** (0.87)	0.763 (1.50)	p<0.001
Weight z-score	**-0.429***** (1.89)	-0.385 (3.87)	-0.134 (1.94)	0.793 (1.42)	p<0.001
BMI z-score	**-0.447*** (2.31)	-0.557 (3.72)	-0.255 (2.40)	0.512 (2.51)	p=0.03
Age	11.5 (5.04)	13.6* (1.67)	11.3 (7.88)	11.0 (5.67)	p=0.03

Data shows median and (IQR). Only SDS (z-scores) were tested for significance using Kruskal-Wallis, and multiple comparisons (Bonferroni). Difference from healthy children significant at * p<0.05, ** p<0.01, *** p<0.001

3.8.1 Height and weight

The pre-treatment median height z-score of children with Crohn's disease and non-IBD patients was significantly lower than healthy children (p=0.009 & p=0.004 respectively)(Table 3.8). Children with UC had a lower height z-score than healthy children (not significant p=0.07). The median weight z-score of children with non-IBD conditions (NS; p=0.34), UC (NS; p=0.25) and Crohn's disease (p<0.001) was less than healthy children. Children with UC had a much greater weight/height/BMI z-score range compared with the other three groups (Table 3.8).

3.8.2 Body mass index (BMI)

The median BMI z-score was lower in all patient groups when compared to healthy children; only reaching significance in children with Crohn's disease (p=0.02) (Table

Table 3.9: BMI SDS z-score categories in patients and healthy children

Category	Crohn's n=32	UC n=9	non-IBD n=11	healthy n=23
clinically very underweight	4	1	0	0
clinically low weight	2	0	0	0
clinically healthy weight	26	5	6	16
clinically overweight	0	1	4	3
clinically obese	0	0	1	3
clinically extremely obese	0	2	0	1

National Obesity Observatory guidelines[285]

3.8). No child with Crohn's disease fell into the overweight or obese category, and six were underweight (≤–2 BMI z-score), four of which fell into the very underweight category (≤–2.67 BMI z-score) (Table 3.9). For UC patients 3/9 (33%) were overweight (≥1.34 BMI z-score); with two of these children being extremely obese (≥2.67 BMI z-score). 1/9 children with UC had low weight, but this child fell under the very underweight category with a (-5.85 BMI z-score). No non-IBD patients had low weight, but five (45%) were clinically overweight, with one falling into the category of obese. Anthropometric measures were available for twenty-three healthy children, showing seven (30%) were overweight; with three falling into the obese category (≥2 BMI z-score) and one into the extremely obese category. No healthy children were underweight. No differences were seen at baseline between boys and girls with Crohn's disease for height, weight or BMI z-scores (Fig. 3.8).

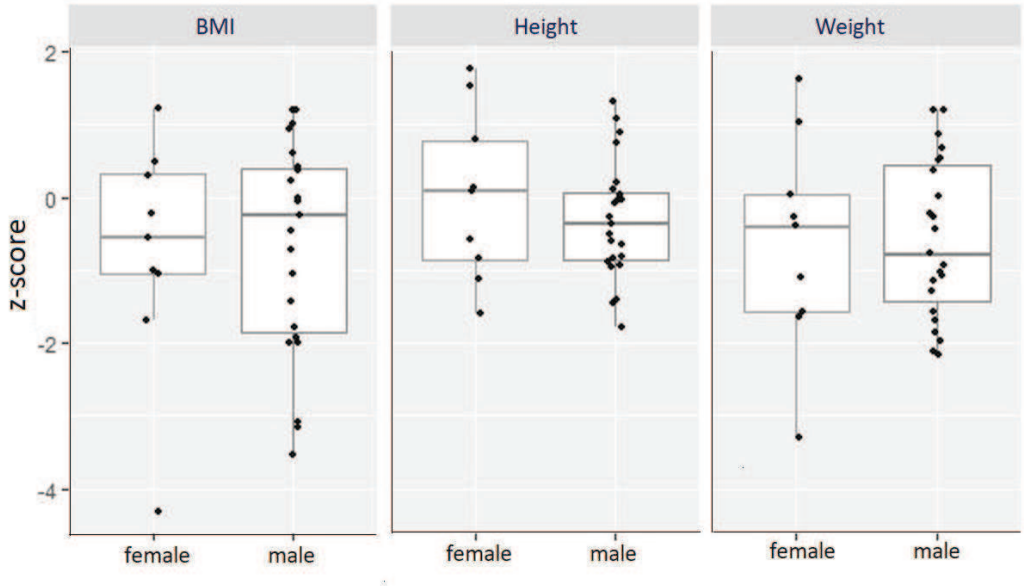


Figure 3.8: Gender differences in height, weight and BMI z-scores at baseline

In children with Crohn's disease at baseline, there was no significant correlation between BMI z-scores and either the wPCDAI or calprotectin as a marker of disease activity (Fig. 3.9), however the disease markers albumin, CRP and ESR did correlate

with BMI (Fig. 3.10) showing disease severity has the potential to lead to poorer growth outcomes.

3.8.3 Change in body composition after treatment with EEN

To explore the effect of 8-weeks of treatment with EEN on growth outcomes in children with Crohn's disease, the change in height and weight between baseline and the end of the 8-week treatment with EEN (0-8wks) was measured; then again after 8-weeks of normal habitual diet (8-16wks); and again after 28-weeks of normal habitual diet (16-36wks). The overall change in height and weight was also measured (0-36wks). Children who failed to enter full remission on treatment with EEN and went onto corticosteroids (non-responders) were compared with those children who went into remission on EEN (responders) (Table 3.10; Fig 3.11).

Table 3.10: Change in body composition after 8-weeks treatment with EEN, then 8-weeks and 28-weeks of normal diet. Median (IQR).

	week 0-8		week 8-16		week 16-36		week 0-36	
Category	non-responder	responder	non-responder	responder	non-responder	responder	non-responder	responder
	n=9	n=22	n=8	n=21	n=8	n=11	n=8	n=12
△ Height (cm)	0.45	1.24	0.20	0.88	1.86	1.15	2.93	3.22
	(1.37)	(1.73)	(0.68)	(1.01)	(3.18)	(1.47)	(3.67)	(1.51)
△ Weight (kg)	0.92	2.74	2.93	0.47	0.51	0.29	6.34	4.58
	(4.85)	(3.07)	(6.70)	(2.42)	(4.38)	(6.21)	(9.49)	(4.48)
△ BMI (kgm^2)	0.13	0.95	1.17	-0.14	-0.31	-0.02	1.92	1.03
	(1.89)	(1.33)	(2.37)	(0.98)	(2.22)	(2.07)	(3.30)	(1.21)
△ Height z-score	0.03	0.06	-0.06	0.00	0.02	**-0.12***	-0.05	-0.04
	(0.15)	(0.17)	(0.12)	(0.16)	(0.20)	(0.15)	(0.22)	(0.21)
△ Weight z-score	0.02	0.41	0.32	**-0.02***	-0.13	-0.18	0.51	0.22
	(0.81)	(0.62)	(0.68)	(0.36)	(0.93)	(0.57)	(1.02)	(0.30)
△ BMI z-score	0.00	0.44	0.47	**-0.08***	-0.18	-0.09	0.61	0.40
	(1.36)	(0.94)	(1.03)	(0.51)	(1.19)	(0.90)	(2.00)	(0.68)

* Differences between responders and non-responder significant at p<0.01

At the end of 8-weeks treatment with EEN (0-8wks), children who did not respond to EEN had the similar height gain as those who responded to treatment (p=0.77)(Fig. 3.11a). However responders to EEN had more weight and BMI gain than children who failed EEN (p=0.056 & p=0.062 respectively)(Fig. 3.11d, 3.11g). Once back onto normal diet for 8-weeks (8-16wks), responders to EEN had slightly more height gain than those who failed EEN (p=0.074). Conversely for weight and resulting BMI, non-responders had a larger weight and BMI increase after 8-weeks normal diet (p=0.005 & p=0.002 respectively)(Fig. 3.11e, 3.11h).

In the following 20-weeks (16-36wks) children who had responded to EEN had a median drop in growth rate in terms of height which was poorer than those who had

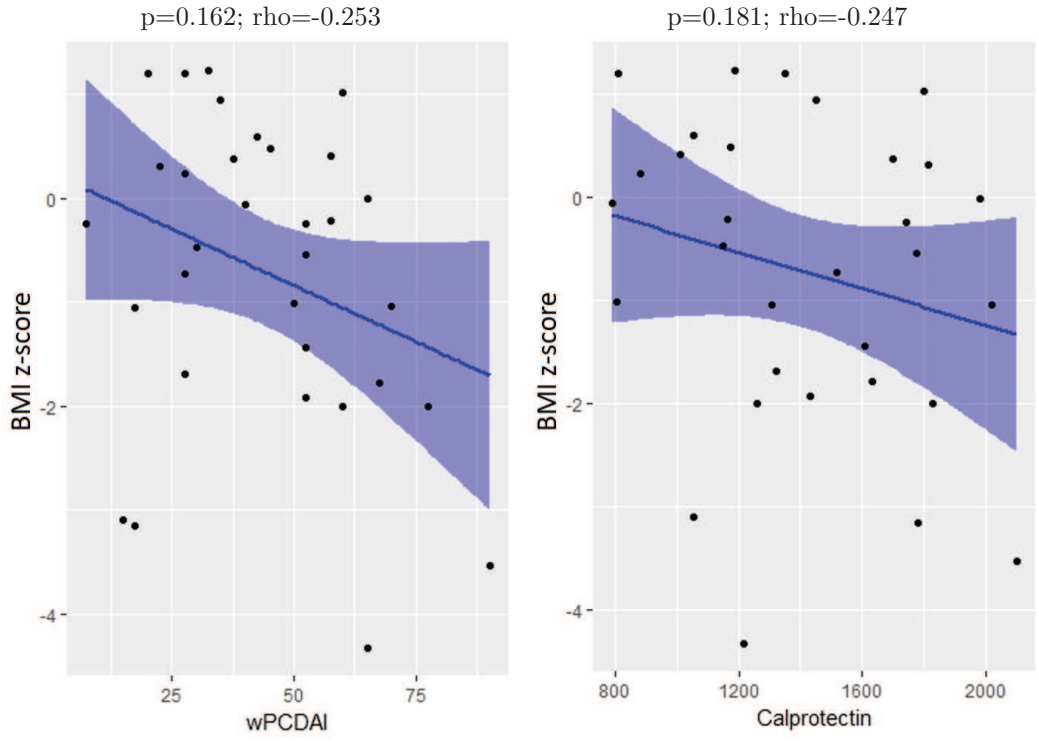


Figure 3.9: Relationship between adiposity (BMI z-score) and disease activity given by (a) wPCDAI and (b) faecal calprotectin as a marker of inflammation.

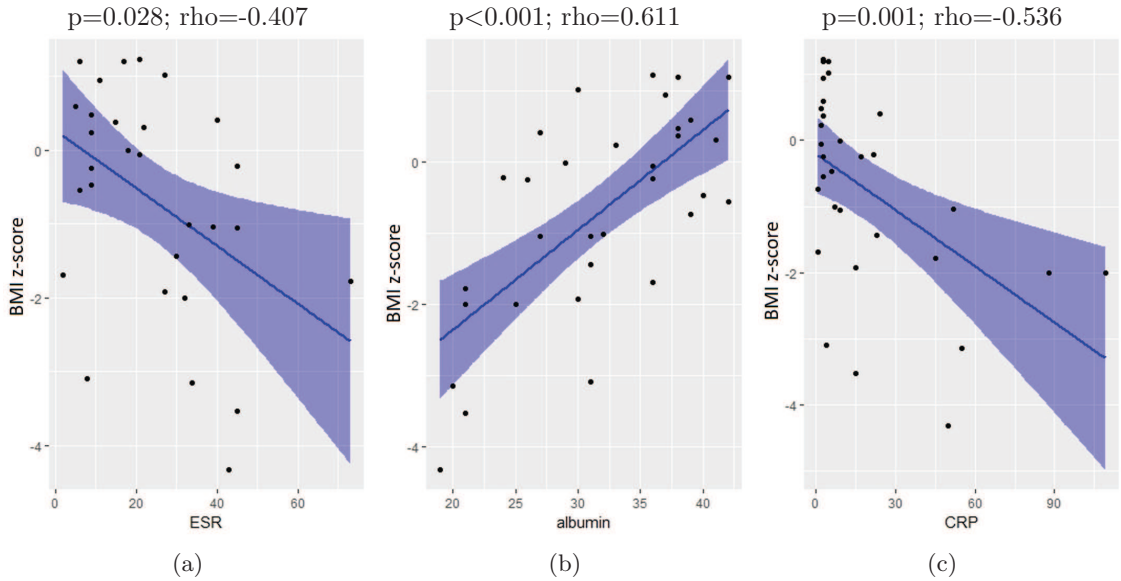


Figure 3.10: Correlation between BMI z-score and disease markers (a) ESR, (b) albumin and (c) CRP. All were significant

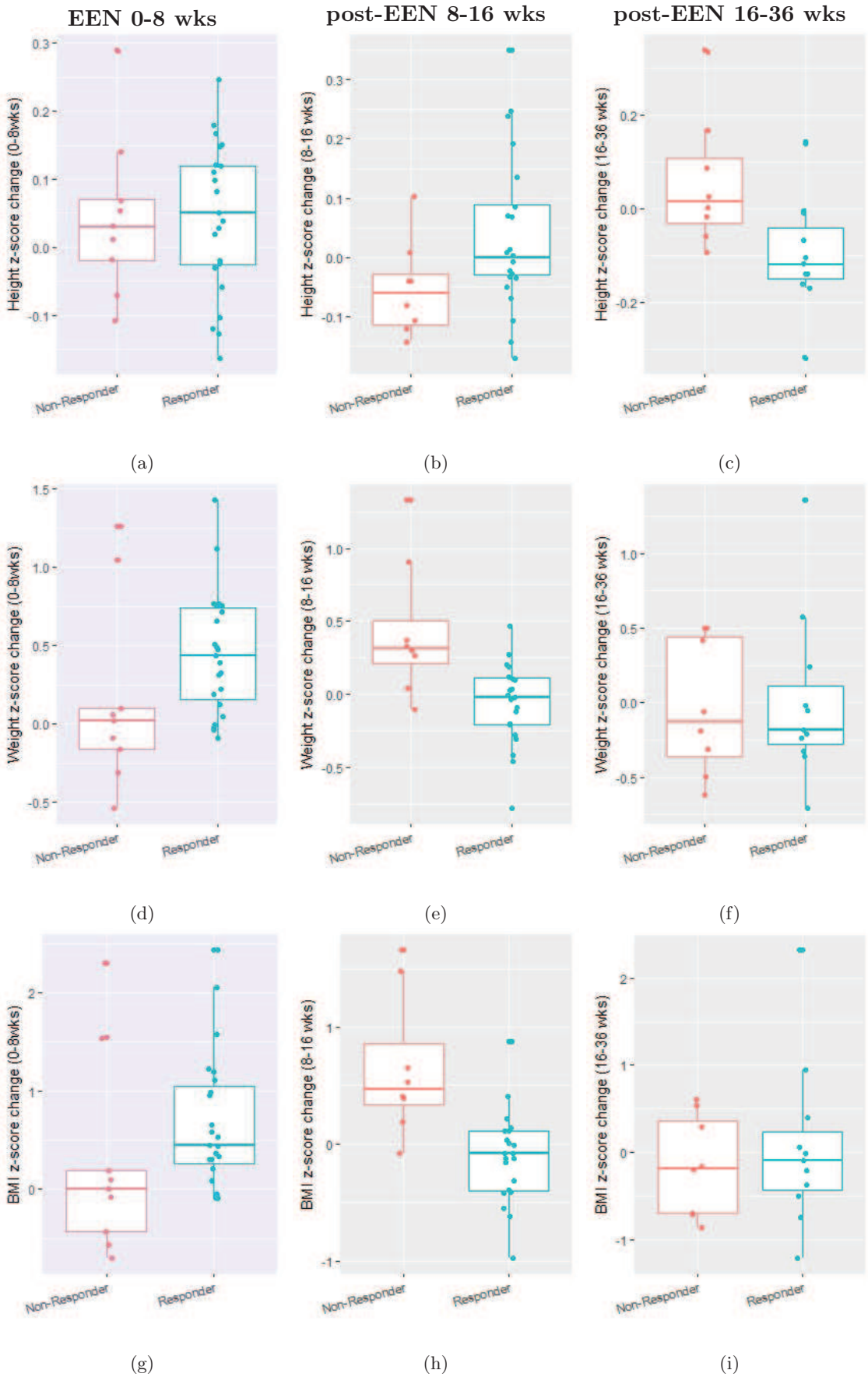


Figure 3.11: Change in body composition (z-scores) at (a)(d)(g) after treatment with EEN; (b)(e)(h) 8-16 weeks post EEN; and (c)(f)(i) 16-36 weeks post EEN for height, weight and BMI. Responder - child who went into remission on EEN; Non-Responder - child who failed to respond to treatment with EEN.

Table 3.11: BMI of children with Crohn's disease at baseline, after EEN and post-EEN

Category	week 0		week 8		week 16		week 36	
	responder	non-responder	responder	non-responder	responder	non-responder	responder	non-responder
very underweight	2	2	0	0	0	0	0	0
low weight	2	0	0	2	0	0	2	0
healthy weight	19	7	21	7	18	7	7	6
overweight	0	0	2	0	2	1	2	1
Total	23	9	23	9	20	8	11	7

National Obesity Observatory guidelines[285]

failed EEN (non-responders)(p=0.013)(Fig. 3.11c). Over this period there was no difference in weight or corresponding BMI gain between the two groups of children (p=0.96 & p=0.84 respectively)(Fig. 3.11f, 3.11i). Overall from pre-treatment to 36-weeks there was no difference in growth outcomes for height (p=0.62), weight (p=0.44) & BMI (p=0.79) between responders and non-responders (Table 3.10).

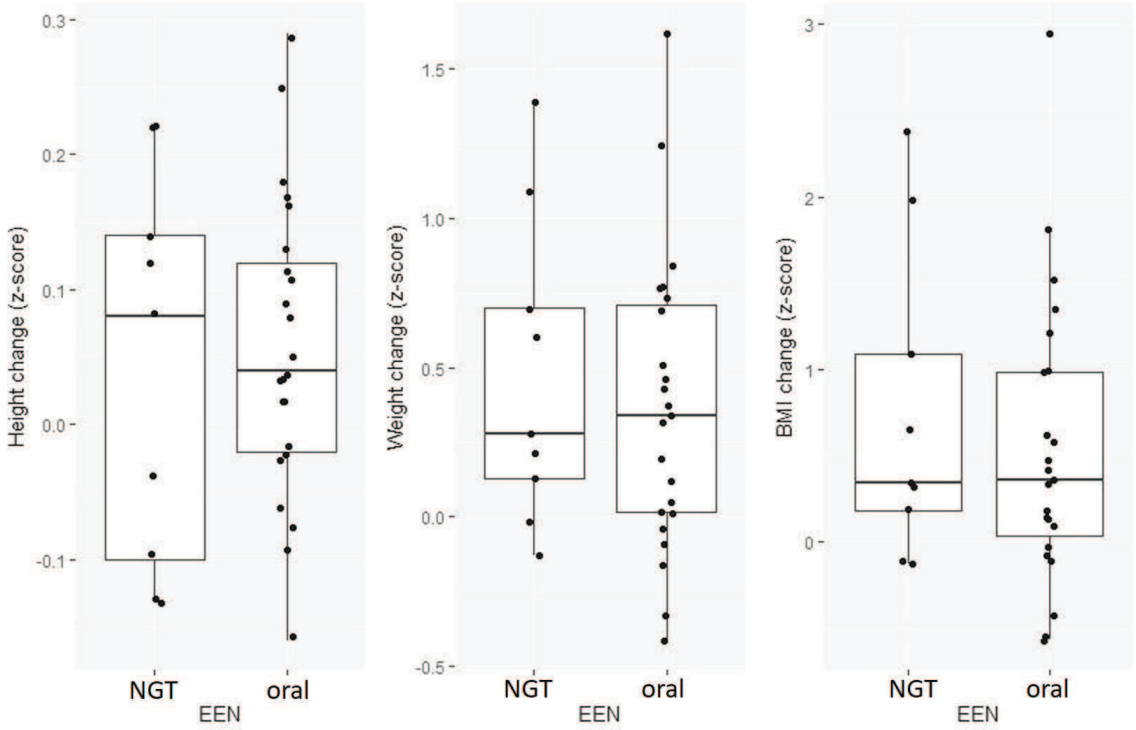
3.8.4 Change in BMI categories after treatment with EEN

For children with Crohn's disease who underwent the 8-weeks treatment with EEN, among those who responded to treatment, none were underweight. However, two children who failed to respond to EEN were in the low weight category; one of these had been very underweight prior to treatment and had only managed to complete 5-weeks of EEN; while the other had been at a healthy BMI pre-treatment but continued to lose weight despite 8-weeks on EEN (Table 3.11). One of the two non-responders who was very underweight at baseline only took EEN for 2-weeks before failing treatment and going onto steroids. At the end of their 2-week course they had moved from very underweight to low weight and it was only after 4-weeks on steroids, this child moved to the healthy weight category. One child who was a healthy BMI at baseline and failed treatment with EEN after only 4-weeks, went on to become overweight by week-16 (8-weeks of normal diet) and remained overweight at 36-weeks.

Among the children with Crohn's disease who responded to treatment with EEN two moved from a healthy BMI at baseline, into the overweight category by the end of EEN. One of these children went back to a healthy BMI once back onto normal diet, however the other was still overweight at week-16 and week-36. Another two children continued a consistent rise in BMI to become overweight, one after 8-weeks (week-16) and the other after 28-weeks (week-36) of normal diet (Table 3.11).

3.8.5 Effect of nasogastric feeding (NG-tube) on growth

To explore whether children who were given EEN via NG-tube had better growth outcomes, changes in body composition z-scores, were compared between children with Crohn's disease taking EEN via NG-tube and those taking EEN orally. No difference was observed for changes in height, weight or BMI z-scores (Fig. 3.12)

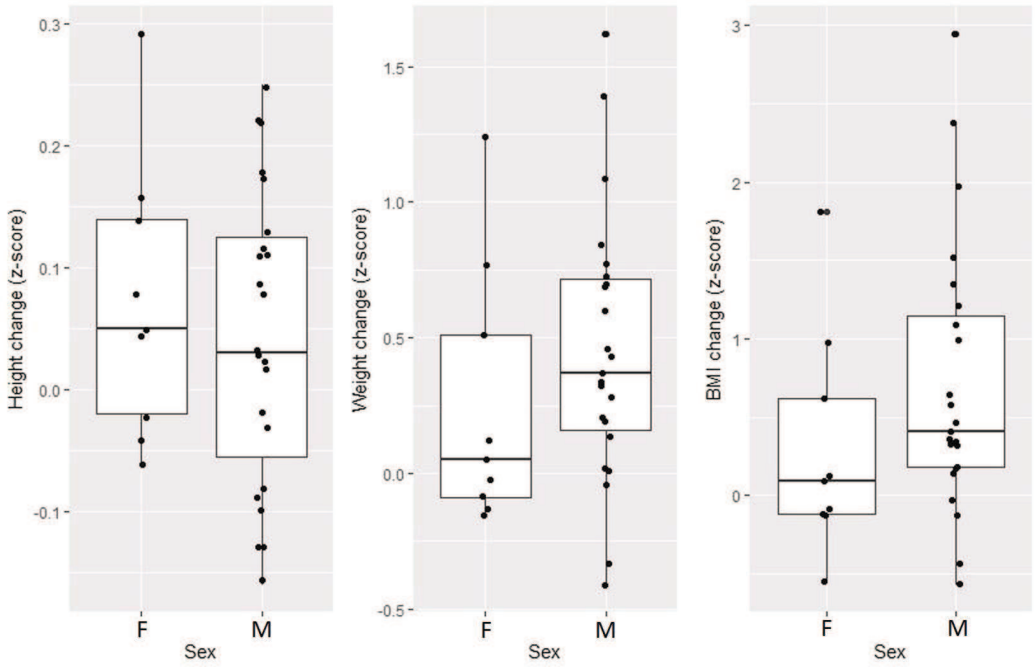


(a) (b) (c)

Figure 3.12: Change in body composition (z-scores) after 8-weeks treatment with EEN for children who took EEN orally or via nasogastric tube (NGT) for (a) height, (b) weight and (c) BMI. No difference between groups.

3.8.6 Gender differences in growth

To explore whether gender might play a role in response to treatment with EEN we looked at gender differences in growth. There was no gender difference observed between girls (n=9) and boys (n=23) for median height increase, before and after treatment on EEN. Although the median weight gain after treatment with EEN for girls (0.91kg; IQR 3.75) was less than boys (2.25kg; IQR 3.05), the difference was not significant (p=0.154). The median BMI gain for girls (0.23kg/m^2; IQR 1.78) was less than boys (0.80kg/m^2; IQR 1.49) but again the difference was not significant (p=0.220). Although boys had higher median z-score increases in weight and BMI after treatment on EEN, these were not significant (p=0.230) (Fig. 3.13).



(a) (b) (c)

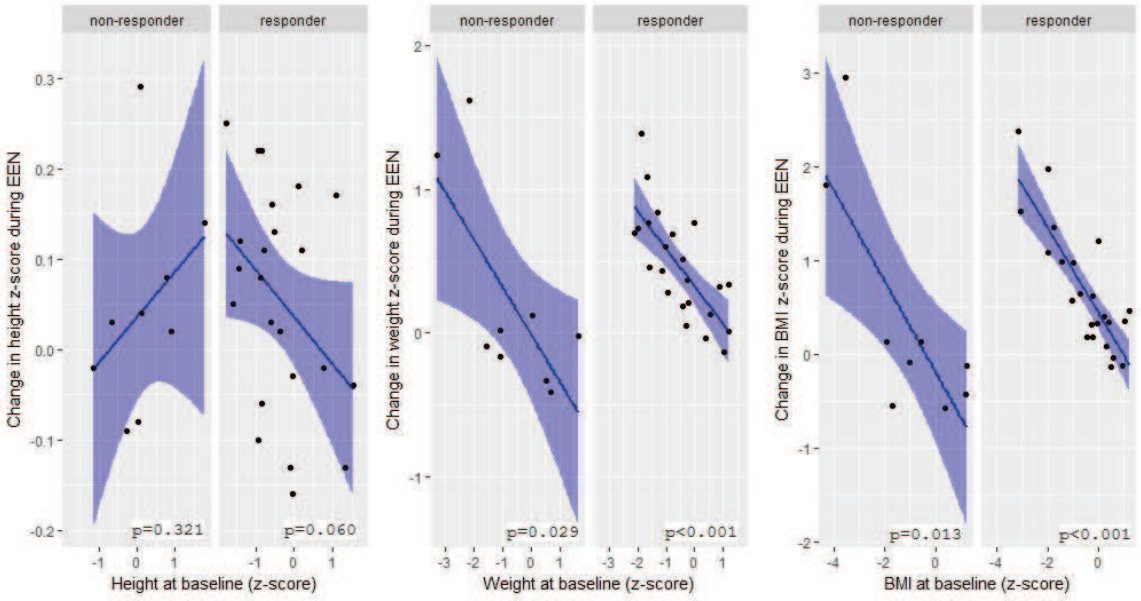
Figure 3.13: Change in body composition (z-scores) for girls (F) and boys (M) after 8-weeks treatment with EEN for (a) height, (b) weight and (c) BMI. No difference between groups.

Baseline body composition as a predictor to growth outcomes on EEN To test if height, weight or BMI at baseline, could predict growth outcomes after 8-weeks of treatment with EEN, the baseline anthropometric measures of children with Crohn's disease were plotted against growth increase after treatment. No correlation was seen between height z-scores of children with Crohn's disease and gain in height during EEN for both children who responded to treatment with EEN (responder)($p=0.060$) and those who failed treatment (non-responder)($p=0.321$)(Fig. 3.14a).

There was however a strong negative correlation between weight z-score at baseline and weight gain during EEN. This was more apparent in children who responded to treatment ($p<0.001$) than in non-responders ($p=0.029$)(Fig. 3.14b). Children who had the lowest weight z-score at baseline had the greatest weight gain during EEN, and children who were less underweight pre-treatment had the least weight gain during the 8-week treatment. The correlation with weight translated to BMI where children with the lowest BMI had the greatest increase in BMI and those with higher BMI had the least increase during treatment. The correlation for BMI was also more apparent in children who responded to treatment ($p<0.001$) than non-responders ($p=0.013$)(Fig. 3.14c).

3.8.7 Impact of maintenance enteral nutrition (MEN) on growth outcomes

To look at whether treatment with MEN post-EEN given with or without an immunosuppressant, could alter growth outcomes, the change in height, weight and



(a) (b) (c)

Figure 3.14: Relationship (Pearson's) between baseline anthropometric data (z-scores) and growth during 8-weeks of EEN for a) height, (b) weight and (c) BMI, for children who failed to respond (non-responder; n=9) and those who did respond (responder; n=23) to treatment with EEN. Shaded area represents the 95% confidence interval.

BMI between week-8 and week-16 was measured. No difference in growth outcomes was seen between treatment groups (Fig. 3.15). However a child in the MEN only group with the largest decrease in z-score for height/weight/BMI was known to be struggling with MEN, and may have failed to comply with the treatment. The sample size was too low to reject the null hypothesis.

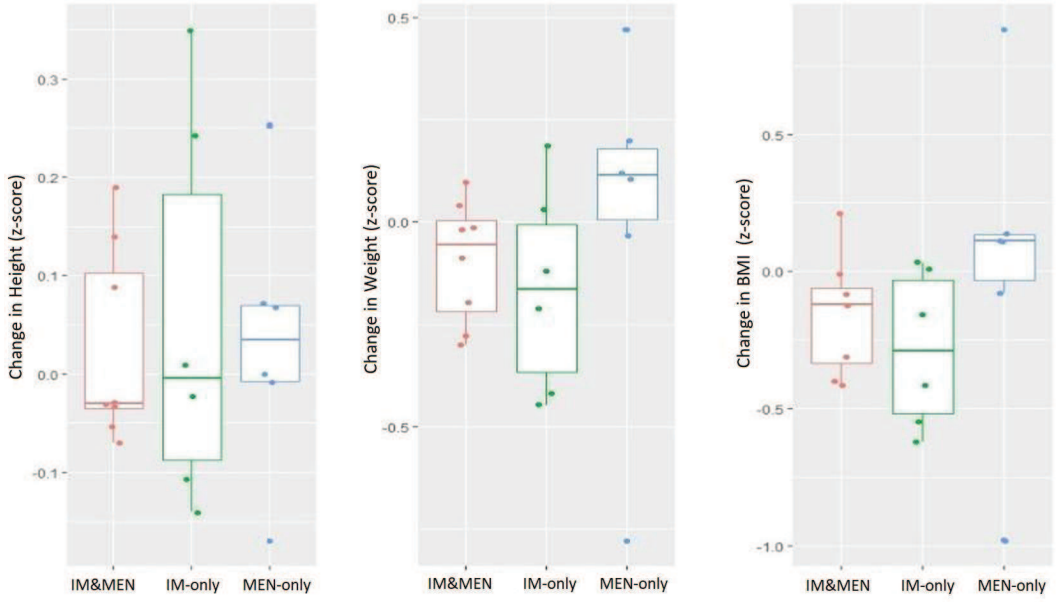
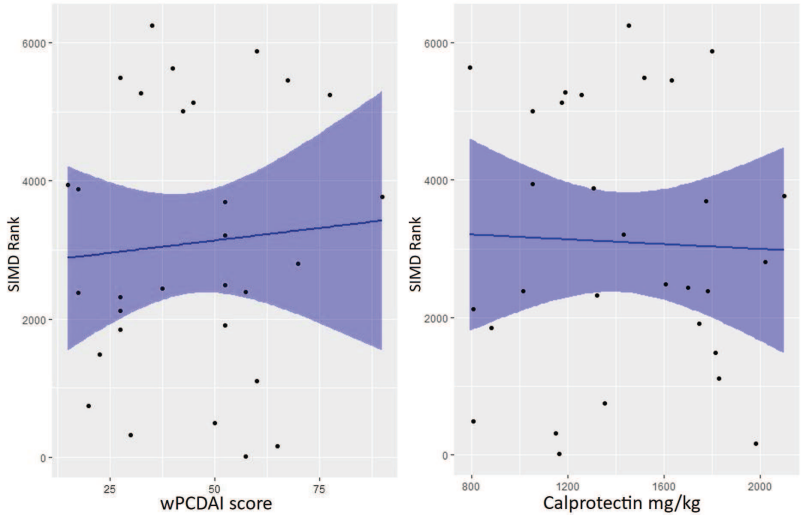


Figure 3.15: Change in height, weight and BMI (growth wk 8-16) in children who took both an immunosuppressant plus maintenance enteral nutrition (IM and MEN)(n=8); an immunosuppressant only (IM only)(n=6); or maintenance enteral nutrition only (MEN only)(n=6). Sample size too low for reliable statistical analysis.

3.9 Predicting response to EEN

Socioeconomic status To explore any potential impact of socioeconomic deprivation on the severity of disease the SIMD scores were plotted against markers of disease activity. No correlation was found between disease activity and socioeconomic status (Fig. 3.16).



(a) (b)

Figure 3.16: Disease activity and deprivation: scatter-plot showing there is no relationship between SIMD rank and either (a) wPCDAI or (b) calprotectin as marker of inflammation for children with Crohn's disease at the time of recruitment.

To explore whether children with Crohn's disease, who were from more socially deprived areas, were more likely to fail treatment with exclusive enteral nutrition (EEN), the SIMD deprivation ranks were compared between children who responded to treatment and those who failed treatment (Fig. 3.17). No difference was seen in median SIMD scores between those who responded and went into remission on 8-weeks of treatment with EEN and those who failed to respond.

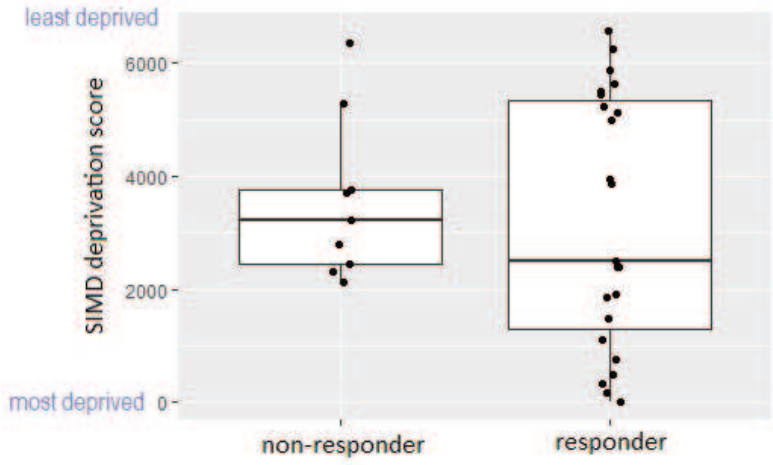


Figure 3.17: Deprivation score (SIMD) of 23 children with Crohn's disease who responded to treatment with EEN (responder) compared with 9 children who failed treatment (non-responder). No difference observed between groups (p=0.609).

Gender In children with Crohn's disease there was no difference in disease activity between girls and boys, either in terms of the wPCDAI score or calprotectin (50; IQR 31.2 and 37.5; IQR 40.0 respectively). Although girls in this study with Crohn's disease were more likely to fail treatment with EEN (5/4; 56%) compared with girls who responded to treatment (4/19; 17%), the sample size was too low to determine if girls were truly more likely to fail EEN. Of the five girls who failed treatment with EEN: one had ileal disease; three had colonic disease and one had ileocolonic disease. Although girls who undertook 8-weeks of treatment with EEN relapsed in a fewer number of days than boys (median 99 days; IQR 265 and 236 days; IQR 277 respectively), this difference was not statistically significant (Fig. 3.18).

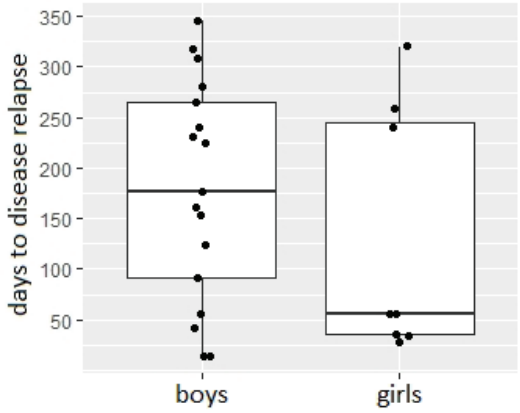
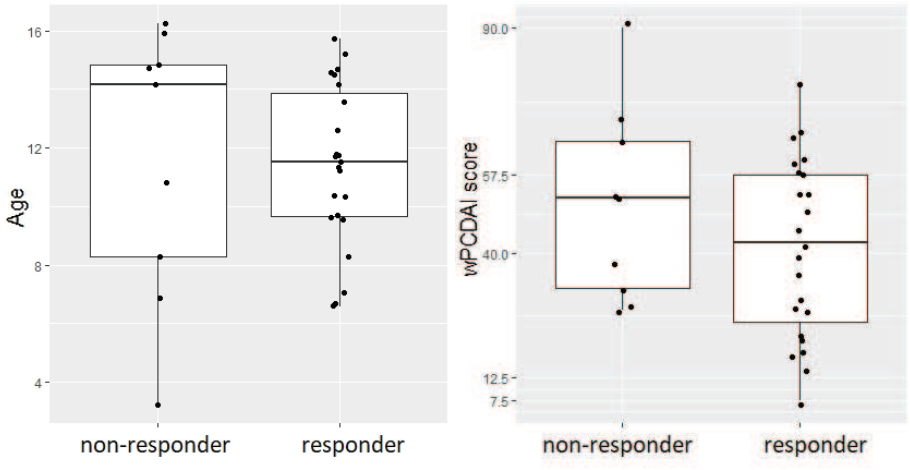


Figure 3.18: Days from start of EEN to disease relapse in boys and girls with Crohn's disease; no difference between groups (p=0.366). 6 boys and 1 girl still in remission at 1-year are not shown.

Age Children across all age groups were just as likely to fail to respond to treatment with EEN. Thus age did not predict response to treatment with EEN (Table 3.13).



(a) (b)

Figure 3.19: (a) Age and (b) disease activity (wPCDAI) at baseline did not predict whether children would respond to treatment with EEN. Responders were compared with non-responders.

Disease activity To see if children with more severe disease activity at baseline were less likely to respond to EEN, the median wPCDAI scores were compared between responders (42.5; IQR) and non-responders (52.5 IQR) (Fig. 3.19b). No difference was seen between groups.

Table 3.12: Disease location in children with Crohn's disease who did and did not attain disease remission after 8-weeks of exclusive enteral nutrition (EEN)

Disease location	total (n=32)	responder (n=23)	non-responder (n=9)
L1 ileal	4 (13%)	3 (13%)	1 (11%)
L2 (isolated colonic)	9 (28%)	3 (13%)	6 (67%)
L3 (ileocolonic)	19 (59%)	17 (74%)	2 (22%)
L4 ⋆ upper GI involvement	23 (72%)	19 (83%)	4 (44%)
Ileal involvement *			
ileal		20	3
non-ileal		3	6

⋆ Can co-occur with L1, L2 & L3. Sample sizes too low to test location categories but when looking at * ileal/non-ileal involvement significant difference were observed between responders and non-responders (χ^2 with Yates correction p=0.009).

Disease location To explore whether disease location could predict children's response to treatment with EEN, children were categorised into disease location groups using the Montreal classification. Of the thirty-two children with Crohn's disease one child with ileal disease (L1) failed treatment with EEN, while six children with colonic disease (L2) failed EEN. Only two children with ileocolonic disease (L3) failed treatment (p=NS) (Table 3.12). A significant difference between response and non-response to EEN was seen between ileal/non-ileal involvement suggesting that children with ileal disease were more likely to response to EEN. Children with ileal involvement also had a greater median number of days to disease relapse than those with isolated colonic disease (Fig. 3.20).

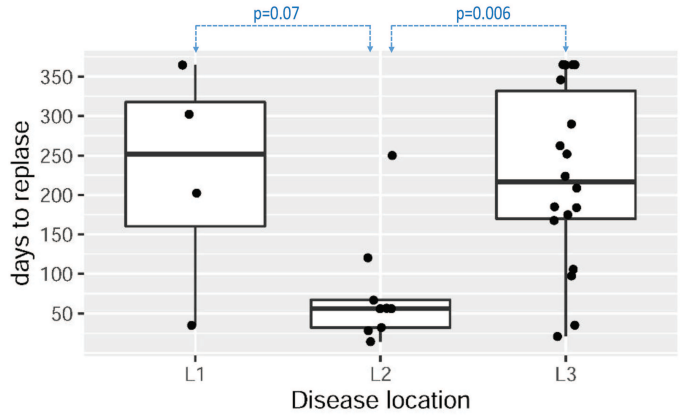


Figure 3.20: Crohn's disease location and days to relapse. L1 -ileal; L2 -isolated colonic; L3 -ileocolonic (Dunn's test of multiple comparisons following a significant Kruskal-Wallis test: p=0.01).

Baseline body composition Baseline height, weight and BMI z-score in children with Crohn's disease who failed to respond to EEN was essentially no different from the group of children who responded well to treatment. As such height, weight and BMI z-scores at baseline, did not predict response to treatment (Table 3.13).

Table 3.13: Summary of baseline body composition in children with Crohn's disease who did and did not attain disease remission after 8-weeks of exclusive enteral nutrition (EEN). Shown as median (IQR)

Characteristic	total (n=32)	responder (n=23)	non-responder (n=9)	p value R vs non-R
height for age †	-0.31 (1.06)	-0.58 (1.03)	0.08 (1.29)	p=0.102
weight for age †	-0.60 (2.05)	-0.43 (1.94)	-1.07 (2.46)	p=0.834
BMI for age †	-0.36 (2.15)	-0.24 (1.85)	-1.04 (3.50)	p=0.502
wPCDAI score	41.2 (31.9)	42.5 (35.0)	37.5 (37.5)	p=0.414
age	11.6 (5.0)	11.6 (4.6)	14.2 (7.8)	p=0.197

No difference seen between children who failed EEN (non-responders) and those who went into remission (responders)(MWU test). † standard deviation z-scores.

3.10 Links between patient characteristics

Looking at the relationship between different patient characteristics can help to identify and understand potential limitations of the study data, helping to better interpret results. To ensure the study included children from a representative range of backgrounds the Scottish Index of Multiple Deprivation 2016 (SIMD) score of the twenty-eight children with Crohn's disease who were able to provide a baseline stool sample was compared with the six children who were unable to provide a stool sample. No difference in SIMD score was seen between the two groups (Fig. 3.21). Therefore those children who were unable to provide a faecal sample came from an even spread of socioeconomic groups.

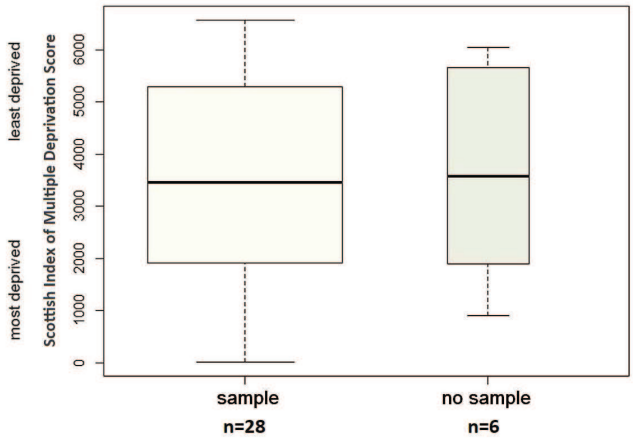


Figure 3.21: The Scottish Index of Multiple Deprivation 2016 (SIMD) score of children with Crohn's disease who provided a baseline stool sample (sample) and those who were unable to provide a sample (no sample).

3.11 Discussion

3.11.1 Recruitment

Hurdles to recruitment and ethical considerations. Approximately one in ten children, all of whom were patients, declined to take part in the study (Fig 3.1). There are a number of reasons why children might decline to take part. Most children were waiting for a diagnosis which is a stressful time and some families said they felt taking part could place an extra unnecessary burden on their child. It was observed that mothers *per se*, were more positive about the idea of taking part in research, and seemed to view research as a potential benefit to the long-term well-being of their child. When fathers were present at recruitment meetings they were, generally speaking, more likely to dissuade their child from taking part in the research study. Fathers more often expressed concerns, and often asked more questions about the potential impact research might have, in the short term, on the health of their child. It might be helpful for future studies to try and record any gender difference in attitudes toward research so these differences, if any, could be addressed in the way recruitment studies are explained to parents and guardians.

Another interesting observation was children themselves, often said that they were happy to take part 'especially if it helped another child'. It was surprising children projected the potential benefits of future research on to 'other children' rather than their own need. This may have been an artefact of the way we explained the study. While explaining the study it was made clear that this research would not benefit them directly so as not to mislead children into thinking the study could find a cure for their condition. Thus children may have taken these comments at face value and assumed there could be no benefit to themselves, even in the long-term.

As the study progressed it became apparent, taking part in research had a positive psychological effect on a number of individual children and their families. As with other documented studies,[343] many parents and children who took part in this study reported they derived comfort from knowing research into IBD was taking place, as well as satisfaction from being directly involved in that process, even although they derived no direct benefit from it.[343] Therefore it would be an interesting add-on to future IBD studies to measure any positive impact that knowledge about research has on patient well-being and health.

There were a number of practical hurdles to recruitment. When planning the study, effort was made to include a number of different centres where follow-up treatment for children with Crohn's disease took place. However, clinics in different locations could overlap making it impossible for one person to carry out recruitment effectively. A substantial part of recruitment time was spent travelling between clinics, hence it would have been more cost-effective to try and include staff who were based on site to help with recruitment at individual centres, or limit the number of centres involved.

Table 3.14: Steps taken to optimise recruitment and suggestions for improvement

Strategy	Techniques employed	Suggestions for improvement
Preparation & planning	Assess study feasibility at start Identify centres in surrounding hospitals Establish links with clinical support staff	Increase speed of ethical approval Shared database of eligible patients
Engendering patient support	Research introduced by patient's doctor Advertise the study for healthy volunteers Detailed participant information sheets Discuss concerns with IBD-nurse Contact for complaints or concerns Use of text messaging for convenience	Reduce time commitment required Better rewards for healthy children
Collaboration with clinicians	Integrated clinical and academic team Research meetings providing updates	Improve links with outreach clinicians Involve NHS staff in recruitment process

Another issue identified during recruitment was the duplication of patient research questions and measures, which were already being done by clinical staff. The team in one of the hospital centres came up with the solution of inviting the researcher into the dietetic clinical appointment which saved the duplication of questions and measurements. This reduced the burden on both parents and children, allowing them to leave the hospital more quickly and missing less school/work time. Reducing the time burden on patients would likely result in more patients completing studies.

Meeting recruitment targets From previous hospital records[283] we estimated around fifty to sixty children would come into the Royal Hospital for Children in Glasgow to undergo colonoscopy over 12-months, and be newly diagnosed with Crohn's disease. Within the 18-month recruitment time frame of this study, we aimed to approach around seventy-five treatment naive patients as well as about twenty children with existing Crohn's disease. With a recruitment success rate of 65% this would have given a potential of sixty-two Crohn's disease participants over 18-months. We predicted a study retention rate of 65% to give a final number of forty-two Crohn's disease patients. Our aim was to age and sex match these with a group of forty-two healthy children as controls. We managed to approach seventy-six patients: 13% declined to take part; 14% were unable to provide a baseline stool sample; 14% had non-IBD conditions and 14% had either UC or IBD-U. This left thirty-four children diagnosed with Crohn's disease which at 55% was 10% less than the original target of 65%. The retention rate was slightly better than the predicted 65%, with 71% of children with Crohn's disease completing the study. This left a final number of twenty-four children with Crohn's disease who completed the study (57% of original target); and twenty-five healthy controls (60% of original target).

Recruiting the target number of research participants was extremely challenging for a number of reasons. Firstly, we needed to ensure a strategy to identify all potentially

eligible patients were invited to join the study. Coming into recruitment from a non-clinical background with no prior NHS experience, it took time to establish links with clinical teams and to gain training and understanding of how hospital systems such as patient databases worked. Obtaining permissions to access hospital databases also took time. During this period of establishing links with clinics, obtaining access and learning how to work NHS databases, a number of potential participants were missed. The study recruitment was also dependant on the involvement of clinical staff who were involved in a number of other research studies. The result being, if staff over several hospitals, including clinicians, nurses, dietitians were not constantly reminded about our recruitment aims, identifying potential participants was missed. This complex and time-consuming reminder system did not always work well due to time constraints, hence future studies may find it more efficient and effective to have the onus of identifying patients based within the NHS clinical setting. Despite these challenges we recruited sixty-six patients and twenty-five healthy children due to the clinicians in Paediatric Gastroenterology at the Royal Hospital for Children in Glasgow having an active interest in research and inviting patients to meet with the researcher. Extending the responsibility of recruitment for this study by having an IBD nurse or dietitian actively involved in carrying out recruitment would also have lessened the burden on patients and helped with patient retention; although it would have a greater financial impact on running costs. Creating a shared research database of potentially eligible patients is one way which the complex situation of dealing with many different staff over a number of clinics could possibly be improved (Table 3.14).[344]

Patient travel times can also be an issue with recruitment and participation. We recruited a number of patients from outside the greater Glasgow area who attended hospital in Glasgow. However for those with Crohn's disease it was often problematic to collect samples, as patients were not able to come into Glasgow to drop these off, hence a huge amount of research time was spent driving around the west of Scotland collecting samples. It would have been more cost effective and time efficient to either only recruit children within the Glasgow area or to have employed someone to collect samples. Using existing lab transport services could also have improved sample collection by facilitating the collection of samples from local GP clinics which might have been a convenient drop off point for many patients.

Retaining participants Over the course of the study participant retention was improved by making telephone reminders to parents, and using text messages where the parent chose this as an option, which all participants with Crohn's disease did. One advantage of using text messages as a reminder was, busy parents could call back at a time that was convenient for them, rather than being disturbed by a phone call while at work, while shopping, or preparing family meals. Text messages also

contained useful information such as dates which were at hand, therefore parents were less likely to forget when the next sample was due. The use of text messages made it easier for patients to communicate with the researcher. They could easily ask questions if their child had problems providing samples or inform us if the child was too fatigued to provide a sample, thus allowing us to be more sensitive to individual child needs. Good participant communication was key to the successful retention of participants over the course of the study.

By keeping a detailed patient log (required by ethics), it was noted that children who failed to provide stool samples and dropped out of the study, tended to be children who gained the least enjoyment from taking part. As the study progressed it was easier for researchers to identify any child who was not enjoying taking part and may wish to drop out; allowing researchers to make leaving the study as easy as possible for the family. Future studies may want to consider identifying why many children gain pleasure, while some find it a burden to take part in research. This might help to identify those likely to drop out at an earlier stage of the study, allowing us to either help them leave the study before it becomes a burden, or to adapt the study to better fit their needs.

3.11.2 Representative sampling

Recruitment is a challenging process and it is important to be aware of the limitations of any recruitment bias. There are a number questions to address in terms of understanding whether the children who were recruited onto the study were a fully representative subset of the Scottish population of children with IBD and healthy children. The focus of this study was to look at children with Crohn's disease hence our recruitment of healthy children was not random, but designed to match, as far as possible, the age and gender with our study group.

Social deprivation By checking whether children from more deprived areas were less likely to partake in the study, it was shown children who failed to provide samples, were just as likely to come from deprived areas, hence there was no bias in socioeconomic status for samples collected (Fig. 3.17).

It can be very challenging to recruit healthy controls on studies for a number of reasons. Firstly many volunteers come from personal friends, children of staff/students, who work within the same environment where the study takes place. This can have the effect of creating a bias towards a less deprived demographic. Based on a previous study,[64] there was a 'preconception' that children with Crohn's disease tended to come from less deprived backgrounds, and hence this assumption influenced the effort which was made to recruit healthy children from this demographic.[64] To avoid this bias, future studies should look at the SIMD scores during recruitment, and

use the SIMD website interactive map,[345] to identify areas for targeting recruitment of healthy controls, in order to better match deprivation scores. This could have avoided the slight difference we had between children with Crohn's where 48% came from more deprived areas (Rank 1 & 2) compared with only 20% of healthy controls coming from these two more deprived ranks (Fig. 3.3). This difference could be particularly important in studies related to nutrition, since low income and differences in access to education might affect dietary choices.

A control group should aim to match the study group not only for sex and age, but also for other factors contributing to differences between the groups unrelated to disease, such as differences in activity levels or diet. It is unlikely that the small difference in deprivation scores in our study, between children with Crohn's disease and our healthy control group, will affect results. However, understanding how confounding factors might affect the interpretation and conclusions drawn from data is very important. It should be noted that deprivation scores are for areas, and not individuals, such that any child who lives in an area of social deprivation, is not necessarily deprived and *vise versa*; i.e. the six children with Crohn's disease who resided in the most deprived areas may have fairly affluent lifestyles.

The national 2016 SIMD ranks show different health boards have marked differences in deprivation scores with Greater Glasgow & Clyde, Ayrshire & Arran and Lanarkshire, having the highest income deprivation and health deprivation in Scotland (Methods Fig. 2.3).[286] Hence the population of children we recruited from these areas may not fully represent the whole Scottish population in terms of deprivation. It would be interesting for future studies to look at patient outcomes in health authorities across Scotland, to see if those under the strain of dealing with more deprived populations, negatively impacts on patient outcomes for children with IBD. Families with low socioeconomic status and restricted income, might have less access to healthy food choices. If dietary choice is a risk factor for Crohn's disease then we might expect to see a higher prevalence at one end of the socioeconomic spectrum. If dietary choice affects the likelihood of relapse we might also expect to see a difference in disease outcomes. However in this study no evidence was seen linking socioeconomic status with risk of Crohn's disease (Fig. 3.3).

Gender The ratio of girls to boys was 1:2.56 for Crohn's disease, matched by 1:1.27 for healthy children. It was harder to recruit healthy boys, who appeared not to be as motivated to take part by our offer of a £10 gift voucher. Increasing the value of the gift may have attracted more healthy boys to volunteer, but for future studies it would be worth looking at any gender differences which might act as a barrier to volunteering, so these can be accounted for at the planning stage.

Age Although we successfully managed to recruit a good match in terms of age for children with Crohn's disease, non-IBD patient controls and healthy controls, the group of children with UC were slightly older than the other three groups (Fig. 3.4). This needs to be kept in mind when interpreting any differences in variables such as gut microbiota, metabolites or dietary intake which are examined in subsequent chapters.

Body composition The study aimed to match at recruitment, the BMI of healthy children with that of children with Crohn's disease. However as the study progressed, it became obvious this was unrealistic, given that children with Crohn's have often lost enough weight prior to diagnosis to be classified as underweight to very underweight. The seven healthy children we recruited that were overweight, have the potential to create a bias in our dataset when comparing data from gut metabolites or bacteria to children with Crohn's disease who were either normal weight or underweight; i.e. any effects attributed to disease aetiology might in fact be due to differences in energy intake or body fat mass, hence this needs to be considered when interpreting data in subsequent chapters.

3.11.3 Patient Characteristics

Gender As reported in European and North American studies, the current study also saw a higher number of boys than girls with Crohn's disease in a ratio 1:2.5. To date no studies have identified whether these gender differences are due to environmental factors such as a gender difference in diet, or if genetic differences related to gender, such as sex hormones, increase the risk of Crohn's disease for boys.

Age In 2012 Henderson *et al.*[84] compared 260 Scottish children with IBD from 1990-1995 with 436 children from 2003-2008 and found the age at diagnosis had fallen from 12.7 to 11.9 years ($p<0.01$). This was driven by a decrease in age at diagnosis of children with Crohn's disease CD from 13.2 to 12.1-years ($p<0.001$). These mean ages are in line with the current study of 12.2-years (n=43) for all children with IBD and 11.8-years (n=34) for children diagnosed with Crohn's disease. This is of concern as earlier onset has more potential to impact on linear growth and pubertal development in children.[10;11]

Body composition Although a measure of height and weight might seem relatively straightforward, when measuring children over the short term who are undergoing height growth in a non-linear manner with 'growth spurts' and the affect of puberty; special consideration needs to be given as to whether height is a good tool to measure whether a child is thriving, and nutritional goals set for the child are being met. This

is not a problem when using height or BMI as a measure of growth over a longer time period such as a year, but in this study measures taken at 4-week intervals using height would produce misleading data. To illustrate this, if we take two children the same age and height, and improve their nutrition, both might gain 1kg in weight over four weeks. If one of these children did not gain in height while the other gained 1cm in stature, the child who grew taller would have a lower BMI and our data might mislead us into thinking the child who grew less in stature is thriving better in terms of BMI than the child who is taller. If we use height as a measure of growth then the reverse would be true. Therefore over the time period of this study weight increase is considered a more accurate indicator of improved nutritional status in growing children.

Also when measuring growth impairment in children, static height measurements may be misleading as they are affected by genetic limitations (parental height) and pubertal status. A child with Crohn's disease might be normally short, or a tall child might not have grown for several years but might still be of average stature for age and gender.

The current study saw no difference in height/weight/BMI z-score between boys and girls at baseline (Fig. 3.8) suggesting both genders were equally at risk of poor growth prior to diagnosis. Failing to reach optimum height for age is of concern, especially in girls, as delayed linear growth has been linked with around 2-year delay in pubertal development.[10;11] If the onset of puberty is significantly delayed beyond the age of 14-years, the final height of girls may be permanently compromised.[10;11] Boys have also been shown to have delayed pubertal growth but this did not appear to be linked with linear growth.[10] In Crohn's disease impairment of linear growth can also precede weight loss and thus be an early indicator of disease.

Body mass index (BMI) BMI is only an estimated measure of body fat, as it makes a potentially inaccurate assumption about how much weight is body fat. Nonetheless studies have shown BMI correlates well with other measures of body fat including underwater weight and dual energy x-ray absorptiometry.[346] Unlike other methods BMI requires only measures of height and weight, making it a reliable, easy and affordable way to record changes in body composition or differences between study groups. Its universal acceptance as a method also has the advantage of allowing researchers to compare historical data with current measures, as well as pool data from different studies and regions across the world.

BMI has limitations, which should be kept in mind when interpreting or using BMI data to reach conclusions from data sets. As well as not actually measuring fat, it does not discern between muscle tissue, bone mass and adipose tissue, genetics and muscle mass, all of which have the potential to alter the accuracy and reliability of BMI as a measure of body fat.[346] For example old people tend to have a larger

proportion of body fat than young adults even although they have the same BMI.[347] Female adults tend to have larger proportions of total body fat than men with the same BMI,[348] and people with an athletic physique such as rugby players, can have a BMI which would suggest they are overweight due to having large muscle mass.[349]

There are additional issues when using BMI for children/adolescents. Factors affecting variation in growth rate such as nutrition and genetics, as well as levels of sexual maturity, alter the relationship between BMI and body fat in children.[346] It has also been shown the reliability of BMI as a measure of body fat varies with percentage of body fat. In obese children with a BMI for age which is $\geq 95^{th}$ percentile, BMI is a reliable indicator for body fat. However in overweight children which have a BMI for age between the 85^{th} and 94^{th} percentiles, an increase in BMI can be due to increased fat-free mass rather than adipose tissue.[346] In addition differences in BMI in relatively lean children can be due to differences in the proportion of fat-free mass.[346] Since a proportion of the children with IBD in this study are underweight, the results of BMI measures when compared with healthy children should be interpreted with caution.

As we might expect, children with Crohn's disease in the current study had a significantly lower height, weight and BMI score than healthy children ($p<0.05$). Only one child with UC was underweight; five normal weight and the rest overweight, while none of the children with Crohn's disease were overweight. Identifying the cause of growth failure in individual children is not straight forward, since a number of factors can be involved such as reduction of food/nutrient intake, malabsorption due to inflammation, as well as side effects of medications like steroids. Although western studies particularly those in North America would suggest the risk of IBD patients being underweight is decreasing, with some studies showing a large number of patients being overweight at diagnosis, our study found 19% (6/32) of children with Crohn's disease were underweight compared with 11% (1/9) of our UC patients. Given the time constraints of this study it was not possible to track the time taken from first GP contact to diagnosis. It would however have been interesting to see if the children who were most underweight had taken longer to be referred by their GP. Future studies could easily monitor these time-scales to see if strategies can be identified which would result in less children have clinically low weight prior to treatment. The current study, in line with large North American studies,[53] found that more UC (3/9) than Crohn's disease (0/32) patients were overweight, but numbers were too small to establish significance. The height, weight and BMI of our healthy cohort had z-scores above the norm with 7/23 children being overweight-obese. The z-scores are based on 1990 norms hence it is unclear if our 2014-16 healthy cohort are representative of overall national increases in child body composition. A large Scottish study on adults with IBD reported 18% were obese in comparison to 23% for the total Scottish population.[55] Another 38% were over-weight which was equivalent

to the general Scottish population.[55] However, we need to be careful when comparing adult studies with paediatric studies as Crohn's disease tends to be more severe in children, which could impact on the rate of weight loss prior to diagnosis.

In the current study it was interesting that for BMI at baseline in children with Crohn's disease, a significant correlation between BMI z-scores and serum disease markers albumin, CRP and ESR were seen (Fig. 3.10). This highlights the importance of using body composition measures such as low BMI as an important early warning sign of potential inflammation and IBD.

3.11.4 Disease Characteristics

In the patient control group diagnosed with non-IBD conditions, the five children who had a first or second degree relative with IBD, none were diagnosed with a gastrointestinal condition. Therefore care needs to be taken looking at results in later chapters, to see if any of this group of five children overlap with our study group who have IBD, suggesting that they might have subclinical IBD.

Disease location in children with Crohn's disease Upper gastrointestinal involvement (L4) in Crohn's disease has been reported in previous studies as anywhere between 30%-80% in children and adult studies.[20] 72% of children in our study had isolated upper disease showing just how important it is to understand upper involvement. None of the patients included on the study had isolated upper disease without concurrent ileal/colonic disease (L1-L3). Normally around 40%-50% of patients have ileal disease (L1) however only 13% of our cohort had ileal disease and the numbers of ileocolonic (L3) numbers at 59% were higher than previously reported figures of around 30%-40%.[20] The number of children with colonic disease (L2) was much closer to previously reported figures at 28%.

Studies linking efficacy of EEN with disease location are limited,[26] with some evidence suggesting children with ileal involvement respond better to EEN.[27] However this Japanese study showing children with active Crohn's disease fail to respond well to EEN when ileal involvement was not present are not supported by an Italian paediatric study[127] or a later Japanese adult study.[140]

In 1990 Teahon *et al.*[350] concluded from a large number of case studies that adult Crohn's patients with isolated ileal involvement were more likely to remain in remission after EEN for longer when compared with patients with colonic, or ileocolonic disease. Gavin *et al.*[153] found, after EEN induced remission children with colonic disease tended to relapse earlier.

3.11.5 Predicting response to EEN

Socioeconomic status and response to EEN The current study saw no correlation between disease activity and socioeconomic status (Fig. 3.16). Children with Crohn's disease who were from socially deprived areas were as likely to fail treatment with EEN as those from less deprived areas, as there was no difference in SIMD scores between the children who responded well and went into remission on 8-weeks of EEN and those who failed EEN (Fig. 3.17).

Gender and response to EEN Although the current study saw a gender difference in response to treatment on EEN, 3/5 girls who failed EEN had colonic disease, which did not seem to respond as well to treatment in both girls and boys. For those children who went into remission on EEN, results show no gender differences in the number of days to disease relapse once children were back onto their normal habitual diet.

Disease location and response to EEN As the sample size of the current study was underpowered in terms of disease location, it was not possible to assess whether ileal, colonic or ileocolonic (L1, L2, L3) disease played a role in how well children responded to treatment with EEN. However figures are in line with other research studies which show a significantly greater number of children with ileal involvement respond well to treatment with EEN, when compared to children with isolated colonic disease (Table 3.12). Results from Kim *et al.* 2016[351] found no difference in disease location between children who were responders (n=58) and non-responders (n=8) to EEN. Although the current study saw a higher number (67%) of children with isolated colonic disease in the non-responder group, Kim *et al.* saw no children with isolated colonic disease failing EEN. A meta-analysis of ten trials was unable to link disease location and phenotype with efficacy of treatment, or time to relapse, despite studies suggesting patients with ileal involvement respond better to EEN.[26] Therefore further studies are needed to clarify whether isolated colonic disease could be a separate subcategory of IBD.

Age and response to EEN The current study explored the possibility that children of a particular age group might be at greater risk of failing treatment with EEN, perhaps due to younger children becoming distressed and not complying with the diet; or teens finding the diet too difficult to maintain among their peer groups at school. However since those who failed treatment on EEN ranged in age from 3-16 (median 14.2) it was concluded that no particular age group appeared to be more at risk of failing treatment (Fig. 3.19a) A 2016 Irish study concluded that EEN was less effective in patients aged under 10-years (p=0.04).[352] However given the fairly low number of children who went into remission (n=18), creating an artificial split

Table 3.15: Age-related response to treatment with EEN in children with Crohn's disease: showing potential error in age categorisation

		Response to EEN		
		responder	non-responder	
Lafferty 2014 [352]	0 -<10	8	9	
	≥10	33	9	p=0.04
BIG study	0 -<10	6	3	
	≥10	16	6	NS
BIG study	0 -<12	15	4	
	≥12	8	5	NS

The current study (BIG) has been split into two different age bands, to show how creating an artificial divide can bias results.

above and below the age of ten was not a robust way to analyse the risk of failing treatment. If the current study analysed data using the same cut-off age similar ratios are produced. However if we were to ask if there is a difference between primary and secondary age children and thus choose a cut-off age of 12-years rather than 10-years, the data would then suggest the converse is true, *i.e.* that children over the age of 12-year respond less well to treatment than younger children (Table 3.15). Hence without a good reason for doing so and a clearly defined research question it is not helpful or advisable to create age categories, but better to analyse as continuous data.[353]

Body composition and response to EEN Knowing whether the baseline height, weight or BMI z-score in children who failed treatment with EEN was different from those who responded was important, since any differences at baseline could introduce a bias, potentially leading us to identify differences between the response groups in research variables which were due to differences in nutritional status at baseline. However height, weight and BMI z-scores at baseline did not predict the response to treatment (Table 3.13).

3.11.6 Effect of 8-weeks treatment with EEN

Weight gain after EEN It is worth considering how using EEN as a treatment option to achieve disease remission in children with Crohn's disease might impact on growth targets for children who fail to respond to treatment with EEN. Of the 9/32 children with Crohn's disease who failed to respond to EEN, only 56% gained weight compared with 95% of responders. Although it might be assumed children who failed EEN put on less weight due to being on EEN for less weeks, this was not the case (Fig. 3.22). It appears that among non-responders, children who managed to stay on EEN for 6 to 8-weeks did not gain any benefit in terms of weight gain compared to those who stopped EEN early. This was explained when looking at the effect of baseline weight (pre-EEN) on weight gain (post-EEN). There was clear

relationship for both responders and non-responders whereby children which were most underweight at baseline put on the most weight over the 8-weeks regardless of how long they were on EEN (Fig. 3.23), (Spearman's rank p<0.001 Rho -0.664).

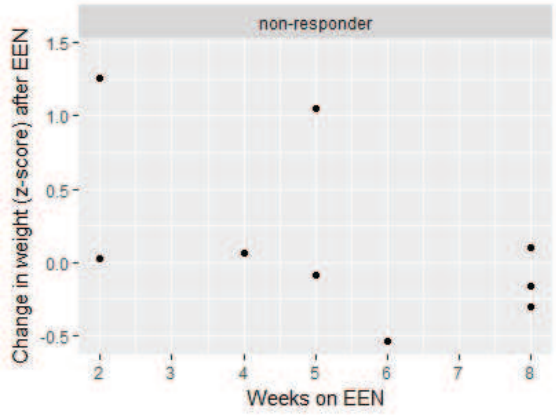


Figure 3.22: Relationship between number of weeks on EEN and weight gain in children with Crohn's disease who failed to respond to EEN (non-responders). The 2 children with most weight gain had very low BMI at baseline (z-score -3.53 and -4.32).

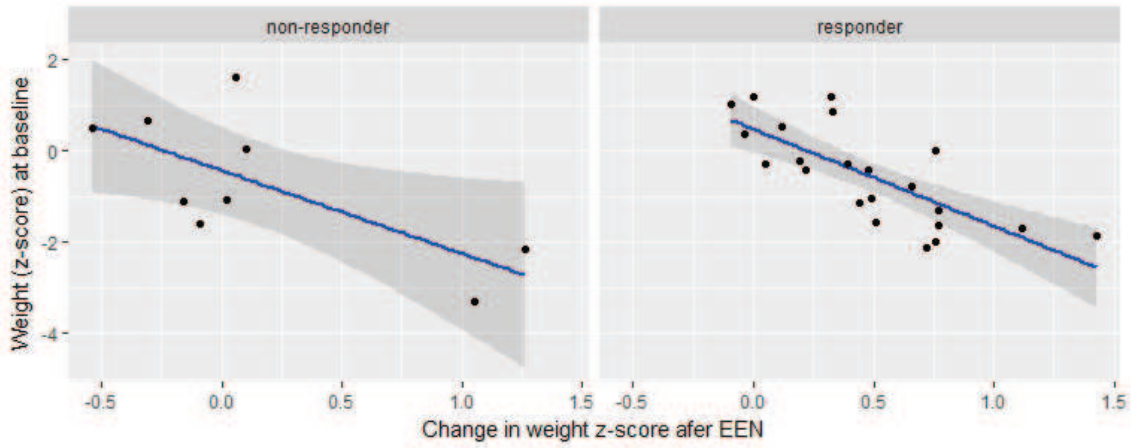


Figure 3.23: Relationship between baseline weight z-score and change in weight z-score after EEN in responders and non-responders. For combined groups Spearman's rank p<0.001 Rho -0.664.

This has important implications for studies such as Gavin *et al.* 2017,[153] where they report a significant gain in BMI among those who took MEN (p=0.004). In the Gavin study, the children who took MEN were those who had a significantly lower BMI at baseline (p=0.004) than those who took normal diet alone; and looking at the strong correlation between baseline weight and weight gain in the current study, it could be argued that baseline BMI is more likely giving rise to this increase than MEN. Gavin *et al.* saw no difference in BMI between children taking EEN and steroid therapy over a 12-month period.[153]

In the current study children who failed EEN, were not more underweight at baseline than children who responded well to EEN; thus weight gain does not give any clues as to whether children who failed EEN might have had compliance issues. When asked at their clinic appointment, all children reported they had fully complied with taking

EEN. It is difficult to know with certainty whether children who fail to respond to EEN as a treatment, fail to adhere fully to the treatment regime. It is possible loss of weight or failure to gain weight was due to malabsorption where EEN had not succeed in reducing disease activity and inflammation in those who failed treatment.

Although the nutritional benefits of EEN as reported in other studies,[283;49;153] led to weight gain (p=0.056) and increased BMI z-scores (p=0.062), inline with these studies, the current study did not see a corresponding increase in height z-scores (p=0.77).[283;49;153] The time frame of 8-weeks is however not long enough to reliably measure changes in height (section 3.11.3). It is believed that corticosteroid therapy is responsible for the negative impact on growth, however some studies have suggested, age at diagnosis, disease activity and possibly disease location in Crohn's disease, may have a larger impact on linear growth than steroid use.[354]

For children who fail EEN, it is important that future studies look at the effect of combining EEN with corticosteroids to establish if these children have the same opportunity to obtain target growth outcomes as well as achieve disease remission.

3.11.7 Effect of treatment with MEN

Since the current study was an observational study, we could not influence the numbers of children choosing to take maintenance enteral nutrition (MEN) post-EEN. It was predicted from previous years at the Royal Hospital for Children that approximately 55% of children would refuse MEN as a course of treatment to maintain disease remission. However only 6/23 (26%) of children who went into remission on EEN opted not to take MEN (Fig. 3.6), one then going onto MEN around 17-weeks post-EEN. Another problem was, although all children who opted not to take MEN were on an immunosuppressant, only 8/17 children taking MEN were taking an immunosuppressant, creating a third subgroup. Children taking an immunosuppressant as well as MEN creates a confounding factor, as it could have an effect on the gut microbiota profile and metabolites, making understanding differences between these three groups more challenging.

It is unclear why more children chose to take MEN than in previous years. It is possible that in explaining the study to parents/guardians and children with Crohn's disease, made them more aware of the possible benefits of MEN as a therapy. It is also conceivable that in discussions with clinical staff, IBD-nurses and dietitians we influenced the likelihood they would recommend MEN as maintenance treatment. A combination of both of these may have influenced the choices participants made when offered MEN.

Although no difference was seen in number of days to relapse between children with Crohn's disease who took MEN, an immunosuppressant or both together (p=0.82), the sample sizes were too low to detect any differences which have been reported in

other studies (Chapter 1, Table 1.6). It is possible that children who are more likely to relapse are more likely to be given more aggressive therapy by clinical staff. In fact, in the current study children with higher disease activity (wPCDAI scores) at baseline were more likely to be given MEN plus an immunosuppressant post-EEN (p=0.01)(Fig. 3.24), therefore the treatment groups have a bias in terms of disease activity, making it impossible to determine if treatment has an effect on days to relapse since those who have more severe disease might relapse sooner. No correlation was seen between disease activity at baseline (wPCDAI score) and days to relapse (Spearman's rank, p=0.480; rho=-0.155); thus having more severe disease at baseline did not affect how quickly children relapsed. Although baseline disease activity affected treatment, baseline height/weight/BMI z-scores did not affect whether children took MEN, an immunosuppressant or both together (p=0.73, p=0.77, p=0.56 respectively).

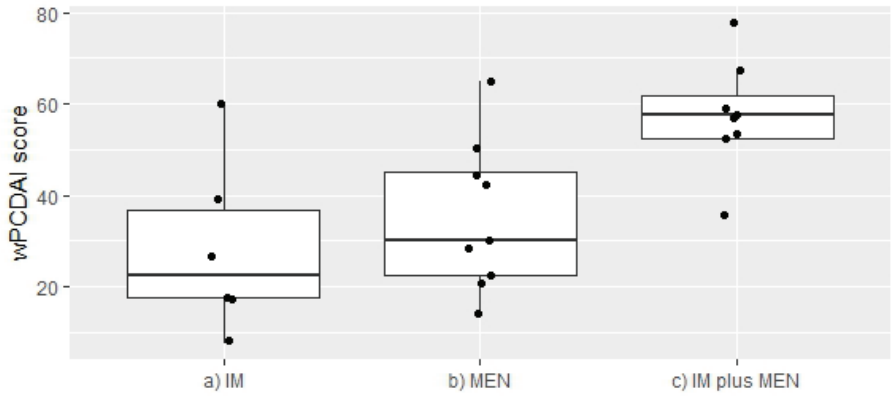


Figure 3.24: Effect of baseline disease activity (wPCDAI score) on treatment with maintenance therapy for children taking a) immunosuppressant (IM); b) maintenance enteral nutrition (MEN) or c) immunosuppressant plus maintenance enteral nutrition (IM plus MEN). IM plus MEN was significantly higher than IM (p=0.01) and MEN (p=0.03). Dunn's test of multiple comparisons was used following a significant Kruskal-Wallis test.

It was important to look at whether treatment with MEN (with or without an immunosuppressant), could affect growth outcomes. There was no overall difference seen between treatment groups (Fig. 3.15). However the child who had the largest drop in z-score for height, weight and BMI over the 8-weeks of MEN was recorded to be struggling with MEN, and may not have complied with the treatment. Although removing this child from the analysis may have given a more accurate assessment of the effect of MEN on growth outcomes, the remaining sample size of five children was too low to be meaningful. It is possible in terms of weight change, that children who took MEN were better able to maintain and gain weight; however as we do not have a non-treatment control group it is also possible that taking an immunosuppressant negatively affects the child's ability to gain weight. A larger sample size is required to enable reliable statistical analysis.

MEN is an attractive therapy option, because evidence to date suggests when com-

pared with normal habitual diet, MEN may be effective in maintaining remission in children with Crohn's disease (Chapter 1, 1.8.3; Table 1.6). It could also be as effective as some medication with one study suggesting MEN can delay the need for corticosteroid therapy[101] as well as improving the child's growth and nutritional status. A problem with interpreting MEN studies is different doses and feeding approaches have been used, such as overnight nasogastric feeds combined with normal eating during the day; short periods of nasogastric feeds several times a year; and a daily supplement drink used alongside normal eating with variable success rates.[151] Since the 2007 Cochran systematic review,[355] suggesting MEN could be effective at extending remission post-EEN, studies have been few, usually with small sample sizes which lack statistical power, making it difficult to draw conclusions.[356] While larger studies are still needed, and given that MEN could be dose dependant,[148] studies using murine models could shed some light on whether lower doses of around 25% MEN are likely to be ineffective.

Medications It is important to consider the medications children take at the start of and during treatment with EEN since these have the potential to influence the gut microbiota profile and metabolites. 23/32 (72%) of children diagnosed with Crohn's disease were medication free at baseline, giving a good study size free of medication as a confounding factor. 7/9 children with UC (77%) and 10/11 (91%) non-IBD children were also medication free (Table 3.5), meaning the study control groups also had a low chance of being affected by any influence of medication.

Treat to Target Although it is evident that inducing and maintaining clinical remission of symptoms for patients with Crohn's disease are the main therapeutic goals, they may not be enough to prevent longer term damage to the gut. As such corticosteroids have been the cornerstone treatment and provide effective control of symptoms in the shorter term; however they fail to prevent the long-term progression of disease.[357] Therefore biochemical markers are becoming a much more important aspect of monitoring disease progression and gut inflammation. More consistent monitoring of inflammatory markers such as calprotectin in children with Crohn's disease could in future be linked with therapeutic doses of MEN to better control the likelihood of relapse.

3.11.8 Summary of key findings

- Children with Crohn's disease had significantly lower height/weight/BMI z-scores than healthy children at baseline.
- In children with Crohn's disease, markers albumin, CRP and ESR, correlated with BMI z-scores, suggesting increased disease severity results in poorer growth outcomes.
- 72% of children with Crohn's disease attained remission after 8-weeks EEN; and all children with moderate to severe disease at baseline had reduced disease activity.
- Of children diagnosed with Crohn's disease, 72% had upper involvement; with 59% having gastric disease.
- Children with Crohn's disease who were underweight pre-EEN put on more weight during EEN than those who were normal weight at baseline.
- No differences in BMI or growth were seen between responders and non-responders to EEN, either at baseline or at 28-weeks post-EEN.
- 1-year after the start of treatment with EEN, only eight of the original twenty-three respondents (35%) were still in disease remission.
- Children with ileal disease were more likely to respond to EEN (NS).

New findings from this study:

- Although children with Crohn's disease had age appropriate growth during EEN, growth velocity was not maintained once children went back onto normal diet.
- No differences in growth were seen between the three types of maintenance therapy (MEN, MEN+IM, IM).

Future research goals:

- Growth can be used as an outcome to measure the effectiveness of paediatric Crohn's disease therapies, however studies to date have focused on short-term growth. There is an urgent need to study not only clinical outcomes, but also the long-term growth outcomes of treatment with EEN and MEN.
- Patients with non-IBD conditions should not be used as healthy controls. The current study shows that these children, when recruited from gastroenterology clinics, have conditions which should exclude them as controls. Nearly half of the non-IBD controls were given no diagnosis, and all of these children had a family history of IBD; hence it is possible some could have subclinical IBD.
- The reality in clinics is therapy for children with Crohn's disease involves multiple strategies, which often take place simultaneously making it impossible to ascertain if changes or outcomes are due to a single therapy or a combination approach; and appropriate controls are unavailable. Animal studies which trial single and combined therapies with controlled diets, using well designed controls, would give more robust results which could then form the basis of more informed human trials.

4 Markers of inflammation in Crohn's disease

4.1 Introduction

Chronic and persistent disease activity in IBD is linked with poor prognosis and hence monitoring inflammatory markers in these patients would allow the potential suppression of sub-clinical inflammation as a method of treatment.[358] Disease markers are also a priority, because not only is monitoring disease activity by endoscopy invasive for the patient, particularly for children who have to undergo a general anaesthetic, but it is also expensive and diverts valuable resources which might better benefit IBD patients if these were redirected to long term treatment and care.

Crohn's disease has historically been managed in a step-wise manner with evaluation of disease activity and treatment mostly based on active symptoms. Failing to keep patients in remission worsens the long-term outcomes of patients with Crohn's disease and places them at risk of bowel damage.[359] Identifying and using specific disease markers for Crohn's disease to regularly monitor disease activity would allow the identification of sub-clinical disease. This concept called 'treat to target' has been beneficial in other autoimmune conditions such as rheumatoid arthritis.[360]

In the current study where treatments and outcomes are assessed, it is important to have reliable markers of inflammation, which are independent of any potential bias.

4.1.1 Stool characteristics and markers of inflammation

Faecal calprotectin Henderson *et al.*[361] have shown that faecal calprotectin can act as a better marker of colonic inflammation than other commonly used blood markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelet count, haemoglobin, total white cell count, and albumin in children with IBD. Faecal calprotectin is a measure of the amount of the protein calprotectin present in the stool of patients. An increase in calprotectin, is an indicator of neutrophil migration into the intestinal mucosa, which occurs during the type of gut inflammation caused by IBD. Testing for calprotectin could reduce the need for repeated and invasive colonoscopy.[362]

Human calprotectin is a 24kDa dimer of the calcium binding proteins S100A8 and S100A9.[363] It can chelate iron,[364] manganese and zinc,[365] and this ability to sequester essential metals gives calprotectin its antimicrobial properties.[363] Calprotectin, makes up around 60% of protein content in the cytosol of neutrophils; it is resistant to enzymatic degradation, and can be easily measured from stool samples using an ELISA.[366]

There are a number of diseases that cause an increased excretion of faecal calprotectin including IBD, coeliac disease, infectious colitis, necrotising enterocolitis, intestinal

cystic fibrosis and colorectal cancer.[367] However, faecal calprotectin is now frequently used as indicator for active disease in IBD.[367] Measurement of faecal calprotectin has been shown to be strongly correlated with 111-indium-labelled leukocytes which are considered the gold standard measurement of intestinal inflammation.[366] However, the European Crohn's and Colitis Organisation (ECCO) suggest that faecal calprotectin only be used as a secondary outcome measure in Crohn's disease due to high variability in results, which leads to low levels of precision and unclear cut-off levels for assessing disease activity.[125]

By measuring faecal calprotectin as a marker of colonic inflammation in the current study, and confirming it correlates with measures of clinical disease remission (wPCDAI), changes in this continuous data set could be used to help answer the current study hypotheses. Stool markers such as calprotectin have emerged as a new diagnostic tool to detect intestinal inflammation. However, stooling frequency and the Bristol stool chart score (Methods, Fig. 2.8), an indicator of water content, also have the potential to act as low cost approximate indicators of gut inflammation in IBD.

4.1.2 Blood markers

C-Reactive protein (CRP) CRP is a pentameric protein present in blood plasma. It is synthesised in the liver and increases in response to macrophage and T-cell IL-6 production.[368] A normal concentration of CRP in the blood serum of healthy humans lies between 5-10mg/L and tends to increase with age. CRP is involved in a number of functions linked with host defence, including its ability to recognise both pathogens and damaged host cells. As such the level of CRP in plasma increases during acute phase response to tissue injury, infection, and other inflammatory stimuli and is therefore routinely used as a marker of inflammation in IBD.[358] It is however less sensitive than faecal calprotectin as a marker of inflammation.[369]

Erythrocyte sedimentation rate (ESR) ESR is the rate that red blood cells sediment in one hour and is used as a non-specific measure of inflammation. Inflammatory processes result in a high level of fibrinogen in the blood which causes red blood cells to clump into stacks called 'rouleaux'. Due to increased density these settle more quickly. However in patients with Crohn's disease, ESR can be within normal limits while CRP is elevated, and CRP tends to return to normal faster than ESR in response to treatment.[370]

Human serum albumin Serum albumin is the most abundant protein found in human blood plasma, making up around half of all serum protein. It is produced in the liver and transports compounds such as hormones, fatty acids, as well as buffering pH and maintaining the colloid osmotic pressure of blood vessels. Serum albumin is a

negative acute-phase protein which is down regulated in inflammatory conditions. As such it can be used as a non-specific marker of disease activity in IBD.[370] Both ESR and albumin are criteria used in the weighted Paediatric Crohn's Disease Activity Index (wPCDAI) (Appendix 3).

Full blood count Results from a full blood count can show inflammation from the white blood cell count and anaemia can be detected via red blood cell results.[370]

Liver function test This test helps to monitor if the liver is functioning properly, and is important in relation to the side effects of some medications used to treat IBD. Methotrexate and Infliximab have been reported to cause liver damage in IBD patients.[371;372] However the prevalence of altered liver function test results in IBD patients has been difficult to establish because various studies have used different methods to collect data, as well as different diagnostic and patient selection criteria.[373;374;375] Issues with liver (hepatobiliary) function can be due to inflammation in patients with IBD but the causal relationship between abnormal liver function test results and IBD is unclear.[376;377] Although Mercaptopurine and Azathioprine induced hepatotoxicity is not common in the adult IBD population, it has been reported in 10-15% of paediatric patients,[378] hence liver function monitoring is important for children taking these drugs as maintenance therapy.

Urea and electrolytes (U/Es) Urea and electrolytes (U/Es) are routinely measured from bloods taken from paediatric IBD patients. Measuring an electrolyte panel including sodium, potassium, chloride, bicarbonate as well as urea and paediatric creatinine (estimates glomerular filtration rate by age and gender),[379] can be used to check for dehydration; the side effects of medication, and toxic megacolon (a rare complication of Crohn's disease).[380]

4.1.3 wPCDAI as an indicator of disease activity in Crohn's disease

Although not a perfect tool, the weighted Paediatric Crohn's Disease Activity Index (wPCDAI) is a standard outcome measure in paediatric Crohn's disease research (Methods, section 2.4.3), and is currently used by clinicians in West of Scotland Paediatric Gastroenterology, Hepatology and Nutrition network to assess disease activity. The wPCDAI form is shown in appendix 3 and includes raised ESR and albumin as part of the scoring index.

4.1.4 Missing observations

One of the limitations of a study looking into inflammatory markers over time is that it can be challenging to obtain bloods close to the study time-points for a number of reasons. This was an observational study and as such we were dependent on children having bloods taken as part of their routine monitoring. On occasions children failed to turn up to clinic or were on holiday and bloods were not taken until a following appointment, which did not then coincide with the study time-points. Sometimes bloods were taken at the planned time but the clinical lab tests were unable to yield a viable result from the sample, and the test was then not repeated within the time-point of interest. This had the effect of creating gaps in the dataset which reduces the power of our ability to look at changes over time.

4.1.5 Hypotheses summary

- Children with Crohn's disease have an inflammatory marker profile which is distinct from that of children with UC, non-IBD conditions and healthy children.
- Inflammatory marker profiles at baseline can predict which children with Crohn's disease will respond to treatment with exclusive enteral nutrition (EEN).
- For children in disease remission post-EEN, supplementing return to normal diet with (20%) maintenance enteral nutrition (MEN) will maintain reduced levels of inflammatory markers associated with remission achieved during EEN.

4.2 Methods

Faecal samples were collected and processed as described in Methods, section 2.4.4. Bristol stool chart scores and stooling frequency were recorded on sample collection (Methods 2.6.1). Faecal water content was calculated by weighing samples before and after freeze-drying (Methods 2.7.1). Faecal calprotectin was measured using the commercial ELISA kit CALP-0170 (CalproLab^{TM} CALPRO, Lysaker, Norway) according to the manufacturer's instructions (Methods 2.6.2).

Data on clinical markers CRP; ESR; serum albumin; as well as full blood count (FBC); liver function test and U/Es were obtained from routine clinical lab results. To explore the relationship between different inflammatory markers the differential expression analysis of the blood data was used. A challenge in research datasets based on biological sampling is that of missing values, which restrict our ability to carry out this type of analysis accurately. Hence a kernel-based method (KMDA) was used to account for missing values.[338] Kernel density estimation is a non-parametric method used to estimate the probability density function of a random variable (Methods 2.10.3). Correlation coefficients were used to group variables into feature sets, thus

allowing us to see the relationship between those variables for each experimental patient group.

Disease activity was also assessed using the weighted paediatric Crohn's disease activity index (wPCDAI)(Methods 2.4.3).[287] Disease remission was defined as a wPCDAI score of <12.5. Mild disease between 12.5-40; moderate disease >40-57.5 and severe disease as >57.5.[287]

4.3 Results

4.3.1 Markers of inflammation at baseline

Collecting markers of inflammation at baseline helps to build a picture of patient characteristics prior to treatment with EEN and MEN, as well as disease severity. Markers provide a useful comparison to compare not only against other inflammatory conditions, but also as a measure of the success of treatment over time.

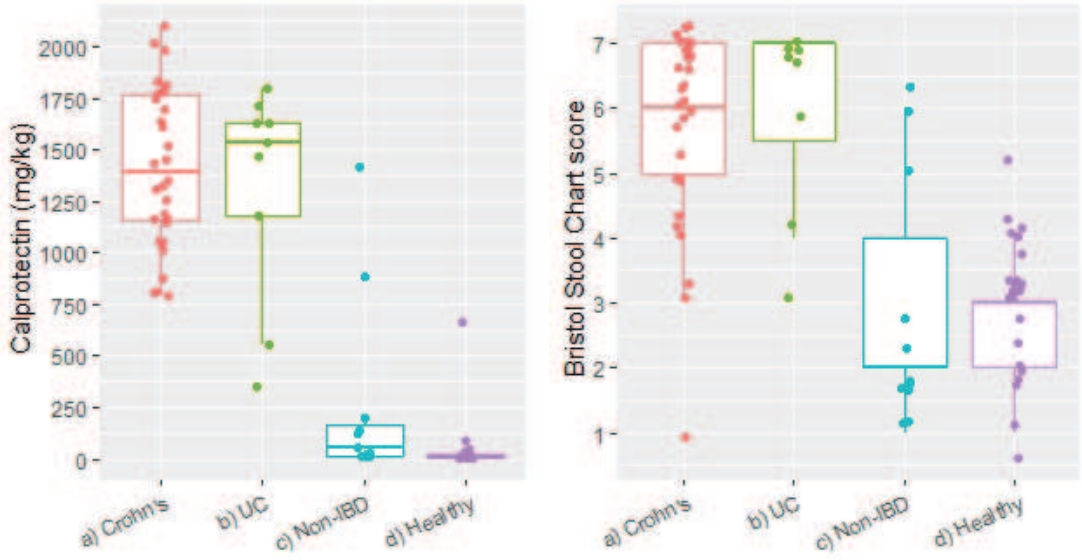
Calprotectin The pre-treatment median calprotectin concentration for children with Crohn's disease was not different from children with UC. Children with Crohn's disease and UC had significantly higher faecal calprotectin levels than healthy children ($p<0.0001$) and patients with non-IBD conditions ($p<0.01$) (Table 4.1; Fig. 4.1a).

Bristol stool chart scores and stool water content Baseline median Bristol stool chart scores for children with Crohn's disease were not different from children with UC. Children with both Crohn's disease and UC had significantly higher median Bristol stool chart scores than both healthy children and those with non-IBD conditions (Table 4.1; Fig. 4.1b).

Table 4.1: Faecal inflammatory markers between patient groups (median; IQR)

	Group				
	Crohn's	UC	non-IBD	Healthy	K-W Test
Calprotectin (mg/kg)	**††**1337** (690) (n=32)	**† **1533** (809) (n=9)	53 (184) (n=11)	18 (31) (n=20)	p<0.001
Water content (%)	**†† **83.5** (11.0) (n=32)	*† **87.0** (11.0) (n=7)	70.0 (11.0) (n=11)	72.0 (9.0) (n=19)	p<0.001
Bristol stool chart	**†† **6.0** (2.0) (n=32)	*† **7.0** (3.5) (n=9)	2.0 (3.0) (n=11)	3.0 (1.5) (n=25)	p<0.001
Daily stooling frequency	** **3.0** (2.0) (n=27)	** **6.0** (7.0) (n=7)	* **3.0** (1.5) (n=6)	1.0 (1.0) (n=19)	p<0.001

Kruskal-Wallis test with Bonferroni correction for multiple testing. Difference from healthy children was significant at * p<0.01, ** p<0.001. Difference from non-IBD patients is significant at † p<0.01, †† p<0.001.



(a) (b)

Figure 4.1: Difference in inflammatory markers (a) calprotectin and (b) Bristol stool chart scores between children with Crohn's disease, UC, non-IBD conditions and healthy children. KW test: children with IBD were significantly different from both the non-IBD control group and healthy children ($p<0.01$).

The percentage of water content of stool samples (Fig. 4.2) shows that the Bristol Stool chart scoring is a good a marker of stool sample water content in patient groups. However for healthy children who had much lower stool chart scores there was no correlation between these lower scores and stool water content.

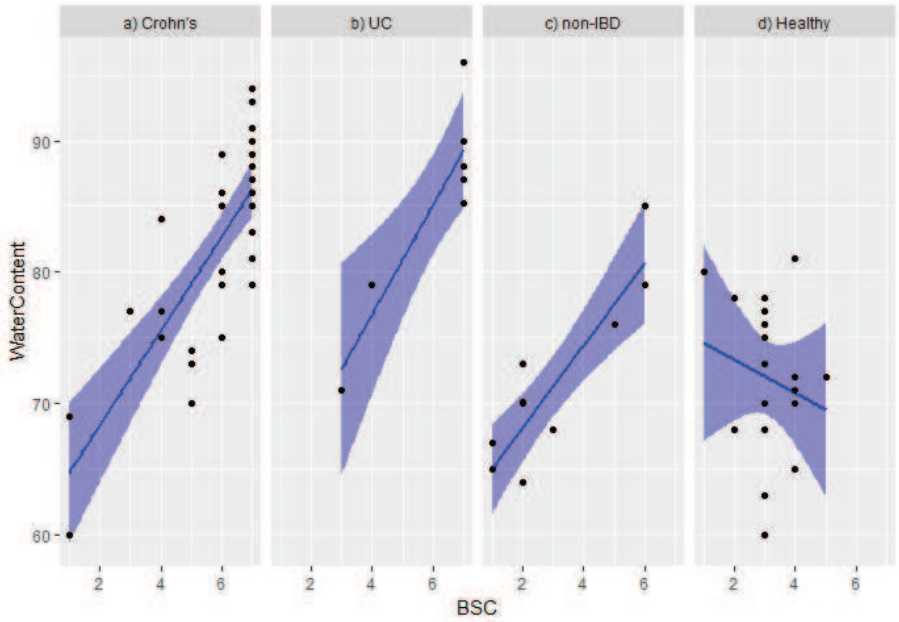


Figure 4.2: The relationship (Pearson correlation) between Bristol Stool chart scores (BSC) and % of water in faecal samples in patients with (a) Crohn's disease (s=0.788; $p<0.001$); (b) UC (s=0.897; p=0.006); (c) non-IBD (s=0.877; $p<0.001$) and (d) healthy children (s=-0.199; NS). Shaded area represents the 95% confidence interval.

Table 4.2: Blood markers measured at baseline, median (IQR)

Test	Crohn's (n=30)	UC (n=9)	non-IBD (n=11)	*reference range*
CRP	6.0 (20.0)	3.0 (3.5)	3.0 (2.2)	0-10 mg/L
ESR	21.0 (26.2)	20.0 (15.5)	5.0 (3.0)	1-9 mm/hr
Full blood count				
white blood count (WBC)	9.20 (4.08)	9.70 (3.85)	6.40 (3.40)	4.5-13.5 $\times 10^9$L
red cell count (RCC)	†****4.86** (0.63)	4.21 (1.24)	4.68 (0.63)	4.0-5.10 $\times 10^{12}$L
haemoglobin	112.0 (27.5)	108.0 (38.0)	127.0 (19.0)	115-155 g/L
haematocrit	0.36 (0.06)	0.33 (0.12)	0.36 (0.09)	0.350-0.450 L/L
MCV	76.7 (13.8)	79.9 (3.4)	82.3 (9.2)	77.0-85.0 fL
MCH	***24.6** (6.2)	26.6 (1.4)	27.4 (3.4)	25.0-33.0 pg
platelet count	****425.0** (218.5)	384.0 (130.5)	301.0 (125.0)	150-400 $\times 10^9$L
neutrophils	***5.4** (3.3)	***5.5** (2.9)	3.4 (1.6)	1.5-8.5 $\times 10^9$L
lymphocytes	◇ †2.0 (0.9)	2.9 (0.8)	2.1 (1.8)	1.5-6.8 $\times 10^9$L
monocytes	***0.80** (0.65)	***1.00** (0.75)	0.60 (0.30)	0.2-1.0 $\times 10^9$L
eosinophils	0.30 (0.55)	0.50 (0.55)	0.30 (0.20)	0.1-1.0 $\times 10^9$L
Liver function test				
total bilirubin	6.0 (3.5)	5.0 (1.0)	7.0 (9.8)	<20 µmol/L
alanine transaminase (ALT)	11.0 (7.0)	16.0 (27.5)	13.5 (5.5)	10-45 U/L
aspartate transaminase (AST)	*†**19.0** (10.5)	24.0 (32.0)	27.0 (16.2)	15-45 U/L
alkaline phosphatase	144.0 (116.5)	208.0 (134.0)	221.5 (64.0)	60-425 U/L
albumin	****32.0** (11.5)	35.0 (7.5)	40.0 (5.2)	35-50 g/L
Urea and Electrolytes				
sodium	138.0 (2.0)	137.0 (2.5)	137.5 (2.5)	133-146 mmol/L
potassium	4.0 (0.3)	4.0 (0.4)	4.2 (0.6)	3.5-5.0 mmol/L
chloride	◇ †104.5 (2.2)	106.0 (2.0)	104.5 (5.5)	95-108 mmol/L
bicarbonate	24.0 (4.5)	22.0 (4.5)	21.5 (3.8)	19-28 mmol/L
urea	3.1 (0.8)	3.1 (1.7)	3.9 (1.2)	2.5-6.5 mmol/L
paediatric creatine	46.5 (12.5)	48.0 (19.0)	41.0 (31.2)	20-50 µmol/L
serum ferritin θ	†25.5 (27.0)	14.0 (16.0)	26.5 (51.3)	7-140 ng/mL

[1] Kruskal-Wallis test with Bonferroni correction for multiple comparisons. Difference from non-IBD patients significant at * p<0.05 ** p<0.01, *** p<0.001. Difference from UC patients significant at †p<0.01, ††p<0.001. θ n= 18,7,11 respectively. ◇ Significance is likely a type 1 error.

Stooling frequency Although children with UC had the highest median number of stools per day, they also had the most variation in number of stools per day (Table 4.1). Healthy children were stooling significantly less than all three patient groups: Crohn's disease; UC and non-IBD patients ($p<0.0001$, $p<0.0001$ and $p<0.001$ respectively). There was no correlation between daily stooling frequency and either faecal water content or the Bristol stool chart scores for any group of children.

Blood markers To explore differences in inflammatory marker profiles between patient groups, standard clinical lab results for C-Reactive protein (CRP); erythrocyte sedimentation rate (ESR); full blood count (FBC); liver function test (LFT) and urea/electrolyte test (U/Es) were obtained from patient records (Table 4.2). Blood test results were not available for the cohort of healthy children who took part in this study.

Although median CRP was higher in children with Crohn's disease compared to children with UC and non-IBD patients, this did not reach significance. With the exception of one non-IBD patient with gastritis, children with non-IBD conditions and UC did not have raised CRP. However 11/30 (37%) of children with Crohn's disease had elevated CRP (>10mg/l).

ESR was higher in both Crohn's disease and UC compared with non-IBD controls but the difference did not reach significance, due to high levels of variability in IBD patients. Only one non-IBD patient with gastritis had raised ESR, compared with 68% Crohn's and 75% UC patients with elevated ESR (>10mm/hr).

Full blood count (FBC) The red blood cell count was significantly lower in children with UC than those with Crohn's disease (p=0.007) and children with non-IBD conditions (p=0.009). Although median haemoglobin and the ratio of the volume of red blood cells to the total volume of blood (haematocrit) were reduced in UC this was not significant. For children with Crohn's disease their median corpuscular haemoglobin (MCH) was lower than non-IBD children (p=0.02) and children with UC (NS)(Table 4.2). White blood cell count was higher in both IBD groups but did not reach significance. The number of neutrophils and monocytes was higher in both children with UC and Crohn's disease ($p \leq 0.05$), compared with non-IBD controls. Although numbers of lymphocytes in children with Crohn's disease are higher than non-IBD controls (NS) and those with UC (p=0.03), this is likely a type 1 error as the non-IBD control group have a range that encompasses both IBD groups (Table 4.2). Platelet count was higher in children with Crohn's disease (p=0.009) and UC (NS) compared to non-IBD controls (Table 4.2).

Liver function test (LFT) Median aminotransferases (ALT & AST) and alkaline phosphatase were lower in children with IBD (Table 4.2), but only reached significance for AST in children with Crohn's disease. Albumin was reduced in IBD compared with non-IBD controls but only reached significance for Crohn's disease (p=0.003). 17/30 (57%) children with Crohn's disease; 3/9 (33%) UC and 2/10 (20%) with non-IBD conditions (one with gastritis; one with intestinal polyps) had albumin levels lower than the reference range (<35g/L).

Urea and Electrolytes (U/Es) Although children with Crohn's disease had a median chloride level that was significantly higher than children with UC, this is likely a Type 1 error 'false positive' as the chloride range in children with non-IBD conditions encompassed both IBD groups. No difference from the non-IBD control group was seen between groups for the other measures in the U/E test (Table 4.2).

Serum ferritin as a measure of iron storage Children with UC had significantly lower median ferritin levels than children with Crohn's disease ($p<0.01$). This difference did not reach significance in children with non-IBD conditions because the range of ferritin levels (IQR) was too high (Table 4.1).

4.3.2 A Kernel approach for differential expression analysis of blood data

Pearson correlation coefficient with a threshold of 0.70 followed by KMDA, showed children with Crohn's disease compared to non-IBD controls, had a higher WBC grouped with an increase in neutrophils ($p<0.001$). ESR and CRP in Crohn's disease also had a correlated increase ($p<0.001$); alkaline phosphatase and albumin were independently lower in children Crohn's disease when compared to non-IBD controls ($p<0.001$ & $p=0.01$ respectively) (Fig. 4.4b). When children with UC were compared to non-IBD controls increased WBC correlated not only with neutrophils and monocytes but also with increased platelet count and ESR ($p<0.05$). In children with UC, a decrease in RCC was correlated with haemoglobin and haemacrit, as well as albumin ($p<0.05$). Children with UC also had significantly more eosinophils ($p<0.05$) and less serum ferritin ($p<0.05$) than non-IBD control patients. Serum ferritin, which was lower in UC ($p=0.003$) was the only marker to be significantly different between Crohn's disease and UC.

The overall blood profile differences between patient groups are shown in figure 4.3. Although 95% confidence intervals for groups overlap, children with Crohn's disease have profiles which are further from the non-IBD control group than children with UC.

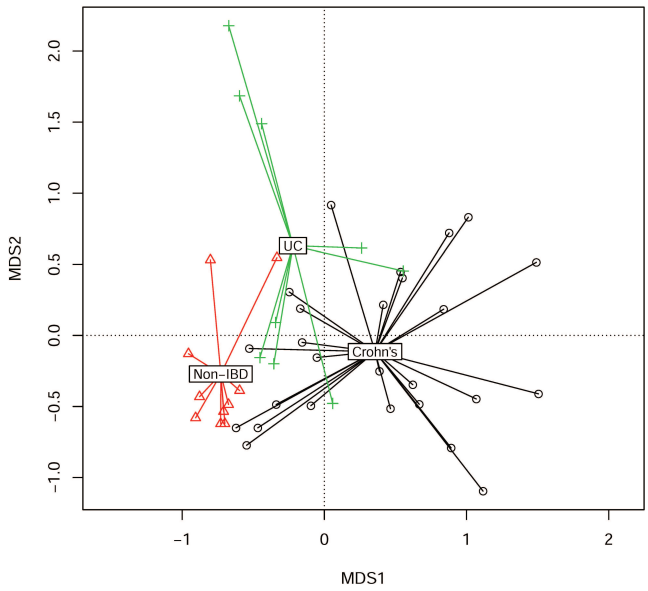


Figure 4.3: Difference in blood result profiles between children with Crohn's disease, UC and non-IBD conditions shown by metric multi-dimensional scaling (MDS) spider plot. PERMANOVA: R2=0.197; **p=0.0001** (*vegan; euclidean*). MDS polygon plot shown in appendix 4.

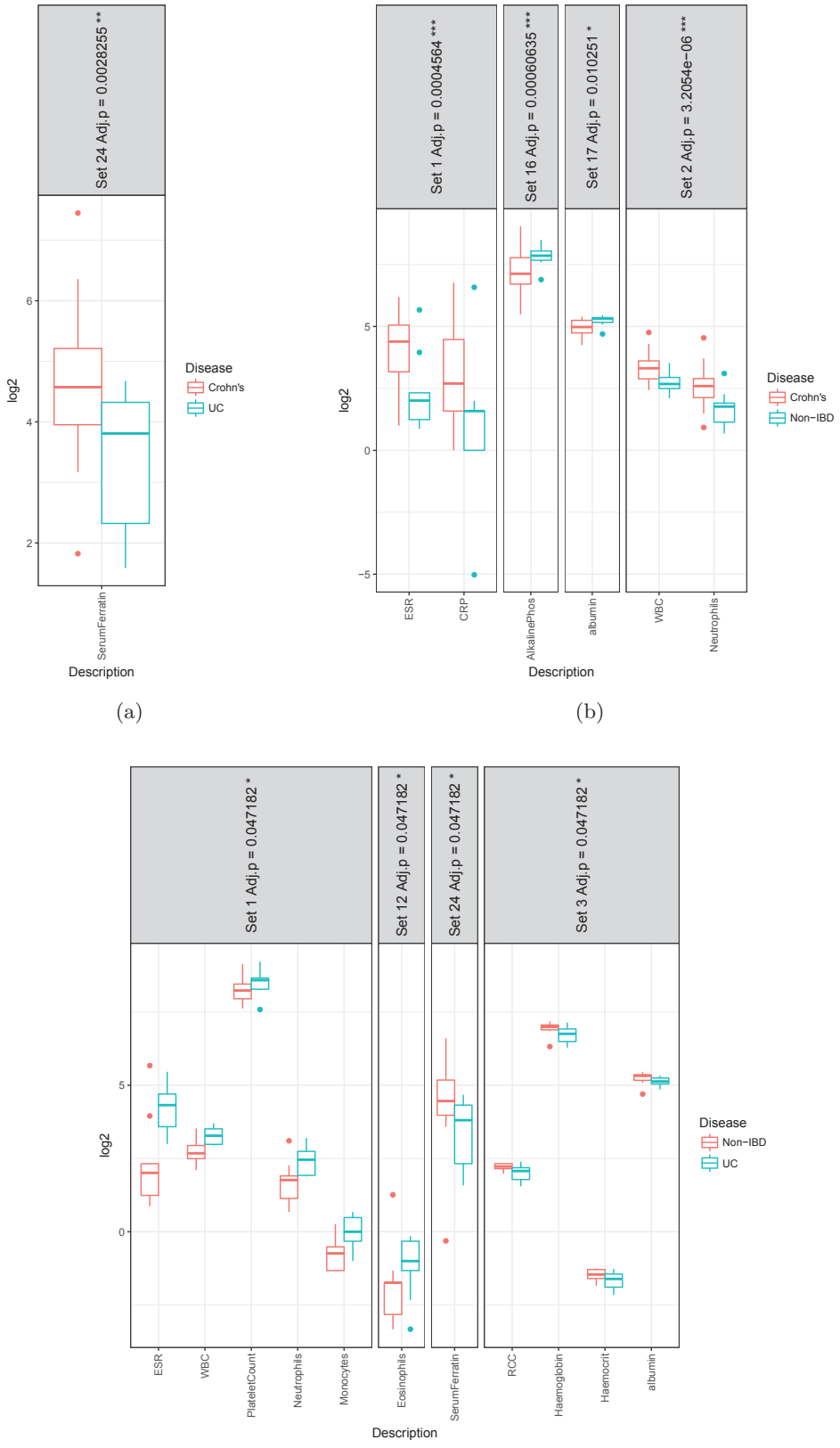


Figure 4.4: Kernel based differential analysis (KMDA) on blood markers at baseline for (a) children with Crohn's vs those with UC, (b) children with Crohn's vs those with non-IBD conditions and (c) children with UC vs those with non-IBD conditions. Correlation coefficients were used to group variables into feature sets, allowing visualisation of the relationship between those variables for each patient group.

4.3.3 Changes in markers of inflammation during EEN

After treatment with EEN, inflammatory markers faecal calprotectin, ESR and CRP were significantly reduced in children with Crohn's disease, along with a significant increase in albumin (Table 4.3). Changes in inflammatory markers were accompanied by an overall reduction in disease activity scores (wPCDAI) (Fig. 4.5).

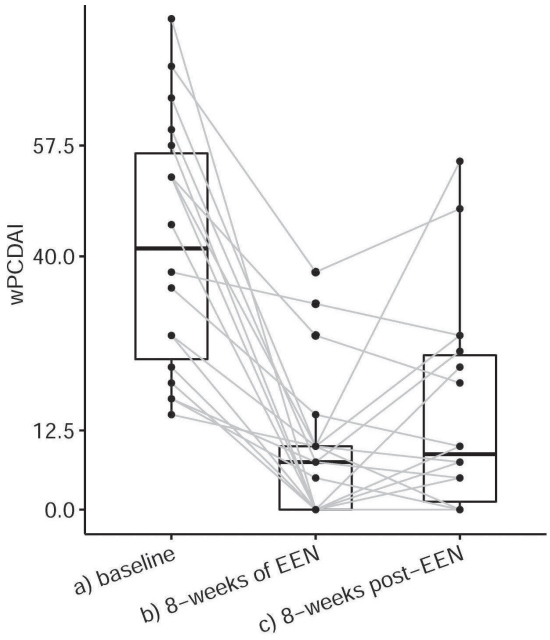


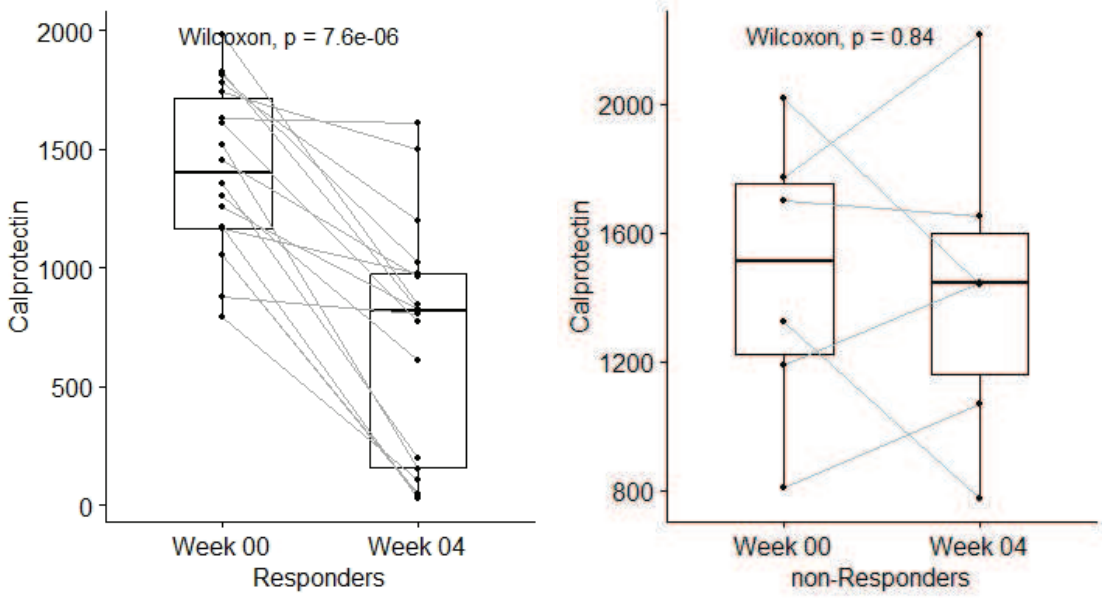
Figure 4.5: Weighted paediatric Crohn's disease activity index (wPCDAI) pre-EEN, after 8-weeks treatment with EEN and 8-weeks post-EEN. Samples are paired across all three time-points (n=18)

Children responding to EEN had a significant reduction in faecal calprotectin, which was not seen in non-responders (Fig. 4.6). Two non-responders had reduced calprotectin after 4-weeks EEN: one of which increased CRP from 1 to 13mg/L during EEN; while the other had very high levels of calprotectin at baseline which only reduced slightly after treatment, and went onto corticosteroids post-EEN (Fig. 4.6a). In the response group one child had no change in calprotectin levels during EEN, however their CRP fell from 45 to 3mg/L, ESR fell from 73 to 39mm/hr and albumin increased from 21 to 38g/L; hence their wPCDAI score fell to zero (Fig. 4.6b).

Table 4.3: Changes in key inflammatory markers in children with Crohn's disease at baseline and after 8-weeks EEN.

	number	Baseline Week-0	EEN week-8	Wilcoxon p value
Calprotectin mg/kg	n=16	1518 (517)	672 (826)	p<0.001
ESR mm/hr	n=17	17 (24)	5 (10)	p=0.002
CRP mg/L	n=13	5 (20)	3 (3)	p=0.023
albumin g/L	n=17	31 (12)	41 (6)	p<0.001

Median (IQR) Differences were analysed with Wilcoxon matched-pairs signed rank test.



(a) (b)

Figure 4.6: Changes in calprotectin during EEN in (a) responders and (b) non-responders to EEN. Wilcoxon paired test. Time-points were (wk-00) baseline and (wk-04) after 4-weeks treatment with EEN.

The Bristol stool chart (Methods Fig. 2.8) was used to measure faecal consistency (Fig. 4.7b) during EEN. The median Bristol stool chart score after 8-weeks EEN showed a reduction in water content (4.5; IQR 4) from baseline (6; IQR 2). Once back onto normal diet for 8-weeks, stool consistency remained similar (4; IQR 4).

The daily stool frequency of children with Crohn's disease reduced after treatment with EEN (Fig. 4.7d): from a median of 3 stools per day (IQR 2.0) at pre-treatment to 1 per day (IQR 0.5) after 8-weeks EEN. Once children were back onto normal diet for 8-weeks there was a slight increase to 1.5 stools per day (IQR 1.0). Daily stooling frequency is one of the indicator questions in the wPCDAI score, and a reduction in water content and stooling frequency was maintained post-EEN (Fig. 4.7).

Blood profiles Clinical blood markers were used to generate a multi-dimensional scaling profile, which in children with Crohn's disease moved towards non-IBD controls after EEN (Fig. 4.8). A kernel based differential analysis between blood markers at baseline and after 8-weeks EEN show changes in CRP and ESR are correlated. Changes in markers from the U/E test including ALT/AST are also correlated in a separate subset (Fig. 4.9)

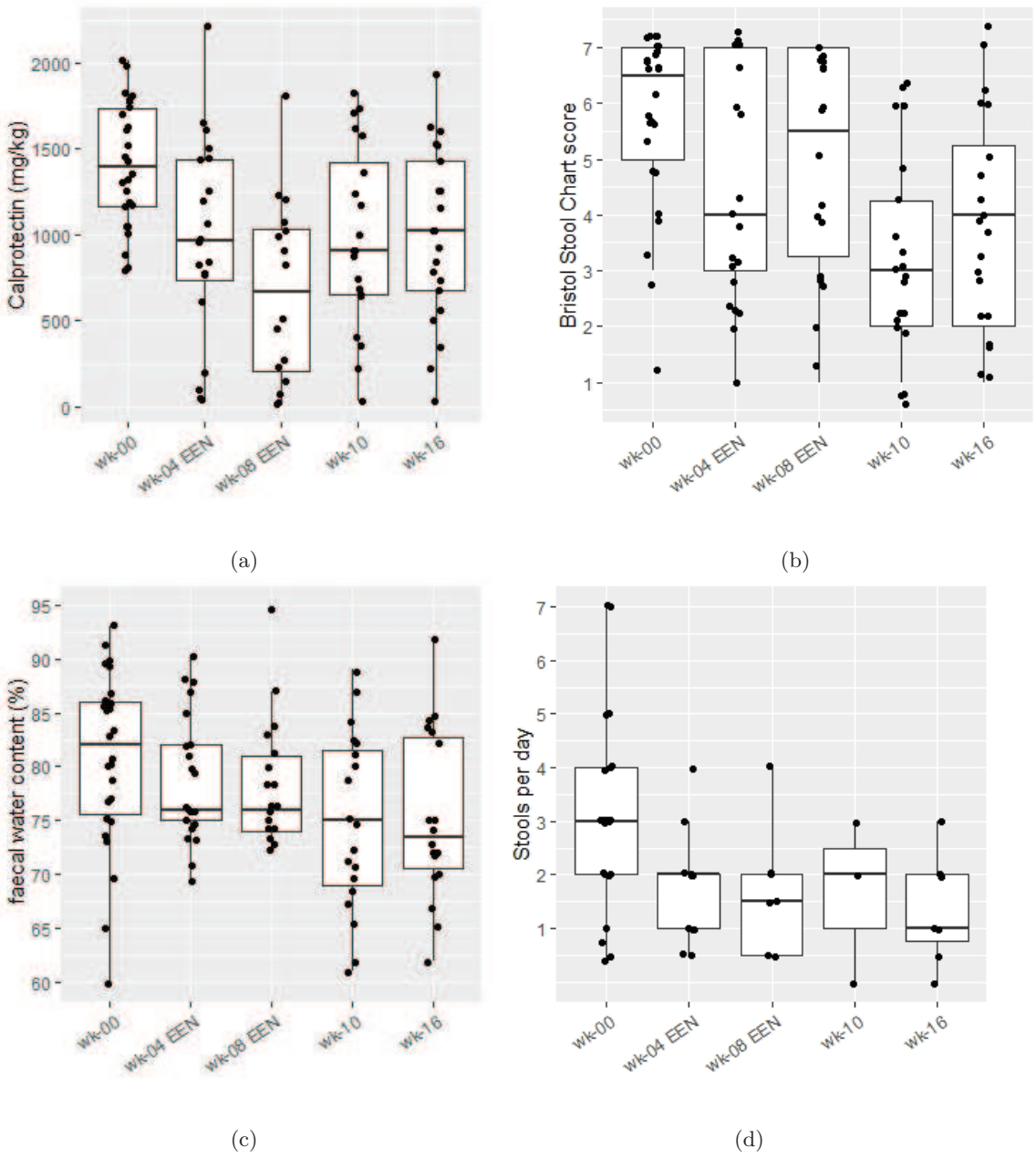


Figure 4.7: Changes in disease activity pre-EEN and post-EEN were measured using a number of indicators: (a) calprotectin; (b) the Bristol stool chart; (c) faecal water content and (d) daily stooling frequency. Time-points were (wk-00) baseline; (wk-04) after 4-weeks treatment with EEN; (wk-08) after 8-weeks treatment with EEN; (wk-10) after 2-weeks and (wk-16) after 8-weeks of normal habitual diet.

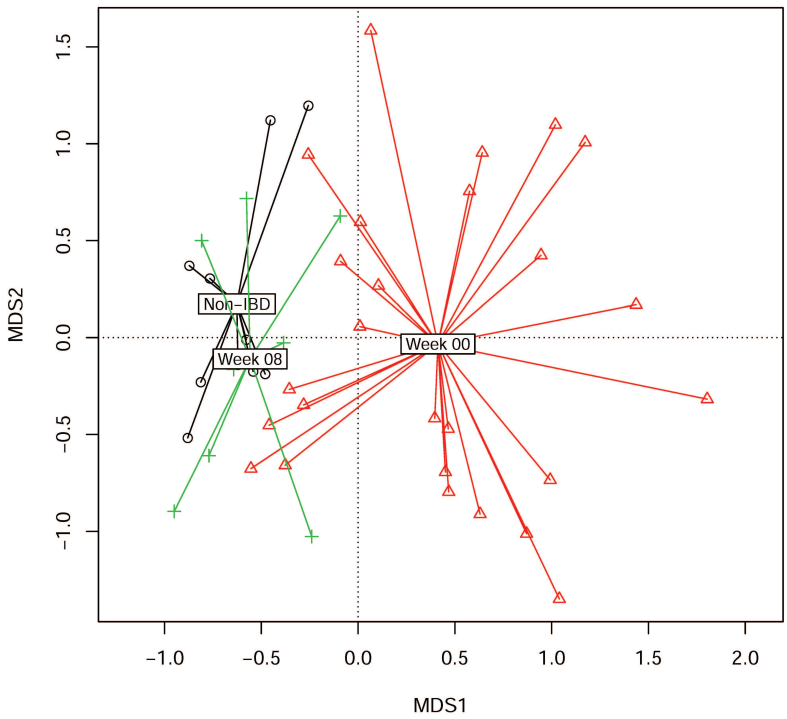


Figure 4.8: Changes in blood result profiles between baseline (week 00) and after 8-week treatment with EEN (week 08) in children with Crohn's disease; shown by metric multi-dimensional scaling (MDS) spider plot. PERMANOVA: R2=0.225; **p=0.0001**; Children with non-IBD conditions (black) are shown for reference. MDS polygon plot shown in appendix 4

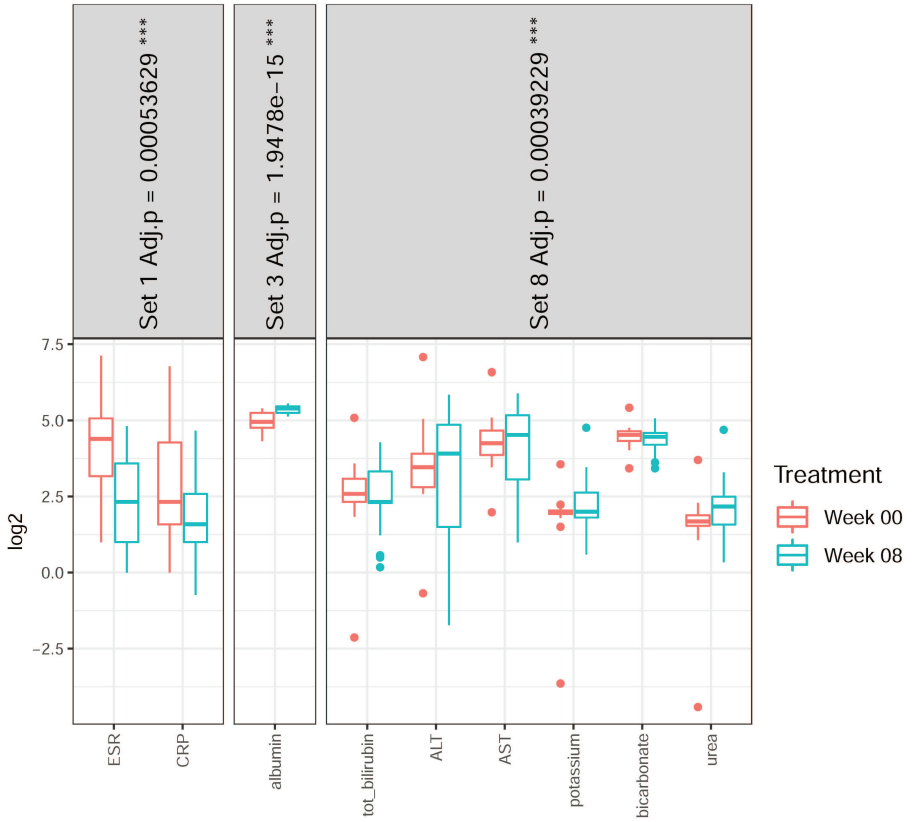


Figure 4.9: Kernel based differential analysis (KMDA) on blood markers in children with Crohn's at baseline (week 00) and after 8-weeks treatment with EEN (week 08). Correlation coefficients were used to group variables into feature sets, allowing visualisation of the relationship between those variables for each patient group.

4.3.4 Predicting response to EEN

The median baseline levels of calprotectin as a marker of inflammation were slightly higher in children who failed to respond to treatment with EEN but the difference was small and did not reach significance (Table 4.4). No significant difference was seen for ESR, CRP or albumin between responders and non-responders to EEN. ESR and albumin are included in the disease activity score index (wPCDAI) which also saw no difference between responders and non-responders (Table 4.4).

Table 4.4: Predicting response to EEN: Difference in key inflammatory markers at baseline between children who responded to EEN and those who failed EEN.

	responder n=22	non-responder n=8	MWU p value
Calprotectin mg/kg	1328 (639)	1566 (547)	p=0.298
ESR mm/hr	21.5 (24)	18 (24)	p=0.430
CRP mg/L	6.5 (19.8)	3 (12)	p=0.395
albumin g/L	31 (11.5)	36 (8.2)	p=0.259
wPCDAI score	42.5 (35.0)	37.5 (37.5)	p=0.414

Median (IQR). No significant difference between responders and non-responders (MWU-test).

4.3.5 Changes in markers of inflammation during MEN

For children who went into remission on EEN, calprotectin significantly increased after only 8-weeks of normal diet post-EEN. ESR and CRP also increased but failed to reach significance for ESR. The median disease activity index score (wPCDAI) had also increased inline with these inflammatory markers after 8-weeks of normal diet, but failed to reach statistical significance (Table 4.5; Fig. 4.7).

Table 4.5: Inflammatory markers in children who responded to EEN; at the end of EEN and after 8-weeks normal diet (post-EEN).

	n value	end of 8-wks on EEN	end of 8-wks normal diet	Wilcoxon p value
Calprotectin mg/kg	n=14	485 (847)	1140 (638)	p=0.004
ESR mm/hr	n=12	5.5 (4.8)	8 (25.8)	p=0.057
CRP mg/L	n=10	3.0 (2.2)	4.5 (5.0)	p=0.050
albumin g/L	n=12	40 (5.5)	39 (7.2)	p=0.157
wPCDAI score	n=14	0 (9.4)	6.2 (19.4)	p=0.066

Median (IQR). Wilcoxon signed rank test with continuity correction.

After children had reached remission on EEN, children chose to either take 20% maintenance enteral nutrition (MEN) or none (non-MEN). The median level of calprotectin was no different between those who took MEN and the non-MEN group after 2-weeks (study wk-10) and 8-weeks (study wk-16) of normal diet (Fig. 4.10). The number of children in the non-MEN group was too low to test significance.

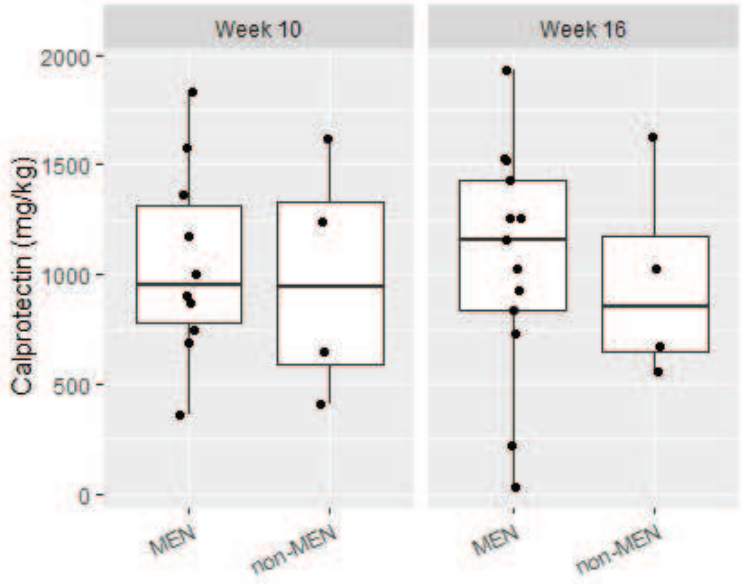


Figure 4.10: Faecal calprotectin as a marker of inflammation during maintenance therapy. Patients were given either 20% maintenance enteral nutrition (MEN) or none (non-MEN). Sample sizes were too low to determine significance

A key factor omitted from most studies on MEN, is that children often take an immunosuppressant (IM) such as azathioprine, as well as MEN as part of maintenance therapy post-EEN. When children were split into groups which factored in both MEN and IM use, it appears possible that children with lower calprotectin at the end of EEN are less likely to be given an IM as part of maintenance therapy (Fig. 4.11).

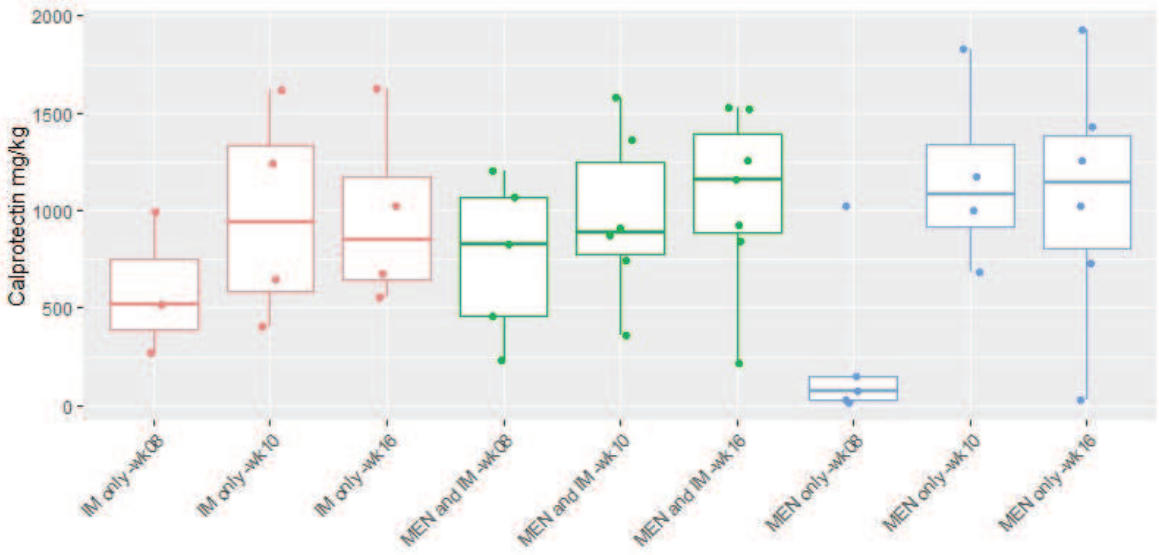


Figure 4.11: Faecal calprotectin as a marker of inflammation during maintenance therapy. Sample sizes were too low to determine significance

4.4 Discussion

The results of the current study from baseline samples show that faecal calprotectin is significantly higher in children with IBD than in healthy children. Daily stooling frequency, faecal water content and Bristol stool chart scores were also significantly increased in children with IBD. These faecal characteristics were not different between children with Crohn's disease and UC. Although not significant, clinical blood marker profiles in children with Crohn's disease were shown to have potential differences from both UC and non-IBD patients.

After 8-weeks of EEN, calprotectin, ESR and CRP were significantly reduced mirrored by a significant increase in albumin. Daily stooling frequency, faecal water content and Bristol stool chart scores were also decreased. Clinical blood profiles after 8-weeks of EEN became similar to that of non-IBD controls. No differences were seen for either baseline faecal or blood markers, between children who responded to treatment with EEN or those who failed EEN.

The current study showed how quickly faecal calprotectin returned to near pre-treatment levels, with calprotectin having a significant increase after only 8-weeks return to normal diet. MEN appeared to have no effect in maintaining reduced faecal calprotectin levels post-EEN.

Markers for disease activity are important in IBD, both for diagnostic and differential diagnostic reasons. They can help to assess disease activity as well as the risk of complications, or predicting time to relapse. They are particularly useful for measuring the effects of therapies, however the choice of marker may influence which patients are classed as responders to a particular treatment. This is a big problem where more than one disease marker is widely used, or a new disease marker is not used universally in all centres.[125]

4.4.1 Indicators of disease activity in children with Crohn's disease

Calprotectin While CRP may be useful in some patients, it has limited specificity compared to faecal calprotectin which correlates much better with active disease. Faecal calprotectin is one of the most reliable, non-invasive diagnostic tools used in the management of Crohn's disease in both adults and children.[381] A study by Schoefer *et al.*[369] showed that when evaluated against the Simple Endoscopic Score for Crohn's disease (SES-CD), calprotectin correlates better with disease activity than CRP, blood leukocytes and the Crohn's disease activity index. The current study was not able to collect SES-CD score data as these were not routinely available from patient notes. Schoefer *et al.* also found that calprotectin was the only marker to discriminate between inactive, mild, moderate and active disease in patients with Crohn's disease. Hence, although not specific to IBD, faecal calprotectin is now

regularly used as indicator for active disease in both Crohn's disease and UC.[367] In this context it is used as a alternative marker of neutrophil influx into the gut lumen and as such is a good non-invasive test for IBD.[382;383] The current study showed that clinical scores of disease activity (wPCDAI) correlated with faecal calprotectin although the relationship was weak (R^2=0.156, p=0.03).

In 2015 Degraeuwe *et al.*[384] carried out a meta study on the faecal calprotectin levels in children with IBD, which included nine individual studies and a total of 853 patients. They established for this data set that faecal calprotectin, as a marker for diagnosing IBD, had a high sensitivity of 0.97 (95% [CI] 0.92-0.99) and a specificity of 0.70 ([CI] 0.59-0.79). A previous meta-analysis[362] which included both children and adults with IBD, concluded that faecal calprotectin was a useful screening tool for identifying which patients to bring forward for endoscopic evaluation. This is particularly important for children where it helps to reduce unnecessary risk from a general anaesthetic required for paediatric colonoscopy, as well as the risk from perforation, bleeding or infection, which although very low are still present.[385] The meta-analysis went on to use individual participant data to create an algorithm showing the probability of having IBD was dependant on faecal calprotectin levels relative to the age of the child.[362]

A draw back with meta-analysis of calprotectin levels is that different studies have used different threshold cut off values.[386] The variation in thresholds varies between centres, as well as changes over time, have affected the numerical thresholds which defines a faecal calprotectin test result as positive or negative[387]. In the current study, calprotectin values are used as continuous data, since using cut-off values for the purpose of assessing inflammation in research, could result in flawed assumptions.

Clinical use of faecal calprotectin for the management of inflammatory bowel disease, as a marker of intestinal inflammation is increasing. A study by El-Matary *et al.* 2017 looked at the impact of faecal calprotectin measurements on the decision making process and the clinical care of children with IBD. They found that when high calprotectin levels were taken into account, it resulted in treatment escalation and clinical improvement for the majority of children.[388]

The competition between the host and gut bacteria for transition metal ions is an important consideration in IBD, with calprotectin being the only known manganese sequestering defence protein in mammals.[363] Understanding the way calprotectin modulates bioavailability of metal ions, and the impact on bacterial growth using *in vitro* models, are key areas for future research in IBD. Metal ion requirements vary between different gut bacteria hence it is important to understand the impact that high levels of calprotectin might have in driving microbiota compositional changes in the gut.[363]

Stooling frequency The current study saw no link between stooling frequency and the water content of stool samples. It is possible that some children with IBD, particularly younger children, are under-reporting stooling frequency. Some older children communicated at recruitment, that they had altered dietary habits by eating less to reduce stooling frequency during school hours. Stooling frequency is one of the questions used in the disease activity index for both Crohn's disease and UC, however it may not be a good measure of disease activity if children are under reporting or fasting to deliberately alter stooling frequency.

Bristol stool chart The current study shows that the Bristol stool chart corresponds well with disease activity. Therefore future studies could investigate whether teaching children with Crohn's disease to use the stool chart, to help them communicate stooling habits with clinical staff, might alert children to potential relapse sooner and allow them earlier access to treatment.

Disease activity index (wPCDAI) A problem with clinical scoring indexes is that some patient questions can be subjective. Particularly where younger children have to describe severity of pain, well-being or stooling frequency. Also clinical staff might report a reduction in pain, not because the pain is less, but because the child reports their pain is no longer interfering with their ability to take part in normal activities. Hence the score can vary to a small extent depending on clinician and patient interpretation of questions.[287] To check for inter-observer variability in the current study questions 1 and 2 of the wPCDAI, which cover pain and general well being (appendix 3), were reviewed via patient and research notes to check for consistency of interpretation across all clinical staff. wPCDAI questions which relate to blood tests (ESR and albumin), or weight loss, perirectal disease and extra-intestinal manifestation were straight forward,[287] and hence scoring was consistent between patients and over time.

4.4.2 Blood markers

Exploring which disease markers correlate with mucosal healing was not within the scope of the current study and has been covered in other studies. The aim was to see if blood markers, which are carried out as part of normal clinical routine, could be used as an inexpensive way to assess disease activity or differentiate disease location in children with Crohn's disease.

Kernel based approach ESR is used as a non-specific measure of inflammation, however it can remain within normal limits even although CRP is elevated.[389] Although our data did not see a significant difference in ESR due to low sample size

when a standard Kruskal-Wallis test was used, the Kernel approach which took into account missing data, did show that when compared with non-IBD controls that ESR was significantly higher in children with Crohn's disease (Fig. 4.4b) and was associated with a rise in CRP, showing the kernel method to be a more powerful tool (Fig. 4.4). ESR was also significantly increased in children with UC when compared with non-IBD controls and was linked with increased white blood cell count (neutrophils & monocytes) and increased platelet count (Fig. 4.4c).

The kernel based approach allows the rejection of more hypotheses at the same level of statistical confidence, potentially providing better insights into the aetiology of disease. Zhan *et al.*[338] developed the KMDA kernel method for use with metabolomics data. Here we have translated its use to look at the relationship between different blood markers, while at the same time accounting for missing data values to strengthen the power of the dataset. It may appear that using an algorithm to fill in blank data with 'smoothed' data could lead to errors based on what are essentially, assumptions. However the error created by missing data is a serious problem, since studies are forced to exclude participants who might only have a single result missing, which in itself creates potential bias or lack of statistical power. The ability of this method to shift the unit of analysis from individual blood markers, to groupings of markers, is a very attractive feature of this kernel-based methodology, since it helps us to think about the biological relationship between these markers. In comparison to the traditional differential analysis where each marker is analysed individually using Kruskal-Wallis followed by a Bonferroni correction for multiple testing to generate a p-value for each blood marker, the kernel approach is less limiting.

Zhan *et al.*[338] tested this method against four other tests and found performance of the Kernel method with a false discovery rate of 0.05 was better other tests including Wilcoxen and 2-sample t-test. In the current study a threshold correlation value of 0.70 was used on blood markers, whereas the metabolite study used 0.95. At 0.95 threshold value the current study had 13-feature sets but no grouping of markers, hence at this cut-off no potential relationships between markers could be seen. The current study tested correlation thresholds from 0.60 to 0.99 and chose 0.70 since it provided four groupings which might give some clues towards relationships between markers without creating errors due to very weak correlations. Ideally this method would work better with a larger data set, where the threshold could be set nearer to an ideal of 0.95; however it was a useful tool despite of the limited size of our data set.

4.4.3 Inflammatory markers during EEN

In the current study the most marked changes seen during EEN were those of reduced faecal calprotectin, followed by blood markers ESR and CRP. This was also mirrored

by an increase in albumin. What was particularly interesting was that blood marker profiles using differential analysis in children with Crohn's disease look similar to patient controls after 8-weeks treatment with EEN.

The effect which EEN has on faecal calprotectin levels in children with Crohn's disease has been shown in a number of studies. One study showed that in fifteen children with Crohn's disease, faecal calprotectin decreased only in children who achieved clinical remission.[390] Another study looked at whether the difference of faecal calprotectin levels between baseline and 2-weeks of treatment on EEN could predict the clinical response in thirty-eight children with newly diagnosed Crohn's disease. However they found that difference in calprotectin at 2-weeks could not predict the clinical response at 6-weeks (p=0.18). Although they found that the difference could predict failure to respond p=0.006 with a sensitivity 58% and specificity 92% for cut-off of faecal calprotectin increase by 486μg/g, the accuracy was too low to be used as a predictor of which patients should stop EEN early and switch to steroid therapy.[391] A review by Day *et al.* also showed an improvement in ESR, CRP and albumin after 8-weeks on EEN in those who achieved remission.[392] Two studies have shown improvements in blood markers ESR and CRP in as little as one week.[142;141]

The current study saw no significant differences for either faecal or blood markers at baseline, between children who responded and those who failed to respond to treatment on EEN. Another study has also shown that changes in calprotectin levels during EEN did not predict length of remission post-EEN.[393]

4.4.4 Maintenance enteral nutrition (MEN) post-EEN

For children who went into remission on EEN in the current study showed inflammatory markers returning to pre-treatment levels after only 2-weeks of return to normal diet. An increase in disease activity (wPCDAI) was also seen after 8-weeks of normal diet, but did not reach significance. After children had reached remission on EEN, children who chose to take 20% MEN showed no difference in calprotectin levels from children in the non-MEN group, although numbers were too low to determine significance. The evidence to support the benefits of MEN is inconsistent (Table 1.6), with the largest of these studies in agreement with the current study, that there is no difference in relapse rates between MEN and non-MEN patients.[153] Treatment with MEN and days to relapse in the current study, is shown in Chapter 3, figure 3.7.

A key factor which is omitted from studies on MEN, is that children often take an immunosuppressant (IM) such as azathioprine, as well as MEN as part of maintenance therapy post-EEN. When children were split into groups which factored in both MEN and IM use, it appears possible that children with lower calprotectin at the end of EEN are less likely to be given an IM as part of maintenance therapy (Fig. 4.11). This is of paramount importance because it suggests that maintenance treatment is

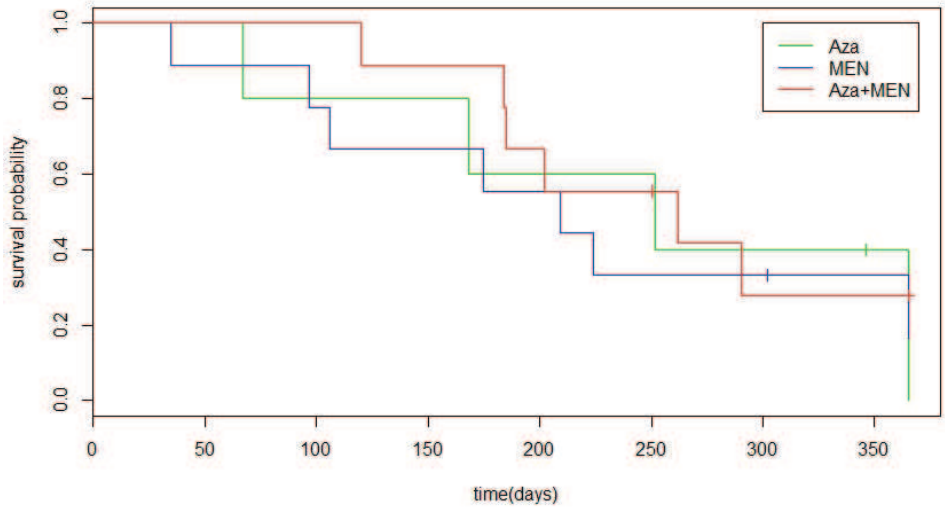


Figure 4.12: Kaplan-Meir survival analysis showing days to disease relapse in children who went into remission after treatment with EEN. Patients were given either 20% maintenance enteral nutrition (MEN)(n=7) an immunosuppressant (AZA)(n=4) or both (MEN+AZA)(n=6). Sample sizes were too low to determine significance

dependent on disease activity post-EEN, thus children on an IM should be put into a separate group of MEN or non-MEN so that treatment with IM is factored into the analysis. Low numbers of participants on maintenance therapy studies make this an unattractive option because it reduces the power of the studies. In the current study no difference in days to relapse (survival rates) were seen between the three groups (Fig. 4.12).

4.4.5 Summary

The current study was unable to reject the null hypothesis that children with Crohn's disease have an inflammatory marker profile which is distinct from that of children with UC, non-IBD conditions and healthy children. Although faecal markers including calprotectin were not different between children with Crohn's and UC, clinical blood marker profiles using differential analysis suggest that it might be possible to develop a test based on multiple markers that could then be tested in larger populations for sensitivity and specificity.

Inflammatory marker profiles at baseline could not predict which children with Crohn's disease responded to treatment with EEN, hence this hypothesis was rejected. For children in disease remission post-EEN, supplementing return to normal diet with (20%) maintenance enteral nutrition (MEN) did not maintain reduced levels of inflammatory markers associated with remission achieved during EEN; hence this hypothesis was also rejected.

4.4.6 Summary of key findings

- Blood inflammatory markers and faecal calprotectin are increased in IBD patients compared with controls.
- Baseline faecal and blood inflammatory markers, did not predict response to EEN.
- EEN is a successful treatment for Crohn's disease induction as shown by reduced disease activity (wPCDAI) and faecal calprotectin.

New findings from this study:

- Differential analysis may be able to use normal clinical blood markers, a cost effective method, to identify Crohn's disease from UC.
- Differential analysis show that clinical blood marker profiles in children with Crohn's disease look similar to patient controls after treatment with EEN.
- A kernel-based approach offers a robust new method to look a blood markers in children with IBD.
- Faecal calprotectin returns to pre-treatment levels more rapidly than previously recognised once children return to normal diet; and that MEN has no effect in maintaining the anti-inflammatory effect of treatment with EEN.

5 The role of gut microbiota in inducing and maintaining remission in children with Crohn's disease

5.1 Introduction

Evidence suggests that IBD is linked to an inappropriate inflammatory response to the gut microbiota in genetically susceptible hosts.[162] Although some bacteria have been linked with Crohn's disease, such as species of *Mycobacterium, Campylobacter, Escherichia* and *Helicobacter*, current evidence does not support the idea that Crohn's disease is caused by a single species or strain of bacteria.[176] Evidence from faecal and mucosal biopsy samples suggests that Crohn's disease is associated with significant differences in gut microbiota at a community-level. Hence understanding these differences along with changes in gut bacterial profiles are crucial to our understanding of Crohn's disease and IBD.

Studies which have gone on to examine the use of exclusive enteral nutrition (EEN) as a treatment for children with Crohn's disease, have highlighted bacterial changes involved in remission and flare up in Crohn's disease. Since studies were underpowered and used technologies which limited bacterial groups/species that could be included, the current study aims to fill this gap using 16S rRNA with Illumina technology to build a wider picture of which bacterial groups, down to species level, are changing during treatment with EEN. This will provide a clearer picture of whether EEN works by reducing pathogens or rather modifies the global balance of bacteria in the gut.

No previous studies had examined gut microbiota changes in children with Crohn's disease post-EEN, treated with maintenance enteral nutrition (MEN) with the aim of preventing disease relapse. The current study design is set up to explore whether MEN could maintain gut bacterial changes achieved during EEN. This is of particular interest since MEN is currently being used as a treatment strategy, given as a supplement in addition to normal diet (usually ∼20% of EAR), with limited understanding of any mechanisms. Understanding differences in the gut microbiota composition at baseline and changes during treatment with EEN and MEN, could help to increase our understanding of the mechanisms that control inflammation in Crohn's disease.

Microbial dysbiosis Dysbiosis refers to any change in the composition of the commensal gut bacterial community relative to the community structure found in healthy people.[394] Our current understanding of what constitutes a healthy gut microbiota composition comes from studies such as a north American study of 129 male and 113 female healthy adults, carried out as part of the Human Microbiome Project.[394] Their study shows that patterns within samples (α-diversity) differ notably from differences between samples (β-diversity) from the same habitat (oral, gut, skin, vaginal) among individuals. The consortium also found that with-in individual variation over time,

was consistently lower than between-individual variation for both bacterial composition and metabolic functionality; showing an individual's microbial composition to be relatively stable over time.[394]

The human gut microbiota is dominated by five bacterial phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. In individuals considered to be healthy, over 90% of species come from the Firmicutes and Bacteroidetes phyla.[176] In 2017 a study looking at 1252 healthy control subjects taken from a cross-study meta-analysis of 3048 public metagenomic datasets (four different studies), showed that the key genera that correlated with the healthy state of control subjects were *Barnesiella*, *Ruminococcaceae UCG-005*, *Alistipes*, *Christensenellaceae R-7 group* as well as a member of *Lachnospiraceae* family genera.[395] However, these results need to be treated with caution as they combined both biopsy samples and stool samples; included both Illumina and 454-sequencing methods; and each of the four included studies used a different set of 16S rRNA primers. This led to some conflicting results between these studies used to make up the meta-analysis.[395] In the last decade studies have recorded significant changes in bacterial community structure in patients not only with IBD, but also other conditions such as diabetes,[396] asthma,[187] allergies and autistic spectrum disorders.[397] This evidence suggests that either changes in bacterial composition, or development of the microbiota during childhood, is a risk factor in a number of diseases, including Crohn's disease. It has been shown that loss in diversity of symbiotic groups such as *Bifidobacterium* and Clostridia species, along with increases in Bacteroidetes and Enterobacteriaceae species associated with inflammation, are key features of Crohn's disease. Understanding these changes is complex because a number of factors can contribute to changes in gut bacterial composition including host genetics, dietary choices, infections and medications such as antibiotics.[162] Not only has research identified dysbiosis in Crohn's disease but it has linked dysbiosis with disease progression.[200] Three categories of dysbiosis have been described:[398] a loss of indigenous beneficial microbiota; overgrowth of less dominant indigenous pathobionts or colonisation by new bacterial pathogens; and loss of overall microbiota diversity, all of which can occur at the same time (Chapter 1, section 1.9.1).

The following chapter will examine differences in gut microbiota community structure seen in the current study, between children with Crohn's disease, UC, non-IBD conditions and healthy children. It will also identify any changes in the gut microbiota in children with Crohn's disease undergoing treatment with EEN; and examine if these changes are maintained once children go back onto normal diet, with or without maintenance enteral nutrition (MEN). It is hypothesised that each of these condition states is associated with specific changes in the gut microbiota and that these changes are associated with disease relapse in children with Crohn's disease.

5.1.1 Hypotheses summary

- Children with Crohn's disease have a gut microbiota which is distinct from UC, non-IBD conditions and healthy children. In addition the microbiota profile in children with Crohn's disease is distinct depending on disease location.
- Treatment with EEN is associated with distinct changes in gut microbiota composition; which can be linked with changes in inflammatory status.
- Gut microbiota profiles at baseline can predict which children with Crohn's disease will respond to treatment with exclusive enteral nutrition (EEN).
- For children in disease remission post-EEN, supplementing return to normal diet with (20%) maintenance enteral nutrition (MEN) will maintain the gut microbiota profile associated with remission achieved during EEN.

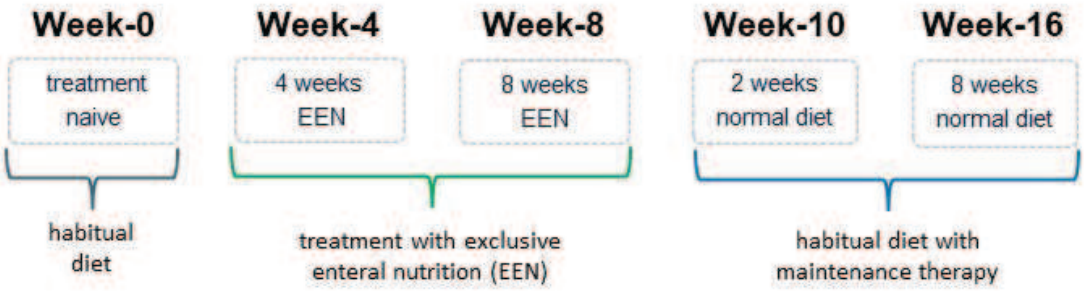


Figure 5.1: In children with Crohn's disease, faecal samples were collected at baseline; 4-weeks treatment with EEN; end of EEN (8-weeks); then after 2-weeks and 8-weeks of normal habitual diet plus maintenance therapy with either MEN, an immunosuppressant or both combined.

5.2 Methods

A detailed description of participants and methods used are given in Methods section 2.2.1. Briefly, children with Crohn's disease (n=34), children with UC (n=9) and a control group of children with non-IBD conditions (n=11) were recruited from the Gastroenterology clinic at the Royal Hospital for Sick Children Glasgow. An age and sex matched group of healthy children (n=25) were recruited from the community. A faecal sample was collected from each participant at baseline, and for children with Crohn's disease at five time-points (Fig. 5.1). DNA extracted from stool samples was sequenced (Illumina MiSeq platform) from the 16S rRNA gene (Fig. 5.2) as described in Methods section 2.8 and 2.9.

Local contribution to beta diversity (LCBD)[330] was used to work out the total sum of squares of species composition for all samples, from which sample-wise local contributions to β-diversity could be shown as a proportion of total β-diversity (Methods section 2.10.3). An aid to understanding the classification of bacteria which are most associated with IBD is given in Appendix 5.

Figure 5.2: Summary of methods used to process faecal samples for 16s rRNA analysis. 16s rRNA sequences were then used to determine bacterial groups from the resulting sequence variants. Full details in Methods section 2.8 and 2.9.

5.3 Results

5.3.1 Microbiota differences between patient groups at baseline

To investigate differences in gut microbiota community structure between children with Crohn's disease and those with UC, non-IBD conditions and healthy children, faecal bacterial profiles determined from 16S rRNA, were compared between groups.

Richness (α-diversity) The number of species (richness) measured as operational taxonomic units (OTUs) and the Shannon index, a measure of richness and evenness were compared between groups (Methods section 2.10.3). Both richness and Shannon index, using OTUs to define species, showed that children with IBD (Crohn's disease and UC) had lower faecal bacterial diversity than healthy children ($p<0.001$). Children with non-IBD conditions also had less bacterial diversity than healthy controls ($p<0.05$) (Fig. 5.3a).

For comparison, divisive amplicon de-noising algorithm (DADA2), a reference free method to infer species from single-nucleotide variants (SNVs) rather than OTUs, was used to examine species level diversity (Methods section 2.10.1). It has previously been shown, using mock communities that it is more accurate than other methods[315] (Methods section 2.10.1). This method confirmed, from bacterial richness and Shannon index, that children with Crohn's disease and UC had significantly less species level diversity than healthy children ($p<0.001$) and that children with non-IBD conditions also had less species level diversity than healthy controls ($p<0.05$) (Fig. 5.3b). Since using DADA2 single nucleotide variants is a better method than using OTUs,[315] all subsequent analyses were done using DADA2 single nucleotide variants (SNVs) as a measure of bacterial species level taxonomy.

Abundance (β-diversity) Bacterial abundance (β-diversity) was used to look at difference in bacterial community structure between groups. Non-metric distance scaling (NMDS) (Methods section 2.10.3) showed a significant difference in species

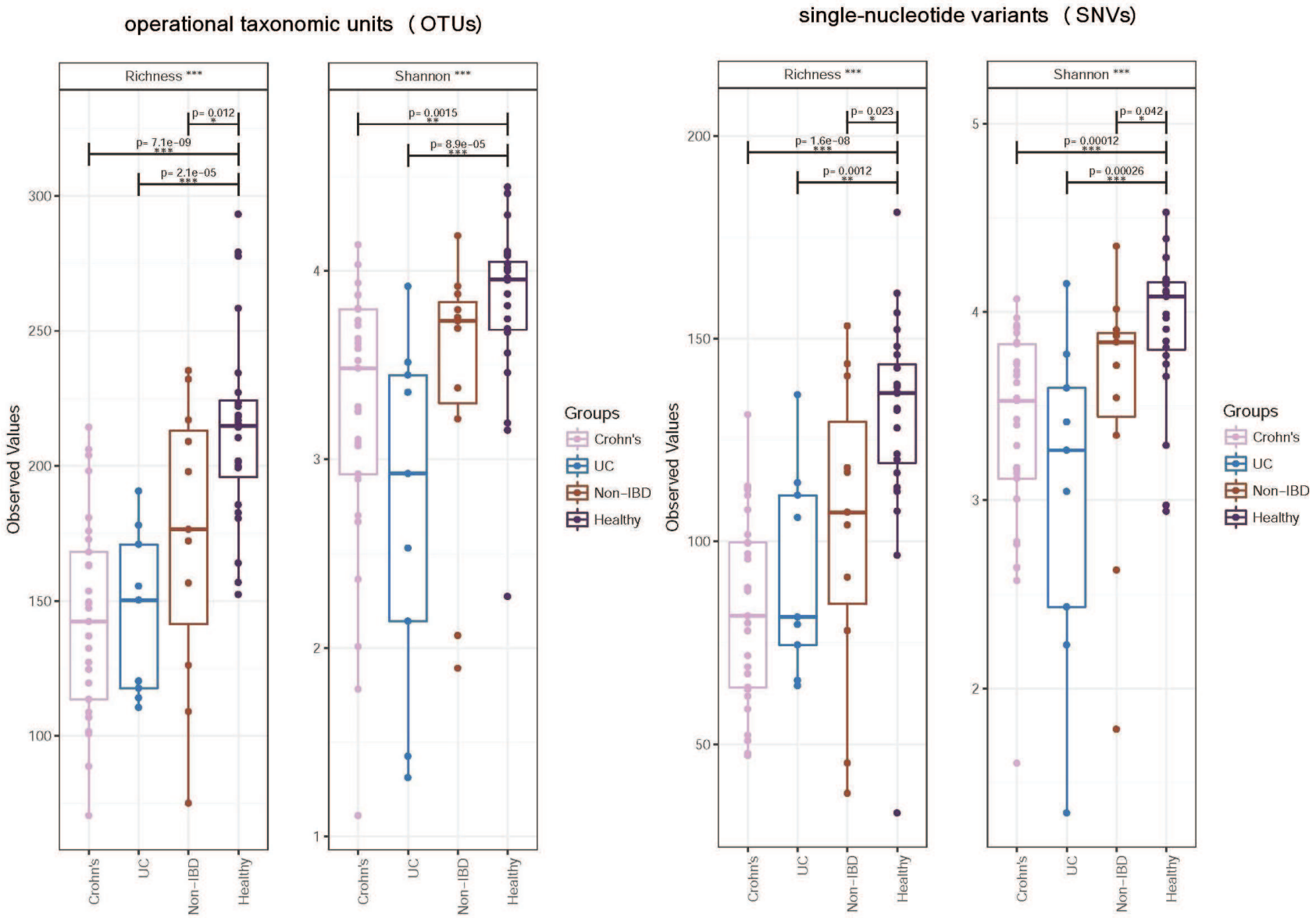


Figure 5.3: Bacterial α-diversity calculated from (a) OTUs and (b) SNVs as a proxy for species; shown by richness (number of species) and Shannon index (species evenness) between patient groups and healthy children. Test for significance (ANOVA). OTUs have 97% nucleotide similarity and SNVs are resolved down to a single nucleotide difference. Both methods show similar results but difference in species richness between non-IBD and healthy children is significant for SNVs but not OTUs.

level community structure between healthy children and both children with Crohn's disease and UC (Fig. 5.4a); which was still significant at phylum level (Fig. 5.4b). Children with Crohn's disease tended to have a greater degree of dysbiosis, clustering further away from healthy children than children with UC (Fig. 5.4a). Children with non-IBD conditions had a wide range of bacteria profiles that overlapped the other groups (Fig. 5.4c), showing that some children in the non-IBD group also have a dysbiotic community structure while others cluster closer to healthy children.

In the current study local contribution to beta diversity (LCBD) shows that β-diversity of individual healthy children as a proportion of total β-diversity had greater similarity to one another (between samples) than children with Crohn's disease and UC ($p=<0.001$ and $p=<0.01$ respectively) (Fig. 5.5). Thus healthy children appear to have microbiota community profiles which are less prone to high levels of LCBD than children with IBD, where higher levels represent outliers which sit well outside the community structure mean.

Taxon differences in microbiota community structure

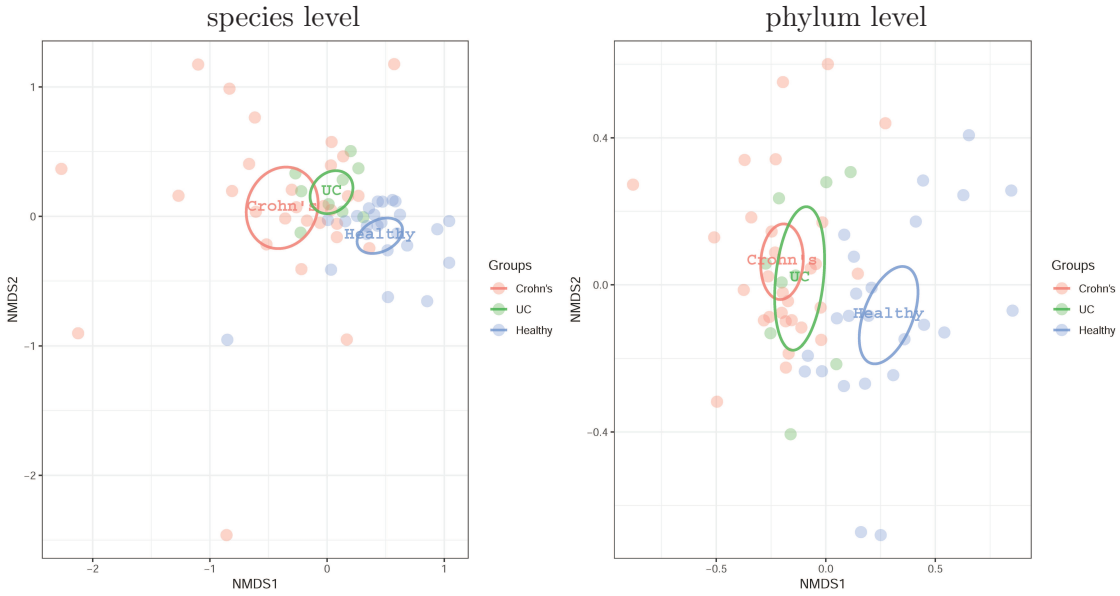
The current study data set generated a total of 2,751 unique bacterial sequences representing species (SNVs), and 161 of these SNVs (5.8%) discriminated Crohn's children from healthy children (Table 5.1). This shows more bacteria at species level were down-regulated in all three patient groups than were increased, and that children with non-IBD conditions also had a degree of bacterial difference from healthy children, with significant decreases in seventy-one SNVs (species level taxa). Thirty-six SNVs were significantly increased and eighteen decreased in children with Crohn's disease when compared with UC (2.0%). Details of which species (SNVs) were up or down regulated are provided in appendix 5.3.

Table 5.1: The number SNVs as a proxy for species, which were significantly different (detected from $\log_2$ mean abundance) between patient groups

	number of SNVs (species)		% of
	increased	decreased	total SNVs
Distinct from Healthy			
Crohn's vs healthy	39	122	(5.8%)
UC vs healthy	17	110	(4.6%)
Non-IBD vs healthy	8	71	(2.9%)
Distinct from Crohn's			
Crohn's vs UC	36	18	(2.0%)

Total number of SNVs was 2,751. Differences in species level abundance was significant at $p<0.001$ (PERMANOVA). A full list of species level taxa (SNVs) are given in appendix 5.3.

Stacked bar plots were used to visualise phylum (Fig. 5.6); family (Appendix 5.1) and genus (Fig. 5.7) level community structure in individual children from each group. The phylum level bar plot (Fig. 5.6) shows that children with Crohn's disease and

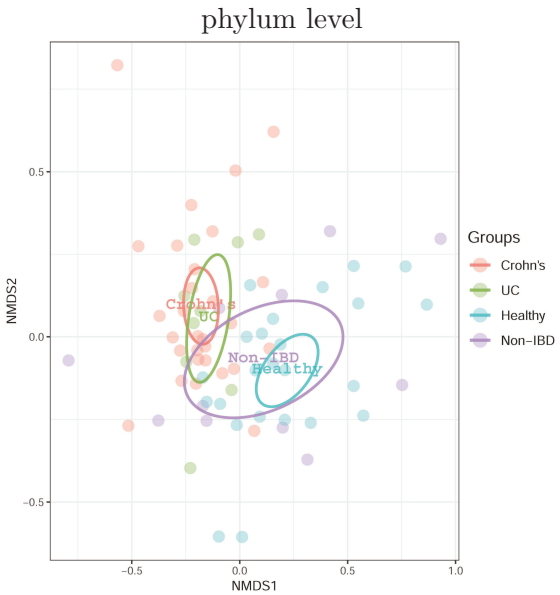


R2=0.074; p<0.001

(a)

R2=0.069; p=0.046

(b)



PERMANOVA Groups:
R2=0.081; p=0.068

(c)

Figure 5.4: Difference in bacterial community structure between patient groups and healthy children shown by NMDS plot in: (a) children with Crohn's and UC at species level (SNVs) (b) and the same at phylum level; showing that differences are still present at higher taxonomic levels. Figure (c) shows that some children with non-IBD conditions overlap with healthy controls while others have a similar profile to children with IBD. Ellipse shows 95% CI.

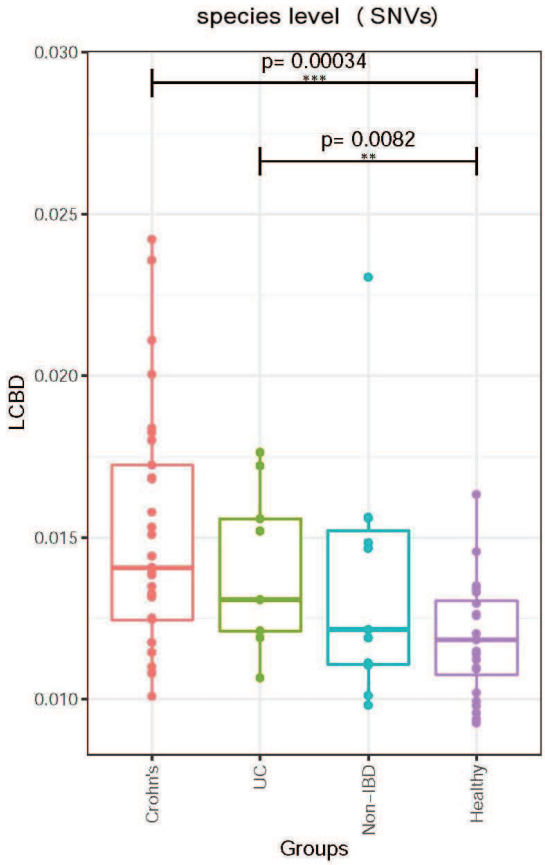


Figure 5.5: Local contribution to β-diversity (LCBD) showing how individuals across groups vary from the β-diversity group mean; in children with Crohn's disease; UC; non-IBD conditions and healthy children; showing that children with IBD have a community structure which has much greater variation from the mean than seen in healthy children. Significance calculated using ANOVA; Bray-Curtis.

UC have higher relative abundance of the gram-negative phyla Bacteroidetes, Proteobacteria and Fusobacteria, as well as lower relative abundance of Actinobacteria and Verrucomicrobia which include *Bifidobacterium* and *Akkermansia* respectively. The taxa plot at genus level clearly shows the large degree of inter-patient variation in children with IBD, as quantified by LCBD bubble size on the base on each bar. To test for a significant difference in $\log_2$ fold change from mean abundance between groups, differential expression analysis was used based on the negative binomial distribution. Results are expressed as the $\log_2$ fold change (Methods section 2.10.3) between patients and healthy controls. The mean abundance of Bacillales Family XI, Fusobacteriaceae and the Proteobacteria families Enterobacteriaceae and Pasteurellaceae, were all increased in both UC and Crohn's compared with healthy children. Neisseriaceae were significantly increased in Crohn's disease but not in UC and the family Carnobacteriaceae, were increased in UC but not in Crohn's disease when compared with healthy children ($p<0.001$). The Carnobacteriaceae were also significantly increased in children with UC when compared with those with Crohn's disease. Fusobacteriaceae were further increased in Crohn's disease compared with UC.

The following were reduced compared with healthy children in both Crohn's disease and UC: Methanobacteriaceae; Defluviitaleaceae; Clostridiales vadin BB60 group; Verrucomicrobiaceae and Rhodospirillaceae. Christensenellaceae and Peptococcaceae were also significantly reduced in Crohn's disease, but not in UC. The family Bacteroidales S24-7 group, were reduced in UC but not in Crohn's disease when compared with healthy children ($p<0.001$). No families were significantly increased and only two families were decreased (Bacteroidales S24-7 group and Rhodospirillaceae) in children with non-IBD conditions when compared with healthy children ($p<0.001$).

In order to show the relatedness of taxa, the phyla, class, order, family, and genera of bacteria which were significantly decreased/increased in abundance in children with Crohn's disease compared with healthy children are shown in a phylogenetic tree (Fig. 5.8); (species level taxa (SNVs) are shown in appendix 5.3). It is noteworthy that the phylogenetic trees formed by health associated and Crohn's disease associated bacteria are separate, with the gram-negative Proteobacteria and Fusobacteria along with genera from the class Negativicutes increased, while most bacteria that are reduced in children with Crohn's come from the phyla Actinobacteria and Firmicutes. Figure 5.7 shows that children with Crohn's disease have higher relative abundance of Veillonella (Negativicutes), a genus which is not prevalent in any of the healthy children.

The bacterial community structure in children with Crohn's disease at baseline was also defined by a significant decrease in abundance of the phylum Euryarchaeota (Archaea), including the family Methanobacteriaceae; reduced Tenericutes consisting of the order Mollicutes RF9; and Verrucomicrobia made up from the family Verrucomicrobiaceae. A number of Firmicutes families from the order Clostridiales were also decreased in children with Crohn's disease, including Clostridiales vadinBB60, Christensenellaceae, Peptococcaceae and Defluviitaleaceae. The family Rhodospirillaceae from the Proteobacteria phylum were also significantly reduced in children with Crohn's disease ($p<0.001$)(Fig. 5.8).

To visualise differences in bacterial community structure between groups, a heatmap of discriminant genera (Fig. 5.9) was created based on sparse projection to latent structure discriminant analysis (Methods section 2.10.3). From faecal samples taken at baseline, clear blocks of differences in bacterial abundance were seen, with healthy children mostly clustering together; a group of children with UC and Crohn's clustering; and a third cluster containing only children with Crohn's disease. Healthy children had higher abundance (red) of commensals such as *Ruminococcus* and Lachnospiraceae which were reduced (blue) in children with IBD. The opposite was true of gram-negative bacteria such as *Bacteroides*, *Prevotella*, *Haemophilus*, *Enterobacter*, *Cronobacter*, *Citrobacter* and *Fusobacterium*, where levels were clearly increased in many IBD patients, especially children with Crohn's disease.

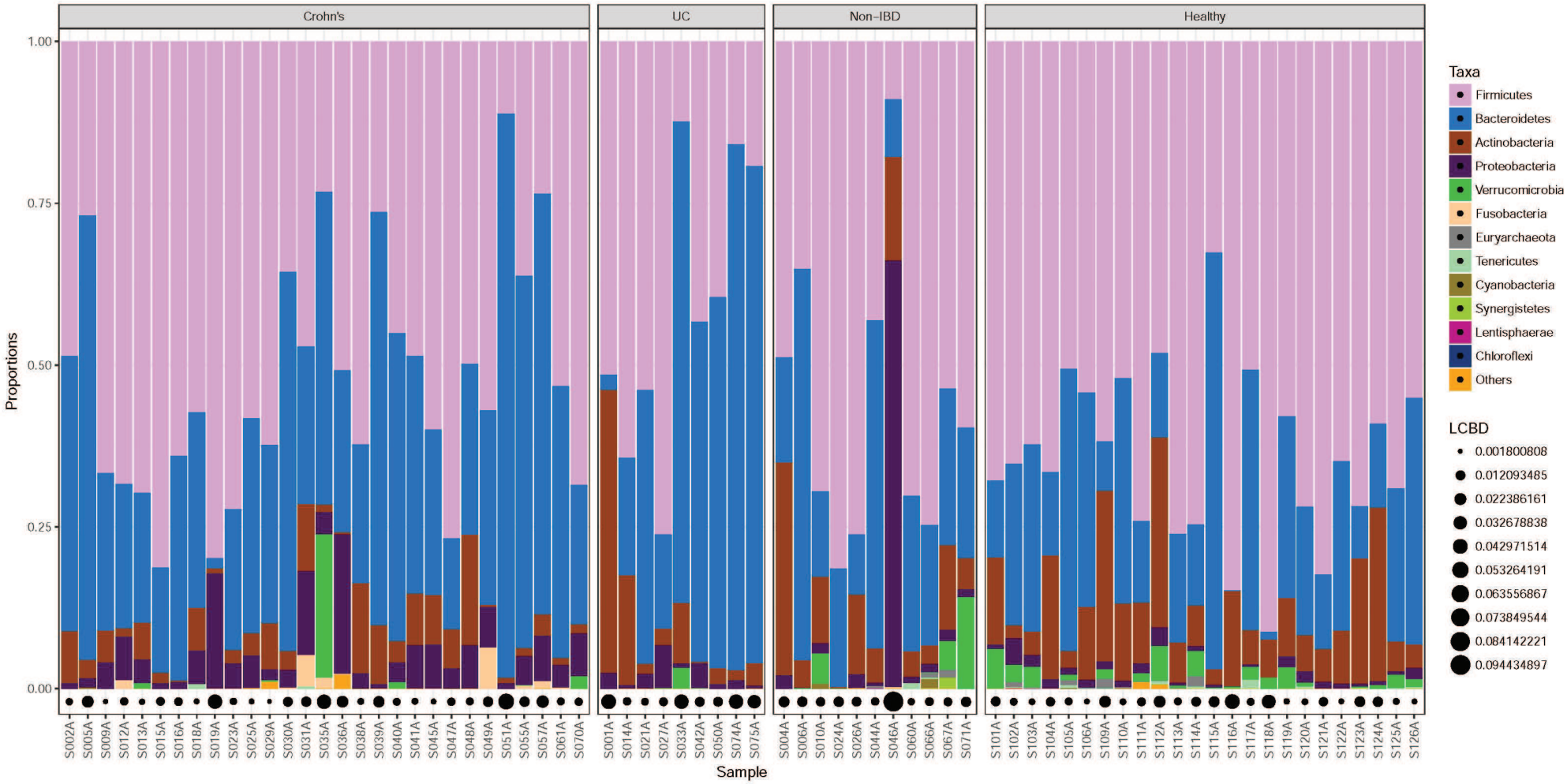


Figure 5.6: Community structure at Phylum level, in children with Crohn's disease, UC, non-IBD and healthy children. Bubble size increase at base of bar plot show local contribution to β-diversity (LCBD). It can be seen that overall children with Crohn's disease have increased Proteobacteria Bacteroidetes and Fusobacteria as well as reduced Firmicutes, Actinobacteria and Verrucomicrobia compared with healthy children $p<0.001$. Non-IBD patient S046 had gastroenteritis hence the high level of Proteobacteria (*Escherichia-Shigella*). Family level taxa is shown in Appendix 5.1

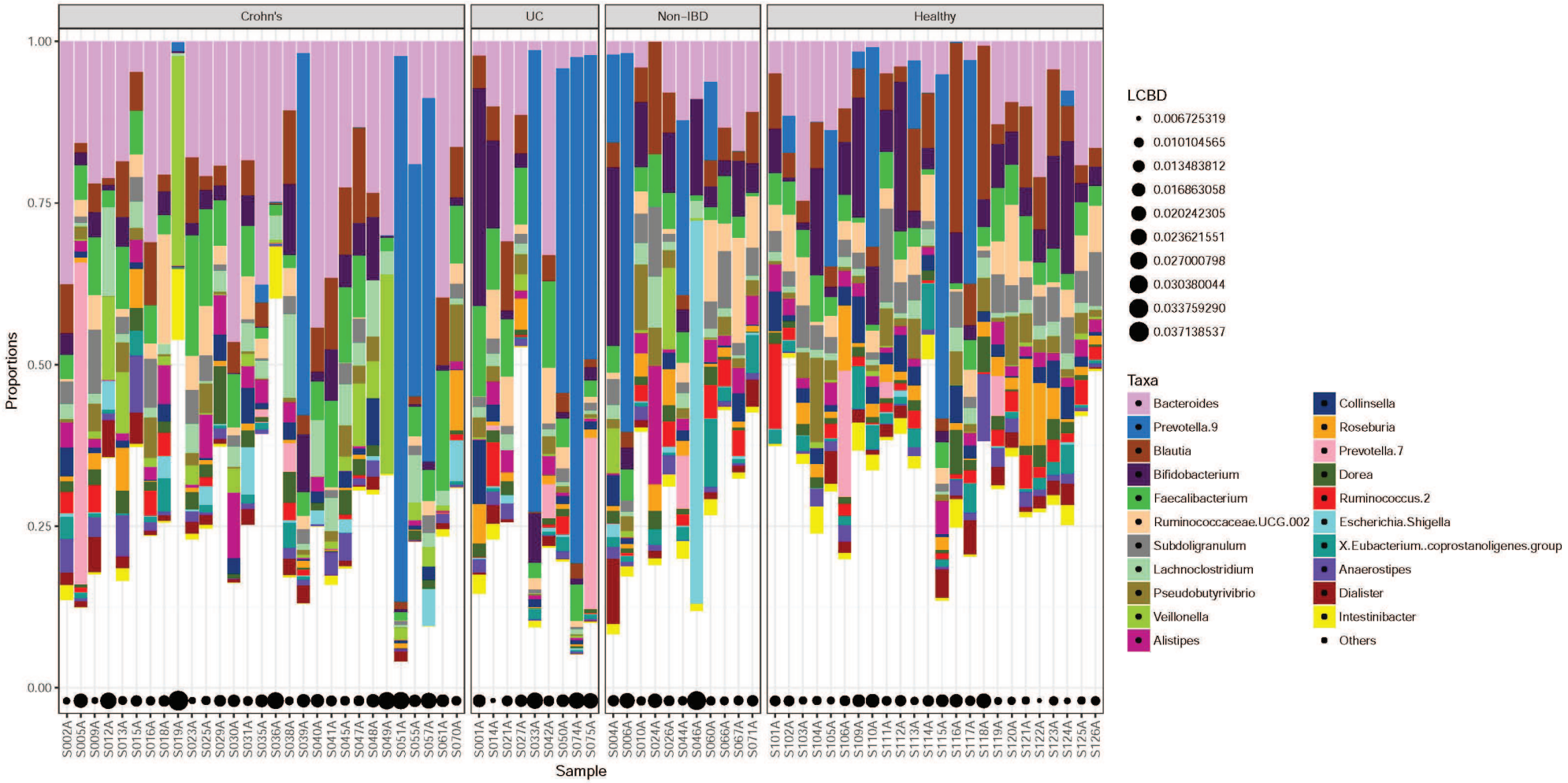


Figure 5.7: Community structure at genus level, in children with Crohn's disease, UC, non-IBD and healthy children. Twenty-one most abundant genera are shown. Bubble size increase at base of bar plot show local contribution to β-diversity (LCBD). Non-IBD patient S046 had gastroenteritis shown by the very high abundance of *Escherichia-Shigella*.

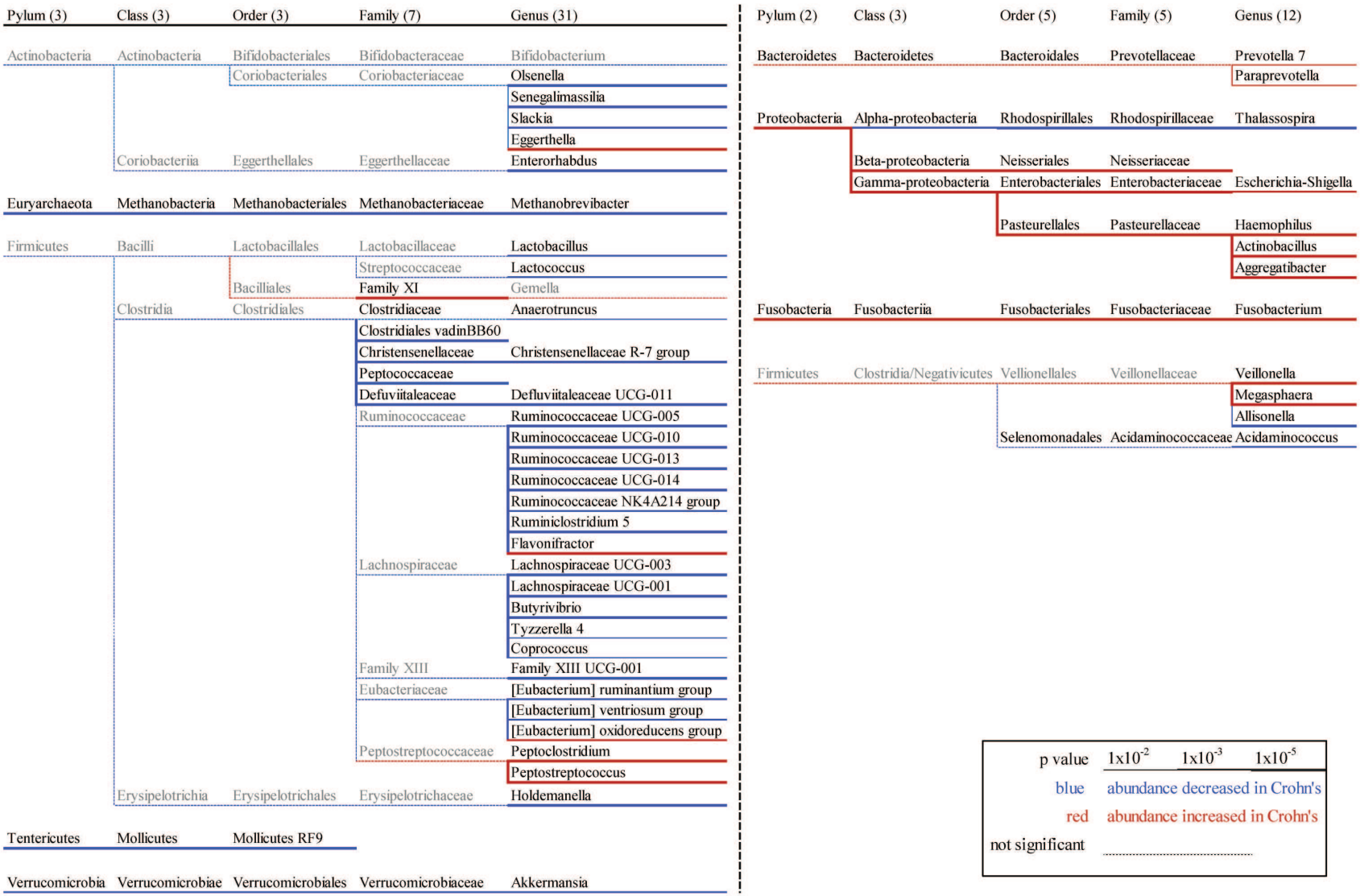


Figure 5.8: Phylogenetic tree showing differences in mean $\log_2$ bacterial abundance between children with Crohn's disease and healthy children at baseline. Significant taxa which were decreased in Crohn's disease are shown in blue and increased taxa in red. Note that the phylogenetic trees formed by health associated and disease associated bacteria are separated, suggesting the evolutionary history and genetic traits of these groups of bacteria are fairly distinct in terms of gut health.

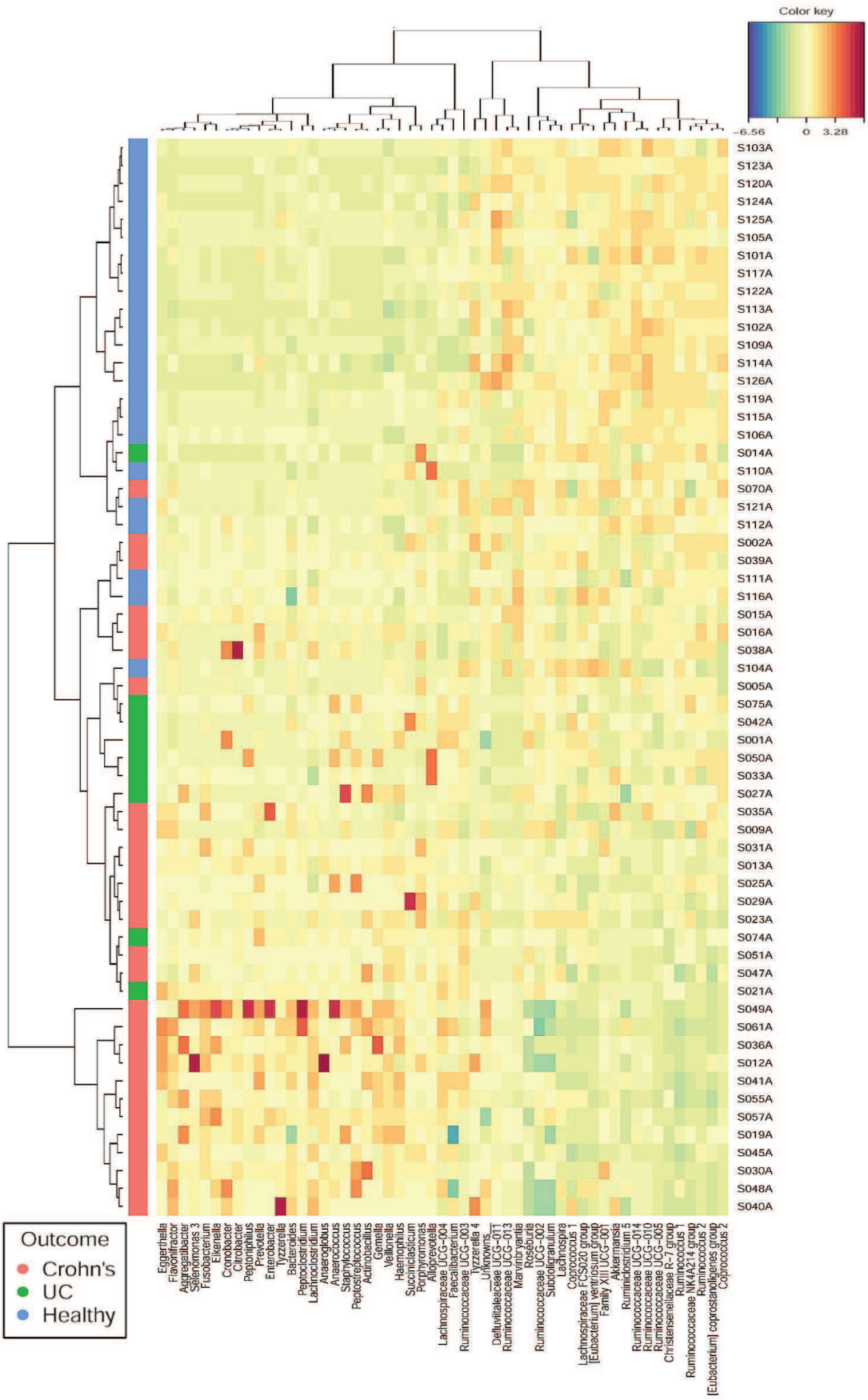


Figure 5.9: Heatmap showing discriminant genera, in children with Crohn's disease, UC and healthy children. Rows and columns are ordered using hierarchical clustering (average linkage) to identify blocks of interest. Heatmap depicts TSS+CLR (total sum scaling + centered log ratio) normalised abundances: high abundance (red); low abundance (blue). Blocks of interest show that children with IBD have lower gram-positive commensals such as *Ruminococcus* spp. and higher levels of gram-negative species such as *Fusobacterium* and *Enterobacter* spp. as well as Negativicutes like *Veillonella*.

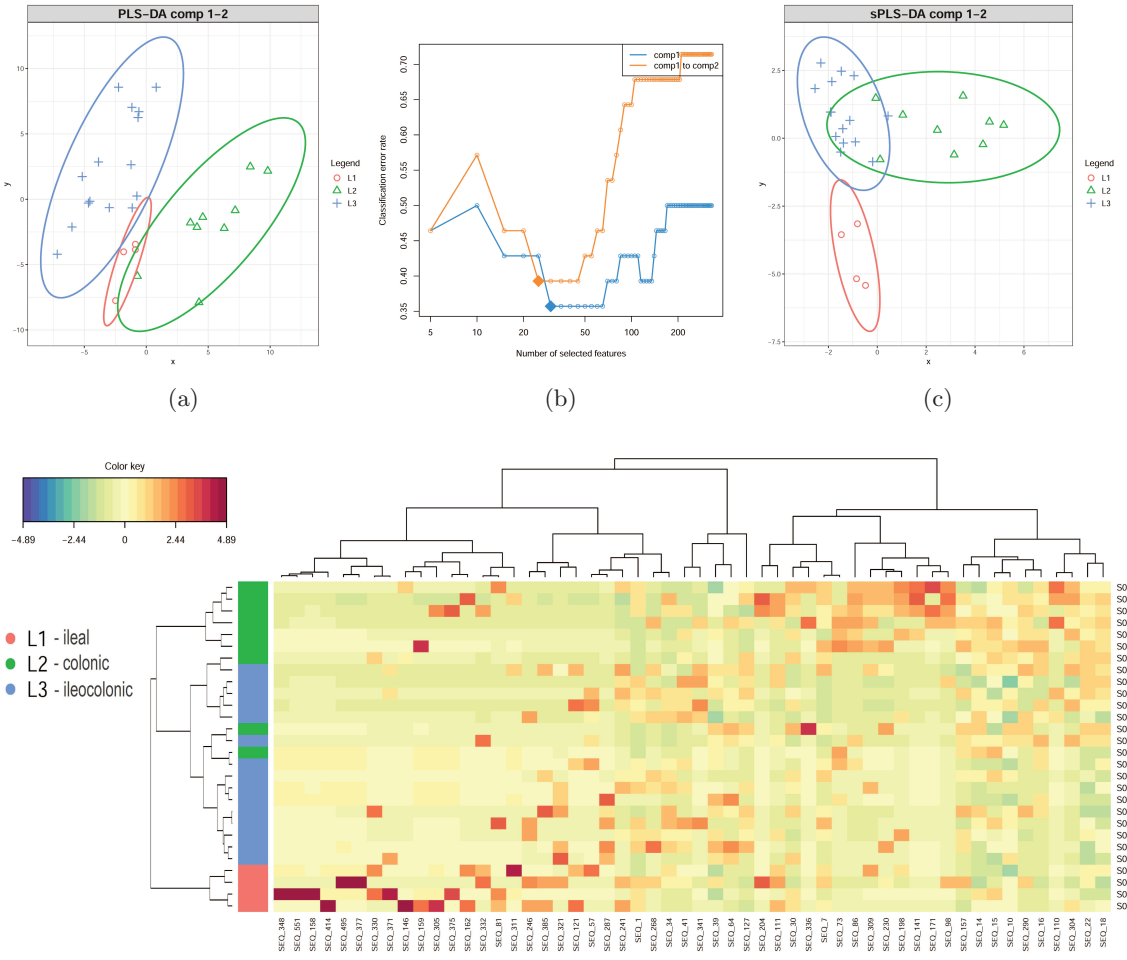
5.3.2 Crohn's disease location and microbiota composition

The Montreal classification was used to subdivide children with Crohn's disease into groups based on disease location (Methods section 2.4.3). These groups were then used to look for differences in gut bacteria community structure across these location groupings. No difference was seen in community structure between disease locations: L1 ileal +/- limited caecal disease (n=4); L2 isolated colonic (n=9); or L3 ileocolonic (n=16) (Fig. 5.10). Sub-set analysis (*BVSTEP* routine), a method for linking multivariate microbiota community structure to environmental variables. This method works by imploding the microbiota abundance table to those essential features that explain β-diversity, highlighting which bacterial genera are the main source of variation between all the samples. A number of genera subsets were found to explain 0.95 variability in microbiota structure for disease location but these were not significant (Table 5.2).

Table 5.2: Subset analysis showing which genera explain the percentage variability in microbiota community structure for disease location in children with Crohn's disease

Subset	Subset of most abundant genera	correlation with full abundance table	PERMANOVA subsets
S1	Veillonella + Haemophilus + Subdoligranulum + Bifidobacterium + Faecalibacterium + Prevotella 7 + Prevotella 9 + Blautia + Bacteroides	0.95	R^2=0.106 p=0.115 (NS)
S2	Veillonella + Haemophilus + Bifidobacterium + Faecalibacterium + Prevotella 7 + Prevotella 9 + Blautia + Bacteroides	0.95	R^2=0.106 p=0.131 (NS)
S3	Veillonella + Haemophilus + Bifidobacterium + Faecalibacterium + Prevotella 9 + Blautia + Bacteroides	0.94	R^2=0.111 p=0.105 (NS)

Subsets were generated using the *BVSTEP* (see detailed Methods section 2.10.3). PERMANOVA of subsets were performed against disease location: L1(ileal); L2(colonic); L3(ileocolonic) in patients, with R^2 explaining the percentage variability in microbiota structure between groupings.



(a) (b) (c)

(d)

Figure 5.10: **sPLS-DA Discriminant Analysis** showing how disease location affects microbiota community structure in children with Crohn's disease. The algorithm is a 2-step process where two components are found reducing the classification error rates (using max.dist) in the algorithm, with (a) showing the ordination of samples using all the species (SNVs) in the first two components (PLS-DA) with ellipses representing 95% confidence interval and percentage variations explained by these components in axes labels. In step two, (b) the number of discriminating species (SNVs) were found for each component, highlighted as diamonds. In (c) the ordination used the discriminants from all two components (sPLS-DA). (d) is a heatmap of discriminant species inferred from SNVs, with rows and columns ordered using hierarchical clustering (average linkage) to show patterns of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red) and low abundance (blue).

5.3.3 Microbiota changes in children with Crohn's disease during EEN

The largest gut microbiota paediatric IBD study, did not follow children over time,[194] therefore this current longitudinal study, chose to look at changes in gut microbiota diversity, and changes in specific bacterial groups associated with Crohn's disease at baseline, by following children during treatment with exclusive enteral nutrition (EEN). The microbial composition was compared, from faecal samples taken at baseline, with those taken after 4-weeks and 8-weeks treatment with EEN (Table 5.3).

Table 5.3: Number of faecal samples available at each time-point during EEN

Time-point	Responded to EEN	Failed EEN
week-0 *(Pre-treatment baseline*	20	8
week-4 *(4 weeks treatment on EEN)*	15	6
week-8 *(8 weeks treatment on EEN)*	15	4

13 children able to provide a faecal sample at all 3 time-points, 4 of these failed EEN.

Richness (α-diversity) During EEN there was no change in median number of species inferred from SNVs (richness); however the median Shannon diversity index (evenness) reduced in children with Crohn's disease after 4-weeks and at 8-weeks EEN but this was not statistically significant (Fig. 5.11). Although there was no significant difference in α-diversity after 4-weeks EEN, in children who responded to treatment with EEN and those who failed to respond, it should be noted that most individual children either saw an increase or decrease in numbers of species (SNVs): with 8/13 responders and 2/6 non-responders having an increase in the number of species (richness) during EEN (χ^2=0.43; p=0.52). Although there was no change in species richness during EEN for children who responded to EEN, children who failed treatment had a non-significant median decrease in species richness (Fig. 5.12).

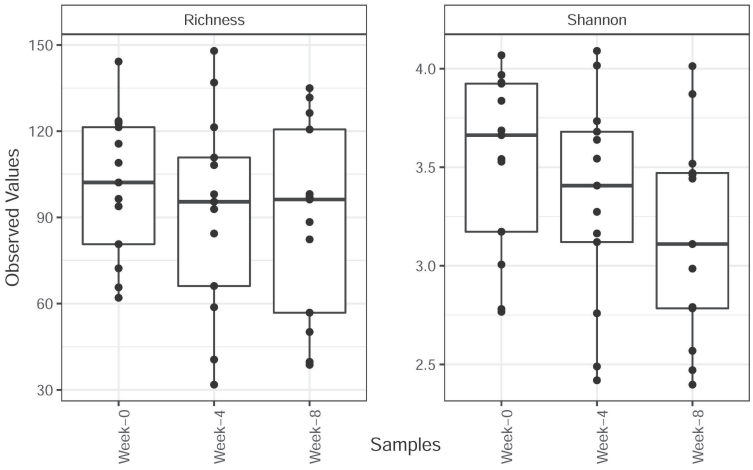


Figure 5.11: Species (SNVs) level α-diversity shown by richness and Shannon index in children with Crohn's disease during treatment with exclusive enteral nutrition (EEN), who provided samples at all three time-points (n=13). Species diversity is shown at baseline (week-0); 4 weeks on EEN (week-4); and 8 weeks on EEN (week-8). Significance tested at p<0.05 ANOVA.

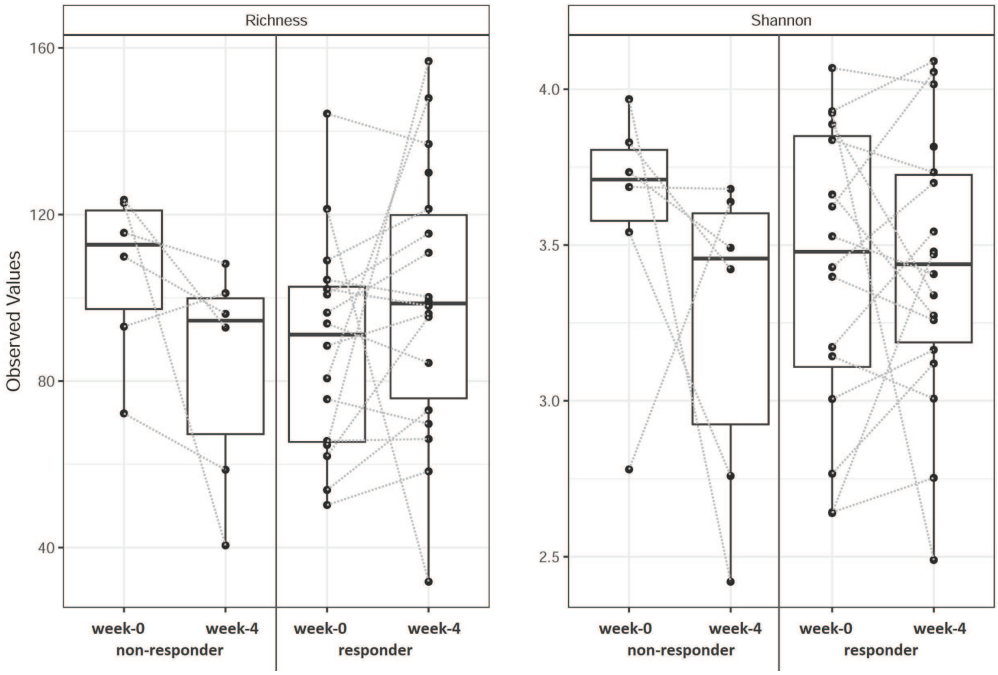


Figure 5.12: Species (SNVs) level α-diversity shown by richness and Shannon index in children with Crohn's disease during treatment with exclusive enteral nutrition (EEN). Paired samples shown for baseline (wk-0) and 4-weeks on EEN (wk-4); for responders (R) and non-responders (NR) to EEN. Difference in diversity between 0-4wks were not significant (ANOVA).

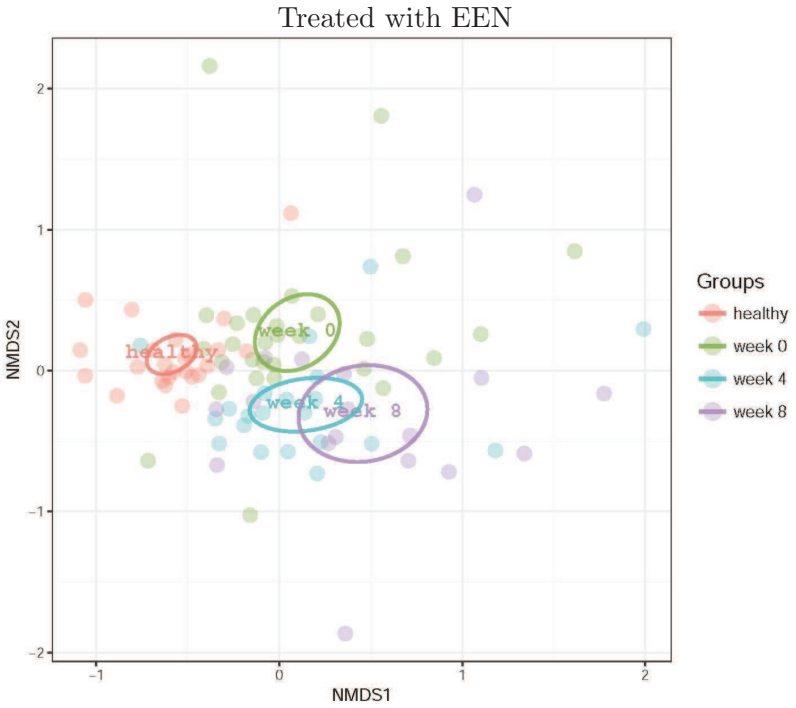
Abundance (β-diversity) Changes in bacterial community structure (β-diversity) was explored using PERMANOVA (Methods 2.10.3; Fig. 5.13a). There was a significant shift in community structure between baseline and 4-weeks of EEN with a further, less distinct, shift at 8-weeks of EEN (R2=0.099; p=0.001). When children with Crohn's disease were split into those who responded to treatment on EEN and those who failed to respond, there was a significant difference in gut bacteria diversity during EEN for responders, but not for non-responders (Fig. 5.13).

Table 5.4: Number of species inferred from SNVs which significantly changed in abundance after 4-weeks of EEN

	number of species inferred from SNVs increased	decreased	% of total bacteria
After 4wks EEN			
Responders	102	47	(5.4%)
Non-Responders	126	41	(6.1%)

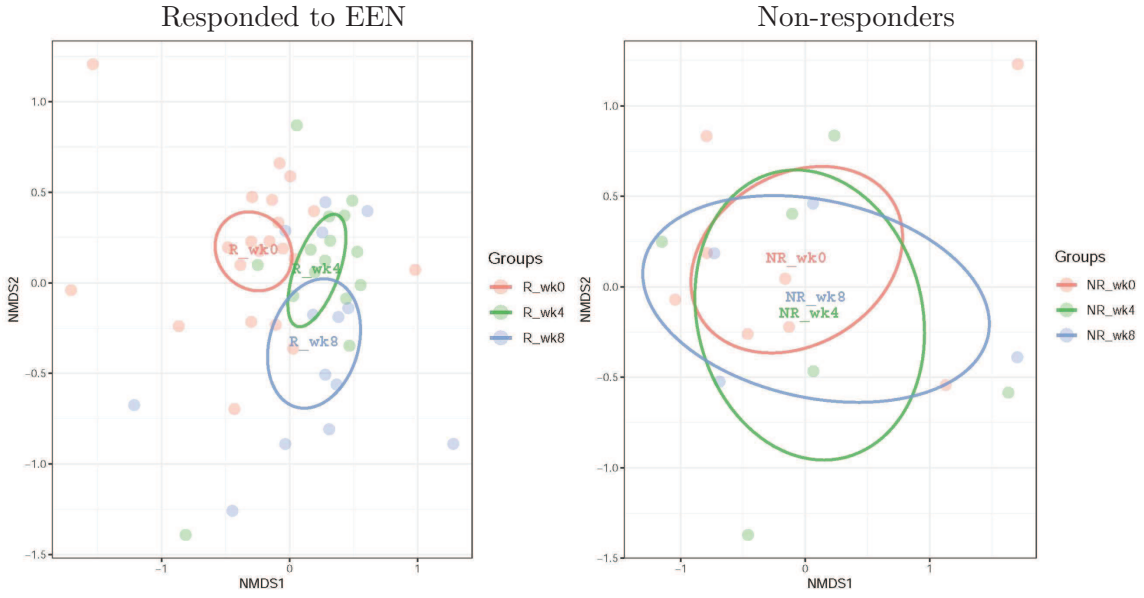
Total number of SNVs was 2,751. Differences in species level taxa were significant at p<0.01. (Differential expression analysis DESeq2)(χ^2=1.58; p=0.21).

Taxon differences The mean abundance in gut microbiota at species level (SNVs) changed after 4-weeks EEN, in both those who responded to EEN and those who failed EEN. There was no difference in the number of bacterial species (SNVs) which increased/decreased between children who responded to or failed treatment with EEN (Table 5.4; Fig. 5.14).



PERMANOVA Groups: R2=0.099; p<0.001***

(a)



R2=0.070; p<0.001***

R2=0.112 ; p=0.56 (NS)

(b)

(c)

Figure 5.13: NMDS plot showing changes in bacterial community structure in (a) children with Crohn's disease at baseline (week-0); after treatment with EEN (week-4) and (week-8), when compared with healthy children; Changes from baseline (wk0), and 4-weeks EEN (wk4) and 8-weeks EEN (wk8) are shown in children with Crohn's who (b) responded (R) to treatment and (c) those who were non-responders (NR). Plots show SNVs (species) level taxa. Ellipse shows 95% CI.

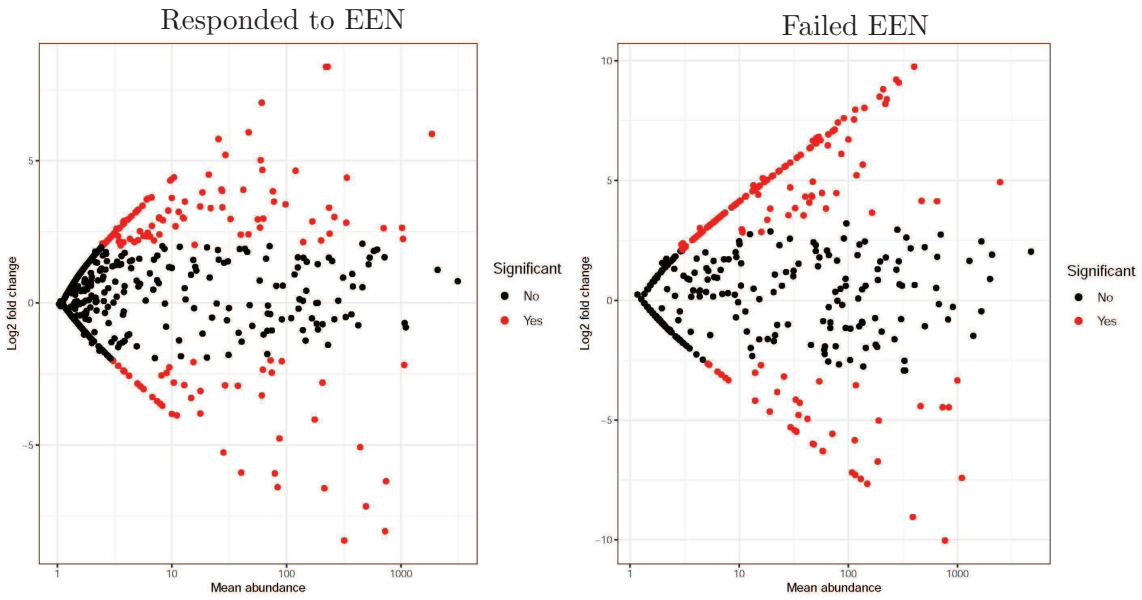
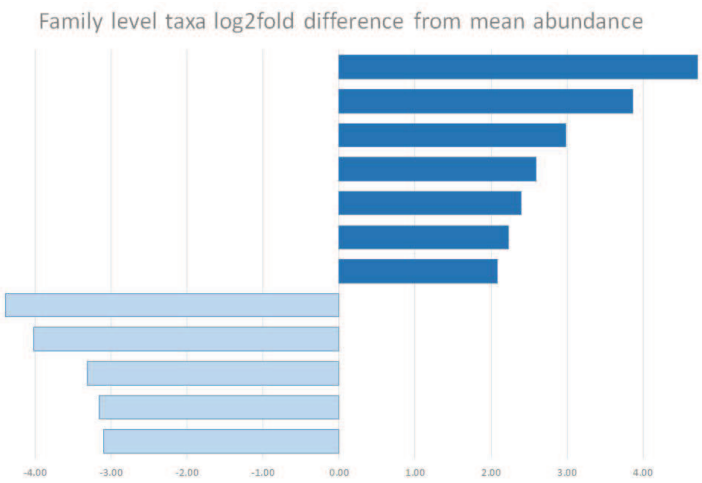


Figure 5.14: SNVs (proxy for species) that are $\log_2$ fold different from mean abundance between baseline and 4-weeks of EEN in (a) children with Crohn's who responded (R) to treatment with EEN and (b) those who were non-responders (NR). Dots shown in black are species (SNVs) similar in abundance between baseline and 4-weeks EEN. Dots shown in red are species (SNVs) significantly increased or decreased in each patient group during EEN where $p<0.001$. (Differential expression analysis DESeq2).

The small number of species which decrease in abundance is surprising, as we might expect a liquid feed with no fibre, mostly absorbed in the small intestine, would result in a large reduction in the abundance of many species and with fewer increases in abundance. However decreases in the number of species (α-diversity) appear to be accompanied by increased abundance (β-diversity) in many bacterial species (SNVs) during EEN.

In-terms of over all changes in abundance at family level during EEN (Fig. 5.15) there was a decrease mainly in gram-negative bacteria. Veillonellaceae which belongs to the subclass Negativicutes was also decreased during EEN with this group of Firmicutes having a cell wall similar to gram-negative bacteria. Although two families of gram-negative bacteria were also increased during EEN, the Enterobacteriaceae and Verrucomicrobiaceae (*Akkermansia*) these were of low abundance. All other bacteria with increased abundance were from the phylum Firmicutes, along with a small increase in Corynebacteriaceae from the phylum Actinobacteria.

Discrimination analysis show that 4-weeks treatment with EEN shifts the microbiota community towards a structure, distinct form both children with Crohn's at baseline and healthy children (Fig. 5.16). This illustrates that EEN did not restore the microbiota towards a state similar to healthy children, but further from it.



	log2 fold change	base mean	p-value adj
Rhodospirillaceae	4.72	4	1.37E-04
Verrucomicrobiaceae	3.86	6	5.21E-03
Enterobacteriaceae	2.98	40	7.22E-08
Family XIII	2.59	65	1.37E-04
Christensenellaceae	2.40	104	1.96E-04
Corynebacteriaceae	2.23	255	4.88E-03
Peptococcaceae	2.09	1217	2.21E-04
Pasteurellaceae	-4.39	10	3.56E-05
Bacteroidales S24-7 group	-4.02	27	2.43E-05
Fusobacteriaceae	-3.32	187	7.22E-08
Prevotellaceae	-3.15	685	4.88E-03
Veillonellaceae	-3.10	864	5.45E-04

Figure 5.15: The $\log_2$ fold difference in family level abundance for children with Crohn's disease after 4-weeks EEN. Only families significant to $p<0.001$ are shown.

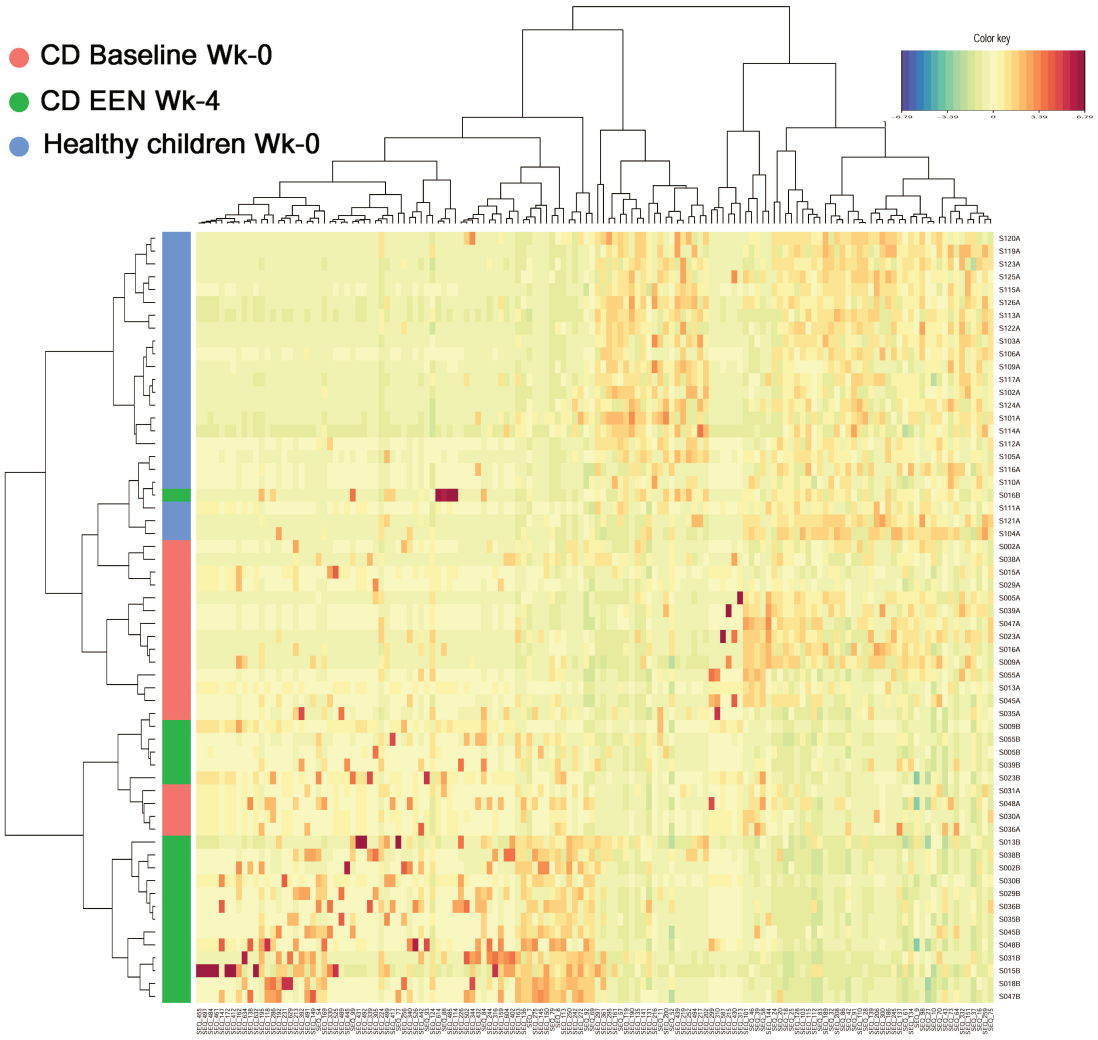


Figure 5.16: Heatmap of discriminant species inferred from SNVs, in children with Crohn's disease at baseline, then after 4-weeks treatment with EEN, and healthy children for comparison. Rows and columns are ordered using hierarchical clustering (average linkage) to identify blocks of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red); low abundance (blue).

5.3.4 Changes in individual children during EEN

To explore individual changes in gut bacterial community structure during EEN two children who failed EEN (non-responders) and four children who respond to treatment with EEN (responders) are shown in figure 5.17 as typical examples of gut microbiota changes. Changes in microbiota community structure at genus level are shown at baseline; 4-weeks EEN; 8-weeks EEN and 2-weeks normal diet.

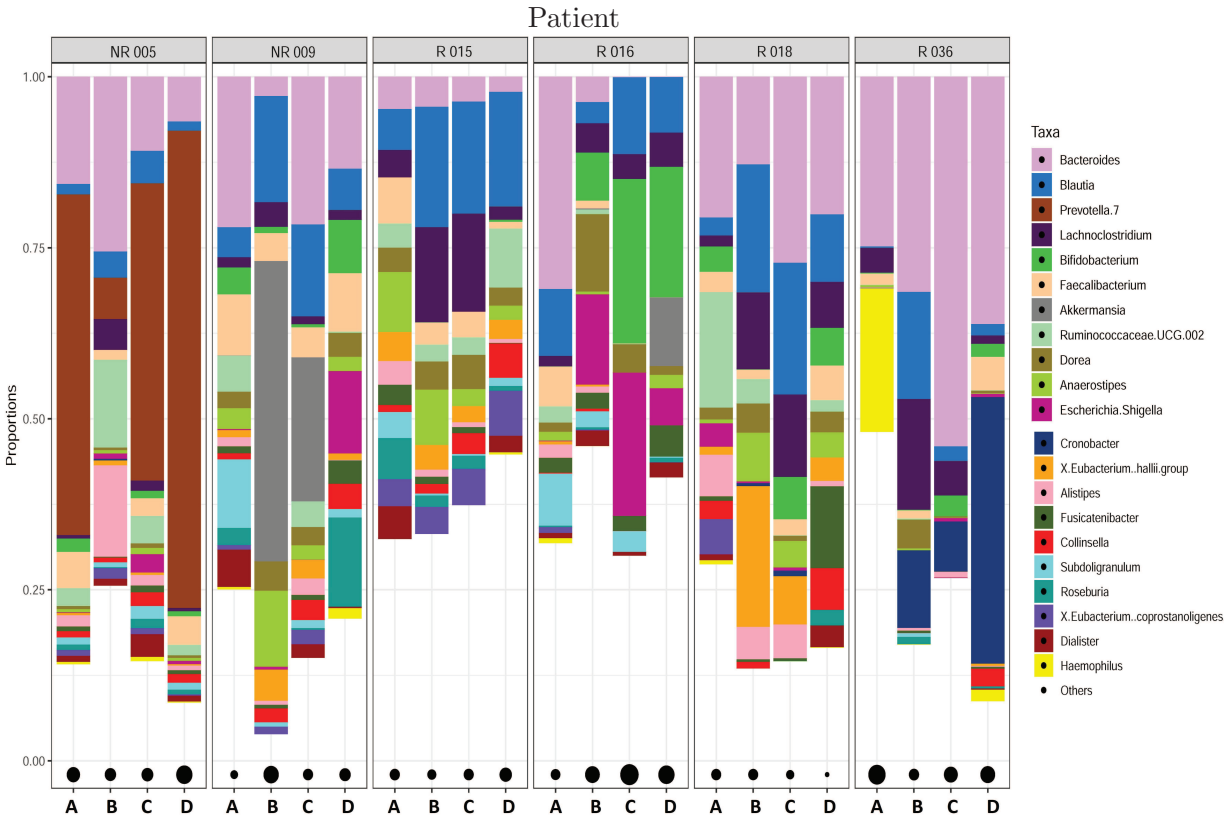


Figure 5.17: Changes in microbiota community structure (relative abundance) for genus level at (A) baseline; (B) 4-weeks EEN; (C) 8-weeks EEN and (D) 2-weeks normal diet: in two children with Crohn's disease who failed to respond to EEN (NR) and four children who respond to treatment with EEN (R). Patient R036 relapsed ∼6-weeks post-EEN.

In participant NR005, a child who completed 8-weeks of EEN but failed to respond and subsequently went onto corticosteroids, the gut microbiota at baseline (S005A) was dominated by the abundance of *Prevotella 7* which was reduced after 4-weeks of EEN (S005B). This was accompanied by an increase in abundance in a number of genera including *Alstipes* and *Rumminoccaceae UGG002*. The gut microbiota community structure at 8-weeks EEN (S005C) was similar to 2-weeks post-EEN (S005D) which is likely because this child was back onto normal diet for 2-days before managing to provide the end of EEN stool sample. In non-responder NR009 who also completed 8-weeks on EEN, the abundance of *Akkermansia*, which was not detected at baseline, dominated the gut microbiota composition during EEN, but was not detected at 2-weeks post EEN (009D). *Escherichia-Shigella* which had not been de-

tected at baseline or during EEN, was present in abundance once this child stopped EEN and started corticosteroid therapy (S009D).

In participant R015, a child who responded to EEN, there was an increase in relative abundance of the butyrate producer *Lachnoclostridium* during EEN, which was reduced once this child went back onto normal diet (S015D). This child also had increased abundance of the butyrate producer *Blautia* during EEN which was sustained when they back onto normal diet (S015D). In participant R018, an other child who responded to EEN, the Firmicutes *Eubacterium hallii* was increased during EEN as well the abundance of another butyrate producer *Lachnoclostridium*. *E. hallii* is important to intestinal metabolic balance because of its ability to utilise glucose, acetate and lactate to form butyrate and hydrogen.[399]

Responder R016 had increased abundance of *Escherichia-Shigella* during EEN which was still present once they had gone back onto their normal diet for 2 weeks (S016D). They also had increases in Actinobacteria *Bifidobacterium* and *Varibaculum*, which were not sustained once the child had returned to their normal diet. *Bifidobacterium* uses a unique fructose-6-phosphate phosphoketolase pathway to ferment oligosaccharides. In this child, *Bacteroides* was the dominant genus at baseline, however abundance was greatly reduced after 4-weeks of EEN and was not present at 8-weeks EEN. Bacteroides was absent from all following samples collected from this child: 2-weeks; 8-weeks; and 28-weeks post-EEN, suggesting that treatment with EEN may have eradicated what is considered a dominant commensal in healthy individuals.

Responder R036 also had increased *Lachnoclostridium* during EEN but this was not sustained once this child went back onto their normal diet (S036D). Patient R036 had a high abundance of the Proteobacteria *Haemophilus* at baseline and although this was not detected during EEN it was detected at 2-weeks post EEN and had increased in abundance further by 8-weeks post EEN. The Proteobacteria *Cronobacter* was absent at baseline but was present during EEN with further increase by 2-weeks post-EEN (S036D). By 6-week post-EEN this child had relapsed and was on corticosteroids. The faecal sample given at 8-weeks post-EEN showed that *Escherichia-Shigella* had replaced *Cronobacter* as the most abundant genera.

Since children who responded saw increases in Proteobacteria during and after EEN while at the same time going into disease remission, it seems unlikely that these strains of Proteobacteria are directly linked with inflammation. It is possible however that less prevalent strains which were not detected could be decreasing during EEN. Looking at the abundance of the most common genera (Fig. 5.17) do not show any obvious differences in bacterial changes during EEN between responders and non-responders.

Given that all children went on to an identical diet during EEN it is surprising that there is such a wide variety in the way bacteria from the same genera respond to the

diet in different individuals. The results from this current study would suggest that the complexity of which bacteria species are present at baseline and/or host genetics, appear to play a larger role in the gut bacteria community structure than diet across different individuals over time.

5.3.5 Predicting Response to EEN

There was no significant difference between responders and non-responders in terms of either richness or Shannon diversity at baseline, hence microbiota diversity did not predict response to EEN (Fig. 5.12).

The genera at baseline which were higher in abundance in responders compared with non-responders, did not appear to show any pattern in terms of Phyla or Family which could explain differences in response to EEN (Table 5.5).

sPLS-DA discriminant analysis (Fig. 5.18) show that twenty-five species (SNVs) could be used to discriminate between responders and non-responders to EEN (ordination ellipses). Children with Crohn's disease that responded to EEN cluster together, where as non-responders although distinct from responders, are also distinct from one another (heatmap fig. 5.18d).

Species richness and response to EEN sPLS-DA discriminant analysis (Fig. 5.19) was carried out between those children who had increased number of species inferred by SNVs during EEN and those children who decreased in number of species (SNVs) to see if these children had differences in microbiota structure at baseline. The ordination ellipses did not over lap suggesting that these two groups of children have distinct differences in microbiota community structure at baseline (Fig. 5.19c). The heatmap (Fig. 5.19d) of discriminating bacterial species level taxa (SNVs) shows that with exception of one participant (S036) these two groups of children show some patterns of differences at baseline which might determine the impact the EEN has on the gut microbiota during EEN. Two children who failed EEN (S045,S048) were among the group that increased species richness during EEN and four children who failed EEN (S002,S005,S009,S029) where in the group who decreased species richness during EEN. The sample size was not large enough to test whether a decline in species richness during EEN was more likely to result in children failing to respond to treatment.

In summary those children who responded to EEN had a more distinct shift in microbiota community structure during EEN and children who failed to respond may have increased in abundance in more species during EEN. Although twenty-five species could distinguish responders from non-responders there was no obvious pattern in term of the phylogeny of these species (Fig. 5.18).

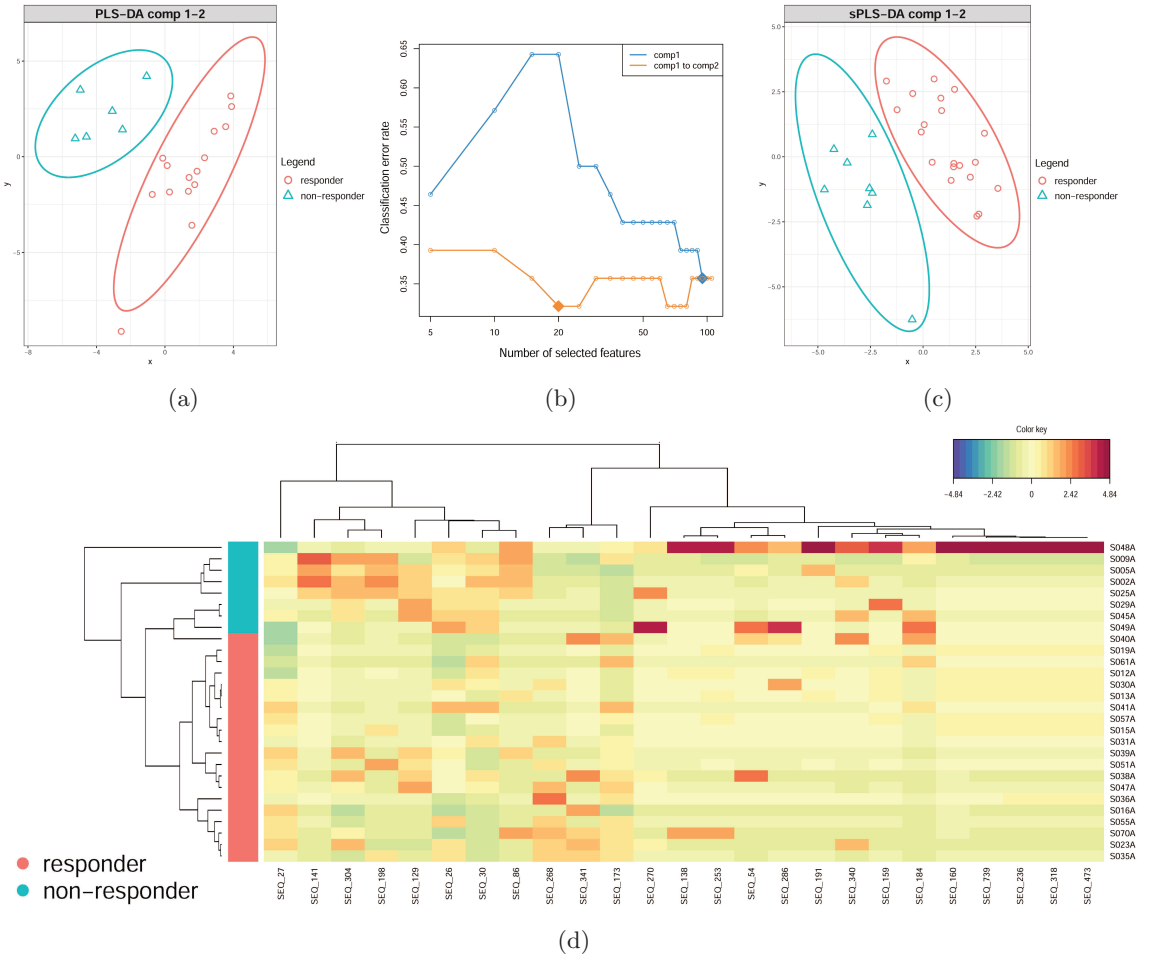


Figure 5.18: **sPLS-DA Discriminant Analysis** showing the difference in microbiota community structure at baseline, between children with Crohn's disease who either responded to or failed treatment with EEN. The algorithm is a 2-step process where two components are found reducing the classification error rates (using max.dist) in the algorithm, with (a) showing the ordination of samples using all the SNVs (species) in the first two components (PLS-DA) with ellipses representing 95% confidence interval and percentage variations explained by these components in axes labels. In step two, (b) the number of discriminating SNVs (species) were found for each component, highlighted as diamonds. In (c) the ordination used the discriminants from all two components (sPLS-DA). (d) is a heatmap of discriminant SNVs (species), with rows and columns ordered using hierarchical clustering (average linkage) to show patterns of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red) and low abundance (blue).

Table 5.5: Genera which were more abundant at baseline in children who responded to or failed treatment (non-responders) with EEN

Genus	base mean	$\log_2$ fold change	p-value adj	up-regulated in
Anaerococcus	5.79	-2.92	2.98E-03	Non-responders
Cronobacter	5.03	-2.91	3.95E-03	Non-responders
Peptostreptococcus	65.60	-3.94	3.95E-03	Non-responders
Actinobacillus	9.72	3.47	9.34E-03	Responders
Aggregatibacter	6.85	3.15	2.86E-02	Responders
Akkermansia	66.00	5.38	4.77E-04	Responders
Fusobacterium	54.72	3.27	2.86E-02	Responders
Phascolarctobacterium	137.18	6.40	3.46E-05	Responders
Prevotella 9	1291.23	7.65	2.84E-06	Responders
Ruminococcaceae UCG-004	9.95	2.80	2.86E-02	Responders
Ruminococcaceae UCG-014	8.98	3.35	1.93E-02	Responders

Differential expression analysis DESeq2.

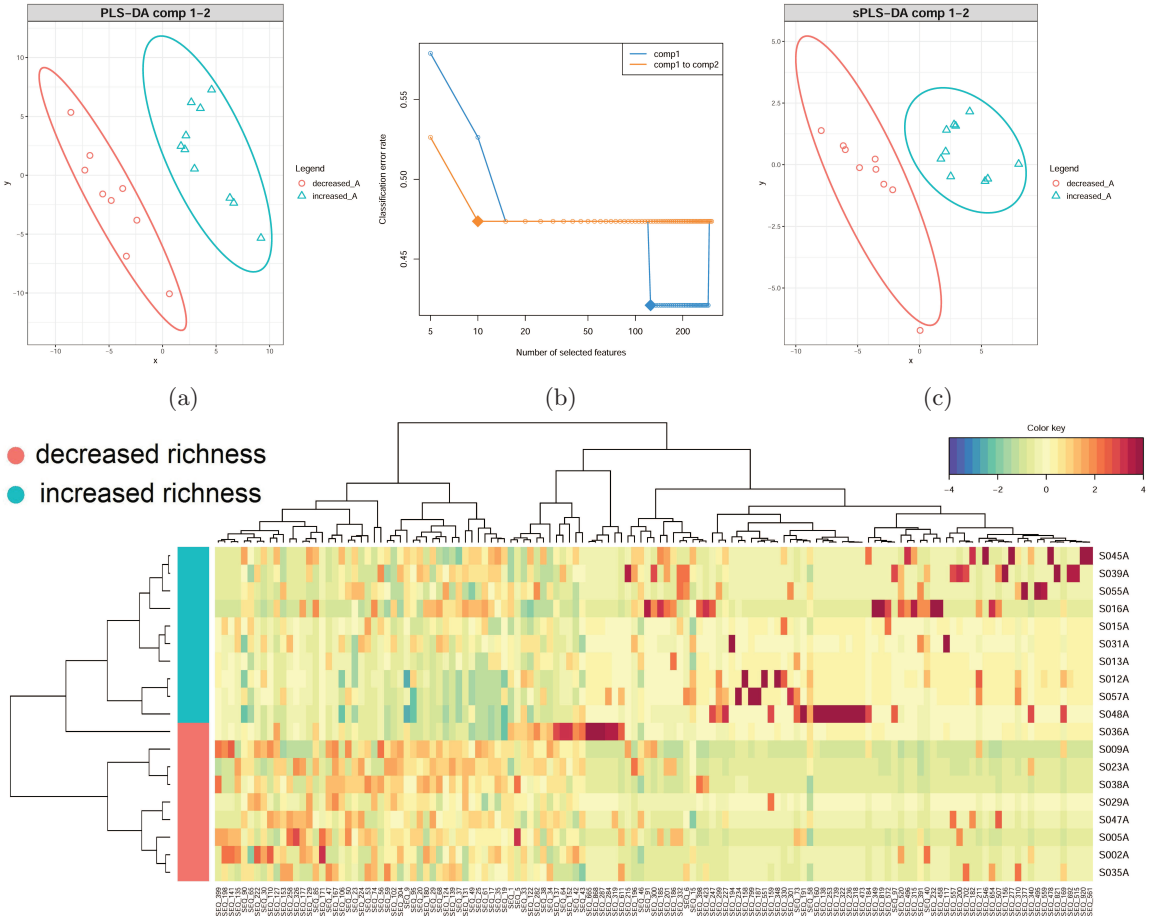


Figure 5.19: **sPLS-DA Discriminant Analysis** showing the difference in microbiota community structure at baseline between children with Crohn's disease who increased species richness or decreased in species richness on EEN. The algorithm is a 2-step process where two components are found reducing the classification error rates (using max.dist) in the algorithm, with (a) showing the ordination of samples using all the species (SNVs) in the first two components (PLS-DA) with ellipses representing 95% confidence interval and percentage variations explained by these components in axes labels. In step two, (b) the number of discriminating species (SNVs) were found for each component, highlighted as diamonds. In (c) the ordination used the discriminants from all two components (sPLS-DA). (d) is a heatmap of discriminant species (SNVs), with rows and columns ordered using hierarchical clustering (average linkage) to show patterns of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red) and low abundance (blue).

5.3.6 Microbiota changes in children with Crohn's disease during MEN

Once children with Crohn's disease had completed 8-weeks of EEN, nine children had failed to respond, and twenty-three children had gone into disease remission and were subsequently placed on maintenance therapy to help maintain remission. Maintenance therapy was either given as maintenance enteral nutrition (MEN); an immunosuppressant plus MEN; or as an immunosuppressant (IM) only. Faecal samples available for analysis in each group are shown in Table 5.6.

Table 5.6: Number of faecal samples available for analysis at each time-point during maintenance therapy

Study time-point	MEN only	MEN plus IM	IM only
week-8 *(end of treatment with EEN*	5	5	4
Back on to normal diet			
week-10 *(2 weeks maintenance therapy)*	4	6	4
week-16 *(8 weeks maintenance therapy)*	6	7	4

IM-immunosuppressant; MEN- maintenance enteral nutrition. All children went onto some type of maintenance therapy (i.e. there was no non-treatment control group).

It is important to note that children who had higher levels of inflammation after 8-weeks of treatment on EEN, as indicated by calprotectin levels, were more likely to be given an immunosuppressant drug as part of maintenance therapy (Fig. 5.20). Therefore any differences in gut microbiota between treatment groups at study week-8, week-10 and week-16 could be linked to differences in disease severity, rather than differences due to maintenance therapy.

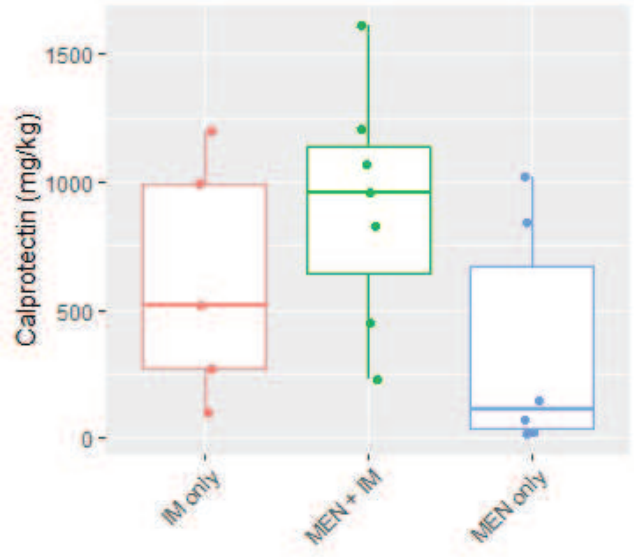


Figure 5.20: Calprotectin prior to maintenance therapy (study week-08) in children with Crohn's disease. Difference seen between groups was not significant p=0.1 (Kruskal-Wallis). IM - immunosuppressant; MEN - maintenance enteral nutrition.

Richness (α-diversity) Although there was a slight increase in the median number of species (SNVs)(richness) and species evenness (Shannon index) after 2-weeks on normal diet (study week-10) and 8-weeks normal diet (week-16) this was not significant in any of the three maintenance treatment groups (Fig. 5.21). When all treatment groups were combined, no difference in species level richness or Shannon entropy was seen as children returned back on to normal diet at week-10 and week-16.

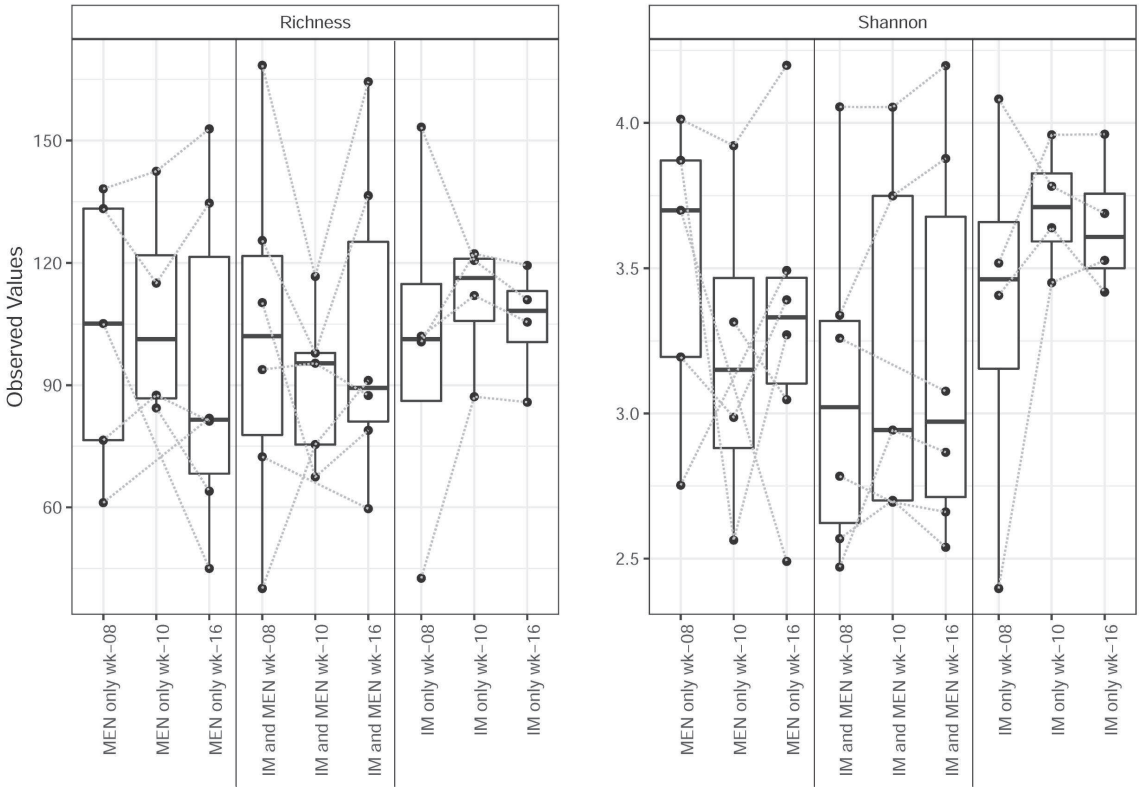
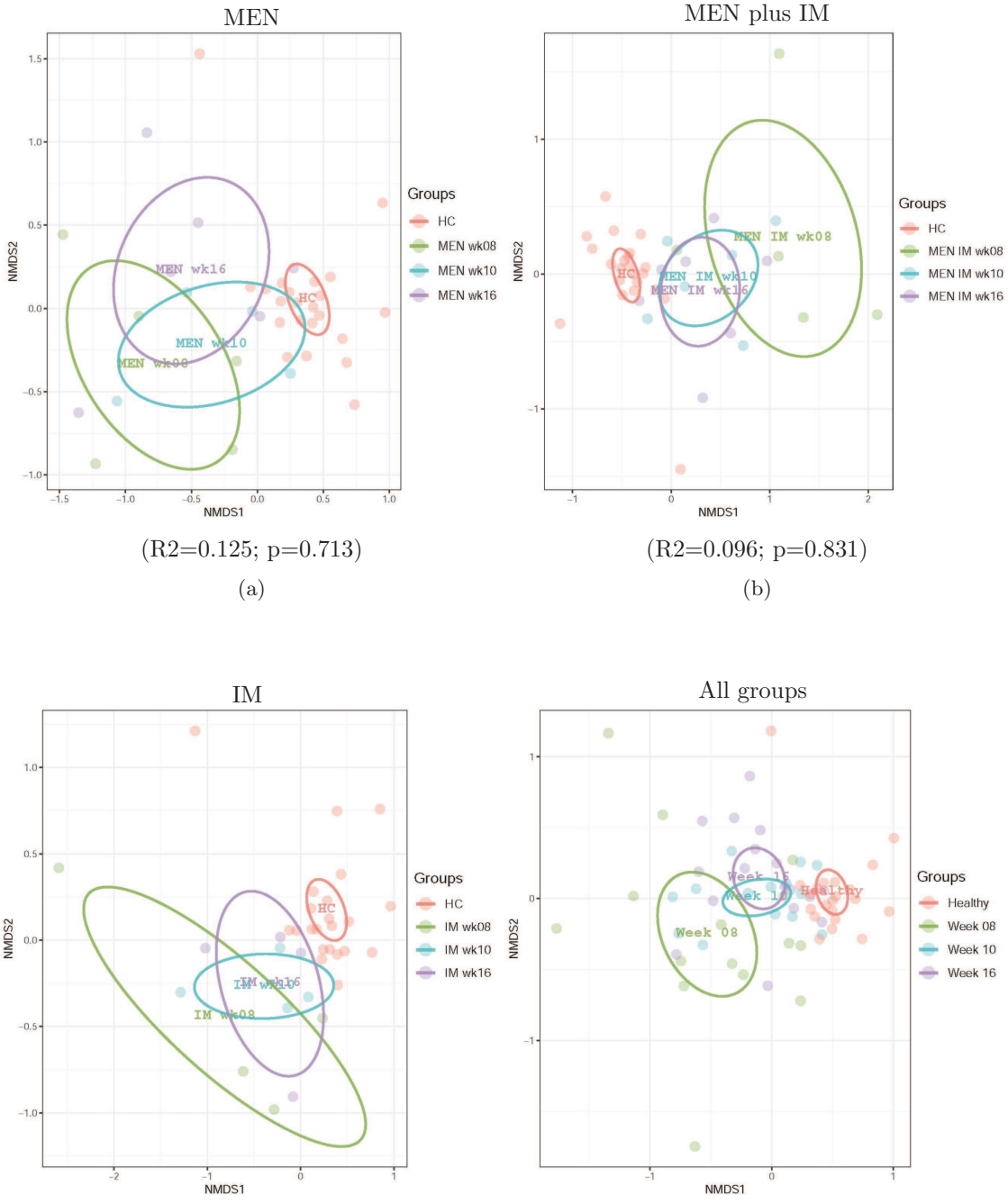


Figure 5.21: Gut bacteria α-diversity shown by richness (no. of SNVs/species) and Shannon index (evenness), in children with Crohn's disease at end of EEN (wk-08); then 2-weeks (wk-10); and 8-weeks (wk-16) of normal diet plus maintenance therapy. Children were treated with an immunosuppressant (IM) only (n=4); IM plus maintenance enteral nutrition (MEN) (n=6 at wk-10; n=7 at wk-16); or MEN only (n=4 at 10-wks; n=6 at 16-wks). No significant difference seen between time-points (ANOVA).

Abundance (β-diversity) Although difference in microbiota community structure was not significant (Fig. 5.22), there is a clear move in all maintenance treatment groups showing that once back onto normal diet, at week-10 and week-16, the gut bacteria community structure starts to move towards a structure associated with healthy children. This is most evident when all maintenance groups were combined (R2=0.055, p=0.112; Fig. 5.22d). Access to a non-treatment control group was unfortunately not possible, since all children who chose not to take MEN were given an immunosuppressant drug; therefore no child on the study returned to normal habitual diet without some form of maintenance therapy.



(R2=0.125; p=0.713)

(a)

(R2=0.096; p=0.831)

(b)

(R2=0.169; p=0.606)

(c)

(R2=0.048; p=0.019)

(d)

Figure 5.22: NMDS showing change in microbiota community structure in children with Crohn's disease during maintenance therapy, who were treated with (a) maintenance enteral nutrition (MEN); (b) MEN plus an immunosuppressant (IM); or (c) IM only. (d) shows all children on maintenance therapy combined. Healthy controls (HC) are shown for comparison but were not included in test for significance. Samples were taken at end of EEN (Week-08); then 2-weeks (Week-10) and 8-weeks (Week-16) after return to normal diet. Change in community structure over time on normal diet was not significant for any treatment group (PERMANOVA). Ellipse shows 95% CI.

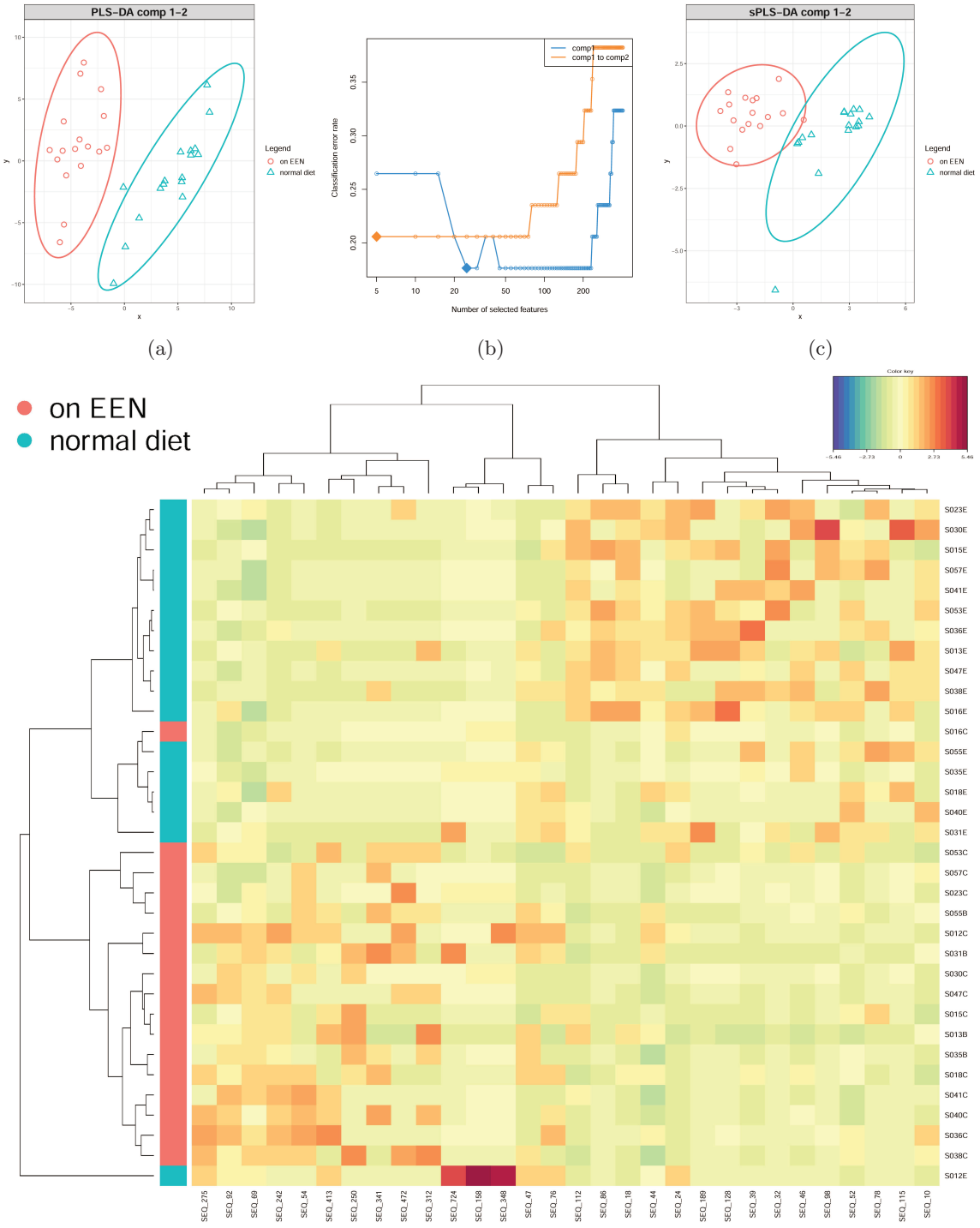


Figure 5.23: **sPLS-DA Discriminant Analysis** showing the change in microbiota community structure at species level taxa (SNVs) between the end of EEN (week-8) in children with Crohn's disease and then once they go onto normal diet plus maintenance therapy (week-16). The algorithm is a 2-step process where two components are found reducing the classification error rates (using centroid.dist) in the algorithm, with (a) showing the ordination of samples using all the species (SNVs) in the first two components (PLS-DA) with ellipses representing 95% confidence interval and percentage variations explained by these components in axes labels. In step two, (b) the number of discriminating species (SNVs) were found for each component, highlighted as diamonds. In (c) the ordination used the discriminants from all two components (sPLS-DA). (d) is a heatmap of discriminant species (SNVs), with rows and columns ordered using hierarchical clustering (average linkage) to show patterns of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red) and low abundance (blue).

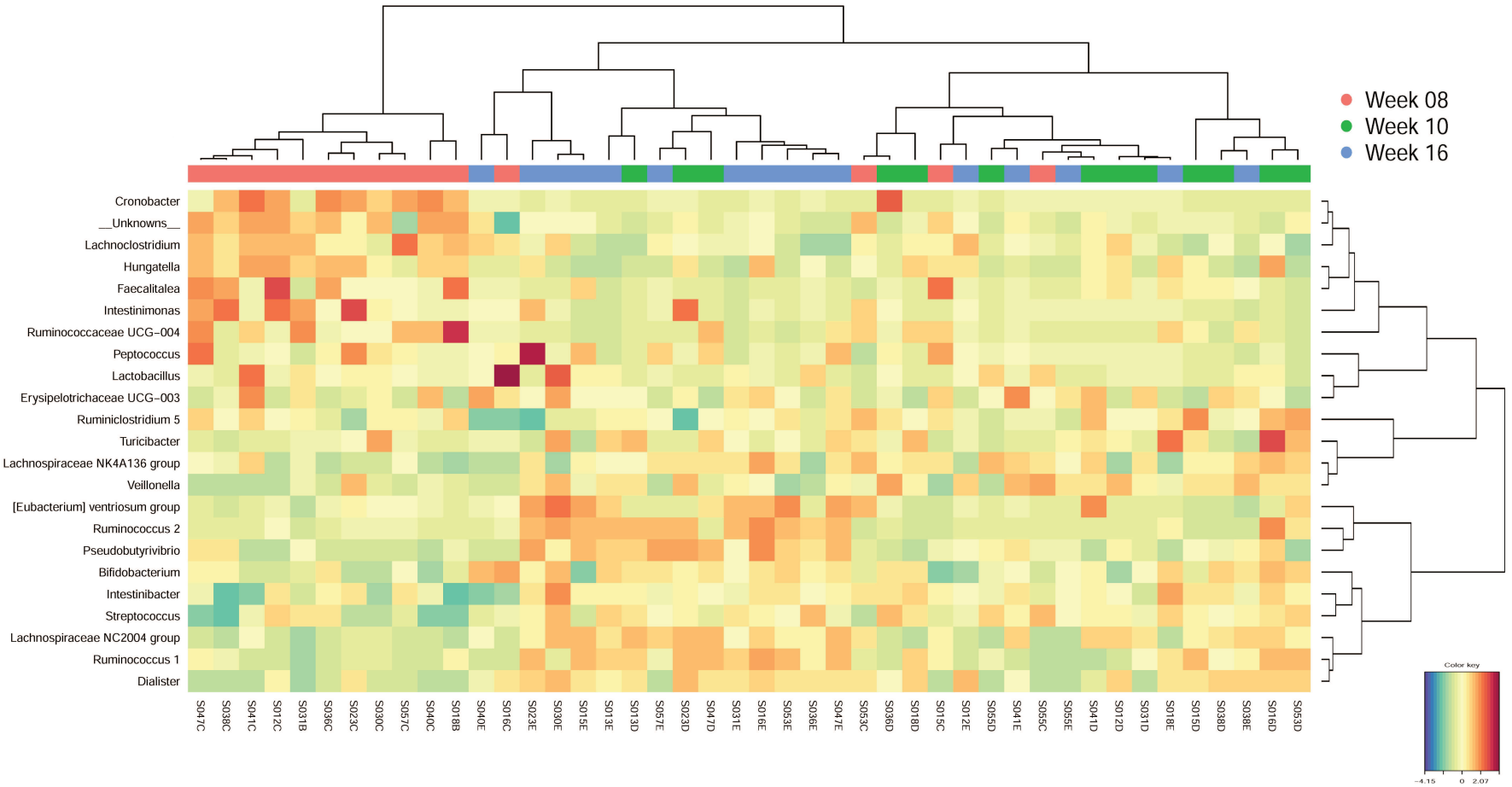


Figure 5.24: Heatmap of discriminant genera, in children with Crohn's disease at end of EEN (study week-08), then after 2-weeks normal diet (study week-10), 8-weeks normal diet (study week-16). Rows and columns are ordered using hierarchical clustering (average linkage) to identify blocks of interest. Heatmap depicts TSS+CLR normalised abundances: high abundance (red); low abundance (blue). A clear shift in which bacteria are most abundant can be seen as children move onto normal diet at week-10 and week-16.

Table 5.7: Number of species level taxa (SNVs) which changed in abundance between the end of EEN (study week-8) and 2-weeks/8-weeks of maintenance therapy (study week-10/week-16)

	MEN number of SNVs		MEN + IM number of SNVs		IM number of SNVs	
	increased	decreased	increased	decreased	increased	decreased
normal diet plus						
2-wks maintenance therapy	49 (1.8%)	37 (1.3%)	68 (2.5%)	45 (1.6%)	50 (1.8%)	53 (1.9%)
8-wks maintenance therapy	70 (2.5%)	73 (2.6%)	45 (1.6%)	70 (2.5%)	50 (1.8%)	79 (2.9%)

Total number of SNVs (proxy for species) was 2,751. Differences shown by $\log_2$ fold change in species were significant at p<0.01. (Differential expression analysis based on negative binomial -DESeq2) (χ^2=0.43; p=0.52). MEN -maintenance enteral nutrition; IM- immunosuppressant

,

The number of SNVs (a proxy for species) which significantly increased in abundance after 2-weeks and 8-weeks normal diet varied between maintenance treatment groups as well as over time (Table 5.7). However all three treatment groups showed a very similar pattern of decreased abundance resulting in between 2.5% (70) and 2.9% (79) of total bacterial species variants (SNVs) having reduced abundance after 8-weeks of normal diet (study week-16) (Table 5.7). Therefore once back onto normal diet, although there was no reduction in the number of bacterial species (α-diversity), there was a significant reduction in the abundance (β-diversity) of many bacterial species (SNVs) in children on all forms of maintenance therapy. This change in abundance is visualised as a heatmap for species level (SNVs) in figure 5.23d and for genus level in figure 5.24.

Using differential expression analysis to look at changes after 8-weeks of normal habitual diet plus maintenance therapy (study week-16), no significant log2 fold change in relative abundance of bacteria was seen at family level taxa. However a number of Proteobacteria genera were significantly (p<0.01) decreased in abundance in the MEN only treatment group including *Citrobacter*, *Cronobacter Enterobacter* and *Pantoea* after 8-weeks of normal diet (Fig. 5.25a). The MEN plus immunosuppressant (IM) group also had decreased Proteobacteria including *Citrobacter*, *Cronobacter*, *Campylobacter* and *Sutterella* (Fig. 5.25b). The IM-only group also had reduced *Citrobacter*, *Cronobacter*, *Enterobacter*, and *Eikenella* (Fig. 5.25c). Not all children saw a reduction in Enterobacteriaceae once they returned to normal diet. Patient S036, a child who took MEN plus IM, had high abundance of Enterobacteriaceae (*Cronobacter*) at then end of EEN (study week-8), which increased in abundance at 2-weeks normal diet (week-10); which was then replaced by a high abundance of *Escherichia-Shigella* at 8-weeks normal diet (week-16). This child had relapsed by 8-weeks normal diet (week-16) and gone onto corticosteroids (Fig. 5.26). Another participant (S012) who took MEN plus IM had a large increase in *Escherichia-Shigella* after 2-weeks normal diet but this had reduced in abundance by 8-weeks normal diet. Participant S016, a child on IM only, had a high abundance of *Escherichia-Shigella* at end of EEN (study week-8), however abundance reduced once the child returned to normal diet

(Fig. 5.26). These patients show that having high abundance of Proteobacteria at the end of EEN does not seem to determine whether this group of bacteria will increase or decrease once children return to normal diet.

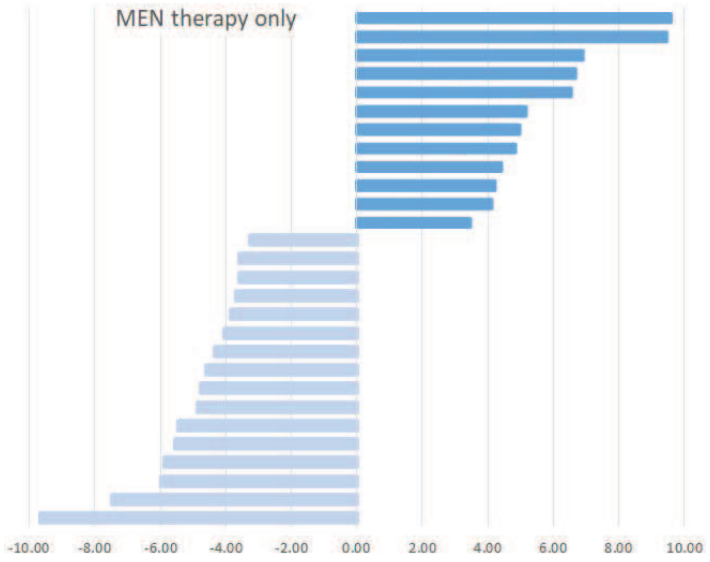
Discriminant analysis (sPLS-DA) at species level (SNVs)(Fig. 5.23) show that most species variants which discriminate between the end of EEN and a return to normal diet were from the order Clostridiales. This was characterised by are a drop in the abundance of five Ruminococcaceae species variants (SEQ 92, 69, 413, 341, 472) and an increase in eight Lachnospiraceae species variants (SEQ 112, 18, 189, 128, 32, 98, 52, 78) once back onto normal diet for 8-weeks (study week-16). The Bacteroidetes species *Alistipes* (SEQ 312, 47) and *Parabacteroides* (SEQ 76) were also reduced along with the Proteobacteria *Cronobacter* and *Parasutterella* (SEQ 54, 724) on normal diet (Fig. 5.23d).

Subset analysis of the microbiota data from children on maintenance therapy (Table 5.8) shows which bacterial genera are the main source of variation from all samples between the end of EEN (study week-8) and return to normal diet (week-16). These included the Actinobacteria, *Bifidobacterium* and *Collinsella*; the Bacteroidetes *Bacteroides*, *Alistipes*, *Prevotella 7* and *Prevotella 9*; the Firmicutes, *Ruminococcaceae UCG-002*, *Faecalibacterium*, *Lachnoclostridium* and *Blautia*; Proteobacteria, *Escherichia-Shigella* as well as the Verrucomicrobia *Akkermansia* (Table 5.8).

Table 5.8: Subset analysis showing which genera explain the percentage variability in microbiota community structure between end of EEN (week-8) and normal diet (week-16)

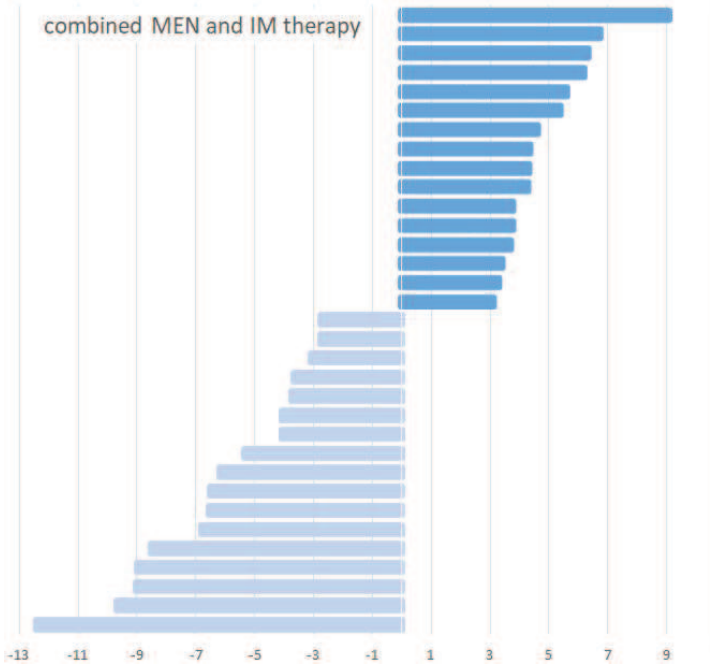
Subset	Subset of most abundant genera	correlation with full abundance table	PERMANOVA subsets
S1	Unknowns + Alistipes + Ruminococcaceae UCG-002 + Escherichia-Shigella + Collinsella + Akkermansia + Bifidobacterium + Faecalibacterium + Lachnoclostridium + Prevotella 7 + Prevotella 9 + Blautia + Bacteroides	0.95	R^2=0.083 p=0.006 **
S2	Unknowns + Ruminococcaceae UCG-002 + Escherichia-Shigella + Collinsella + Akkermansia + Bifidobacterium + Faecalibacterium + Lachnoclostridium + Prevotella 7 + Prevotella 9 + Blautia + Bacteroides	0.95	R^2=0.083 p=0.004 **
S3	Unknowns + Ruminococcaceae UCG-002 + Escherichia-Shigella + Akkermansia + Bifidobacterium + Faecalibacterium + Lachnoclostridium + Prevotella 7 + Prevotella 9 + Blautia + Bacteroides	0.94	R^2=0.087 p=0.003 **
S4	Unknowns + Ruminococcaceae UCG-002 + Escherichia-Shigella + Akkermansia + Bifidobacterium + Faecalibacterium + Lachnoclostridium + Prevotella 9 + Blautia + Bacteroides	0.94	R^2=0.090 p=0.009 **

Subsets were generated using the *BVSTEP* routine in **R**. PERMANOVA of subsets were performed against children on EEN and the same children 8-weeks post EEN (normal diet) with R^2 explaining the percentage variability in microbiota structure between groupings. Where the SNVs had no genera assignment, they were binned together in the category 'Unknowns'.



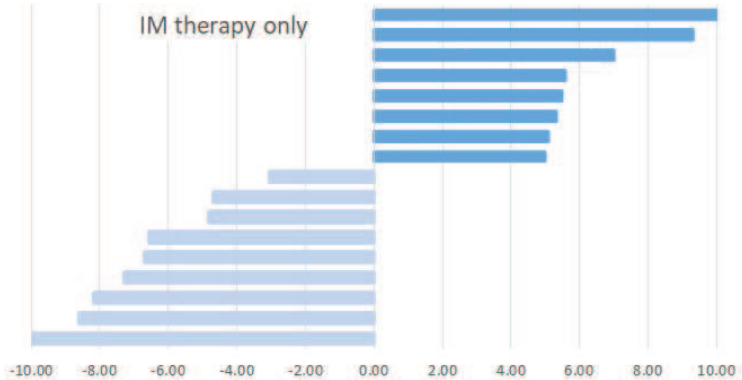
	base mean	log2 fold change	p adj
Prevotella 7	1734.8	9.59	1.54E-04
Anaeroglobus	510.4	9.46	4.23E-05
Ruminococcus 2	171.8	6.88	5.72E-04
Haemophilus	114.6	6.67	4.59E-04
Veillonella	67.2	6.53	4.48E-05
Dialister	452.9	5.17	1.16E-02
Lachnospira	55.1	4.95	1.42E-02
Fusobacterium	21.5	4.85	8.88E-03
Pseudobutyrivibrio	384.6	4.39	2.14E-02
Selenomonas 3	14.0	4.21	1.73E-02
Paraprevotella	13.3	4.11	2.14E-02
Ruminococcaceae UCG-003	33.0	3.45	4.78E-02
Family XIII AD3011 group	69.6	-3.25	3.36E-02
Erysipelatoclostridium	60.2	-3.58	2.56E-02
Hydrogenoanaerobacterium	8.4	-3.60	1.73E-02
[Eubacterium] brachy	8.9	-3.67	2.14E-02
Citrobacter	9.8	-3.83	1.87E-02
Pantoea	11.2	-4.03	1.64E-02
Lactococcus	13.7	-4.34	4.97E-03
Hungatella	186.3	-4.60	1.64E-02
Intestinimonas	18.1	-4.76	3.30E-03
Eisenbergiella	19.3	-4.85	3.29E-03
Enterobacter	28.8	-5.45	3.07E-03
[Eubacterium] nodatum	30.9	-5.55	1.45E-03
Faecalitalea	268.1	-5.87	1.84E-03
Staphylococcus	41.0	-5.97	5.72E-04
Clostridium sensu stricto 13	114.6	-7.46	2.48E-04
Cronobacter	518.8	-9.66	1.22E-09

(a)



Ruminococcus 2	510.8	9.10	2.62E-08
Ruminococcus 1	103.1	6.79	5.04E-05
[Eubacterium] coprostanoligenes	227.6	6.35	5.22E-04
Pseudobutyrivibrio	238.7	6.23	1.18E-04
Lachnospiraceae NC2004 group	47.0	5.64	1.50E-04
Ruminococcaceae UCG-005	40.3	5.42	1.48E-03
Dialister	568.2	4.64	1.31E-02
Coprococcus 3	219.7	4.40	1.61E-02
Roseburia	608.7	4.35	2.41E-02
[Eubacterium] hallii group	520.5	4.33	1.31E-02
Bifidobacterium	1774.3	3.83	3.99E-02
[Eubacterium] ventriosum group	13.7	3.81	1.24E-02
Subdoligranulum	57.2	3.73	4.21E-02
Anaerostipes	153.9	3.46	3.33E-03
Lachnospiraceae FCS020 group	10.0	3.34	4.21E-02
Terrisporobacter	8.9	3.15	2.37E-02
Varibaculum	3.8	-2.76	2.87E-02
Lactococcus	3.8	-2.77	2.86E-02
Ruminococcaceae UCG-004	4.7	-3.11	1.88E-02
Butyricimonas	12.2	-3.67	1.62E-02
Faecalitalea	7.0	-3.74	9.35E-03
Citrobacter	74.4	-4.08	2.37E-02
Coprobacillus	8.7	-4.08	5.81E-03
Campylobacter	20.3	-5.36	5.22E-04
Sutterella	29.0	-6.20	2.67E-04
Peptostreptococcus	57.7	-6.52	6.87E-05
Allisonella	37.3	-6.56	1.50E-04
Paraprevotella	53.9	-6.80	6.87E-05
Romboutsia	178.2	-8.54	4.67E-07
Fusobacterium	517.9	-9.01	9.43E-07
Tyzzerella	365.9	-9.04	9.79E-07
Cronobacter	393.3	-9.69	1.08E-10
Prevotella 9	2622.5	-12.42	1.47E-10

(b)



Ruminococcus 2	870.7	10.45	2.20E-09
Coprococcus 2	388.7	9.26	1.08E-05
Lachnospira	80.2	6.98	2.29E-03
Ruminococcus 1	367.4	5.55	2.17E-03
Prevotella 7	28.4	5.46	1.61E-02
Pseudobutyrivibrio	837.2	5.31	9.49E-03
Terrisporobacter	44.2	5.07	4.58E-03
Proteus	20.5	4.98	2.67E-02
Lachnoclostridium	4096.4	-3.03	1.95E-02
Eikenella	18.2	-4.66	4.22E-02
Faecalitalea	91.2	-4.80	4.20E-02
Staphylococcus	411.8	-6.53	9.49E-03
Enterobacter	71.8	-6.69	4.86E-03
Cronobacter	107.2	-7.27	2.60E-03
Citrobacter	198.8	-8.17	1.10E-03
Lactobacillus	262.0	-8.57	7.04E-04
Varibaculum	1276.3	-10.85	2.16E-04

(c)

Figure 5.25: The $\log_2$ fold change in genera abundance for children with Crohn's disease after 8-weeks maintenance therapy on (a) MEN only n=5; (b) MEN plus and immunosuppressant (IM) n=6 and (c) an immunosuppressant (IM) only n=4. Bacterial genera shown were all significant to $p<0.001$.

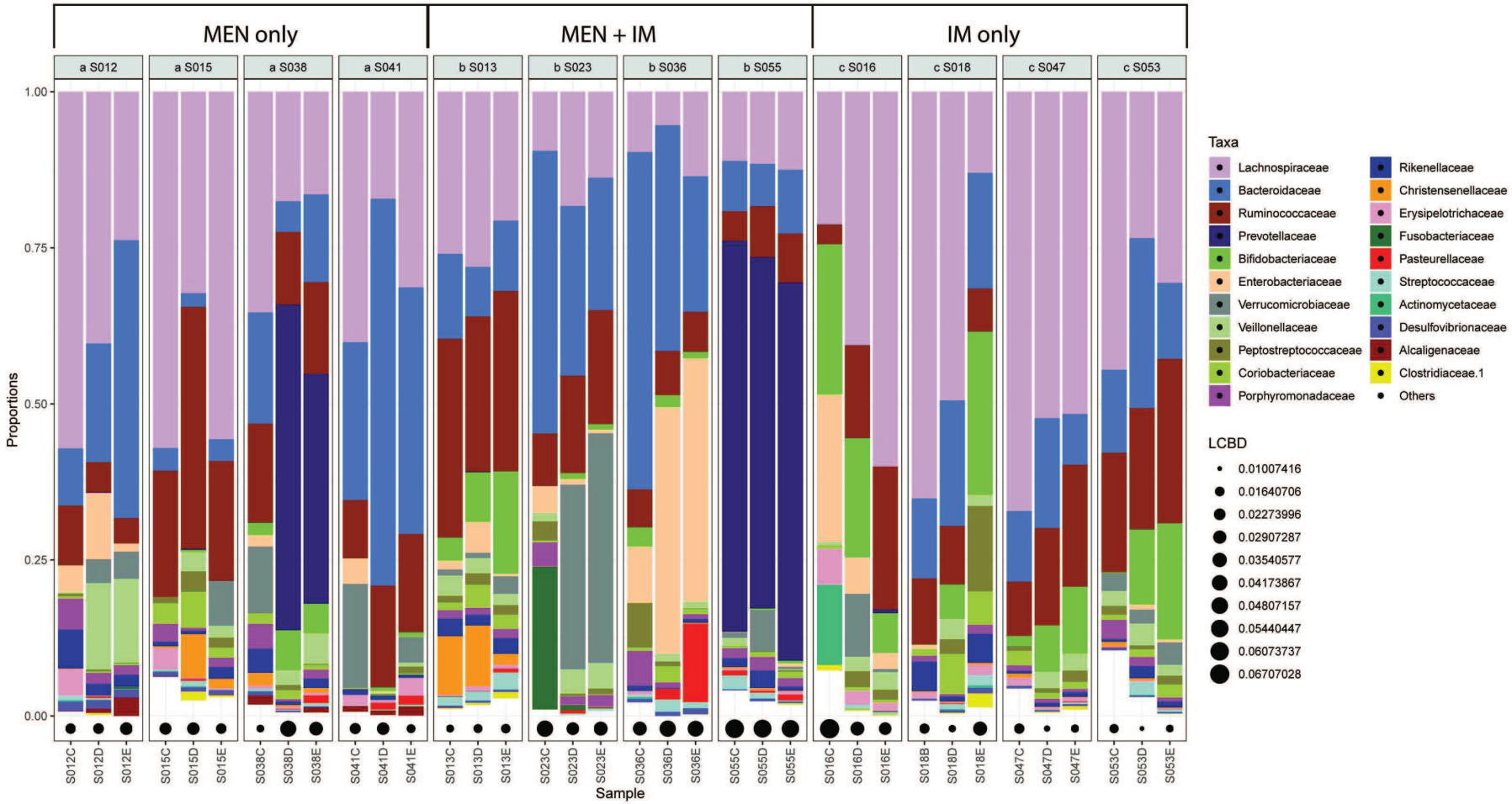


Figure 5.26: Community structure at family level, in children with Crohn's disease on maintenance therapy. MEN -maintenance enteral nutrition; IM- immunosuppressant. C- end of EEN (study week-8); D- 2-weeks normal diet (study week-10); E- 8-weeks normal diet (study week-16). Twenty-one most abundant families are shown. Bubble size increase at base of bar plot show local contribution to β-diversity (LCBD).

5.4 Discussion

5.4.1 Summary of results

The results of the current study from baseline samples highlight some key features of interest when comparing children with Crohn's disease with healthy children. Children with both Crohn's and UC have a significant reduction in bacterial diversity (Fig. 5.3). The results looking at β-diversity between children with Crohn's disease and healthy children show that differences in community structure are not limited to species but are also significantly different at phylum level (Fig. 5.4). Phyla level changes are indicative of more widespread change across the gut microbiota community. Local contribution to β-diversity also highlighted a key feature of Crohn's disease, that dysbiosis as well as being distinct from healthy children, also has the feature of being distinct from other children with Crohn's disease; such that inter-patient variability is very high among Crohn's patients (Fig. 5.5). Results also suggest children with UC have less extreme dysbiosis than those with Crohn's disease (Fig. 5.5). Taxa differences in children with Crohn's disease showed a distinct community structure which is characterised by reduced healthy commensals, particularly gram-positive Clostridia from the Firmicutes phylum. This was accompanied by an increase in gram-negative bacteria including Fusobacteria, Enterobacteriaceae and the Negativicutes (Fig. 5.8).

It was not possible to reliably address questions about differences in gut microbiota profiles by Crohn's disease location, since dividing the study group up resulted in low sample numbers, particularly for isolated ileal disease which is less common. However the discrimination analysis (Fig 5.10) hints that ileal disease might have a community structure with features which are different from children with colonic involvement, warranting further research with larger samples sizes.

Results from the current study suggest it is not possible to predict response to EEN from baseline microbiota profiles; and although twenty-five species (SNVs) were distinct between responders and non-responders, no obvious pattern in species type could be identified. Results went on to show that successful treatment with EEN changes the microbiota community structure, but rather than correcting baseline dysbiosis, EEN pushed the gut bacteria profile further away from that of healthy children (Fig 5.12). However no difference was seen in species richness, except for a non-significant decrease in children who failed to respond to EEN. (Fig 5.13). More interestingly all the family groups which were reduced during EEN were gram-negative bacteria including Pasteurellaceae, Bacteroidales S24-7, Fusobacteriaceae; or Veillonellaceae (Negativicutes). Increases in abundance mainly came from gram-positive Clostridiales families and Corynebacteriaceae (Actinobacteria).

Gut bacterial changes which occurred during EEN, were not maintained once children went back onto normal habitual diet, even when children were given a dietary

supplement of enteral nutrition (MEN) to try and maintain remission. In individual patents using MEN, increases in gram-negative bacterial families such as Veillonellaceae, Enterobacteriaceae Bacteroidaceae, Prevotellaceae and Verrucomicrobiaceae could be seen increasing on normal diet (Fig 5.26).

5.4.2 Results in the context of wider evidence

As increasing evidence in the aetiology of IBD points towards a link between environmental factors and the gut microbiota in genetically susceptible individuals, a great deal of recent effort has gone into investigating the composition and function of the gut microbiota. Although reduced bacterial diversity is a key feature of IBD, it is still unclear if this microbial dysbiosis is a symptom of an inappropriate immune response, or is actively driving inflammation in the gut.

Although some studies have suggested specific bacterial species such as *Mycobacterium; Campylobacter; Escherichia* and *Helicobacter* cause Crohn's disease, evidence does not support this theory.[176] Comprehensive studies using both faecal and mucosal biopsy samples, suggests that Crohn's disease is associated with significant differences in gut microbiota at a wider community level. The current study in line with previous studies (Table 5.9) saw decreased abundance of Clostridia and *Bifidobacterium* species; along with increased Proteobacteria, in Crohn's disease patients. This was accompanied by a non-significant increase in *Bacteroides* also reported by Andoh *et al.*[400] and Kaakoush *et al.*[223] who saw a significant increase in Bacteroidetes, in children with Crohn's disease. However, an equal number of studies have reported a decrease in Bacteroidales. Enough studies have also reported no change in Bacteroidetes (Table 5.9), to suggest that either, there are changes in both directions among genera within Bacteroidetes which is picked up as natural variation between studies; or that possible variation in storage times and freezing of faecal samples, prior to DNA extraction, known to alter the detection rate of Bacteroidetes genera, may make it difficult to detect true differences in some studies[300] (Methods section 2.8). Therefore a loss in gut bacterial diversity of symbiotic groups of gram-positive bacteria, particularly the Clostridia and Bifidobacteriaceae; along with increases in the gram-negative bacteria Bacteroidetes and Enterobacteria are key features of Crohn's disease (Table 5.9).

In 2014 the Crohn's and Colitis Foundation of America ran a prospective study (RISK study), which reported on 447 treatment naïve children with Crohn's disease and 221 non-IBD controls from twenty-eight different sites in USA and Canada between 2008-2012, which has lead to further analyses of this cohort by Gevers *et al.*;[194] Wang *et al.*[401] and Haberman *et al.*[402] (Table 5.9). An interesting aspect to the Haberman study was that they compared the mucosal microbiota between Crohn's disease children with and without ileal inflammation, and found that the identified

pattern of Crohn's disease dysbiosis, was no different between the two groups; and thus independent of inflammation. They suggest therefore, that bacterial dysbiosis precedes inflammation and is not caused by inflammation.[402]

5.4.3 Reduced diversity in Crohn's disease at diagnosis

Ecology research has shown that complex multi-species communities are by their nature, vulnerable to destabilisation.[403] The human gut microbiota manages to maintain a stable state in healthy individuals[175] despite high levels of species diversity,[404] however it is clear from this current study and other studies that children with IBD have lower than normal species diversity, which in itself could leave the microbiota community more vulnerable to destabilisation.[175] Low bacterial diversity has also been identified in a number of other conditions including IBS,[405] arthritis[218] and obesity,[406;407] and although Crohn's disease and IBD have reduced bacterial diversity at baseline in this ($p<0.001$) and other studies, it should be noted that the non-IBD group in the current study also had reduced diversity when compared to healthy children ($p=0.042$). Therefore clearly defining the specific pattern of diversity, will become important in distinguishing Crohn's disease and IBD from other conditions, before microbiota profiles can be used as a markers of disease.

5.4.4 Significance of taxa which are reduced in Crohn's disease

Some of the taxa which have low abundance in children with Crohn's disease compared with healthy children, are known to provide positive metabolic functions in the gut. Bacteria in the order Clostridiales, such as *Roseburia*, *Blautia*, and *Faecalibacterium prausnitzii* are important butyrate producers, providing essential energy for epithelial cells in the gut.[243] Many of the predominant healthy commensal bacteria also produce antimicrobial activity which can help to maintain the overall homoeostasis of the many species of bacteria in the gut, preventing the overgrowth of potential pathogens and increasing overall diversity. Future research may go on to reveal, if probiotic treatment or improved diet could increase clostridial diversity in children with Crohn's disease, replacing this loss in metabolic function with the aim of reducing inflammation. However, if loss of diversity is a product of an inflammatory state, a probiotic or pro-clostridial diet would have little or no effect on patient outcomes.

Table 5.9: Studies showing differences in gut bacteria composition between Crohn's disease and healthy people

Study	Number	Method	Sample	α-Diversity	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria
This study UK	32 CD 25 HC child	Illumina MiSeq 16S rRNA V4	faecal	↓	↓Bifidobacterium spp.	↓Bacteroides spp. ↑Bacteroides spp. ↑Prevotella spp. ↑Paraprevotella spp.	↓Defluviitaleaceae ↓vadin BB60 ↓Christensenellaceae ↓Peptococcaceae ↑ Family XI (Gemella) ↑ Veillonella spp.	↑ Fusobacteriaceae	↓Verrucomicrobiaceae ↓Rhodospirillaceae ↑ Enterobacteriaceae ↑ Pasteurellaceae ↑ Neisseriaceae
Ma *et al.* 2018[408] China	15 CD [a] [b] 14 healthy adult	Illumina MiSeq 16S rRNA V4	faecal	↓		decrease not significant			increase not significant
Zhou *et al.* 2018[409] China	72 CD [c] 73 non-IBD [e] adult	Illumina MiSeq 16S rRNA V4	faecal	↓	↑ Intrasporangiaceae	↓ Bacteroidales ↓ Prevotellaceae	↓ Clostridiales ↓ Ruminococcaceae ↓ Lachnospiraceae ↑ Veillonella ↑ Lactobacillales		↑ Enterobacteriaceae ↑ Pseudomonadales ↑ Burkholderiaceae
Kolho *et al.* 2015[410] Finland	68 CD [a] 26 non-IBD [e] (18 JIA) [d] child	Illumina MiSeq 16S rRNA V4	faecal		↓ Bifidobacteriaceae		↓ Clostridiales ↓ Erysipelotrichaceae ↓ Lachnospiraceae ↑ Veillonellaceae ↑ Gemellaceae	↑ Fusobacteriaceae	↑ Enterobacteriaceae ↑ Pasteurellaceae ↑ Neisseriaceae
Quince *et al.*[144] 2015 UK	23 CD 21 HC child	16s rRNA; NGS	faecal	↓	↓Bifidobacterium ↑Atopobium		↓Faecalibacterium spp ↑Eubacterium rectale ↑Ruminococcus obeum ↑Streptococcus spp ↑Peptostreptococcus		↑E.coli/Shigella
Haberman *et al.* 2014[402] RISK cohort USA -focused on ilium	240 CD; 163 non-IBD[e] child	Illumina MiSeq 16S rRNA V4;	tissue (TI)		↓ Bifidobacteriaceae		↓ Clostridiales ↓ Erysipelotrichaceae ↓ Lachnospiraceae ↑ Veillonellaceae ↑ Gemellaceae	↑ Fusobacteriaceae	↑ Enterobacteriaceae ↑ Pasteurellaceae ↑ Neisseriaceae

Studies showing differences in gut bacteria between Crohn's disease and healthy people (continued)

Study	Number	Method	Sample	α-Diversity	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria
Gevers *et al.* 2017[72] and 2014[194] RISK cohort USA	447 CD 221 non-IBD[e] child	Illumina MiSeq 16S rRNA V4	tissue (rectum, TI)	↓	↓ Bifidobacteriaceae	↓ Bacteroidales	↓ Clostridiales ↓ Erysipelotrichales ↑ Veillonellaceae ↑ Gemellaceae	↑ Fusobacteriaceae	↑ Enterobacteriaceae ↑ Pasteurellaceae ↑ Neisseriaceae
Gevers *et al.* 2014[194] RISK cohort USA	33 CD 10 non-IBD [e] child	Illumina HiSeq2000 NGS	faecal	↓		↓ Bacteroidales	↓ Clostridiales ↑ Veillonellaceae		
Wang *et al.* 2016[401] method paper; LOG10 transformed RISK cohort data	(TI) 245 CD 184 non-IBD [e] (stool) 187 CD 311 non-IBD [e] child	Illumina MiSeq 16S rRNA	tissue	↓	↓ Bifidobacterium adolescentis ↓ Micrococcaceae	↓ Bacteroidales ↑ S24-7	↓ Clostridiaceae ↓ Christensenellaceae ↓ Erysipelotrichales ↓ Lachnospiraceae ↓ Turicibacteraceae ↓ Ruminococcaceae ↑ Veillonellaceae ↑ Gemellaceae ↑ Staphylococcaceae ↑ Enterococcaceae	↑ Fusobacteriaceae	↑ Enterobacteriaceae ↑ Pasteurellaceae ↑ Campylobacterales ↑ Neisseriaceae ↑ Rhodocyclales ↑ Burkholderiales ↑ Xanthomonadaceae ↑ Sphingomonadales ↑ order RF32
Lewis *et al.* 2015[73] UK	86 CD 26 HC child	Illumina HiSeq shotgun	faecal	↓	↑ Eggerthella	↓ Prevotella ↓ Odoribacter ↓ Parabacteroides ↓ Alistipes	↓ Eubacterium ↓ Roseburia ↓ Ruminococcus ↑ Veillonella ↑ Enterococcus		↑ Escherichia ↑ Klebsiella
Kaakoush *et al.* 2015[223]	19 CD 18 HC child	454 Pyro V1-V3	faecal	↓		↑ Bacteroidetes	↓ Clostridia ↓ Coprococcus ↓ Roseburia ↓ Ruminococcaceae		↑ Enterobacteriaceae
Kennedy *et al.* 2014[411] UK	40 CD 32 non-IBD [e] adult	Illumina MiSeq 16S rRNA V1-V3	faecal	↓			↓ Ruminococcaceae ↓ Faecalibacteria		↑ Enterobacteriaceae

Studies showing differences in gut bacteria between Crohn's disease and healthy people (continued)

Study	Number	Method	Sample	α-Diversity	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria
Morgan *et al.* 2012[195] PRISM cohort	121 CD 27 non-IBD [e] adult	454 FLX Titanium 16S rRNA V3-V5	tissue; faecal	↓			↑ Clostridium ↓ Roseburia ↓ Ruminococcaceae ↓ Phascolarctobacterium		↑ Enterobacteriaceae
Frank *et al.* 2011[412;189] USA	35 CD 54 non-IBD [e] adult	MegaBACE 1000 SEQ[f]	resected tissue from surgery	not given	↑ Actinobacteria	↓ Bacteroidetes	↓ Lachnospiraceae		↑ Proteobacteria
Andoh *et al.* 2011[400] Japan	31 CD adult	T-RFLP V4	faecal	↓		↑ Bacteroidetes	↓ Clostridium		
Andoh *et al.* 2012[413] Japan	67 CD 121 HC adult	T-RFLP V4	faecal	↓	↓ Bifidobacterium	↑ Bacteroides	↓ Clostridia ↓ Faecalibacterium		
Hansen *et al.* 2012[220] UK	12 CD 12 HC child	RT-PCR & 454 pyro V3-V6	tissue	↓	↓ Coriobacteriaceae		↑ Faecalibacteria prausnitzii		
Willing *et al.* 2010[414]	29 CD 35 HC all twin pairs adults	454 Pyro	faecal	↓			↓ Faecalibacterium ↓ Roseburia ↑ Ruminococcus gnavus		↑ Enterobacteriaceae

Differences were significant to $p<0.01$. **TI** - terminal ileum biopsies; **CD**- Crohn's disease; **non-IBD**- non IBD conditions used as 'healthy' controls; **454 Pyro** -pyrosequencing; **T-RFLP** - Terminal restriction fragment length polymorphism; **V1-V6** - 16S rRNA region which was sequenced.

[a] not treatment naïve

[b] 4 patients were in disease remission

[c] 7 patients were in disease remission

[d] most children in control group had juvenile idiopathic arthritis (JIA)

[e] study used non-IBD patients as 'healthy' controls.

[f] a fixed concentration of DNA was not used therefore PCRs will have been subject to concentration dependent inhibition.

Actinobacteria (Bifidobacteriaceae) A number of studies have recorded reduced abundance of the family Bifidobacteriaceae,[410;402;194] in Crohn's disease patients while others have reported a decrease at genus level (*Bifidobacterium*).[144;413] The current study saw a significant decrease in *Bifidobacterium* at species level. Frank *et al.*[412] reported an increase at phylum level (Actinobacteria), but this has been the only study to report an increase. Their methods were different from other studies, in that they used tissue samples taken from resected surgery; patients were not treatment naïve; and they used MegaBACE 1000 rather than Illumina to sequence their samples. *Bifidobacterium* gut colonisation involves vertical transmission from mother to child, with recent studies showing that specific *Bifidobacterium* strains are shared between mother and child.[415] It is known that strains of *Bifidobacterium*, via production of acetate, can prevent the colonisation of pathogenic *E. coli* by inhibiting Shiga toxin,[416] hence reduced levels of *Bifidobacterium* may open a niche for some genera of Enterobacteriaceae such as *Escherichia-Shigella* to increase in number. Results from mouse models have suggested that exo-polysaccharides produced by a strain of *B. longum* induced IL-10 secretion in macrophages as well as lower levels of TNF-α secretion.[417] An earlier study has also suggested that exo-polysaccharides from a *B. adolescentis* strain can increase cell division activity in murine splenocytes and Peyer's patch cells.[418] Therefore *Bifidobacterium* species play a range of different roles in immunomodulation potentially affecting inflammatory processes in IBD.

Bacteroidetes Although Bacteroidetes have been reported to be increased in three studies looking at Crohn's disease, these studies tended to have small sample sizes, and used older technologies such as 454 sequencing or T-RFLP.[223;400;413] The larger better quality paediatric USA studies which use Illumina sequencing,[194;412] report a decrease in the Bacteroidales. Bacteroidetes have also been reported to be decreased in Chinese adults with Crohn's disease (NS), with the same study finding that the relative abundance of Bacteroidetes showed a negative correlation with Crohn's disease activity index scores.[408;409] Although the increase in Bacteroidetes in the current study was not significant perhaps due to sample size it is clear that our findings agree with the larger studies which suggest a reduction in *Bacteroides* species.

Bacteroides fragilis, a prominent gut commensal, has been shown to protect against intestinal inflammation.[419] Round *et al.* showed that monocolonisation of a germ-free mouse model with *B. fragilis* increases the suppressive ability of T_{reg} to induce anti-inflammatory cytokine production from Foxp3$^+$ T-cells in the gut. *B. fragilis* expresses polysaccharide-A, which aids the conversion of CD4$^+$ T-cells into Foxp3$^+$ T_{reg} cells that produce IL-10 during commensal colonisation. Polysaccharide-A also produces functional Foxp3$^+$ T_{reg} cells during gut inflammation, and toll-like receptor 2 signalling is required for both T_{reg} induction and IL-10 expression. Their study showed that Polysaccharide-A produced by *B. fragilis* was not only able to prevent,

but also cure experimental colitis in mice,[182] which suggests that *B. fragilis* and other related bacteria could have a potentially vital role in regulating inflammation in humans.

Mouse models suggest that low *Bacteroides* abundance is both a risk factor and a potential driver of Crohn's disease.[420] One study has shown that reconstitution of germ-free mice with a diverse microbiota which did not contain the phyla Bacteroidetes failed to restore full immunity, suggesting that some Bacteroidetes species could have the ability to modulate pro- and anti-inflammatory responses in the gut.[420] Thus, uncovering the immunological role of individual *Bacteroides* species, could be an important avenue of research in understanding inflammation in Crohn's disease.

Firmicutes; Clostridiales Studies have shown that in children with Crohn's disease the relative abundance of genera within the class Clostridia: including *Roseburia*; *Faecalibacterium*; members of the family Lachnospiraceae and Ruminococcaceae are decreased in abundance[223;195;401] (Table 5.9). The current study saw significantly reduced abundance in six genera of Ruminococcaceae and five genera of Lachnospiracae as well as *Eubacterium*. The Christensenellaceae were also significantly reduced in children with Crohn's disease (Fig. 5.8).

A recent study has gone on to show that T_{reg} cell-inducing strains of Clostridia species, can be used to attenuate disease in mouse models of colitis and allergic diarrhoea.[183] This suggests that the loss of Clostridia species, a key feature of Crohn's disease in this and other studies, could be a factor in maintaining inflammation. It is therefore possible that the future clinical use of isolated strains might provide a tailored way to reduce inflammation in patients with IBD.

Verrucomicrobia In the current study there was a significant decreased abundance in the family Verrucomicrobiaceae made up from the genus *Akkermansia*, in children with Crohn's disease, a finding also seen by Haberman *et al.*[402] and Lewis *et al.*[73] *Akkermansia muciniphila* is a gram-negative bacterium able to use mucin as a sole source of carbon and nitrogen, a specialisation which allows it to colonise the gut mucosa, making it a key player at the interface between the gut lumen and epithelial cells.[421] Although isolated fairly recently, it has been of significant interest to microbiota research due to reduced levels being observed in patients with IBD[422] and obesity, as well as gut *Akkermansia* abundance been shown to be dramatically reduced in a number of knock-out or diet-induced mouse models that develop obesity, including mice fed a high fat diet.[423;424;425] Researchers restored normal levels of *A. muciniphila* by feeding the same mice live *A. muciniphila* along with a prebiotic diet. In comparison to controls, mice lost weight, had reduced insulin resistance and interestingly a thicker layer of intestinal mucus, which suggests *A. muciniphila* may play an important role in maintaining the mammalian gut mucosal layer which protects

the gut epithelium from damage and potential pathogens. This could have clinical importance as it suggests that a probiotic containing *A. muciniphila* may have the potential to promote mucosal healing in patients with IBD.

5.4.5 Significance of taxa which are increased in Crohn's disease

As seen in the current study the majority of taxa that are increased in Crohn's disease come from the class γ-proteobacteria, many being known as opportunistic pathogens.[226] Studies have also recorded, as seen in this current study, increases in Fusobacteriaceae from the phylum Fusobacteria, and a number of Firmicutes families mainly from the class Negativicutes and Bacilli (Table 5.9).

Firmicutes Although as a phylum Firmicutes are decreased in Crohn's disease, some families within the Firmicutes are often reported to increase, particularly the Veillonellaceae (Negativicutes).[194;402] Veillonellaceae although belonging to the gram-positive Firmicutes phylum, have a peculiar cell wall with a lipopolysaccharide outer membrane which stains gram-negative,[426] which may have been laterally acquired from Proteobacteria.[427] The 2017 study by Mancabelli[395] which combined data sets from three previous studies also recorded a higher relative abundance in *Veillonella* ($p<0.001$). Although the current study did not see a significant overall increase in Veillonellaceae, two children with Crohn's disease did have much higher levels of Veillonellaceae than any healthy children on the study (Appendix 5.1, S012A and S049A).

Other Firmicutes families and orders including Family XI/Gemellaceae (class:Bacilli);[194;402] Lactobacillales; Staphylococcaceae (class: Bacilli)[401;144] and Enterococcaceae (class: Bacilli)[401;73] have also been reported to increase in Crohn's disease (Table 5.9). The current study also saw a significant increase in the abundance of Gemellaceae, which although a normal part of the mucous membranes of the oral cavity and upper gut in healthy humans, they have the ability to become opportunistic pathogens in non-healing wounds.[428] Hence these increases in Gemellaceae and Veillonellaceae may be opportunistic, filling a niche left by decreasing numbers of other Firmicutes. These families have been overlooked in terms of immunological research and future research should investigate whether these bacteria have the potential to increase inflammation in the gut.

Proteobacteria In 2016 Wang *et al.*[401] using data from the 'RISK' study, $\log_{10}$ transformed bacteria abundance and found a number of Proteobacteria families were increased in children with Crohn's disease, which had not been detected by the original 'RISK' study by Gevers *et al.*[194] Both of these studies, in line with the current study, recorded a significant increase in Neisseriaceae in children with Crohn's disease

when compared with healthy children. The genus *Neisseria* is best known for the human pathogens, *N. meningitidis* and *N. gonorrhoeae*. However this genus is an abundant part of the normal commensal oropharyngeal microbiota as well as being successful at finding niches over a number of body sites including the gut. Although commensal *Neisseria* species are less virulent than *N. meningitidis* and *N. gonorrhoeae* they have been shown to be opportunistic pathogens in humans.[429] Hence the family Pasteurellaceae, particularly *Haemophilus*, which are significantly increased in the gut microbiota of children with Crohn's disease in four previous studies, as well as the current study,[410;402;72;401] should be examined further as potential drivers of inflammation in Crohn's disease.

Given that patients with Crohn's disease and UC have an inappropriate response to altered gut microbiota, leading to a protracted immune response and chronic inflammation, it is not surprising that opportunistic species of Proteobacteria are able to thrive in this environment. Strains of the family Enterobacteriaceae have been shown to do well in the presence of inflammation, out-competing the healthy microbiota in a colitis mouse model.[430] A 2004 study of Crohn's disease patients found from ileal tissue, that adherent invasive *E. coli* (AIEC) strains were present in 36.4% ileal mucosa and 22.2% of healthy control mucosa (p=0.034). However in colonic mucosa, AIEC strains were only found in 3.7% of Crohn's disease patients; 0% of UC patients, and 1.9% of control patients, therefore suggesting that AIEC strains are associated with the ileal mucosa of Crohn's disease patients.[431] Proteobacteria such as *E. coli* are incapable of digesting complex polysaccharides, and since beyond the ileocaecal valve most unabsorbed carbohydrates are complex polysaccharides, this could explain why Proteobacteria are more often associated with ileal Crohn's disease. The question that remains to be answered, is whether these *E. coli*, either as individual species/strains or as part of symbiotic relationships with other bacteria, are part of the aetiology of Crohn's disease or simply opportunistic pathogens that thrive in inflammatory conditions in the gut. The finding of this current study that Enterobacteriaceae, Neisseriaceae and Pasteurellaceae were increased in children with Crohn's disease, is in line with the findings of other studies including the 'RISK' study[72] (Table 5.9).

Fusobacteria As seen in other paediatric studies[194;410] (Table 5.9), the current study saw a higher abundance of Fusobacteria in children with Crohn's disease compared with healthy children. Interestingly although Fusobacterium is a known butyrate producer, when its butyrate production pathway composition was compared with commensal Firmicutes such as *Roseburia*, Lachnospiracea species and *Faecalibacterium*, it has been shown that glutarate and lysine pathways were utilised by Fusobacteria rather than the the pyruvate pathway used by commensal Firmicutes associated with a healthy gut. The loss of the pyruvate pathway for butyrate production in Crohn's

disease may play an important role, however the pathways utilised by Fusobacteria releases ammonia as a by product which may also have an negative impact on gut health.[432] It has also been shown that *Fusobacterium* has an increased number of putrefaction pathways which can lead to the production of a number of harmful metabolites in the gut.[276] *Fusobacterium* has also recently been identified as a possible risk factor for colon cancer;[433] while a study looking at oral periodontal infections has suggested that *Fusobacterium* can aggravate periodontal infection via protein fermentation.[434] Therefore increases in Fusobacteria may play a role not only in maintaining gut dysbiosis but also in gut epithelial cell damage. This makes Fusobacteria of particular interest for further study.

Patterns of change The small intestine is rich in nutrients which can be used by both the host and the microbiota to sustain growth. Proteobacteria, mainly from the family the Enterobacteriaceae; and the Firmicutes, Lactobacillales and Erysipelotrichales, are dominant groups within the normal small intestine. Increases in these groups of bacteria appear to be a common feature of Crohn's disease (Table 5.9). A feature highlighted from the 'RISK' study, is that bacteria associated with the ileal mucosa (tissue samples) in healthy children tended to proliferate in the stool of children with Crohn's disease, whereas bacteria prevalent in stool samples of healthy children are decreased in abundance in children with Crohn's disease. Therefore in the microbiota profile of Crohn's patients, it appears that a loss of normal colonic microbiota is being replaced with microbiota more typical of the small intestine. This shift can also be viewed as an overall decrease in gram-positive bacteria being replaced with gram-negative bacteria.

Although it is true that 16S rRNA and metagenomic sequencing provide a detailed view of the gut microbiota, turning this data into meaningful clinical information has been difficult.[435] It has been challenging to separate larger scale functional effects due to taxa differences at phylum and order level, with smaller scale differences at genus or species level, which could also have direct effects on epithelial cells in the gut. Given that the gut microbiota has a huge variation in abundance between different species, where species have low abundance, especially in smaller scale studies these bacteria may not generate enough statistical power for us to identify differences which may be important in Crohn's disease. Although, where larger cohorts are available, studies such as Wang *et al.*[401] have shown that the phylogenetic trees of bacteria associated with Crohn's disease and those associated with healthy individuals do not overlap and therefore have distinct lineages. The current study backs up this finding (Fig. 5.8), however the large inter-patient variability in IBD makes it impossible to fit individuals to a clear model of IBD at this stage.

Drivers of behavioural changes in bacteria Different taxa within the gut microbiota when found together can enhance inflammatory effects. A rat model for intra-abdominal sepsis has shown that obligate anaerobes such as *Bacteroides fragilis* or *Fusobacterium varium* when combined with facultative aerobes like *E. coli* or the Bacilli *Enterococcus faecalis* result in peritonitis and abscess development[436] where neither alone lead to abscess formation. Mice infected with a combination of *E. coli* and *B. fragilis* in the peritoneal cavity saw an increase in TNF-α production in the peritoneal tissues which was not seen when infected by these bacterial species independently.[437] This is important because these are genera we see increased in Crohn's disease, thus further work identifying the behaviour of specific strains from Crohn's patients, in mixed communities could shed light on what is driving inflammation.

This group also showed when mice were co-infected with *E. coli*, expression of cytokine-KC increased; however when co-infected with a *Lactobacillus* species, cytokine-KC mRNA expression decreased. It is therefore possible that increased Proteobacteria along with reduction of *Lactobacillus* species could potentially alter the behaviour of *Bacteroides* species in such a way as to drive inflammation. Further studies have also shown that a *B. fragilis* by-product can impair the ability of host neutrophils to kill *E.coli* by phagocytosis[438;439] and suppress the *E. coli* associated LPS-induced cell adhesiveness for neutrophils in the gut.[439] Therefore understanding the changing behaviour of dominant commensal bacteria in the presence of other bacterial groups could be key to understanding inflammation in the gut in Crohn's disease.

5.4.6 Gut microbiota profile as a predictor of Crohn's disease

Like the current study, the larger scale studies that have focused on treatment naïve children, which have less confounding factors than adults,[194] have confirmed the findings of other studies that dysbiosis is present not only in well established Crohn's disease, but also at disease onset. Given that these recent studies identify Crohn's disease as having decreased abundance of the phyla Firmicutes and Bacteroidetes, along with an increase in abundance of Proteobacteria, the obvious question is, whether these differences in microbiota community structure can be used to diagnose IBD. We also need to know if the microbiota profile from faecal samples can have the same predictive power as tissue biopsy samples.

The idea that we can use the gut microbiota community structure to identify patients with IBD is based on the concept that either a loss or gain in specific bacteria is responsible for IBD, and this may not be the case. The results of the current study show that the local contribution to β-diversity is significantly higher in children with Crohn's disease and UC showing that their gut bacteria profile tends to be highly dissimilar from other children with the same condition. This might suggest that the risk factors or causative agents that drive inflammation could be unique to individuals

or sub-groups of IBD patients, making it very challenging to use community structure as a marker for IBD. It is possible that large scale future research will be able to focus in and identify a discriminatory group of bacteria, or specific bacterial genes associated with inflammation in the gut. However if inflammation associated with Crohn's disease and IBD is due to changes in bacterial behaviour, rather than presence or absence of bacterial groups, this will make clinical disease profiling from bacteria impossible at present.

Although at baseline tissue samples appear to be more indicative of specific dysbiosis, faecal samples are picking up the same pattern of reduced Firmicutes, Bacteroidales and increased Proteobacteria (Table 5.9). In contrast to the Gevers *et al.*[194] study, Wang *et al.* found that both faecal and ileal tissue samples had equal predictive power, while Papa *et al.*,[440] a study using adults with established Crohn's, found that stool samples were more predictive than ileal tissue samples. However, we need to be cautious when comparing biopsies with stool samples, as tissue samples can contain mucus in relatively greater amounts than found in faecal samples, possibly accounting for differences in some studies, since mucus is known to inhibit PCR reactions. Not amplifying enough bacterial DNA from tissue samples could lead to a sampling bias which may not be a true representation of mucosal bacteria. Another issue is that studies often use different DNA extraction methods between faecal and tissue samples to try and maximize DNA yield from biopsies, which could also account for differences in results.[304] Faecal samples are a non-invasive way to assess the gut microbiota, thus hold the key to diagnosing IBD, if they can be shown to have predictive power. Papa *et al.*[440] showed a predictive power of AUC 0.83 from faecal samples, however much of their cohort were not treatment naïve thus, treatment and perhaps prolonged inflammation, may affect the reliability of their results. Gevers *et al.*[194], a study which did use treatment naïve children, concluded that faecal samples were a poor predictor of IBD (AUC 0.66). Therefore more work needs to be done to identify specific bacterial profiles which will have much greater predictive power.

5.4.7 Limitations in study designs and data

Non-IBD controls One potential issue with cross comparing IBD studies is that the so-called 'healthy' controls used for many studies, especially those using tissue biopsy samples, are actually non-IBD patients[441;195;409] with a range of other conditions including inflammatory conditions such as juvenile idiopathic arthritis.[410] The current study clearly shows that as a collective, children in the non-IBD patient group also have dysbiosis and reduced diversity, and therefore do not represent the typical healthy gut microbiota of truly healthy children. As such it is not correct, as many studies do, to refer to this patient control group as 'healthy children'.

Sample bias and size The biggest issue with most studies investigating the role of the gut microbiota in IBD, including this one, is that they are unable to obtain high enough numbers of patients, and thus can become insufficiently powered to link differences in gut microbiota with variables such as disease severity, disease location and response to treatment. Like other studies, participation in this study was optional, and it is possible that children who were the most unwell, were the same children who declined to take part. For ethical reasons we could not look at the records of those who declined to take part, and hence cannot be sure if children who participated, fully represent, for disease activity or disease location, all children in the West of Scotland with IBD. More robust data could be gathered if small studies using the same methods were linked together over a large number of sites to remove sampling bias.

Different methods lead to differing results A 2014 study by Kennedy *et al.*[441] demonstrated the significant effect on DNA yield and bacterial DNA composition when comparing DNA extracted from a faecal sample using different DNA extraction kits. Thus researchers need to be cautious when cross-comparing studies that use different DNA extraction methods. Another issue is sequencing method. The large Japanese study by Andoh *et al.* 2012[413] used Terminal restriction fragment length polymorphism (T-RFLP) analysis. Although this method does not have the high resolution of using a 16S rRNA clone library,[442] it can be useful for assessing a large number of samples as it allows faster comparison of gut bacteria profiles between samples.[443] However we can see that this method did not detected as many taxa differences as seen in smaller 16S rRNA studies, including the current study (Table 5.9), making it less useful as a research tool. The Andoh *et al.*[413] study did however have an excellent approach to collecting control samples, as they selected individuals from five different regional districts of Japan (matched with the five IBD centres), to test for the presence of regional differences due to environmental or dietary differences, and concluded that any differences in gut microbiota profile between districts was negligible.

Having used region V4 primers (515F-806R) to amplify the 16S rRNA in the current study, recent studies have shown that V4 can result in an under representation of the phylum Actinobacteria, including *Bifidobacterium*;[444] hence it is possible that along with other studies, we may not have detected a true representation of *Bifidobacterium* species due to our choice of PCR primer region. Choosing primers can be very challenging as getting the balance right between maximising the detection of bacterial groups and comparing results with other studies are important considerations, and future studies may begin to combine a number of primers aimed at different 16S regions to obtain a truer representation of taxa.

Age affects gut bacteria The gut microbiota research community may need to be cautious when comparing adult studies with paediatric studies because ageing has been associated with continual changes in the gut microbiota, including a gradual decrease in *Bifidobacterium*.[195;445;446] It is possible that adult Crohn's disease studies do not detect the same differences in *Bifidobacterium* seen in paediatric studies, due to children having a higher abundance of this bacterial group (Table 5.9). It would be useful to carry out adult and paediatric studies together in the same analysis for comparison, to avoid assumptions about differences which might be due to methods or researcher based bias. However, this type of combined study can be difficult, due to service provision for children and adults being located in different hospitals, and research sometimes funded from different sources.

Dysbiosis is not unique to IBD By studying the gut microbiota at baseline in this new-onset paediatric population, we have shown along with other studies,[194] that faecal dysbiosis is present prior to treatment. However, data from other studies into non-IBD conditions, would suggest that dysbiotic states reported for Crohn's disease are not unique, and occur in a number of intestinal diseases and other inflammatory conditions. It may be that some aspects of dysbiosis such as reduced Clostridia is a risk factor for a wide range of conditions. Comparing the gut microbiota in children with Crohn's disease with those of healthy children does not identify features unique to Crohn's disease, or ascertain if dysbiosis is the consequence of inflammation as opposed to causing the disease. To achieve these goals, groups studying various inflammatory conditions such as rheumatoid arthritis, psoriasis, Type-1 diabetes, and other gastrointestinal conditions such as IBS and coeliac disease need to come together, and using the same metagenomic methods, link studies to see how dysbiosis varies between these conditions. This would help us to understand which bacteria might be driving inflammatory conditions and which bacterial changes are a result of inflammation, as well as leading to the identification of unique bio-markers which differentiate Crohn's disease from other dysbiotic diseases, including UC.

Other microbial members The term 'microbiota' should include single celled eukaryotes, fungi and viruses all of which are important functional components of the gut micro-environment. However, the majority of microbiota studies have focused on the bacterial community, and future research will need to unravel the interaction between all of these players, as other gut inhabitants such as bacteriophages, could be driving a reduction in commensals or reducing diversity. Interestingly as a commensal species, *Clostridium* bacteriophage diversity[447] is also increased in Crohn's disease patients with increased bacteriophages being higher in non-ulcerated mucosal samples.[448] Understanding bacterial homoeostasis in the gut may be dependant on uncovering the role that bacteriophages and other members of the gut microbiota play in altering the normal healthy bacterial composition.

This and other studies show that although there is no single species associated with Crohn's disease there are some clear potential culprits; mainly increased abundance of Enterobacteriaceae, Pasteurellacaea, Veillonellaceae and Fusobacteriaceae, accompanied with a decreased abundance of commensal Firmicutes, especially from the class Clostridia.

5.4.8 Microbiota changes during EEN

The majority of the studies looking at the impact of treatment with EEN on gut microbiota have been on children, with many having small sample sizes which limit our ability to draw real conclusions from datasets. It has also been difficult to draw comparisons across studies due to the high level of inter-individual variation, as well as having to compare results from different sample types (faecal and tissue biopsies). However, overall, these studies see a decrease in bacterial diversity during treatment with EEN.[223;145;144;216] Although one small study of four children reported an increase in diversity during treatment with EEN, this was only seen in one child who failed to go into remission on EEN.[449] It is worth pointing out that although most children have a reduction of species richness on EEN, around 1/3 of children on the current study had an increase in diversity during EEN, with these children having a gut bacterial profile distinct from children who decreased species richness. Children who had increased richness during EEN tended to have higher levels of Firmicutes especially Lachnospiraceae species such as *Dorea* and Ruminococcaceae species, than children who had reduced species richness. This difference at baseline did not predict either response to treatment with EEN or time to relapse once children went onto normal diet. Hence it cannot be suggested that these bacterial differences which are associated with an increase or decrease in species richness during EEN, are linked with reduced inflammation and healing.

Changes in bacterial richness (α-diversity) during EEN

In the current study there was a no decrease in the number of species (richness) and in evenness of diversity in children after 4-weeks and 8-weeks on EEN. It should be noted however that species richness increased in some children while decreasing in other children independent of clinical response to EEN. This is curious given that these children are on identical diets. This would explain why small studies have different findings when reporting diversity during EEN. A small study[450] of only five patients reported decreased diversity, as did a later larger study.[145;144] Another study, again with a small sample size of only eight adult patients, saw no difference in diversity during EEN.[219] Dunn *et al.*[227] followed nine children with Crohn's disease who went into remission after 12-weeks of EEN, for a further 12-weeks on normal diet and found that in five patients who maintained remission α-diversity (Chao-1) had increased

between baseline and 12-weeks EEN, whereas in the four patients who relapsed at 24-weeks there had been a decrease in diversity during EEN. It is surprising that if later relapse is really associated with reduced diversity during EEN, that Dunn *et al.*[227] did not find or report the same pattern in diversity between responders and non-responders to EEN. The current study did not find any relationship between decreased diversity during EEN and time to relapse; and was able to show that the children who increased in species richness during EEN had distinct differences in gut microbiota at baseline which discriminated from those children who decreased in species richness during EEN (Fig. 5.19). Although two children who failed EEN were among the group that increased species richness during EEN, and four patients who failed EEN where in the group who decreased in species richness during EEN, it is possible that with larger sample sizes, a correlation between change in species richness and response to EEN may emerge.

Further research with larger sample sizes, perhaps including patient genomics, and environmental data, will need to be done to establish what other factors affect whether species will decrease or increase during treatment with EEN. Evidence shows a link between a number of diseases and reduced bacterial diversity in the gut. Therefore it is important for future studies to determine, where diversity is further reduced by treatment with EEN, if long-term damage could be occurring in a similar way that antibiotics have been implicated to harm normal gut homoeostasis. On a reassuring note, studies that have reported decreased diversity during EEN, have also reported a return to pretreatment diversity levels once children are back onto their normal habitual diet.[145] Although the current study also saw a returned increase in diversity once children returned back onto normal diet, the species composition was often altered, and in some children normal healthy commensals such as *Bacteroides* were lost. Longer term monitoring of gut bacteria post-EEN, would elucidate whether this type of commensal bacteria loss is permanent or indeed harmful to those individuals affected.

Table 5.10: Studies showing evidence for changes of microbiota during exclusive enteral nutrition (EEN)

Study	Subjects	Samples	EEN	Method	Results
Ashton *et al.* 2017[449]	4 CD (3 on EEN) 1 UC 1 IBD-U (11-15yr)	baseline EEN wk 2,6	6-wks EEN polymeric	16S rRNA V4 Illumina MiSeq	Diversity increased during EEN moving towards healthy diversity at remission (NS).
Dunn *et al.* 2016[227]	9 CD (10-17yr) 5 HC (9-14yr)[a]	faecal: baseline EEN wk 12	12-wks (formula not given)	16S rRNA V6-V8 NGS	Responders saw increase in genera: *Dorea*, *Parabacteroides* & *Akkermansia* while non-responders saw increases in: *Prevotella*, *Sutterella* & *Klebsiella*
Schwerd *et al.* 2016[224]	8 CD	faecal: baseline EEN wk 2, & 6-8	EEN polymeric	16S rRNA V3-V4 Illumina MiSeq NGS	EEN decreased relative abundance of Bacteroidetes from family: Bacteroidaceae; Porphyromonadaceae; Rikenellaceae ($p<0.05$); and increase in Firmicutes (Clostridiales): Ruminococcaceae & Christensenellaceae ($p<0.05$)
Kaakoush *et al.* 2015[223]	5 CD (yrs) 5 HC	faecal: baseline EEN wk 8	90% EEN polymeric	MiSeq 16S rRNA V1-V3 & NGS HiSeq	decreased diversity; 6 families of Firmicutes found to correlate with disease activity. (NS).
Lewis *et al.* 2015[73]	90 CD (10-15yr) 52 anti-TNF 21 EEN 26 HC (7-19yr)	faecal: baseline EEN wk 1, 4, & 8	90% EEN (formula not given)	NGS Illumina HiSeq	Microbiota composition changed within 1-week of EEN, moving farther from HC ($p=0.05$). Abundance of 6 genera changed after 1-week (NS). HC data taken from a previous study.
Quince *et al.* 2015[144]	23 active CD (7-15yr) 21 HC (4-16yr)	faecal: baseline EEN wk 2, 4, 8 post-EEN wk 4, 16	8-wks EEN polymeric	MiSeq 16S rRNA & NGS HiSeq	Decrease in species seen after 15-days EEN ($p=0.037$). During EEN, diversity in children with CD decreased and community profile moved further away form HC than at baseline. For every 10-days on EEN, was a loss of 0.6 genus diversity. 34 genera decreased during EEN (including *F. prausnitzii*); only *Lactococcus* increased with EEN.
Gerasimidis *et al.* 2014[145]	15 CD (Med. 13yr) 11 treatment naive 21 HC (Med. 10yr)	faecal: baseline EEN wk 2, 4, 8 post-EEN wk 4, 16	8-wks EEN polymeric	MiSeq 16S rRNA & RT-qPCR	After EEN, bacterial diversity & abundance decreased ($p=0.037$); returned to normal on free diet ($p=0.041$). During EEN, conc. of *F. prausnitzii* ($p=0.002$) & *Bifidobacterium* decreased ($p=0.053$); re-increased once on normal diet for *Faecalibacterium* ($p=0.006$) and *Bacteroides/Prevotella* (NS), but still lower than HC.
Guinet-Charpentier *et al.* 2016[222]	4 CD EEN 12 CD non-EEN (Med. 6-21yr)	faecal: baseline EEN wk 2 & 6	8-wks EEN polymeric	MiSeq 16S rRNA NGS	Decreased *Escherichia/Shigella*, *Sutterella* (Proteobacteria); Increased *Alistipes*. Low sample size.

Studies showing evidence for changes of microbiota during EEN (continued)

Study	Subjects	Samples	EEN	Method	Results
Shiga *et al.* 2012[219]	8 CD 17 HC (15-47yr)	faecal; baseline EEN wk 4 HC 6-wks apart (n=12).	8-wks EEN elemental formula (n=8)	T-RFLP analysis of 16S-rRNA. RT-qPCR.	No change in diversity. Ratio of *Bacteroides fragilis* (p=0.03) reduced by EEN .
Jia *et al.* 2010[218]	20 CD, 21 IBS 14 UC, 18 HC (adults)	faecal: baseline EEN wk 2	2-wks EEN elemental formula (E028) Extra	PCR of *F. prausnitzii* DNA (A2-165 and M21/2 subgroup)	In CD 2 strains of *F. prausnitzii* (A2-165 & M21/2) decreased after 2-wks EEN ($p<0.05$ & $p=0.61$). Both *F. prausnitzii* strains in CD were significantly lower than other patient groups before and after 2-wks EEN. Hence EEN did not appear to restore healthy levels of *F. prausnitzii*.
Leach *et al.* 2008[216]	5 CD (2-13yr) 7 HC (2-12yr)	faecal; baseline, EEN wk 1, 2, 4, 6, 8 post-EEN wk 16, 26 HC 8-wks apart	8-wks EEN (formula not given)	PCR 16S rRNA gene followed by DGGE	CD had greater degree of change in bacterial composition during EEN compared to controls on normal diet ($p<0.05$). Reduction seen in *Eubacteria*, *Bacteroides–Prevotella* and *Clostridium* ($p<0.05$). Study only looking at a few selected species.
Lionetti *et al.* 2005[215]	9 CD (9-17yr) 5 HC (10-15yr)	faecal during EEN	8-wks EEN	TGGE analysis of 16S rRNA V6-V8	EEN characterised by changes in banding patterns in all CD patients; HC stable over time. Only show evidence for 4 patients and 1 healthy child; fail to provide data or analysis of bands.

CD - Crohn's disease; HC - healthy controls; UC - Ulcerative colitis; NGS - next-generation sequencing; T-RFLP - Terminal restriction fragment length polymorphism; TGGE - temperature gradient gel electrophoresis; DGGE - denaturing gel electrophoresis

[a] healthy controls were siblings of Crohn's patients

Changes in bacterial abundance (β-diversity) during EEN

In the current study, although the number of species reduced on EEN, there were many species which significantly increased in abundance during EEN. This is interesting since we hypothesised that Modulen, a liquid feed with no fibre, which is mostly absorbed in the small intestine, might lead to a reduction in abundance for most species. One reason we might see this increased abundance, is if these species are resident in the small intestine where they are able to take advantage of the low residue feed, while colonic bacteria reduce in number and abundance as the feed is mostly absorbed by the time it reaches the colon.

As seen in previous studies,[73;144;145] the current study saw a marked shift in community structure (β-diversity) after 4-weeks of EEN which continued to shift further away from healthy controls by 8-weeks EEN. When children with Crohn's disease were split into those who responded or failed EEN, the current study saw a significant change in diversity for children who responded to EEN not seen in non-responders (Fig. 5.13). Although the small sample size for non-responders makes it impossible to draw any firm conclusion from this, the data for both number of species (α-diversity) and abundance (β-diversity) suggest that non-responders to treatment were less likely to have reduced diversity or a change in abundance during EEN. It cannot be easily ruled out that children who fail EEN perhaps struggle to fully comply with the liquid diet regime, thus explaining why we see less change in bacterial diversity and abundance in these children.

5.4.9 Changes in bacteria during EEN

If bacterial richness and overall abundance is reducing during EEN, it is important to know which groups of commensal bacteria are reducing in order to understand how these changes might lead to reduced inflammation. It is also important, particularly in children, to know if any potentially pathogenic bacteria which are normally of low abundance in the gut are increasing to fill a niche left by commensals during EEN.

The current study found after 4-weeks of EEN, children saw a decreased abundance in gram-negative bacteria from Proteobacteria and Fusobacteria. Veillonellaceae (class: Negativicutes), notable for having a cell wall similar to gram-negative bacteria, were also decreased after treatment with EEN. However two families of gram-negative bacteria were also increased during EEN, the Enterobacteriaceae and Verrucomicrobiaceae. All other bacteria which increased in abundance during EEN were from the Firmicutes. It is difficult to pick out why some bacteria are increasing in abundance while others are reducing since we might expect Firmicutes to be decreasing due to the loss of fibre in the liquid feed diet, however this is not the case for all species.

Changes in Firmicutes It had been hypothesised that *Faecalibacterium prausnitzii* might play a role in mucosal healing during EEN. However the current study inline with others saw a significant reduction in the relative abundance of *Faecalibacterium* during EEN (p=0.02). Another study focusing on two strains of *F. prausnitzii*, showed the low levels recorded at baseline were not restored after 2-weeks of treatment with EEN.[218] Gerasimidis *et al.*[145] also showed that *F. prausnitzii* was reduced along with *Bifidobacterium* during EEN (p<0.01) and was therefore unlikely to play a role in reducing inflammation and mucosal healing seen during treatment on EEN. An Australian study[223] which focused on six families of Firmicutes (Erysipelotrichaceae, Ruminococcaceae, Lachnospiraceae, Streptococcaceae, Veillonellaceae and Peptostreptococcaceae) found an overall reduction in relative abundance of these families correlated with clinical improvement; however there was a great deal of variation between study subjects. These studies suggest that reduced abundance of predominant healthy commensals, including *F. prausnitzii*, during EEN could be linked with reduced inflammation, supporting the theory that Crohn's disease is caused by an inappropriate response to normal commensal bacteria.

Changes in Bifidobacteriaceae The current study, in line with four previous studies,[145;144;216;219] found Bifidobacteriaceae were reduced during EEN in both children who responded to EEN and those who failed EEN. *Bifidobacterium* are healthy commensals with anti-inflammatory properties, which are normally depleted in active Crohn's disease.[194] However like *F. prausnitzii* and other Firmicutes it is reducing during EEN in patients who have reduced inflammation and go into remission, suggesting it does not have a direct role in healing.

Changes in Bacteroidetes Specific Bacteroidetes do not seem to follow a clear pattern of change during EEN as seen across different studies. Dunn *et al.*[227] report an increase in *Parabacteroides* during EEN, while Schwerd *et al.*[224] report a decrease in abundance of three Bacteroidetes families: Bacteroidaceae; Porphyromonadaceae and Rikenellaceae. Shiga *et al.*[219] reported that *Bacteroides fragilis* was reduced by EEN (p=0.03). Leach *et al.*[216] reported that the Bacteroides-Prevotella group were reduced in six children during EEN, which they linked with clinical improvement, while Gerasimidis *et al.*[145] reported the same decrease in fifteen children who had clinical improvement. In the current study family level decreases were seen during EEN in Bacteroidales S24-7 and Prevotellaceae. Overall, studies appear to be reporting a decrease in different Bacteroidetes families during EEN.

Bacteroides, early colonisers of the gut, play a positive role in regulation of the immune system.[451;452] However a recent study has suggested a role for a strain of *Bacteroides vulgatus* in the development of IBD via activation of the NF-κB signalling pathway.[453] Another study investigating *Bacteroides* species found some act

as colitis-promoting species while others are colitis-protective in murine models.[454] If composition of *Bacteroides* species are able to shape the balance between immune regulatory T_{reg} cells and pro-inflammatory T-cells, an imbalance in *Bacteroides* species could result in a loss of immunological homeostasis. Therefore, given that the phylum Bacteroidetes contains a huge number of species, it is likely some will be vital in immune regulation while other species and strains could be drivers of inflammation. Further detailed research into Bacteroidetes species and others known to be involved in immune regulation, could help to reveal how management of these bacteria might be used to control inflammation in a clinical setting.

Given that Bacteroidetes are the largest phylum of gram-negative bacteria, which as part of the normal healthy microbiota are considered major players in maintaining homoeostasis in the gut, it is perhaps also concerning that EEN appears to be reducing the abundance of these important commensals in this and other studies.

Changes in Proteobacteria Given that studies looking at gut microbial diversity have consistently shown an expansion and increase of Proteobacteria species in patients with IBD,[226] it might be assumed that treatment with EEN works by reducing this group of bacteria, particularly Enterobacteria. However studies seem to show the converse is true. Dunn *et al.*[227] reported in nine children who responded to EEN that in the five children who then relapsed within 12-weeks, an increase in the Proteobacteria family Enterobacteriaceae during EEN. The current study also found an increase in Enterobacteriaceae and other gram-negative families in both patients who responded to EEN and those who failed to respond during EEN. The larger study by Quince *et al.*[144] also reported a increase in the genera *Escherichia/Shigella* for all children on EEN, while a small study of only four children on EEN reported a decrease in *Escherichia/Shigella* and *Sutterella*.[222] An Australian study[223] clearly illustrated how Proteobacteria were increasing in some patients during EEN while in others abundance fell. It is likely that low sample size along with high inter-patient variation leads to opposing results, but perhaps the key point is that changes in Proteobacteria in individual children during EEN are not consistent.

Although gram-negative Enterobacteriaceae such as *E.coli* are a normal part of the healthy gut, many are opportunistic pathogens which are held in relatively low abundance in the healthy gut. If treatment with EEN is increasing Enterobacteriaceae species in some individuals, this may not be an issue for most children, since levels appear to reduce once children are back onto normal diet. However some children may have more virulent strains of Enterobacteriaceae species, which during EEN are able to gain a previously unavailable advantage. The increase of pathogenic species could in turn lead to increased inflammation and explain why some children fail to respond to treatment on EEN.

There is a normal oxygen gradient throughout the colon which affects gut microbiota composition. A greater abundance of Proteobacteria is found at the mucosal surface due to greater environmental ratios of oxygen.[455] Epithelial inflammation which causes cell damage and bleeding in active Crohn's disease is likely to provide higher ratios of luminal oxygen, thus favouring members of the Proteobacteria, particularly the Enterobacteriaceae. It is known that some Enterobacteriaceae species can utilise inflammatory compounds as terminal electron acceptors, further promoting their growth and giving them an edge under inflammatory conditions.[456] Understanding functional pathways such as nitrate reductase activity, the genomes of which are more commonly found in Enterobacteriaceae, may in future lead to better dietary treatments including EEN which are adapted to keep Enterobacteriaceae abundance in check by interfering with the availability of nitrate.

Community structure Although we see changes in bacterial abundance during EEN there is no overall pattern of change common to all children. Since all children are taking the same diet during EEN, it would appear the niches created by this liquid feed which contains no fibre, are being filled by different genera in each child, perhaps dependent on which bacteria are present at baseline, environmental factors and host genetics. Given that 1:5 children fail treatment with EEN, and dietary treatment in itself appears not to determine which bacterial genera will increase or decrease, makes it difficult to predict who will respond to treatment.

The large study into microbiota changes during EEN by Quince *et al.*,[144] reported a decrease in relative abundance of thirty-four genera coming from a range of different phyla, which is in line with the current study which found a decreased abundance in twenty-one bacterial genera. As yet, we have no understanding of the long term outcomes for children with Crohn's disease using diet (EEN) as a medical treatment to drive major bacterial changes in their gut. It is important that future studies start to track the long term progress of children who take EEN to better understand if bacterial changes that happen during EEN, have an a positive or negative impact on disease progression into adulthood.

5.4.10 Predicting response to EEN

Given that studies have associated active Crohn's disease with decreased abundance of Firmicutes and Bacteroidetes and increased Proteobacteria, what can the baseline gut microbiota structure tell us about which patients will respond well to treatment and which might need more aggressive therapies? Although studies have tried to link the microbiota with treatment outcomes there is poor evidence from these studies that microbiota profiling either before or during EEN could be used to accurately identify which patients are more likely to fail EEN. Studies have also been unable to

reliably link the reduction of specific groups or species of bacteria during EEN with a reduction in inflammation and mucosal healing.

Dunn *et al.*,[227] using ten children with Crohn's disease on EEN and five healthy children, compared the microbiota profile between five patients that maintained remission for 6-months after EEN, and four patients who did not achieve or maintain remission. They reported that during EEN, Proteobacteria increased further in those who did not maintain remission. They also found that the genus *Bacteroides* (including *B. fragilis* and *B. ovatus*), was one of the most predominant species associated with sustained remission, however *Bacteroides* (including *B. plebeius*) were also associated with failure to sustain remission.[227] The Dunn study is however seriously flawed, as only 4/10 patients relapsed and two of these children had been on antibiotics prior to the 12-week sample collection time-point. Treatment with antibiotics therefore will have contributed to results and it was misleading of the authors to claim that what they measured was due to treatment with EEN.

One issue with most studies investigating the gut microbiota is that they are insufficiently powered to link the microbiota with disease severity and treatment over time. This makes it difficult to identify any relationships between bacteria and specific disease traits such as disease location or severity and treatment outcomes. The 'RISK' study,[194] which included 447 Crohn's disease patients and 221 non-IBD patients tried to identify multiple factors (genetics, microbiota and immunology) in children that could help predict response. This study focused on biopsies (terminal ileum and rectum) with a subset of children who gave faecal samples. They did not however collect dietary data which could have helped assess the relationship between diet and gut microbiota and Crohn's disease. Follow-up biopsies were also not available, making it impossible to asses changes in gut bacteria during treatment. They concluded from this study that the gut microbiota profile at baseline did not play a role in disease progression. However another study by Shaw *et al.*[457] did concluded that it might be possible to predict response to treatment with 76.5% accuracy (AUC=0.75).

For ethical reasons repeat colonoscopy in order to understand serial changes of mucosal bacteria and mucosal healing in children with Crohn's disease is not possible, and this lack of knowledge about pre/post treatment, impacts our understanding of the mechanisms that underlie the success of EEN to induce remission. Therefore studies are reliant on faecal samples to try and understand changes in gut microbiota over time. Although faecal samples may not be fully representative of the mucosal microbiota, they may be able to tell us enough about global gut microbiota changes to act as a reliable marker of changing gut mucosa health.[194]

In summary, current understanding has shown that during treatment with EEN, bacterial diversity is further decreased and the community structure moves further from that of healthy children. Also the abundance of key Firmicutes species such as *F. prausnitzii* are reduced. Therefore previous suggestions that EEN worked by

improving the functionality of the gut by restoring a more healthy gut microbiota, paradoxically seems not to be true. It might be that a reduction in availability of bacterial substrate, either as a whole, or by targeting specific members of the bacterial community in the gut lumen, which is driving a reduction in inflammation. Studies to date including this current study have failed to provide a meaningful link between the gut community structure at baseline and response to treatment on EEN, and given the huge amount of variability in inter-patient community structure in patients with Crohn's disease, a feature of the disease, it may not be possible to predict response from baseline gut microbiota alone. However as we develop more complex models of analysis, a combination of data from microbiota structure, host genetic risk, and gut bacteria metabolites may not only allow us to predict response to EEN, but also to tailor treatment with prebiotics and probiotics to individual patient needs. Gaining a better understanding of the mechanisms of how EEN works will lead to improved outcomes for children with Crohn's disease and it may become possible to extend the benefits to include maintenance of remission.

5.4.11 Microbiota changes associated with MEN

Remission induced by EEN is not maintained, with 60-70% of children relapsing within 1-year,[4] and although drug therapy to maintain remission is effective, it is not risk free and can have side effects. As such, finding a dietary solution is extremely attractive as a safe alternative to maintain remission over the long term. Current evidence suggests that maintenance enteral nutrition (MEN) might be as effective as some medication in maintaining remission,[356] which would make it an ideal therapy option, particularity for growing children. Therefore after completion of EEN, when the normal habitual diet is slowly re-introduced, enteral nutrition has been trialled as a supplement to try and maintain remission for longer periods of time[154] as well as improving linear growth,[146] with mixed results.

Given that treatment with EEN has been shown to alter the gut microbiota, the follow-on question was to look at whether the gut microbiota profile would return to pre-treatment levels once children revert back onto their normal diet post-EEN. Studies such as that by Gerasimidis *et al.*[145] show that the gut microbiota does return to pretreatment levels within 2-weeks post EEN. The next logical step was therefore to explore whether the benefit of EEN could be extended post EEN, and as such studies have attempted to follow changes in the gut microbiota in patients with Crohn's disease, while using MEN, to try and establish if microbial changes during EEN can be maintained. A study on nine children with active Crohn's disease, at end of treatment with EEN, had children return to normal diet with a supplement of 40% of daily energy intake from a polymeric formula.[215] Using 16S-rRNA PCR and TGGE, changes in bacterial composition seen after treatment on EEN, was maintained for several months once children went back onto normal diet. However a major drawback

Table 5.11: Studies showing the effect of maintenance enteral nutrition (MEN) on gut microbiota.

Study	subjects	samples	MEN	Method	Results
Lionetti *et al.* 2005[215]	9 CD (9-17yrs) 5 HC (10-15yrs)	faecal; during MEN	polymeric MEN (40%)	TGGE analysis of 16S rRNA	They only show evidence for 4 patients and 1 healthy child. They also failed to provide any data or analysis of bands and hence their results were very subjective.
Leach *et al.* 2008[216]	6 CD (2-13yrs) 7 HC (2-12yrs)	faecal: baseline 8, 18wks post-EEN. HC 2-samples 8-wks apart	formula not given	PCR of 16S rRNA followed by DGGE	CD children had a greater degree of change in the bacterial composition during EEN compared to controls on a normal diet ($p<0.05$). The greatest change was seen in Ruminococcaceae ($p<0.001$) and the least in the Bacteroides-Prevotella group ($p<0.01$). Study was underpowered.
Kaakoush *et al.* 2015[223]	1 CD MEN (13yrs) 4 CD non-MEN (7-10yrs)	4, 8, 12wks post-EEN	polymeric MEN (Osmolite) amount unknown	454 sequencing	All children were on different maintenance therapy. Thus unable to make proper comparisons.

CD - Crohn's disease; **HC** - healthy controls; **NGS** - next-generation sequencing; **T-RFLP** - Terminal restriction fragment length polymorphism; **TGGE** - temperature gradient gel electrophoresis; **DGGE** - denaturating gel electrophoresis **Modulen** - Polymeric enriched with TGF-β2

was that this study did not include a non-MEN control group and thus it is impossible to tell if MEN had any effect that would not have also been seen in children who were returning to normal diet without MEN supplementation.

A small 16S-rRNA study using DGGE with six Crohn's disease children who had completed EEN, followed children for a subsequent 4-months of MEN.[216] It was interesting however that in the five children who continued on MEN, although there was a partial return to the bacterial profile found at the start of treatment (40% similarity), the gut bacteria composition was still to some degree altered at 4-months post-treatment with EEN. The study suggested that *C. leptum* stability after EEN, was associated with a reduction in gut inflammation and disease activity.[216] Although five children went onto MEN, two began medication on aminosalicylates while two started azathioprine, hence we cannot be sure whether medication use contributed to these results. This study was also limited in that it only looked at five species of bacteria using PCR: *Eubacteria*; *Bifidobacteria*; *Bacteroides-Prevotella*; *C. coccoides* and *C. leptum*.

The Kaakoush *et al.* study[223] included one child taking MEN, and reported that this child had a large drop in the abundance of Erysipelotrichaceae, Ruminococcaceae, and Lachnospiraceae which correlated with both treatment on EEN and improvement in disease activity. This was accompanied by a large increase in the abundance of Veillonellaceae once back onto normal diet plus MEN, which corresponded with a mild exacerbation of symptoms at 12-weeks therapy. However given that the five children on this study were given a different type of maintenance therapy it is impossible to draw any meaningful conclusion from their results (Table 5.11).

The current study also saw an increase in Negativicutes from the family Veillonellaceae such as *Veillonella*; *Dialister*; *Anaeroglobus* and *Selenomonas* particularly in children who took MEN once back onto normal diet (Fig 5.25). Given that children with Crohn's disease tend to have higher abundance of Negativicutes at baseline compared with healthy children, it is possible they play a role in inflammation. A study looking at mucosal biopsies from patients with Ankylosing Spondylitis[458] found that degree of inflammation was positively correlated with abundance of the genus *Dialister* (Spearman's rho=0.62; q<0.01) in each individual, and was further supported by a significantly lower abundance of *Dialister* in non-inflamed ileal and colonic biopsies from both patients and healthy controls. Therefore the pro-inflammatory potential of bacteria from the Negativicutes needs to be investigated as possible drivers of inflammation post-EEN.

The Firmicutes, *Ruminococcus*, genera from Lachnospiraceae and *Roseburia*, were also among the bacteria which increased in abundance, suggesting that levels of this genera which were reduced during EEN were being restored once children were back onto normal diet. This is not surprising as these bacteria are associated with the fermentation of plant based complex carbohydrates found in the normal diet, which are lacking in enteral nutrition.

Many of the bacteria that significantly reduced in abundance once children went back onto normal diet were gram-negative bacteria: Enterobacteriaceae such as *Citrobacter*, *Cronobacter*, *Enterobacter* and *Pantoea*; as well as other Proteobacteria such as *Sutterella*, *Campylobacter* and *Eikenella*. The Actinobacteria *Varibaculum* which stains gram-negative, was also decreased on normal diet in children who took an immunosuppressant (Fig 5.25). Many of the Enterobacteriaceae species are associated with the fermentation of sugars in the small intestine. It is not surprising therefore that the EEN feed containing glucose syrup, casein and sucrose leads to an increase in Enterobacteriaceae species during EEN, which then reduce once the diet is stopped. Many studies have suggested that adhesive invasive *E. coli* (AIEC) may be directly involved in the aetiology of Crohn's disease,[459] however if this were true it is difficult to explain why an increase in Enterobacteriaceae during EEN leads to a reduction in inflammation rather than increasing symptoms.

A study by Lewis *et al.*[73] evaluating changes in gut microbiota from faecal samples taken at 1-week, 4-weeks and 8-weeks EEN, also gave a group of children partial enteral nutrition which made up 53% of the child's daily intake. When they compared the gut bacteria profile in children treated with EEN, with those children treated with partial enteral nutrition the same pattern of change was not seen (p=0.83), hence they concluded that either changes to bacterial profile seen during EEN are dose dependant, or that only total removal of normal diet altered the microbiota composition during treatment. Setting up research studies to answer these questions would not be easy to achieve as large groups of patients would need to take various

different doses of enteral nutrition to try and establish if EEN and MEN are dose dependant. However if 50% partial enteral nutrition fails to have an impact on gut microbiota composition, as Lewis *et al.* suggest,[73] it might imply that MEN, which is usually around 20% of daily calorific intake, is unlikely to alter the gut microbiota enough to effectively sustain remission. However trails comparing the use of polymeric formula (Modulen; 50g twice/day) with the aminosalicylate anti-inflammatory drug 5-ASA to maintain remission, suggest that MEN may be as good as 5-ASA. 42% (18/43) of patients using MEN relapsed at 6-months compared with 55% (22/40) of patient who took mesalamine as maintenance therapy (RR 0.76; 95% CI0.49-1.19).[460] A study by Hani *et al.* also looked at an elemental feed used as MEN compared with 6-MP and found 38% (12/32) of MEN patients relapsed by 12-months compared with 23% (7/30) of patients who took 6-MP as maintenance therapy (RR 1.61; 95% CI0.73-3.53).[152] A recent Cochrane review[356] has concluded that studies to date have not been able to show any firm evidence about the efficacy and safety of enteral nutrition in quiescent Crohn's disease. Therefore more work needs to be done to establish the efficacy of different maintenance therapies so that children with Crohn's disease have the best chance to maintain disease remission in the longer term.

Bacterial changes in individuals returning to normal diet In a sub-group of eight children on this current study who went on to an immunosuppressant; MEN; or both, we can see that once children go back onto their normal diet (Fig. 5.26) the gut bacteria are distinct, not only from treatment with EEN, but also from their baseline gut bacterial profile. Given that *Bacteroides* is considered a major part of the normal healthy gut microbiota, it raises questions about the long term benefits of using EEN therapy if important bacterial species are lost from the gut microbial system as seen in patient S016 (Fig. 5.26). This was matched with an increase in *Escherichia/Shigella* during EEN, and still present at 2-weeks and 8-weeks of normal diet. *Escherichia/Shigella* was not detected at baseline in this patient, suggesting that the shift in *Escherichia/Shigella* could be due to dietary treatment. However in contrast to this, another patient (S018) who had *Escherichia/Shigella* at baseline, had reduced abundance during EEN, with this genus not being detected once they returned to normal diet.

In another child (S023) we see a notable change in gut microbiota profile both during EEN and then again during the maintenance phase, with increased *Cronobacter* (Enterobacteriaceae) and Fusobacteriaceae during EEN, which was reduced during normal diet. Abundance of *Akkermansia* became high once the child went onto normal diet but had not been detected at baseline or during EEN. It is not clear if these changes are part of normal variation within gut community structure, or if diet therapy is driving changes in the bacteria profile which could have either beneficial or deleterious consequences for the health of the gut. Careful questions need to be

raised, as it appears that treatment with EEN, and possibly both dietary and pharmaceutical maintenance therapies may have medium to long term changes in the gut microbiota, which we cannot determine at this stage as beneficial to individual patients. There is an assumption that because EEN is not a drug, that its use to induce remission in children with Crohn's disease is safe, but this has yet to be fully established from long-term patient outcomes.

Based on previous years, the current study calculated that around 55% of children once they had completed 8-weeks EEN, would refuse MEN as maintenance therapy. However out of the twenty-three children who went into remission on EEN only six (26%) children opted not to take MEN and two of these children failed to provide faecal samples during maintenance therapy leaving the study with a control group of only four patients. It is possible that by introducing the nature of the study to patients, subsequent knowledge about gut microbiota influenced their decision to take MEN as a supplement post-EEN. Human observational studies are limited as they cannot determine treatment and hence can lack good quality controls. Using an animal model would allow the use of clear non-treatment controls to see the effect of both enteral nutrition and immunosuppressants on the resident gut bacteria. The use of such controls in humans would be unethical, making it almost impossible to design MEN studies which eliminate confounding factors such as habitual diet and immunosuppressant drug therapy.

Another issue with the current study was that Crohn's children with mild disease at diagnosis were more likely to be given MEN only as maintenance therapy, where as children with severe disease at diagnosis were more likely to be given combined MEN and immunosuppressant therapy (Table 5.12). Therefore it could be argued that observed differences between groups in both outcome and microbiota changes could be due to disease severity rather than treatment group.

Table 5.12: Number of patients on each maintenance therapy showing disease severity at diagnosis

	disease activity		
treatment group	mild	moderate	severe
MEN only	5	3	1
IM only	4	1	1
MEN and IM	1	2	5

Disease severity was measured at baseline using the weighted paediatric Crohn's disease activity index (wPCDAI) (Methods section 2.4.3).

5.4.12 Summary of key findings

The current study:

- Supports previous studies which show reduced microbiota diversity in children with Crohn's disease and UC, compared with healthy children.
- Supports previous findings that commensals are decreased in abundance while gram-negative potential pathobionts are increased in children with Crohn's disease.
- Supports a recent finding that, despite reducing inflammation, EEN pushes dysbiosis further from that of healthy children.

New findings from this study:

- Although at baseline twenty-five species (SNVs) were distinct between responders and non-responders to EEN, no pattern in species type was seen; suggesting it is not possible to predict response to EEN from the microbiota profile.
- Show that MEN does not sustain microbiota profiles achieved during EEN.

6 Changes in short chain fatty acids in children with Crohn's disease during treatment with enteral nutrition

6.1 Introduction

Data produced by the Human Microbiome Consortium has shown that despite a huge deal of variation and diversity in gut microbiota profiles between different individuals, the metabolic profile which includes short chain fatty acids (SCFA) is much more constant among groups of individuals.[234] This is because biochemical pathways are consistent across different bacterial species, even up to the level of phyla.[235] SCFA as a product of bacterial metabolism, are also an additional source of energy to host cells. Thus dietary components which the host is unable to digest directly in the small intestine are converted by bacteria in the colon to SCFA where around 90% of these are absorbed by the host.[236]

SCFA are carboxylic acids with between one and six carbon atoms with attached functional groups such as hydroxyl or dicarboxyl. In the human gut, they arise from bacterial fermentation of carbohydrate, peptides, protein or glycoprotein precursors,[232] and are important sources of fuel since they produce large quantities of ATP when metabolised. The groups of bacteria which typically ferment fibres in the colon to produce SCFA, have been shown to be reduced in abundance in both the mucosa and faeces of patients with IBD in comparison to healthy controls. This feature of IBD is important because the SCFA, particularly acetate, propionate and butyrate, have been shown to be involved in maintaining intestinal homoeostasis.

It is important to note that individuals with IBD as well as having reduced abundance of dominant butyrate producing bacteria like *Faecalibacterium prausnitzii* and *Roseburia intestinalis*, have been shown to have reduced levels of SCFA, especially butyrate, detected in faeces compared to healthy individuals.[225;461;462;463]

6.1.1 SCFA and gut barrier integrity

Bacteria-derived butyrate has been shown to improve gut epithelial barrier function via epithelial oxygen consumption leading to stabilisation of hypoxia-inducible factor-1 (HIF-1), a transcription factor which regulates gut barrier integrity.[464] In both antibiotic treated and germ-free mice, HIF-1α expression and butyrate levels were reduced, but could be restored with butyrate supplementation.[464]

Table 6.1: Studies showing evidence for differences in SCFA between Crohn's disease and healthy controls

Study	subjects	samples	Method	Results
De Preter *et al.* 2014[465]	40 HC; 83 CD; 68 UC Adults	faecal	GC-MS	The levels of MCFAs: valerate (C5), hexanoate (C6), heptanoate (C7), octanoate (C8) and nonanoate (C9) were significantly decreased in patients with CD, UC compared with HC. Hexanoate levels were inversely correlated to disease activity in CD (correlation coefficient=-0.157, p=0.046).
Machiels *et al.* 2013[466] Belgium	87 HC; 127 UC Adults	Faecal, expressed as dry weight.	GC-MS	SCFA were reduced in UC patients (p=0.014), but no direct correlation between SCFA and the identified bacteria was found.
Walton *et al.* 2013[467] UK	22 CD; 20 UC; 19 HC	faecal	headspace sampling; GC-MS	Median acetate lower in CD than healthy controls. Propionate and butyrate higher in CD. (NS) This study is flawed as many patients had zero propionate and butyrate suggesting the group had technical issues with either extraction or detection of SCFA.
Midtvedt et al. 2013[468] Sweden	4 CD; 5 HC Adults	faecal	GC	Decreased propionate and increased butyrate in CD. Mean Hexanoate (C6) was reduced in CD (0.9) compared with HC (2.6)
Huda-Faujan et al. 2010[469] Malyasia	50 HC and 8 IBD (UC = 6, CD = 2); (2/6 with active disease) Adults	faecal	HPLC	Levels of acetate, butyrate, and propionate were decreased in IBD when compared with HC. Authors noted that % of butyrate was higher than propionate in all participants and suggested this was due to the high rice starch content of the Malaysian diet.
Gerasimidis 2009[470] UK	20 CD (11 treatment naïve); 20 HC Children	faecal (wet sample calculated)	Ether extracts: GC	No significant difference in acetate, propionate and butyrate between CD and HC. Conc. of valerate, hexanoate and octanoate were significantly reduced in CD; % of valerate and octanoate were also reduced in CD.
Takaishi *et al.* 2008[471]	10 HC; 39 UC; 12 CD Adults	faecal (50% had inactive disease)	HPLC	Conc. of butyrate and propionate were significantly decreased in IBD patients compared with HC. Acetate in IBD was not significantly lower than in HC but acetate in CD lower than in UC.
Van Neunen *et al.*[472] 2004	6 HC; 8 IBD (5CD; 3UC) Adults	faecal	*in vitro* colonic model	*in vitro* SCFA & BCFA production higher in IBD samples than in HC samples
Vernia *et al.* 1988[473]	16 HC; 18 UC; 20 CD Adults	faecal	GC	In UC, concentrations of each SCFA, especially butyrate, were decreased compared with HC and CD. All CD patients had disease restricted to the colon. They saw a negative correlation between the severity of UC and butyrate concentration.

Studies showing evidence for differences in SCFA between Crohn's disease and healthy controls (continued)

Study	Subjects	Samples	Method	Results
Hove & Mortensen 1995[474]	70 HC*;103 UC;127 CD Adults	faecal	steam distillated; (GC)	No difference in total SCFA between IBD and HC samples. Acetate was reduced in both UC and CD compared with HC P <0.05). In contrast to other studies SCFA (including butyrate) did not correlate with disease activity or location for either UC or CD. NOTE: HC were non-IBD patients with gastrointestinal symptoms.
Treem et al. 1994[475]	12 HC; 17 UC; 22 CD Children	CD with ileocolonic involvement	vacuum-distillation; GC	Patients with UC and CD had a decrease in the concentration of acetate (p<0.05) and an increase in butyrate (p <0.01) compared with HC. Faecal SCFA concentrations correlated with disease activity.
Stein et al. 1992[476]	10 HC; 8 CD Adults	faecal	acid vacuum transfer; HPLC	Acetate, propionate and butyrate were all reduced in patients with CD (NS). The reduction in total SCFA in patients with CD was however significant compared with HC.

CD - Crohn's disease; HC - healthy controls; UC - Ulcerative colitis; MCFA - medium chain fatty acid
GC - gas chromatography; MS - mass spectrometry; HPLC - high performance liquid chromatography;
* non-IBD patient controls

Butyrate is know to improve gut barrier function via activation of transcription factors such as STAT3 and SP1 which control tight-junctions. It has been shown butyrate can increase trans-epithelial electrical resistance in human cell models[253;254;255;256] and murine small intestine cells.[240] Butyrate has also been shown to improve trans-epithelial electrical resistance in piglet small intestine cells when exposed to inflammatory conditions,[477] showing it can mitigate the ability of bacterial lipopolysaccharide (LPS) to damage epithelial integrity. This effect has also been shown *in vitro* using a supplement of butyrate producing bacteria added to the microbiota of a Crohn's disease patient, to enhance gut epithelial barrier integrity.[478] These studies support the idea that butyrate has the potential to restore intestinal barrier function in IBD, where gut epithelial healing is a key therapeutic target.

6.1.2 The role of SCFA in intestinal immunoregulation

Butyrate has been associated with beneficial anti-inflammatory effects on epithelial cells,[479] with a 2007 study linking the down-regulation of the butyrate transporter with inflammation in the colonic mucosa of IBD patients.[480] In addition, butyrate-producing bacteria were shown to be decreased in colonic gut mucosa and in faecal samples, of patients with IBD compared to control patients.[189] This might suggest that butyrate insufficiency, could play a role in the pathogenesis of IBD. Butyrate enemas, either given alone, or with a mix of other SCFA, have decreased colonic inflammation in patients with IBD.[262;481] However, the underlying mechanisms behind this improvement are not understood. In addition studies have linked butyrate to gut microbiota-mediated induction of functional T_{reg} cells in the gut mucosa, providing a potential mechanism of how metabolites produced by colonic bacterial fermentation in the gut could bring about gut immune homoeostasis via host-microbiota crosstalk. Bacterial cross-feeding is the foundation of most butyrate production in the colon, thus variation in dietary substrate availability to gut bacteria may be driving changes to microbiota and consequent metabolic composition, which could in turn play a role in gut inflammation.

It is known that SCFA exert anti-inflammatory effects in the gut mucosa via histone deacetylases inhibition.[267;268] The discovery that SCFA act as natural ligands for receptors across a wide range of cell types has led to the current interest in SCFA as signalling molecules between the gut microbiota and host immunoregulation, but these have yet to be demonstrated *in vivo*.

6.1.3 SCFA levels in IBD and Crohn's disease

A 2008 Japanese study[471] reported that the concentration of butyrate and propionate were significantly decreased in twelve adult patients with Crohn's disease compared to ten healthy controls (Table 6.1). In 2010 a small Malaysian study of only eight

patients with IBD (2 Crohn's; 6 UC) also reported significantly less butyrate and propionate than seen in fifty healthy controls ($p<0.05$).[469] Another study by Vernia *et al.* found that in forty-eight adults with IBD, concentrations of all SCFA, especially butyrate, were decreased but only in UC when compared with healthy controls and those with Crohn's disease. It should be noted that 8/20 Crohn's patients were on corticosteroids and the cohort were not treatment naive, which may account for differences between Crohn's disease and UC in this study.[473] It has also been reported that in those Crohn's disease patients who have ileal disease, butyrate producing Ruminococcaceae, particularly the genus *Faecalibacterium* were reduced compared to patients without ileal involvement.[482] Decreases in propionate and butyrate are supported by metagenomic and metaproteomic studies which have confirmed a decrease in butyrate and propionate metabolism genes in ileal Crohn's disease[195] along with a measured reduction in butyrate and other SCFA.[483]

6.1.4 Changes in SCFA during exclusive enteral nutrition (EEN)

Two studies have recorded faecal SCFA in children with Crohn's disease undergoing treatment with EEN. Tjellstrom *et al.* 2012[129] used the pattern of SCFA to explore the functional status of the gut microbiota by collecting samples from eighteen children with Crohn's disease, before and after 6-weeks EEN. The pattern of SCFA was used to create two indices: index A represented the fermentation of carbohydrates (acetate minus butyrate plus propionate as a proportion of total SCFA); and index B represented fermentation of proteins (iso-butyrate plus iso-valerate). Therefore they suggest index A reflects the pro-inflammatory properties and index B anti-inflammatory properties of bacterial fermentation via SCFA.[484] Tjellstrom *et al.* reported a significant decrease in pro-inflammatory index A after treatment with EEN, as well as a rise in anti-inflammatory index B, suggesting this might explain the anti-inflammatory effect of EEN.[129] However, the use of these indices is an oversimplification of fermentation in the gut, and may not be an accurate model of pro-/anti-inflammatory conditions in the colon. A potential factor is that children with serve disease who are feeling unwell may have an altered dietary intake at baseline, thus differences in SCFA between Crohn's disease children and healthy controls, as well as before and after treatment with EEN, may be a product of dietary differences not directly linked with inflammation. It should also be noted that although Tjellstrom *et al.* reported median data for a total of eighteen children, only eight gave paired samples at the start and end of EEN. Given the high interquartile ranges of their SCFA results, their findings are flawed.

In 2014 Gerasimidis *et al.*[485] showed, in twelve children with Crohn's disease that the concentration and ratio of butyrate decreased after 4-weeks and 8-weeks of EEN; with median concentration of butyrate falling to 50% pre-treatment levels ($p<0.01$). No significant decrease was seen in either acetate or propionate during EEN. The decrease

in butyrate returned to pre-treatment levels once children went back on their normal habitual diet as measured at 8-weeks post EEN. This group also reported that an increase in the inflammatory marker, faecal calprotectin, was positively linked with the concentration of propionate and total SCFA (Spearman rho=0.58; p=0.039), once children went back onto their normal habitual diet. Faecal pH as a marker of reduced bacterial fermentation capacity increased from a median 7.1 to 7.8 ($p<0.001$) after treatment with EEN.[486]

Whelan *et al.* has also shown that EEN negatively reduced concentrations of total SCFA and butyrate; as well as concentration of *F. prausnitzii* in non-IBD patient controls.[487] A follow up study in 2016 on seventeen adult patients with Crohn's disease saw a significant reduction in the production of toxic microbial metabolites, as well as SCFA, after 2-weeks of EEN.[488] This leads on to the hypothesis that it is the reduction of any potentially toxic metabolites after EEN, rather than SCFA which makes the diet effective at reducing inflammation in Crohn's disease patients.

6.1.5 Changes in SCFA post-EEN

The only study to look at changes in short chain fatty acids post-EEN is interesting because, both the concentration and the proportion of faecal butyrate at 15-days post-EEN was higher in seven children with Crohn's disease who failed to achieve clinical remission. This group of children also did not achieve the same reductions in short chain fatty acids during EEN as those children who did achieve disease remission.[489] The total SCFA for all children on EEN was significantly increased post-EEN (15-days normal diet) back towards pre-treatment levels.[489] As yet no studies have looked at the effect of maintenance enteral nutrition (MEN) on the production of SCFA in the gut.

6.1.6 Hypotheses

Given that differences are seen in gut bacteria both at baseline and during treatment with EEN in children with Crohn's disease, the current chapter will go on to hypothesise that:

- The pattern of SCFA as a product of fermentation in the gut will be distinct in children with Crohn's disease and UC compared with healthy children and non-IBD controls.
- Along with reduced numbers of Firmicutes species, butyrate will be reduced in children with IBD.
- Due to lack of fibre, and a reduction in bacterial commensal species, total SCFA production in children with Crohn's disease during treatment with exclusive enteral nutrition (EEN) will be reduced.
- The loss of bacterial commensal species, which contain many butyrate producers will result in a disproportionate loss of butyrate during EEN.
- Treatment with maintenance enteral nutrition (MEN) will maintain SCFA profiles achieved on EEN.

The chapter will go on to discuss the role that SCFA may play in driving or reducing inflammation in the gut, and how this might help researchers to design more robust experiments, which could in turn lead to better long term treatments for children with Crohn's disease.

6.2 Methods

There is no practical method to measure the precise amount of SCFA produced by gut bacteria in the colon of humans. SCFA measured from faecal samples are the result of both bacterial production and host absorption in the colon, and therefore do not accurately measure true amounts produced. They can however act as a proxy for SCFA production in the gut.

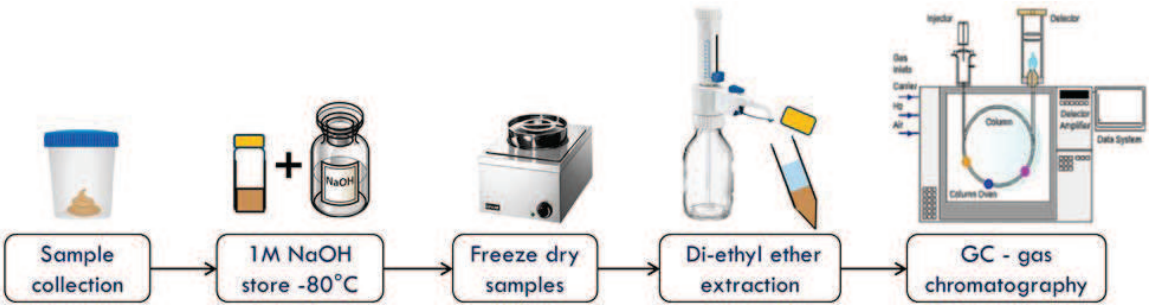


Figure 6.1: Methods used to process faecal samples for SCFA analysis. Results were expresses as $\mu mol/g$ of wet faecal sample or as a ratio of total fatty acid concentrations.

A summary of methods is shown in figure 6.1, with detailed methods being described in Methods section 2.7. In brief faecal samples were collected as described in Methods section 2.4.4 with SCFA being stabilised for storage using sodium hydroxide (NaOH) and immediately stored at -80°C. Samples were then freeze dried (Edwards apparatus Micro Modulyo, Thermo Scientific®) for 36-hours. Water content was determined by weighing samples before and after freeze drying.

To extract SCFA, 100mg of freeze dried faecal sample was weighed out into corning tubes with 300µL of distilled water. 100µL of orthophosphoric acid and 100µL of 2-ethyl butyric acid (internal standard) was added. Di-ethyl ether was added to the tube and shaken for one minute. The upper ether phase was removed to a separate tube and the process repeated three times. Extracted supernatant was transferred to glass vials and sealed with silicone seal crimp tops, before being analysed using gas-chromatography. All samples were extracted and analysed in duplicate; where duplicates did not agree the extraction and analysis was repeated. Results for Heptanoate (C7) were not included in the analysis because concentrations were too low to be reliably detected using this method. The concentration of SCFA was expressed as $\mu mol/g$ of wet faecal sample, calculated from the percentage of water in individual samples, and as a percentage of Total SCFA (C2-C8).

6.3 Results

6.3.1 SCFA differences between patient groups and healthy children

At baseline children with Crohn's disease and UC had a lower median concentration of total SCFA than both non-IBD patients and healthy children (Table 6.2). However as all groups of children had a very large IQR, differences were not close to significance. No differences were seen between groups for the SCFA acetate (C2), propionate (C3) and butyrate (C4). The reduced concentration and ratio of valerate (C5), seen in children with Crohn's disease and UC at baseline was significant when compared with healthy children. The concentration and ratio of the medium chain fatty acids (MCFA), hexanoate (C6) and octanoate (C8), were also significantly lower in children with Crohn's disease compared with healthy children. Children with UC also had less hexanoate and octanoate than healthy children but this did not reach significance for hexanoate. No difference was seen between any of the patient groups and healthy children for the branch-chain fatty acids, iso-butyrate (iC4) and iso-valerate (iC5) (Table 6.2).

The SCFA/MCFA profile of healthy children and those with Crohn's disease was visualised using a metric multidimensional scaling (MDS) plot (Fig. 6.2). It shows that the SCFA/MCFA profile of children with Crohn's disease, was dissimilar to that seen in healthy children (R2=0.07; p=0.011).

Table 6.2: Median (IQR) short chain fatty acids (SCFA) from the faecal samples of patient groups and healthy children

Fatty acid	carbons	Crohn's n=29	UC n=7	non-IBD n=8	Healthy n=19	KW
Amount ($\mu mol/g$)						
Acetate	C2	58.4 (43.7)	54.6 (52.3)	92.9 (52.2)	69.0 (35.8)	p=0.33
Propionate	C3	14.1 (13.6)	13.4 (12.8)	17.4 (14.0)	14.8 (8.9)	p=0.82
Butyrate	C4	8.6 (11.2)	7.3 (6.2)	14.3 (12.8)	8.6 (8.8)	p=0.77
Valerate	C5	*0.9 (2.1)	*0.2 (1.0)	1.9 (1.4)	2.0 (1.7)	**p=0.02**
Iso-butyrate	C4	2.3 (2.1)	2.2 (1.5)	2.0 (1.7)	3.0 (1.9)	p=0.37
Iso-valerate	C5	2.9 (2.6)	2.7 (0.9)	2.8 (2.4)	3.8 (3.0)	p=0.31
MCFA						
Hexanoate	C6	***0.1 (0.1)	†0.1 (0.3)	0.2 (0.3)	0.6 (0.9)	**p<0.001**
Octanoate	C8	***0.2 (0.3)	***0.0 (0.2)	0.4 (0.3)	0.5 (0.4)	**p<0.001**
Total		88.2 (72.9)	82.1 (78.3)	140.6 (87.6)	109.7 (63.3)	p=0.41
Ratio						
% Acetate	C2	67.2 (12.6)	68.3 (4.6)	69.5 (7.3)	68.23 (6.9)	p=0.89
% Propionate	C3	15.3 (10.4)	14.4 (8.0)	14.15 (6.6)	13.7 (5.2)	p=0.65
% Butyrate	C4	9.7 (4.5)	9.8 (2.4)	9.8 (1.6)	8.6 (3.1)	p=0.75
% Valerate	C5	*1.1 (1.6)	*0.6 (1.1)	1.8 (0.7)	1.8 (1.0)	**p=0.02**
% Iso-butyrate	iC4	2.2 (2.4)	2.4 (1.6)	2.0 (0.8)	2.3 (1.4)	p=0.65
% Iso-valerate	iC5	2.8 (2.7)	3.1 (2.6)	2.6 (1.2)	3.6 (2.4)	p=0.52
% Hexanoate	C6	***0.1 (0.1)	† 0.1 (0.1)	0.1 (0.3)	0.6 (0.6)	**p<0.001**
% Octanoate	C8	***0.2 (0.2)	**0.1 (0.2)	0.4 (0.5)	0.5 (0.6)	**p<0.001**

Amount is expressed per g of wet faecal sample. KW- Kruskal-Wallis test (Dunn's test of multiple comparisons was used following a significant Kruskal-Wallis test). Difference from healthy children is significant at * p≤0.05; ** p≤0.01; *** p≤0.001; † p=0.07

6.3.2 Linking SCFA at baseline with gut bacteria

A Kendall rank correlation (Methods 2.10.3) showed a non-significant relationship between the number of bacteria and the ratio of faecal SCFA, mostly from the Firmicutes phylum (Fig. 6.3). Only five genera were from the Proteobacteria and only two of these (*Aggregatibacter* and *Thalassospira*) reaching statistical significance in children with Crohn's disease.

The correlation of acetate, propionate, and butyrate with a number of Firmicutes genera, was different between healthy children and children with Crohn's disease, but was not significant. For some genera the association of valerate (C5) and octanoate (C8) was different between healthy children and children with Crohn's disease (NS) but these differences were across a number of different groups of bacteria and did not show any obvious pattern. There was however a significant difference between healthy children and children with Crohn's disease for hexanoate (C6) suggesting that children with Crohn's disease have a significant positive correlation across a number of genera, mostly Firmicutes, which was not seen in healthy children (Fig. 6.3). However this positive correlation is associated with a significant reduction in the ratio of Hexanoate (Table 6.2) as well as a reduced abundance of these genera in children with Crohn's disease when compared with healthy children.

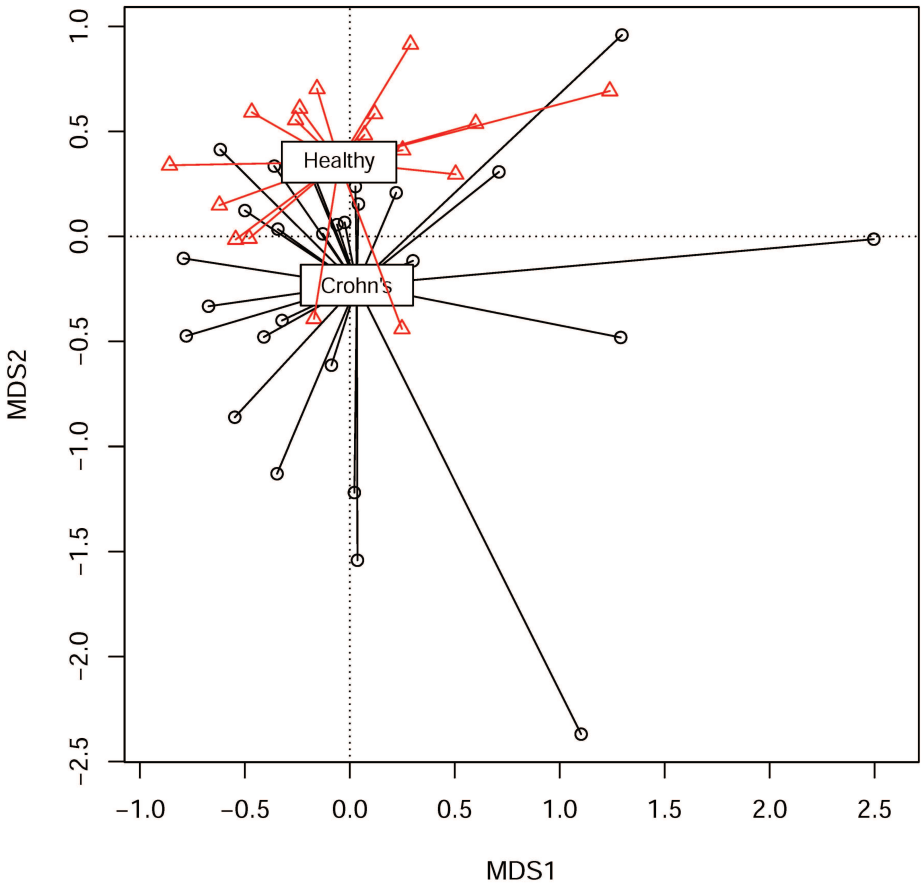


Figure 6.2: Difference in faecal SCFA profiles between children with Crohn's disease (black) and healthy children (red) as shown by metric multidimensional scaling (MDS) spider plot. PERMANOVA: R2=0.07; p=0.011. MDS polygon plot is shown in appendix 6

Clear non-significant differences could be seen in the ratio of iso-butyrate (iC4) and iso-valerate (iC5) between healthy children and children with Crohn's disease (Fig. 6.3). A lot of these differences were in gram negative Proteobacteria such as *Aggregatibacter*, *Thalassospira* and *Escherichia*; the gram negative *Fusobacterium*; the gram-negative Bacteroidetes genera *Prevotella-7* and *Paraprevotella*; as well as *Acidaminococcus* from the class Negativicutes. These bacteria which showed a negative non-significant correlation with the ratio of iC4 and iC5 in healthy children, show a non-significant positive correlation in the group of children with Crohn's disease.

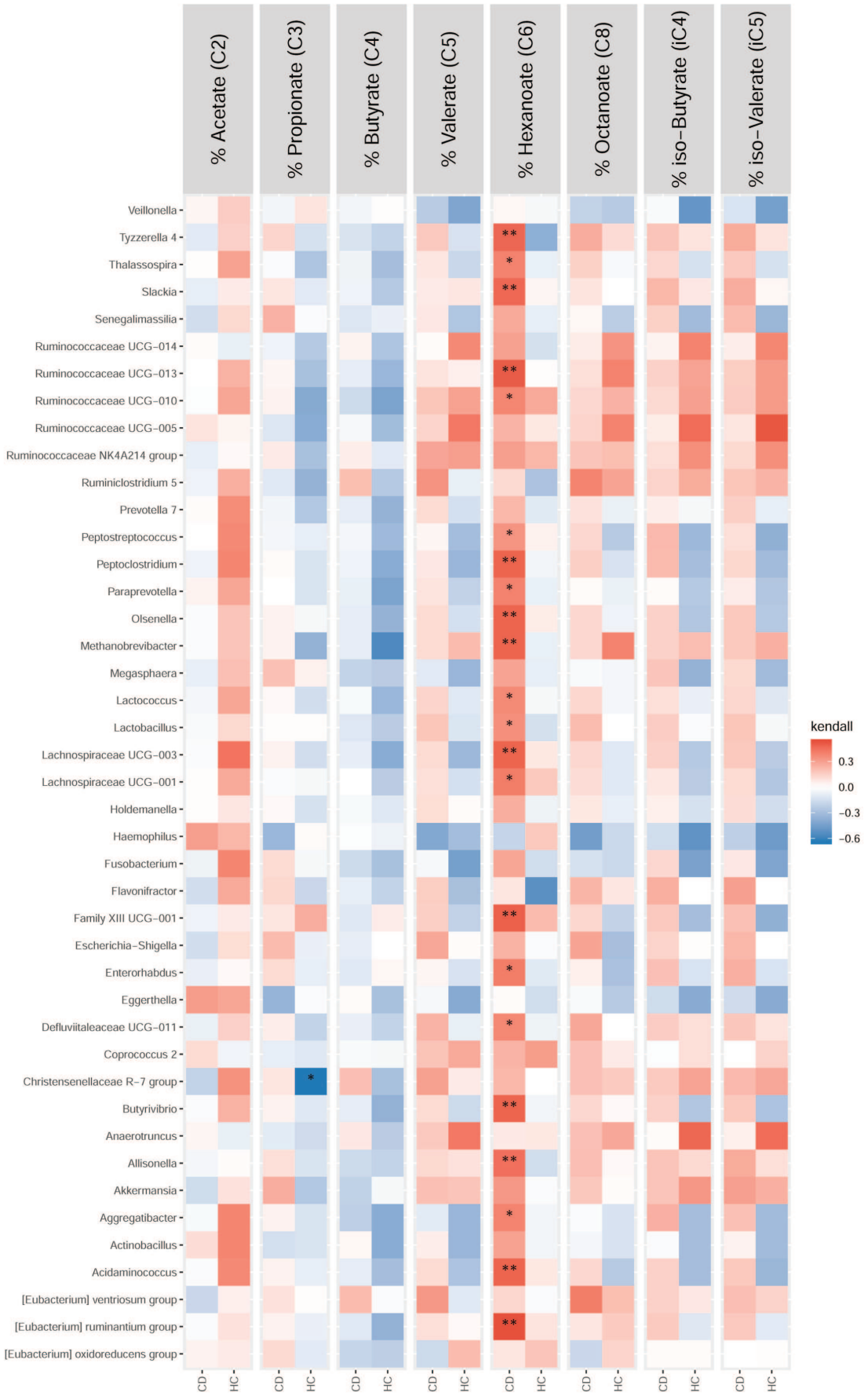


Figure 6.3: Correlation between the ratio of faecal SFCA and gut microbiota genera in children with Crohn's disease (CD) and healthy children (HC) at baseline. Kendall rank correlation significant at * $p \leq 0.05$; ** $p \leq 0.01$ (Multiple comparison - Benjamini-Hochberg method). The positive correlation for Hexanoate is associated with a significant reduction in Hexanoate in children with Crohn's disease when compared to healthy children.

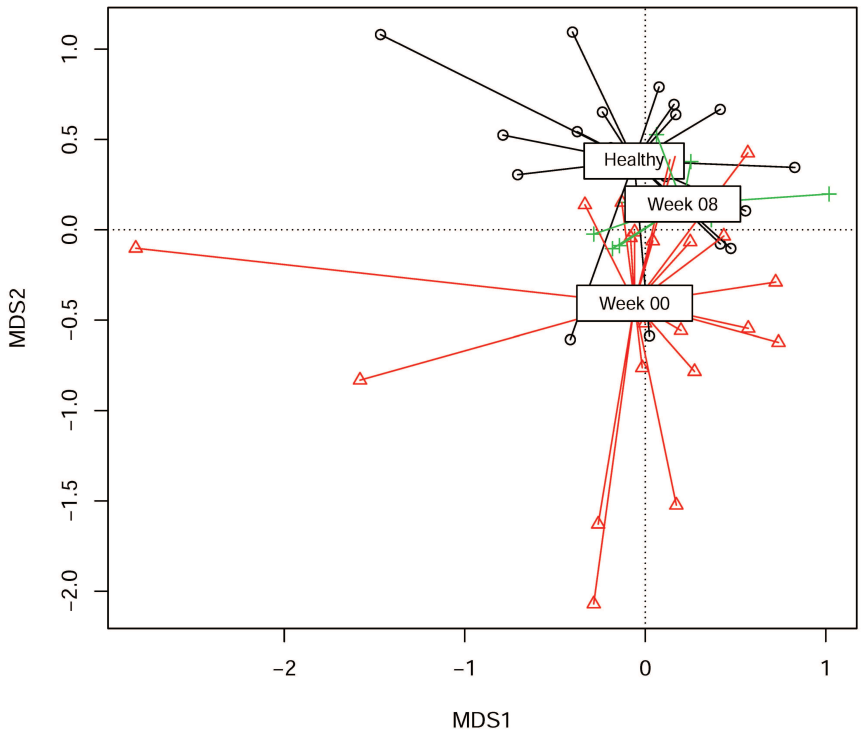


Figure 6.4: Changes in SCFA profiles in children with Crohn's disease after 8-weeks treatment with EEN (week 08; green) compared with baseline (week 00; red), shown by metric multidimensional scaling (MDS) plot. PERMANOVA: R2=0.108; p=0.002 Healthy children (black) are shown for comparison. MDS polygon plot is shown in appendix 6

6.3.3 Changes in SCFA during treatment with EEN

To look at how SCFA levels changed during treatment with exclusive enteral nutrition (EEN), the difference between baseline concentration in paired samples, was compared with SCFA concentration after 4-weeks EEN. (Table 6.3). Once children with Crohn's disease had been on treatment with EEN for 4-weeks, all fatty acids combined (total) fell significantly (p=0.012). [Changes which include all children with Crohn's disease, i.e. unpaired samples, are shown in figure 6.5a p=0.007]. The median concentration of acetate (C2), propionate (C3) and butyrate (C4) for paired samples, were significantly reduced in children with Crohn's disease after 4-weeks of treatment (Table 6.3). The medium chain fatty acids hexanoate (C6) and octanoate (C8) were also reduced after 4-weeks on EEN, but octanoate did not reach significance (Table 6.3). However the ratio of individual fatty acids to total fatty acid, showed there was no difference in the ratio of acetate and propionate. The ratio of butyrate (C4) however saw a marked reduction during EEN, also shown in figure 6.5b p<0.001. The ratio of valerate (C5) increased during EEN but did not reach significance (p=0.074)(Table 6.3). The ratios of SCFA suggest, that SCFA production with the exception of butyrate (C4) are falling as a product of an overall reduction in colonic fermentation. The ratio of branched chain fatty acids iso-butyrate (iC4) and iso-valerate (iC5) increased slightly on EEN, but the difference did not reach significance (p=0.086 & p=0.070 respectively). The SCFA/MCFA profile was visu-

Table 6.3: Difference in median (IQR) fatty acids measured from faecal samples of children with Crohn's disease at baseline (week-0) and after 4-weeks of treatment with EEN.

Fatty acid	carbons	week-0	week-4	Wilcoxon
Amount ($\mu mol/g$)				
Acetate	C2	73.5 (53.1)	50.4 (37.5)	**p=0.006**
Propionate	C3	15.1 (15.8)	10.2 (4.7)	**p=0.015**
Butyrate	C4	11.7 (12.0)	5.1 (3.7)	**p=0.001**
Valerate	C5	1.2 (2.3)	1.2 (1.6)	p=0.538
BCFA ($\mu mol/g$)				
Iso-butyrate	C4	2.5 (2.2)	1.8 (1.7)	p=0.920
Iso-valerate	C5	3.3 (3.1)	2.7 (2.2)	p=0.732
MCFA amount ($\mu mol/g$)				
Hexanoate	C6	0.1 (0.12)	0.0 (0.10)	**p=0.025**
Octanoate	C8	0.2 (0.40)	0.2 (0.10)	p=0.092
Total fatty acids	C2-C8	123.6 (77.9)	68.3 (43.4)	**p=0.012**
Ratio				
% Acetate	C2	67.5 (9.0)	68.8 (8.1)	p=0.313
% Propionate	C3	14.8 (6.4)	14.0 (3.4)	p=0.701
% Butyrate	C4	10.1 (5.5)	6.4 (2.4)	**p=0.010**
% Valerate	C5	1.5 (1.6)	1.9 (1.5)	p=0.074
% Iso-butyrate	iC4	2.2 (1.8)	3.2 (1.7)	p=0.086
% Iso-valerate	iC5	2.9 (2.2)	4.2 (1.8)	p=0.070
% Hexanoate	C6	0.10 (0.12)	0.05 (0.10)	p=0.189
% Octanoate	C8	0.20 (0.30)	0.25 (0.12)	p=0.977

Amount is expressed per g of wet faecal sample. All samples are paired n=20. Significant results are shown in **bold**.

alised using a metric multidimensional scaling (MDS) plot (Fig. 6.4) and shows that the SCFA/MCFA profile, after 8-weeks EEN, moved towards that seen in healthy children (R2=0.108; p=0.002).

6.3.4 Return to normal diet

As children with Crohn's disease returned to normal habitual diet at 8-weeks post-EEN (study week-16), all fatty acids combined (total) had a median increase which did not reach significance (p=0.074 for paired samples) (Table 6.4). The median concentration of propionate (C3) and butyrate (C4) significantly increased after 8-weeks normal diet (p<0.001), however only the increase in the ratio of butyrate reached significance (p<0.001). The ratio of acetate fell from 71.6% to 66.7% after 8-weeks normal diet but did not reach significance (p=0.070) while the ratio of propionate increased from 12.8% to 16% (p=0.140). The concentration and ratio of octanoate (C8) had a significant increase after 8-weeks normal diet (p=0.006 and p=0.035) (Table 6.4).

Treatment with MEN Children with Crohn's disease who were taking maintenance enteral nutrition (MEN) along with normal diet post-EEN (n=11), had a lower median ratio of acetate (C2) and a higher ratio of propionate (C3) than children who

Table 6.4: Difference in median (IQR) fatty acids measured from faecal samples of children with Crohn's disease at end of EEN (week-8) and again after 8-weeks of normal diet (week-16).

Fatty acid	Carbons	week-8	week-16	Wilcoxon
Amount ($\mu mol/g$)				
Acetate	C2	51.8 (26.5)	55.8 (48.1)	p=0.252
Propionate	C3	9.8 (8.2)	12.9 (7.6)	**p<0.001**
Butyrate	C4	3.4 (3.3)	9.2 (9.9)	**p<0.001**
Valerate	C5	1.2 (1.1)	1.3 (1.2)	p=0.821
BCFA amount ($\mu g/g$)				
Iso-butyrate	C4	2.3 (1.7)	2.2 (1.5)	p=0.900
Iso-valerate	C5	2.9 (2.0)	3.0 (1.9)	p=0.528
MCFA amount ($\mu g/g$)				
Hexanoate	C6	0.04 (0.11)	0.03 (0.06)	p=0.505
Octanoate	C8	0.12 (0.14)	0.38 (0.39)	**p=0.006**
Total fatty acids	C2-C8	76.1 (40.2)	85.3 (61.4)	p=0.074
Ratio				
Acetate %	C2	71.6 (9.1)	66.7 (14.4)	p=0.070
Propionate %	C3	12.8 (3.9)	16.0 (6.6)	p=0.140
Butyrate %	C4	5.05 (1.2)	10.2 (3.8)	**p<0.001**
Valerate %	C5	1.9 (0.8)	1.5 (1.2)	p=0.258
Iso-butyrate %	iC4	3.2 (0.8)	2.6 (1.5)	p=0.339
Iso-valerate %	iC5	4.2 (0.7)	3.6 (0.7)	p=0.393
Hexanoate %	C6	0.10 (0.12)	0.05 (0.10)	p=0.212
Octanoate %	C8	0.25 (0.12)	0.50 (0.30)	**p=0.035**

Amount is expressed per g of wet faecal sample. All samples are paired n=16. Significance differences are shown in **bold**.

went onto normal diet without MEN (non-MEN; n=4) (Fig. 6.6). The median ratio of butyrate (C4) was also higher in the MEN groups but this difference disappeared by 8-weeks normal diet. The median ratio of valerate (C5) was higher in the MEN group at 8-weeks normal diet but no differences were seen for the medium chain fatty acids (C6-8) or the branch chain fatty acids (iC4-iC5). Due to this being an observational study, the lack of non-MEN participants, resulted in the sample size being too low to carry out reliable statistical analysis.

6.3.5 Changes in SCFA and markers of inflammation

To test the effect that EEN had on all children with Crohn's disease samples are shown at baseline (study week-00), 4-weeks and 8-weeks of EEN, and then after 2-weeks and 8-weeks of normal diet (study week-10 and week-16). Figure 6.5a shows that overall faecal SCFA was significantly reduced during EEN but started to return towards pre-treatment levels once back onto normal diet. This effect was most pronounced for the ratio of butyrate (Fig. 6.5b). Faecal pH, as a marker of fermentation in the gut, showed the same pattern with a highly significant increase in pH at 4-weeks and 8-weeks on EEN (Fig. 6.5c). The pH again decreased once back onto normal diet at study week-10 and week-16, showing increased colonic fermentation. Calprotectin as a marker of inflammation (Fig. 6.5d), decreased during EEN (week-4 and week-8) in

most children and increased gradually as children returned to normal diet (week-10 and week-16).

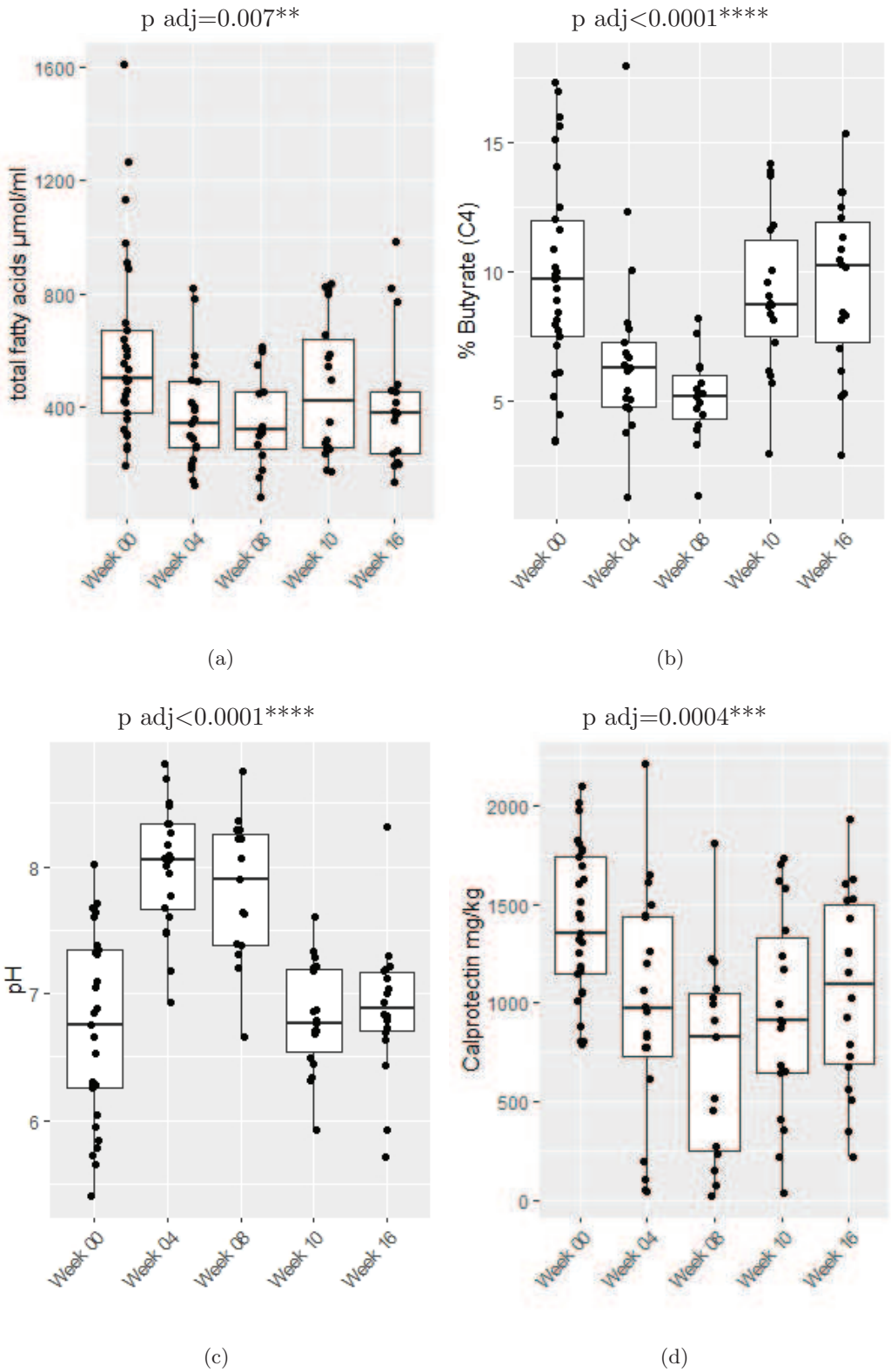


Figure 6.5: Differences in SCFA during EEN (week-4 & week-8) and on return to normal diet (week-10 & week-16) for (a) the concentration of all (total) fatty acids and (b) for the ratio of butyrate. As a marker of fermentation (c) feacal pH and (d) calprotectin as a marker of inflammation were measured at all time-points. Some patients were unable to provide a faecal sample for every time-point. ANOVA used to test for significance.

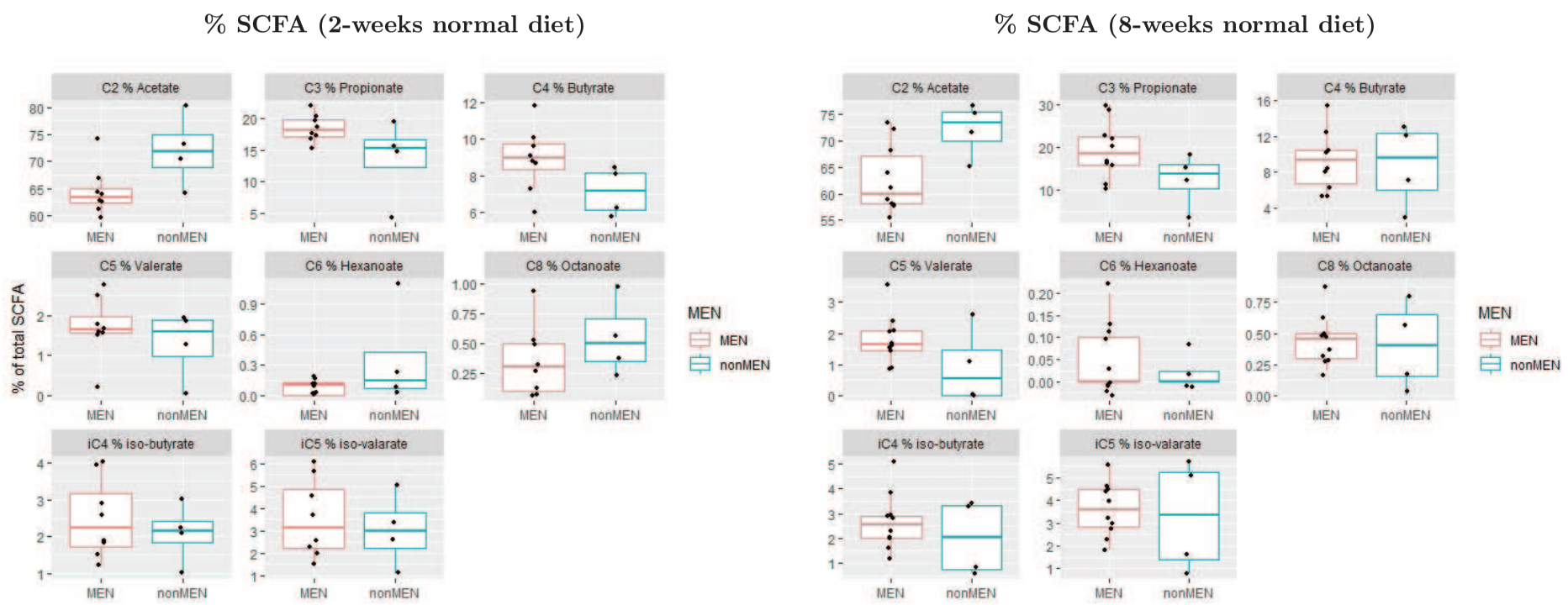


Figure 6.6: Difference in ratio of SFCA/MCFA between children with Crohn's disease taking maintenance enteral nutrition (MEN) along with normal diet, and those children who went onto normal diet without MEN (non-MEN); at (a) 2-weeks normal diet and (b) 8-weeks normal diet. The number of children in the non-MEN group was too low to calculate significance.

6.4 Discussion

6.4.1 Summary of results

The current study results show at baseline, both the amount and ratio of valerate (C5), hexanoate (C6) and octanoate (C8) were all significantly reduced in children with Crohn's disease compared to healthy children. Hexanoate was shown to positively correlate with a number of bacterial genera (mostly Firmicutes), but only in children with Crohn's disease.

During EEN SCFA/MCFA profiles moved towards that of healthy children, although production of SCFA fell, particularly Butyrate (C4). Within 2-weeks post-EEN SCFA had returned to pre-treatment levels. Although some possible differences in SCFA/MCFA were seen between children who took MEN or declined MEN (non-MEN) post-EEN, the non-MEN group of only four children in this observational study was too low to show significance.

6.4.2 Differences in short chain fatty acids (SCFA) in IBD

Despite low samples sizes in some studies, and high levels of inter-group variation, there appears to be a clear consensus that SCFA levels are reduced in Crohn's disease and UC compared with healthy controls.[474;465;466;469;471;475] However the current study along with two other studies, did not find significantly lower levels of acetate, propionate and butyrate.[465;470] SCFA are very volatile, especially acetate and propionate, thus some methods/studies may be better at extracting all SCFA, while some detection methods are more sensitive; therefore increasing the chances of differences becoming significant.[465] Three studies have recorded an increase in butyrate in patients with Crohn's disease[475;468;467] with one of these, Walton *et al.* also recording an increase in propionate.[467] However, the latter study[467] appears to have had problems with either extraction or detection as many samples show zero amounts of propionate and butyrate.

An early *in vitro* study showed an increased capacity via *in vitro* fermentation of Crohn's disease microbiota to have increased total SCFA (particularly acetate) when compared with healthy controls.[475;490] However this finding has not been supported by studies directly analysing SCFA from faecal samples (Table 6.1).

The current study along with other studies[465;470] have reported significantly reduced amounts of valerate (C5) in Crohn's disease. SCFA in the form mono-valerin and tri-valerin which are esters of valeric acid, when given as feed additives to chickens have been shown to reduce the colonisation of *Salmonella enteritidis*,[273] as well as reducing the incidence of necrotic enteritis caused by *Clostridium perfringens*;[274] which suggests valerate needs to be properly investigated in terms of the role it might play in protecting the gut from both pathogens and inflammation.

A recent study has suggested that valerate may play a role in gene expression, being a potential contributor to the histone deacetylase inhibitory effect.[271] Valerate has a structure similar to the inhibitory neurotransmitter GABA, with a study showing valerate can act as a GABA-agonist in rats.[272] The mechanism of action of valeric acid could be similar to its analogue, valproic acid, which is known to increase production of GABA, resulting in decreased succinic acid synthesis.[491] This is relevant because succinic acid has been identified as a metabolite in innate immune signalling, which enhances IL-1β production during inflammation.[280]

6.4.3 Differences in medium chain fatty acids (MCFA)

Interestingly, medium chain fatty acids (MCFA; C6–C12) have been shown to possess antibacterial properties at much lower concentrations than SCFA (Chapter 1, section 1.10.1).[492;277;278;279;279] Of three studies which measured MCFA (C6-8) in Crohn's disease patients (adults and children), all three reported reduced hexanoate (C6),[465;470;468] while two reported reduced concentrations of octanoate (C8).[465;470] These findings were supported by the current study which also saw significantly reduced concentration and ratios of hexanoate and octanoate in children with Crohn's disease.

Although the current study does not support the hypothesis that the faecal SCFA, acetate, propionate and butyrate in patients with Crohn's disease are distinct from healthy children; our findings do support the hypothesis that the SCFA valerate and the MCFA hexanoate and octanoate are reduced in children with Crohn's disease when compared with healthy children.

6.4.4 The impact of reduced fermentation capacity in Crohn's disease

The essential role of the gut bacteria in producing SCFA have been highlighted by studies using germ-free mice, whereby the lack of bacteria result in low levels of SCFA production when compared with normal mice;[493] showing how little SCFA is obtained directly from the diet. The current study saw the loss of species of SCFA producing bacteria from the genera *Anaerostipes*, *Blautia*, *Coprococcus*, *Faecalibacterium*, *Lachnospira*, *Ruminococcus* and *Roseburia* from faecal samples of patients with Crohn's disease. These findings are in line with previous studies.[494;495;466;217;463] Species from the genus *Roseburia* are among the most abundant butyrate-producing bacteria in the gut ($\sim$0.9 to 5.0% of total bacteria),[496] and reduction of this genus could explain why some studies record reduced faecal butyrate in Crohn's disease. A metagenomic and proteomics study looking at the microbiota in ileal Crohn's disease, has shown that genes for SCFA production are reduced, along with a decrease in proteins from the important butyrate producers *Faecalibacterium prausnitzii* and *Roseburia* species.[495]

Acetate, butyrate and propionate as products of bacterial fermentation are involved in shaping the colonic environment by affecting gut transit; host nutrient uptake; pH levels and bacterial homeostasis within the gut.[241] Where a consistent reduction in these bacterial groups is seen, suggests there may be a corresponding loss of SCFA available to patients with Crohn's disease to help with functions such regulation of inflammatory processes or tissue repair.[241]

Mucosal damage A high concentration of butyrate has been used to create a reliable colitis model in adult mice,[497] and studies in rats using colon histological injury scores have shown that SCFA have the ability to damage normal mucosa.[498] The effect of SCFA administration was however minimal in post-weaned rats of 23-24 days old, suggesting that bacterial maturation of the gut protects against these effects.[498] Thus although the current study did not see increased amounts of SCFA in patients with Crohn's disease, it is possible that dysbiosis and low bacteria diversity create an environment where SCFA can damage the mucosa. Given that in healthy individuals about 90% of SCFA from the digestion of foods are absorbed in colon, with the rest excreted in faeces,[236] SCFA never reach high concentrations in the gut lumen. If malabsorption and inflammation affect the ability of colonocytes to take up SCFA such as butyrate, it might be possible for the concentration of SCFA to build up in the gut, to high enough levels to cause further damage. However the results of the current study suggest this is not the case since children with IBD did not have higher concentrations of faecal SCFA than healthy children (Table 6.2).

Butyrate derived from commensal bacteria has been shown to increase mitochondrial-dependent oxygen consumption in enterocytes, as well as stabilising the hypoxia inducible factor involved in gut barrier protection.[464] SCFA have also been shown to directly drive lower mucosal oxygen concentrations, leading to a less favourable environment for aerobic pathogens to grow.[499] Thus low butyrate concentrations in the gut could in theory lead to reduced mucosal protection. Although some studies have reported a reduction in butyrate in patients with Crohn's disease,[469;471;475] these findings were not supported by five studies including the current study.[489;465;468;474]

Pro/anti-inflammatory effects It is known that SCFA exert anti-inflammatory effects in the gut mucosa via histone deacetylases inhibition.[267;268] The discovery that SCFA act as natural ligands for receptors across a wide range of cell types has led to the current interest in SCFA as signalling molecules between the gut microbiota and host immunoregulation. SCFA also have immunomodulatory and anti-inflammatory effects, via mediating homeostasis of colonic T_{reg} cells,[241;267;269;500] although the anti-inflammatory effect of SCFA could be via other mechanisms. However it is also known that metabolites produced by *F. prausnitzii* can block NF-κB activation and IL-8 production.[217] This might suggest that SCFA producing species have the ability

to shift in the direction of an inflammation promoting microbiota, possibly driving inflammation towards more severe Crohn's disease. As such, there is a need for well designed human studies which could reveal the significance that SCFA play in regulating host immunology and inflammation in the gut.

6.4.5 How might SCFA be used to treat Crohn's disease

Metabolic profiling including SCFA could become an effective exploratory tool for understanding metabolic pathways and how these might be altered in conditions such as Crohn's disease and UC. It has been suggested that supplementing patients with either butyrate producing bacteria or butyrate could be used as an intervention strategy.[268]

Schulthess *et al.*[501] have shown via intracellular staining of calprotectin proteins, a marked up-regulation of calprotectin in macrophages after 5-days of differentiation with butyrate *in vitro*. The same group then tested results in a murine model and found that butyrate treated-mice exhibited a significantly higher antimicrobial activity against *Salmonella* and *Citrobacter* compared to controls.[501] This study has important implications in Crohn's disease and UC, given that some studies have found reduced butyrate levels in patients with these conditions (Table 6.1); and Schulthess *et al.* have suggested a valid model by which low levels of butyrate could lead to increased pathogenic bacteria, which in turn drive inflammation.

It has been shown that the abundance of *Roseburia inulinivorans* along with other butyrate producing species is reduced in patients with Crohn's disease compared to healthy individuals.[463] An *in vitro* study has shown that the introduction of six butyrate-producing species including *F. prausnitzii*, *Roseburia hominis* and *R. inulinivorans*, to the microbiota of Crohn's patients, increased the level of butyrate in the dysbiotic bacterial community as well as improving the barrier function in a *Caco-2* epithelial cell model.[478] This opens up the potential for butyrate-producing bacteria to be used as a therapeutic probiotic in a clinical setting. However more work needs to be done to understand how probiotics might affect individual Crohn's disease patients, since the pattern of dysbiosis varies greatly between individuals.

The administration of butyrate by giving human patients butyrate tablets or enemas, have only been carried out on UC patients. At a molecular level butyrate enemas have been shown to decrease NFκB nuclear translocation in lamina propria macrophages, in tissue taken from UC patients.[258] It has also been shown to decrease lipopolysaccharide induced cytokine expression and NFκB activation of lamina propria mononuclear cells; as well as peripheral blood mononuclear cells (PBMCs) in tissue taken from Crohn's disease patients.[502] However administering butyrate in human trials have not been successful for a number of reasons, including delivery issues and poor patient compliance.[281] Butyrate has been shown to be less effective in pro-

ducing an anti-inflammatory response in a colitis mouse model when compared to inoculation with *F. prausnitzii*. However both *F. prausnitzii* and butyrate were each shown to increase IL-10 production, and reduced IL-12 and TNF-α production.[217] It is likely there would need to be a constant production and delivery of SCFA to the gut mucosa, in order to gain a sustained anti-inflammatory effect. Therefore using butyrate producing bacteria to increase butyrate production in the gut could be a better target for potential therapy.[503]

Future research Subsequent research will need to find good candidate probiotic bacteria, which have good tolerance to stomach and small intestinal conditions to ensure they reach the colon, as well as demonstrating potency in terms of butyrate production in the colonic microbiota. It might also be possible that bacteria which are not in themselves butyrate producers, but rather stimulate butyrate production among the gut microbiota, could also be a good candidate to improve butyrate levels in the gut of IBD patients.[504;505]

SCFA have the potential, not only to increase our understanding of Crohn's disease and UC, but also diagnose and treat these conditions. SCFA/MCFA profiles and other metabolites could possibly be used as biomarkers, easily measured from non-invasive urine, faeces or blood samples.

6.4.6 Changes in SCFA during EEN

The lack of understanding as to how exactly exclusive enteral nutrition (EEN) works has discouraged its use in some centres.[506] There have been many suggestions as to how EEN might work, but it now appears that the idea of bowel rest[507] or the reduction of possible food allergens[508;509] are not correct. It might seem unlikely that EEN works via immunosuppression, because patients with UC would be expected to benefit if this were the case. However EEN is ineffective in the treatment of UC.[510]

There is increasing evidence that inflammation in Crohn's disease is linked with an inappropriate immune response against the gut microbiota. Therefore it is possible that changes in the metabolic activity of certain members of the microbiota might play a role in regulating this immune response.[467;511]

Gerasimidis *et al.*[145] saw a reduction in total SCFA during EEN along with a significant reduction in both the amount and ratio of butyrate during treatment with EEN in seven children who went into remission. Although this study suggested these differences were not seen in nine children who either failed EEN, or did not achieve complete clinical remission; there were clear, although less pronounced reductions in all SCFA, especially butyrate during EEN in these nine patients. It should also be noted that patients who responded to EEN had a higher median concentration and ratio of butyrate at baseline (15.4, 16.9%), compared with patients who failed EEN

(7.2, 8.3%). It is therefore not surprising that on a fixed diet of EEN for 2-weeks, those with higher baseline amounts, had a larger reduction of butyrate on EEN (9.1, 9.3%) than children who failed EEN (4.0, 6.8%). After 8-weeks on EEN, children who responded actually had a higher ratio of butyrate (9.3%), compared with those who failed to achieved remission (7.8%).[470]

In 2012 Tjellstrom *et al.*[129] proposed the use of a SCFA fermentation index; where acetate which they argue has pro-inflammatory properties, less anti-inflammatory propionate and butyrate, expressed as a percentage of total SCFA, could be used to asses the pro/anti-inflammatory capability of the gut microbiota. The study showed that in nine children who went into remission on EEN that there was a significant decrease in the SCFA index. They also showed that five children with perianal disease who failed EEN, that the index significantly increased during EEN. Their study failed to show data for another four children who failed EEN hence it is not clear if the difference in SCFA index was due to these patients having perianal disease or an indicator of having failed treatment with EEN. However, it is interesting that in the current study figure 6.4 clearly reflects the findings of Tjellstrom *et al.* in that acetate (C2), propionate (C3) and butyrate (C4) are associated with a move towards active Crohn's disease, and a shift in the ratio of these SCFA during EEN brings profiles towards that of healthy children. The findings of the current study do not support the use of this SCFA index. The index based on the current study data (n=(19), contrary to the findings of Tjellstrom *et al.* (n=9), showed a slight non-significant increase in median index value (Fig. 6.7). It is possible that the low samples sizes in the Tjellstrom study have created a Type I error (false positive).

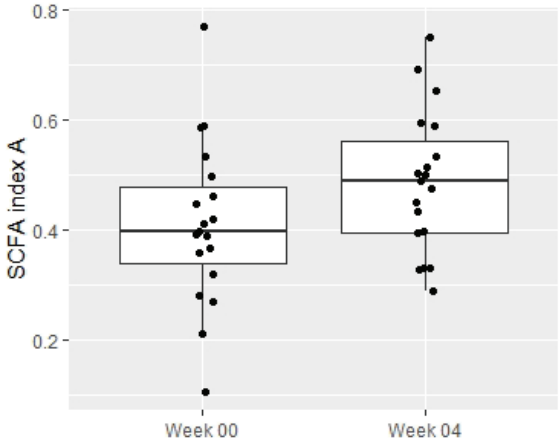


Figure 6.7: Boxplot showing SCFA inflammatory index proposed by Tjellstrom *et al.*[129] using the current study data. Contrary to Tjellstrom the current study did not see a decrease in SCFA index A after EEN. Index A=(C2-C3-C4)/total SCFA

The current study after 4-weeks EEN, using paired samples, also saw a reduction in total fatty acid. The median concentration of acetate, propionate and butyrate, were reduced along with the medium chain fatty acids hexanoate and octanoate. Only the ratio of butyrate however was markedly reduction during EEN. The ratios of SCFA

in the current study suggest that SCFA production with the exception of butyrate fell as a product of a reduction in colonic fermentation.

6.4.7 Drivers of SCFA changes during EEN

Although this and other studies see a reduction in faecal butyrate during EEN, it is possible that this is due to better absorption and utilization of butyrate by colonocytes as a result of reduced inflammation and mucosal healing in the gut. It has been proposed that the uptake of butyrate is impaired during active disease in IBD.[512] If this were the case then we would expect to see only a decrease in butyrate among those children who responded to treatment. However this is impossible to accurately measure, since EEN is a residue free diet with no fibre, which leads to a reduction in butyrate producing bacteria. Reduced faecal concentrations of butyrate could be due to both reduced production by bacteria and malabsorption in different proportions in different individuals. Interestingly a recent study looking at the effect of 7-days EEN on healthy adult volunteers saw a similar significant reduction in SCFA, as that seen in children with Crohn's disease on EEN.[513] This study also showed that a low residue ordinary solid food diet with a similar nutrient composition to EEN, was able to achieve similar reduction of SCFA to EEN. However post-treatment amounts of SCFA in the solid food diet, with the exception of propionate, were not as low as those seen with EEN.[513] If SCFA play in role in regulating the immune response to gut microbiota, this type of solid food diet, could potentially have an important role to play, not only in attaining remission in children with Crohn's disease but also in maintaining long term remission and preventing disease relapse.

Treatment with EEN leads to reduced fermentation, and thus potentially a reduction of toxic metabolites, such as 1-propanol, p-cresol, phenol, 1-butanol, dimethyl disulphide and fatty acid ethyl esters. This could explain why EEN leads to mucosal healing, and is supported by a study showing these faecal toxic metabolites were present in Crohn's disease patients but not in healthy controls.[488] Similar metabolites have also been identified in UC patients, but interestingly unlike Crohn's patients, these did not reduce after treatment with EEN.[467] This finding supports the idea that dysbiosis in patients with UC differs from Crohn's disease, which the current study findings support at a bacterial species level. It is therefore possible that toxic metabolites produced from abnormal bacterial metabolism could be an important factor in Crohn's disease.

A 2011 study[514] comparing peripheral blood mononuclear cells (PBMCs) from twenty-two Crohn's and nine UC patients with twenty healthy controls, showed that to inhibit the release of IL-12/23p40 from PBMCs after activation via TLR2-agonist, larger amounts of butyrate were needed in patients with Crohn's and UC compared to healthy controls. It is possible that toxic metabolite signalling could be impairing the

sensitivity of PBMCs to butyrate; thus impaired sensitivity to the inhibitory effect of butyrate in Crohn's disease could play a role in disease aetiology. Further work using cell models could go on to examine whether toxic metabolites such as ammonia or phenol have the ability *in vitro* to alter the sensitivity of PBMCs to butyrate.

6.4.8 SCFA and maintenance enteral nutrition (MEN)

As yet no studies have looked at the effect of maintenance enteral nutrition (MEN) on the production of SCFA in the gut. The current study is the first study which attempted to look at SCFA levels in patients taking MEN post-EEN. After 2-weeks on normal habitual diet the median ratio of acetate was lower (NS), while propionate and butyrate were marginally higher (NS) in children taking MEN as a supplement. For butyrate this difference was no longer seen at 8-weeks normal diet. Due to this being an observational study, and possibly because clinical staff and patients had information about MEN, only four patients on the study chose not to take MEN. In the previous 2-years 54% of children had chosen not to take MEN. The numbers were therefore too low to carry out any statistical analysis and ongoing studies would need to factor in ways to ensure a larger non-MEN control. This could be challenging as it may be deemed unethical not to offer MEN to some children.

When all children on normal diet in the current study were combined, a significant increase in the concentration of propionate and butyrate was seen after children had been on normal diet for 8-weeks post-EEN. There was also a significant increase in octanoate. Total SCFA was also increased but the difference did not reach significance. Only the ratio of butyrate and octanoate were significantly increased 8-weeks post-EEN. In a previous 2009 study the total SCFA for all children on EEN was shown to be significantly increased at 2-week post-EEN.[489] Both these studies show that once children go back onto normal diet that SCFA increase back towards pre-treatment levels and are not maintained, even when MEN is added as a supplement to the diet. This is not surprising as the increase in dietary fibre leading to an increase in numbers of SCFA-producing bacteria will result in increased SCFA production regardless of whether the diet is supplemented with residue-free MEN.

A 2006 study[123] using partial enteral nutrition (50% EDA) to induce remission in children with Crohn's disease (n=24), compared with those taking EEN (n=26), found that EEN was associated with a significant rise in serum albumin and reduced ESR, which was not seen in those taking partial enteral nutrition. This study suggests that partial enteral nutrition of 50% or less does not have the same immunomodulatory properties as EEN, and gives some indication as to why the dose of MEN may be too low to have an effect in maintaining remission. It would be interesting for future studies using murine models, to look at the effect enteral nutrition dose has on SCFA production in the gut and how this in turn relates to inflammatory processes.

6.4.9 SCFA and the microbiota

A decrease in Firmicutes and an increase in Proteobacteria have been a consistent outcome from IBD gut microbiota studies.[515] The reduced abundance of Firmicutes mainly coming from the families Ruminococcaceae (*clostridial cluster IV*) and Lachnospiraceae (*clostridial cluster XIVa*) is a key feature of dysbiosis in Crohn's disease, particularly in active disease.[515;516;517;518] These families are important functional members of the gut microbiota, because the majority of butyrate producing bacteria come from these families, thus reduced abundance in these groups can be linked to reduced butyrate producing capacity of the gut microbiota in patients with Crohn's disease.[519] Hence if butyrate has a protective effect on the gut, these benefits are also lost along with butyrate-producing bacteria in Crohn's disease.

The substantial production of butyrate by *F. prausnitzii* makes this bacterium of particular interest. Butyrate being the major source of energy for colonocytes[520] also plays a role in modulating the immune system by inhibiting NF-κB transcription factor activation, up-regulating PPARγ, and inhibiting IFNγ, potentially reducing inflammation.[521] It is important to understand the interdependency of many bacteria and that these anti-inflammatory properties of bacteria such as *F. prausnitzii* rely on cross feeding with other members of the microbiota.

In vitro studies show *Bifidobacteria* degrade oligofructose to produce acetate, utilised via cross-feeding by commensal bacteria including *F. prausnitzii*, *Anaerostipes* and *Roseburia* species, which produce butyrate.[520;522;523;524] *B. thetaiotaomicron* (acetate producer), has been shown to increase goblet cell differentiation, expression of mucus genes and the ratio of sialylated/sulfated mucins in rats; suggesting that *B. thetaiotaomicron* stimulates mucus production.[525] However when *B. thetaiotaomicron* was mixed with *F. prausnitzii*, (acetate consumer/butyrate producer), the effect on goblet cells and mucus glycans were diminished. This murine model suggests an imbalance of butyrate and acetate producers in the gut reduces the ability of the mucus layer to act as a barrier against pathogens, allowing pathogenic bacteria to access gut epithelial layers, triggering inflammation.[525] Increases in specific bacteria, overall dysbiosis and a loss of cross-feeding could lead to an imbalance in SCFA production. It is therefore possible, that loss of functional homeostasis in the form of SCFA rather than bacteria themselves, lead to mucosal damage and inflammation. Evidence from the current study supports this idea since SCFA profiles of children with Crohn's disease move towards that of non-IBD controls after treatment with EEN (Fig. 6.4). This would explain why EEN works despite further losses of what would be considered healthy commensals such as Firmicutes and an increase in Proteobacteria. It is a change in the ratios of SCFA which is driving a reduction in inflammation. This would also explain why inflammatory markers increase once children return back onto habitual diet post-EEN as SCFA levels start to increase.

A recent elegant study by Schulthess *et al.*[501] has shown that macrophages which differentiated in the presence of butyrate had enhanced antimicrobial activity *in vivo* and increased resistance to pathogenic species of bacteria including *Salmonella*; adherent-invasive *Escherichia coli* (AIEC); *Staphylococcus* species and *Citrobacter*. They concluded that increased gut butyrate could be used as a strategy to increase host defence. However studies looking at children taking EEN as therapy see a marked reduction in butyrate accompanied with increased Enterobacteria species, which is associated with a reduction in inflammation. Mucosal healing and reduced inflammation may be more dependent on reduced bacterial abundance than levels of butyrate. It is therefore possible that increases in pathogenic species of bacteria during EEN could explain why some children fail EEN; and treatment with butyrate may increase the success of EEN. However, boosting the diet of children with Crohn's disease with butyrate to try and reduce inflammation would need further research.

Looking at the effect of butyrate on normal healthy cells in the colon, butyrate has been shown to illicit effects via the G protein receptors GPR109A; GPR41 and GPR43. Transported into cells via the SMCT1 (SLC5A8) a co-transporter for H^+ and butyrate and MCT1 (SLC16A1) a co-transporter for Na^+ and butyrate. If a lot of butyrate is present and Na^+ is transported into cells water tends to follow; and for this reason butyrate is regarded as an anti-diarrhoeal agent. However during treatment with EEN, the converse appears to be true since a significant decrease in the amount and ratio of butyrate is associated with a reduction in diarrhoeal samples.

The majority of research into butyrate suggests it has anti-inflammatory properties, yet paradoxically, treatment with EEN where butyrate is significantly reduced, is associated with decreased inflammation as shown by inflammatory markers such as calprotectin and mucosal healing. It is therefore possible, as some studies suggest, that normal pathways associated with butyrate are dysfunctional in some way.

6.4.10 Future research

In 2015 an interesting study[526] looking at a much wider range of metabolites as volatile organic compounds (VOC) in exhaled air, which includes esters of SCFA has suggested that these can be used not only as a marker of Crohn's disease, but also a marker of disease activity. Taking this work further by assessing the sensitivity and specificity of this method against other inflammatory conditions, and working out how to make the test kit inexpensive, should be a focus of future research. This type of testing has the potential to make clinical diagnostics much easier, and could possibly be used by GP practices, as well as be developed for home testing, allowing patients to monitor disease activity in a non-invasive manner. A breath test kit could in the long term save money by helping patients maintain remission by accessing treatment strategies before their disease condition is severe.

Studies looking at the composition of the gut microbiota in IBD are much more numerous, than studies into the functional capability of these communities. The role of gut bacteria metabolites in IBD are still poorly understood and more needs to be done to explore the role that metabolites such as butyrate have in controlling or driving inflammation in the gut. It would be easy to overlook the role that these ubiquitous bacterial molecules play but they are in fact important signalling molecules between the gut bacteria and host as well as the role they play in regulating host cellular metabolism in many different tissues around the body.

There are metabolic mechanisms which might explain the link between dysbiosis and Crohn's disease. It is possible that dysbiosis drives inflammation via the loss of SCFA producing bacteria. Changes in SCFA could limit resources to gut epithelial cells, increase production of inflammatory cytokines, as well as decrease the suppression of pathogenic Proteobacteria. On the other hand it is possible that dysbiosis is caused by the gut inflammation in Crohn's disease. Proteobacteria are facultative anaerobes which have a high resistance to the reactive oxygen species produced by inflammation, thus giving them a selective advantage over the obligate anaerobes from the Firmicutes and Bacteroidetes phyla. However it should be noted that these mechanisms are not mutually exclusive. It is likely that bacterial dysbiosis both contributes to, and is further driven by, inflammation in the gut of patients with Crohn's disease. The future challenge is not only to identify bacteria but also the compounds they produce which might either drive or protect the gut from inflammation. This also leads to the possibility of using SCFA produced by bacteria to manipulate the gut microbiota profile towards a healthy state.

It is possible that within the gut microbiota individual bacteria secrete particular profiles of SCFA/MCFA which work to inhibit the growth of competing species. Future studies should be designed to address the role of SCFA and MCFA on biofilm formation and maintenance, as this might be key to understanding the lack of bacterial diversity in IBD. It is also important that future studies identify the impact SCFA have, either directly or indirectly, on mucosal maintenance and integrity (Table 6.5).

It is important to remember that the metabolism of the gut microbiota is not driven by a clear set of linear pathways but rather a complex web of interconnected reactions facilitated by enzymes that connect multiple molecules across many pathways.[527] Hence the development of more complex analysis models which can look at multiple factors will likely help to unravel the complex processes which are driving inflammation in Crohn's disease and UC.

6.4.11 Summary

Evidence is now beginning to show that gut bacteria not only play a fundamental role in energy homoeostasis but is also necessary for a healthy homeostasis of the immune system which has been educated toward appropriate and non-appropriate responses. Gut bacteria which ferment fibre to produce SCFA, especially butyrate, can play a role in regulation of innate and adaptive immune cell generation; trafficking and function. Butyrate inhibits recruitment and pro-inflammatory activity of macrophages; neutrophils; dendritic cells and effector T-cells as well as increasing the amount and activity of T_{reg} cells.

Causative links between gut bacterial dysbiosis and Crohn's disease has been difficult to prove. Reduced numbers of SCFA-producing bacteria along with reduced butyrate is linked with an increase in the amount of pro-inflammatory immune cells present in the gut mucosa of patients. Therefore understanding the relationship between bacterial dysbiosis along with reduced butyrate production in Crohn's disease could lead to new novel therapies.

Table 6.5: Summary of key factors from SCFA research which could directly or indirectly explain inflammation in Crohn's disease

Summary of key points from SCFA research
SCFA have been shown to affect mucosal thickness.[498]
SCFA can improve epithelial integrity and tissue repair.[498;464;477;240]
At high concentration SCFA can damage normal mucosa.[497]
SCFA can alter pH level and oxygen availability in the gut.[464;499]
SCFA/MCFA have direct and indirect antimicrobial properties.[501;271;278;279]

6.4.12 Key findings

The current study:

- Support recent findings that low levels of MCFA C5, C6 and C8 differentiate IBD from healthy individuals.
- Support recent findings that SCFA, specifically ratios of butyrate are reduced during EEN but return to pretreatment levels once back on normal diet.

New findings from this study:

- In children with Crohn's disease, hexanoate (C6) is positively associated with a number of bacterial genera, particularly from Firmicutes; both of which are significantly reduced in patients with Crohn's disease.
- During EEN the overall ratios of SCFA move towards that of healthy children.
- SCFA return to pre-treatment levels once children return to normal diet; and treatment with MEN post-EEN does not maintain SCFA profiles achieved during EEN.

7 The role of diet in inducing and maintaining remission in children with Crohn's disease

7.1 Introduction

Recent research is changing our understanding of the role diet might play in IBD. Awareness of susceptibility not directly related to host genetics, has highlighted that development of IBD has an environmental component. Microbiota studies have shown that gut bacteria have an important role in IBD pathogenesis, with diet likely impacting the composition and functionality of the gut bacteria. Clinical studies show that diet, particularly exclusive enteral nutrition (EEN), can induce remission in Crohn's disease, and possibly aid maintenance of remission reducing the need for surgery.

The following chapter will look at composition of dietary intake at baseline in children with Crohn's disease, UC, non-IBD controls and healthy children and will examine whether any differences can explain gender bias in the incidence of Crohn's disease. Whether dietary intake at baseline can predict response to treatment with EEN; as well as how the composition of EEN differs from the child's normal habitual diet will also be explored. The effect of treatment with EEN on post-EEN dietary choices will also be explored in children with Crohn's disease. This chapter will then go onto explore any links between diet and the gut microbiota since children with Crohn's disease are also known to have reduced bacterial diversity, particularly Firmicutes. As a consequence, this chapter will examine whether low fibre intake in children with Crohn's disease could explain the reductions in bacterial species driving dysbiosis.

7.1.1 Diet as a risk factor for IBD

Although several studies have shown associations between the consumption of specific foods or dietary patterns and the risk of developing IBD, the dietary collection methods, mostly food frequency questionnaires (FFQs), are varied making it difficult to compare and assess results (Table 7.1). However there is consistency across all four of these studies which suggest low fibre, low fruit and vegetables and high fat (particularly animal fat) are risk factors for Crohn's disease. A Japanese study also reported that vegetable protein was protective against Crohn's disease.[528]

In infants, the first dietary option is between breast milk or a milk formula typically derived from cow's milk. Breastfeeding has been linked with lower incidence of IBD in a recent meta-analysis (odds ratio, 0.69),[529] and is more likely due to a protective effect of breast milk,[530] rather than a negative effect of formula milk, as IBD is rare before weaning.[531] Although studies have also shown that breast milk changes composition of the gut microbiota in neonates,[532] there are significant changes in the gut microbiota profile after weaning irrespective of whether a child was breast

or formula fed.[533] This would suggest that solid food is important for early risk of IBD.A study looking at the metagenomic analysis of faecal samples from ninety-eight Swedish infants has shown, in comparison to vaginally delivered infants, the gut microbiota of those delivered by C-section had significantly less resemblance to their mothers. Interestingly, maturation into an adult-like microbiota profile was associated with the end of breast-feeding, rather than the introduction of solid food.[534] The cessation of breast milk had a profound effect on the bacterial profile taken from faecal samples of 12-month old infants, shifting the gut bacteria profile towards a more adult composition which was enriched with the genera *Bacteroides*, *Roseburia*, *Clostridium*, and *Anaerostipes*.[534] In contrast, the gut bacteria of infants which were still being breast fed at 12-months were dominated by *Bifidobacterium*, *Lactobacillus*, *Collinsella*, *Megasphaera*, and *Veillonella*; bacteria which have been shown to be present in breast milk.[535] This might have implications for IBD risk since two meta-studies[529;536] have suggested a possible protective effect of breast milk; with a more recent study showing being breast-fed for more than 12-months decreased the adjusted odds for Crohn's disease in an Asian population (aOR 0.10; CI 0.04-0.30).[537] This leads to the possible conclusion that very early dietary exposures are involved in disease aetiology and that an altered gut microbiota due to diet could increase the risk of Crohn's disease later in life.

Fibre Observational studies have reported an inverse relationship between intake of fibre and risk of Crohn's disease; with one prospective study showing long term intake of dietary fibre being linked with a reduced risk of Crohn's disease (odds-ratio 0.59, 95% CI 0.39–0.90).[540] Reduced risk was greater for fruit based fibre, whereas no protective effect was seen from fibre from cereals, whole grains or legumes.[540] The protective effect of fibre has been proposed to come from SCFA, derived from the fermentation processes of gut bacteria, inhibiting the transcription of pro-inflammatory mediators.[568] Fibre also helps to maintain gut barrier integrity as well as reduce translocation of pathogen such as *E. coli* across Peyer's patches *in vitro*.[569] Studies dating from the 1970s noted that IBD patients at baseline ate less fibre, in the form of raw fruits and vegetables compared to healthy controls.[570] Depriving gut bacteria of fibre in murine models, has been shown to favour bacterial taxa able to use alternative carbon sources from within the gut mucosal layer; which in turn depletes the thickness of mucus and hence barrier function; resulting in immunological activation and tissue damage.[571] Two more recent studies[539;547] also show reduced fruit and vegetable intake to be risk factors for Crohn's disease (Table 7.1).

Protein Protein intake has also been linked with Crohn's disease risk, particularly animal protein.[538;528;544] A study which positively linked animal protein intake with risk of Crohn's found that milk was especially high, but that fish did not contribute to risk. The study also found a negative correlation with vegetable protein.[528]

Table 7.1: IBD dietary risk factors based on epidemiology studies

study	cohort	methods	results
Jantchou *et al.* 2010[538] France	30 CD; 43 UC; 67,504 controls (adult women)	self-completed dietary questionnaire (Cox proportional hazards)	High animal protein intake is an increased risk for CD (p=0.04) and IBD in general (p=0.007) but not dairy or eggs.
D'Souza *et al.* 2008[539] Canada	149 CD; 251 HC (children)	FFQ -factor analyses & unconditional logistic regression (adjusted)	A diet of meat, fried food, fast food, snacks and desserts were positively associated with CD (p=0.03); whereas a diet of vegetables, fruit, yoghurt, dairy, eggs, olive oil, fish, white rice, tofu, grains, and nuts is negatively associated with CD (p=0.02).
Shoda *et al.* 1996[528] Japan	242 CD; 68,000 control (adults)	dietary interview for 5-days annually (19-yrs)	Positive correlation between increase in total fat and incidence of CD (p<0.001); also with animal protein (especially milk) but not fish protein (p<0.001); negative correlation between vegetable protein and CD (p<0.001).
Ananthakr-ishnan *et al.* 2013[540] USA	269 CD; 338 UC (adult women, nurses)	FFQ every 4-yrs (over 26-yrs)	Higher intake of fibre associated with lower incidence of CD, but not UC. Strongest negative correlation was with fruit fibre and CD (p<0.05). No significant correlation between vegetable, whole grain, bran and legume fibre and CD.
Costea *et al.* 2014[541] Canada	182 CD; 250 controls (children)	FFQ -logistic regression using energy-adjusted nutrients	Higher consumption of dietary ratio of n-6/n-3 PUFA was susceptible for Crohn's if also carriers of specific variants of CYP4F3 and FADS2 genes. NB// Western diets tend to have high n-6 and low n-3 PUFA intake,[542] which may influence PUFA metabolic pathway in favour of n-6 pathway, leading to gut inflammation in genetically susceptible people.
Chan *et al.* 2014[543] (8 European countries)	EPIC-IBD cohort: 110 CD; 244 UC (adults)	self-completed FFQ to measure carb, sugar, starch	No significant risk association for carb, sugar or starch for CD or UC. Study did not look at fibre.
Tragnone *et al.* 1995[544] Italy	51 CD; 53 UC; 208 control	recall questionnaire by interview	Increased intake of protein associated with increased risk of CD (p=0.04) and UC; increased intake of mono- and polysaccharides associated with increased risk of CD; no difference in pre-intake levels of fat.
Reif *et al.* 1997[545] Israel	33 CD; 54 UC; 144 control	quantified questionnaire similar to FFQ (180 items) by interview	Although not significant due to small sample size, findings suggest low fibre and high animal fat were risk factors while high fruit, Vit C, vegetable and fibre were protective.
Sakamoto *et al.* 2005[546] Japan	128 CD; 111 UC; 219 control	self-completed semi-quantitative FFQ	Higher intakes of sugars and sweeteners, as well as fats and oils, fish and shellfish (p<0.05) were associated with an increased risk of CD; total fat intake increased risk for CD in dose-response relationship(p=0.002).
Amre *et al.* 2007[547] Canada	130 CD; 202 control	validated youth adolescent FFQ; 151 items	Lower fibre intake increased risk of CD (p=0.01); carbohydrates without fibre were not a risk factor with CD (p=0.67); high vegetable (p=0.03) and fruit (p=0.02) intake decreased risk of CD.

CD -Crohn's disease; UC -ulcerative colitis; FFQ -food frequency questionnaire; carb -carbohydrate

Table 7.2: Evidence from animal and *in vitro* studies that link diet with immunological function in the gut

Diet	Study	Model/method	Effect
High fat/high sugar diet	Martinez-Medina *et al.* 2014[548]	CEABAC-10 mice	Decreased MUC2 expression Depleted Goblet cells Increased intestinal permeability Increased TNF-α secretion
High fat/high sugar diet	Agus *et al.* 2016[549]	CEABAC-10 mice	Decreased FoxP3 T_{regs} in mesenteric lymph nodes Decreased butyrate production Reduced expression of the butyrate GPR43 receptor Dysbiosis Significantly decreased SCFA concentrations
High fat diet	Ma. *et al.* 2008[550]	WT C57BL/6 mice	Higher numbers of non-CD1d-restricted natural killer T cells in the colonic IEL Increased TNF-α and IFN-γ expression Decreased levels of colonic Tregs
High fat diet	Suzuki. *et al.* 2010[551]	LETO and OLETF rats; human Caco-2 cells	Increased intestinal permeability Decreased T-junction proteins expression (claudin-1, claudin-3, occludin and junctional adhesion molecule-1)
High fat diet	Gruber *et al.* 2013[552]	TNFδARE/WT mouse and WT C57BL/6	Aggravation of ileal inflammation Reduced expression of occludin Increased translocation of endotoxin Increased pro-inflammatory markers Recruitment of dendritic cells and Th17-biased lymphocyte into the lamina propria
Sodium caprate (constituent of milk fat)	Söderholm *et al.* 1998[553]	Rat ileum	Increased tight junction permeability
Sodium caprate	Söderholm *et al.* 2002[554]	Specimens from distal ileum of CD patients	Rapid increased in paracellular permeability
Gluten	Ejsing-Duun *et al.* 2008[555]	NOD and BALB/c mice	Decreased the occurrence of T_{regs} by 10%–15% ($p<0.05$)
Gluten	Wagner *et al.* 2013[556]	TNFδ ARE/WT mouse	Increased intestinal permeability Reduced occludin expression Induced chronic ileitis
Gluten (gliadin)	Lammers *et al.* 2008[557]	Human intestinal epithelial cell, Caco2 cells, IEC6 cells	Zonulin released, causedtight junction disassembly
Gluten (gliadin)	Hollon *et al.* 2015[558]	Ex vivo human duodenal biopsies	Increased intestinal permeability Higher concentration of IL-10 in controls compared with coeliac disease in remission or gluten sensitivity
Wheat (ATIs)	Junkers *et al.* 2012[559]	Human cells and biopsies	ATIs are potent activators of human innate immune responses in monocytes, macrophages and dendritic cells
Wheat (ATIs)	Zevallos *et al.* 2017[560]	TLR4-responsive mouse and human cell lines	Activation of dendritic cells in mesenteric lymph nodes Released inflammatory mediators
Soluble fibres and resistant starch	Bassaganya-Riera *et al.* 2011[561]	C57BL/6J WT mice IL-10-/- mice	Decreased ileal and colonic inflammatory lesions Decreased IFN-γ production by effector CD4+ T cells from Peyer's patches Resistant starch increased the IL-10-expressing cells Suppressed gut inflammation
Dietary pectin	Ye *et al.* 2010[562]	IL-10-/- mice	Reduced expression of TNF-R and GATA-3 Lower levels of IgE, IgG and IgM expression Modulation of production of pro-inflammatory cytokines and immunoglobulins
Fermentable fibre (guar gum, partially hydrolysed GG)	Hung *et al.* 2016[563]	BALB/c mice aged 7 weeks	Increased expression of occludin and claudin-3, claudin-4 and claudin-7 (reduced permeability) Greater total faecal SCFA concentrations Reduced inflammation score
Multifibre mix diet	Wang *et al.* 2016[564]	IL-10-/- mice	Reduced disease activity index score Decreased CD4+CD45+ lymphocytes, IFN-γ/IL-17A, TNF-α /TNF-R2 mRNA expression Increased Tregs and SCFA production Increased epithelial expression and correct localisation of tight junction proteins
Alcohol	Forsyth *et al.* 2017[565]	Mice	Colonic (but not ileal) hypermobility Decreased butyrate/total SCFA ratio in stool
Dietary salt	Tubbs *et al.* 2017[566]	IL-10-/- murine model of colitis	Exacerbation of inflammatory pathology Enhanced expression of numerous pro-inflammatory cytokines TLR4 activation

CD - Crohn's disease; UC - ulcerative colitis FFQ - food frequency questionnaire
Table adapted from Levine 2018[567]

Fats Increased intake of fats have also been linked with risk of Crohn's disease in Japanese studies,[528;546] but only an increased ratio of omega-6/omega-3 polyunsaturated fatty acids (PUFAs) has been associated with risk in a North American study; and only in those who carried particular genetic variants.[541] Although an IL-10 knockout mouse model has suggested that saturated fat is associated with pro-inflammatory T-helper type 1 immune response,[572] this association has not been seen in prospective human studies.[573]

A 2014 European study[543] which included 110 adult Crohn's patients saw no increased risk association for any dietary macronutrients. However, the study failed to look at fibre intake. It is also possible that by spreading their questionnaire across eight different countries with potential cultural differences in diet, might have masked dietary differences seen in one country not found in another, and hence may not be an accurate assessment of dietary risk.

7.1.2 Using diet to treat IBD

Studies have examined links between diet and IBD symptoms, with patients often reporting that specific food groups aggravate their symptoms.[574] However, despite dietary connections being apparent to many clinicians in individual patients, it is challenging in humans to prove dietary links via qualitative and quantitative means. Evidence from animal studies however support the idea of dietary components having immunomodulatory effects (Table 7.2). EEN is known to be effective at bringing about remission and reducing gut inflammation in children with Crohn's disease as well as maintaining their linear growth; but a review based on forty-eight studies which isolate specific food groups from habitual diets, suggests that individuals react differently on exposure or exclusion, to particular foods.[574] The main aim of dietary research in Crohn's disease is to identify ways to prolong remission particularly in children, using dietary measures, thus reducing the need for medication and surgical intervention.

Exclusive enteral nutrition (EEN) It has been long understood that EEN, since it contains no fibre, it is easily digested and absorbed in the small intestine thus suppressing the growth of bacteria in the colon (Chapter 1, section 1.9.6). It is thus thought to provide 'bowel rest', and has been shown to be efficacious in achieving disease remission especially in children with Crohn's disease.[120] Metagenomic studies have shown that EEN alters the gut microbiota (Chapter 5, Table 5.10) with one study showing that changes in the gut bacteria happen as soon as 1-week after treatment initiation.[73] However, changes due to EEN push the gut bacteria profile even further away from that of healthy children.[145] The use of EEN is likely dose dependant, since changes to the gut microbiota profile was not seen in children using

partial enteral nutrition where the diet consisted of 50% formula and the rest normal diet.[138] This might suggest that exclusion of normal diet could be the key factor in driving changes which lead to remission in Crohn's disease. Since the effectiveness of clinical and mucosal remission is greatest when 100% EEN is used,[116;132] and because many patients feel that EEN is overly restrictive and interferes with normal family mealtimes, research is moving towards the goal of creating a whole food diet that will have the same benefit as EEN.

Maintenance enteral nutrition Although using EEN to induce remission in children with Crohn's disease is of clear benefit, it is not feasible to use such an extreme exclusion diet to attain the long-term maintenance of remission. Studies have gone on to look at supplementing the normal diet with enteral nutrition (MEN) post-EEN (Chapter 1; Table 1.6) and although results from these studies might suggest MEN is comparable with immunosuppressants at maintaining remission, out of ten studies only one was a randomised controlled trial.[151] Sample sizes are small (∼20 MEN patients) and patients with less severe disease are often more likely to be treated with MEN. Eight of these studies took place in Japan[575;576;148;577;45;578;150] and hence the same treatment may not be as successful in other countries where habitual diet is different. Therefore the efficacy of enteral nutrition to maintain remission in children with inactive Crohn's disease has yet to be determined.

Specific exclusion diets The specific carbohydrate diet (SCD) is an exclusion diet with reported success in maintaining remission in IBD. The SCD diet was created in the 1920s for children with coeliac disease, excluding all grains, corn and rice; starchy tubers; all dairy containing lactose and any processed sugars. In 2016 Suskind *et al.*[579] developed an online anonymous survey for patients (both children and adults) with Crohn's disease (n=188) and UC (n=174) who had started using SCD as dietary therapy at home. After 2-months on the diet 33% reported remission and after 6-months and 12-months 42% reported they had reached remission. Although patients symptoms improved, it is possible they had ongoing inflammation; however 47% of patients who reported reaching remission on the diet also reported improved clinical blood markers but the study did not give details.[579] A recent North American study reported clinical remission in 8/10 children with active Crohn's disease after initiation of the SCD diet without use of concomitant treatment, which were also linked with significant changes in gut bacterial composition.[580] The Crohn's Disease Exclusion Diet (CDED) was created by a research group in Israel in 2014,[581] following the hypothesis that efficacy of EEN is due to exclusion of dietary constituents, which either increase gut permeability or a pro-inflammatory gut microbiota composition. It excludes: gluten, dairy, animal fat, processed meats, emulsifiers and all processed foods. In a study of patients with mild/moderate Crohn's disease, treatment using 50% polymeric formula plus the CDED exclusion diet, the combined diet was suc-

cessful in achieving clinical remission in 21/29 children and 6/11 adults; including a reduction in inflammatory markers.[581] Interestingly a group of seven patients on this study, who refused the polymeric formula, achieved either clinical remission (6/7) or response (1/7) using CDED alone.[581]

A study in adult Crohn's patients has also shown that after induction of remission on EEN, that in forty patients who excluded foods which they perceived as 'triggering' symptoms, had extended remission along with reduced inflammatory markers when compared with thirty-eight patients who remained on their normal habitual diet.[582]

Although not as well studied, low-lactose and FODMAP (low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) diets have been reported to reduce symptoms of IBD.[583;584] In one study fifty-two adult Crohn's disease and twenty UC patients were given FODMAP dietary advice with telephone follow-up on compliance and symptoms.[583] Around 70% of patients adhered, while around 50% responded to diet ($p<0.02$). For Crohn's disease, efficacy was associated with dietary adherence ($p=0.033$) and inefficacy with non-adherence ($p=0.013$).[583] In another study of thirty-nine adult Crohn's disease patients using a FODMAP diet 72% reported a reduction in gastrointestinal symptoms.[584] Although neither of these studies looked at whether the diet had an impact on inflammatory markers, and data showing reduced gut mucosal inflammation is lacking, low FODMAP diets, in a similar way to EEN, have been shown to reduce colonic healthy commensal bacteria such as *F. prausnitzii* within the colon, as well as faecal butyrate.[585] Achieving adequate fibre intake on a low FODMAP diet is challenging as it excludes a number of high fibre foods, thus any mechanism to reduce inflammation on a FODMAP diet may simply be due to lack of fibre in the colon.

Excluding sugar has shown little evidence of amelioration in Crohn's disease[586] while omega-3 fatty acids have shown some promise in reducing symptoms.[587] Although exclusion diets may have a role in maintenance of remission in Crohn's disease, it will be necessary to characterise their effect on mucosal healing, as well as gut microbiota and metabolite composition. Careful nutrient assessment of potential exclusion diets will also be necessary for children who are still growing.

Diet is important in gut homeostasis; interacting with the microbiota; host barrier function and immune sensing.[588] It therefore makes sense to explore the potential role of dietary factors in the pathogenesis of Crohn's disease. Modifying diet as a target for prevention and treatment of IBD is attainable, however understanding its role in Crohn's disease in terms of ability to modify gut microbiota composition and the production of metabolites, is much more complex. The following chapter, using estimated diet from food frequency questionnaires (FFQs), will look at differences in diet between healthy children and children with Crohn's disease; before going on to examine how EEN is nutritionally different from normal habitual diet, and what impact this might have on controlling inflammation in the gut.

7.1.3 Hypotheses

- The composition of dietary intake at baseline in children with Crohn's disease is different from that of healthy children. Given that children with Crohn's have reduced bacterial diversity, particularly among the Firmicutes, it is predicted they will also have reduced intake of dietary fibre.
- The incidence of Crohn's disease is higher in boys. It is therefore predicted that dietary intake in boys with Crohn's disease is different from that of girls.
- That dietary intake at baseline predicts response to treatment with EEN.
- That attaining remission using EEN for 8-weeks will affect dietary choices post-EEN i.e. That habitual dietary intake post-EEN will differ from dietary intake at baseline.
- Diet at baseline is associated with the gut microbiota profile and inflammatory status of children with Crohn's Disease.

7.2 Methods

The detailed composition of enteral nutrition (Modulen IBD) is given in appendix 7.2, and details of treatment with exclusive and maintenance enteral nutrition are given in Methods 2.3. To establish the child's normal habitual diet, additional information on dietary intake was gathered using child specific food frequency questionnaires (FFQ) and 3-day food diaries which were collected at each time-point over the course of the study.

Details about other dietary methods which were used at the start of the study, along with detailed FFQ methods are described in Methods 2.5. In brief the FFQ, a constructed list of foods and drinks with a frequency response tick-box section, was used to report the frequency of all foods eaten based on the previous 2-weeks diet. Calculations for nutrient intake were estimated by the diet survey team at the University of Aberdeen (calculations multiply frequency of item eaten by amount of nutrient in each serving).[290] Paper copies of the Scottish Collaborative Group validated FFQ (Appendix 7.1.2) were used and include ∼150 commonly eaten UK foods or drink.[291] Two versions were used: one which is designed for children aged between 3-11 and normally completed by parents/guardian; a second version designed for young people aged between 12-17, which includes coffee and alcohol, and is completed by the young person themselves.

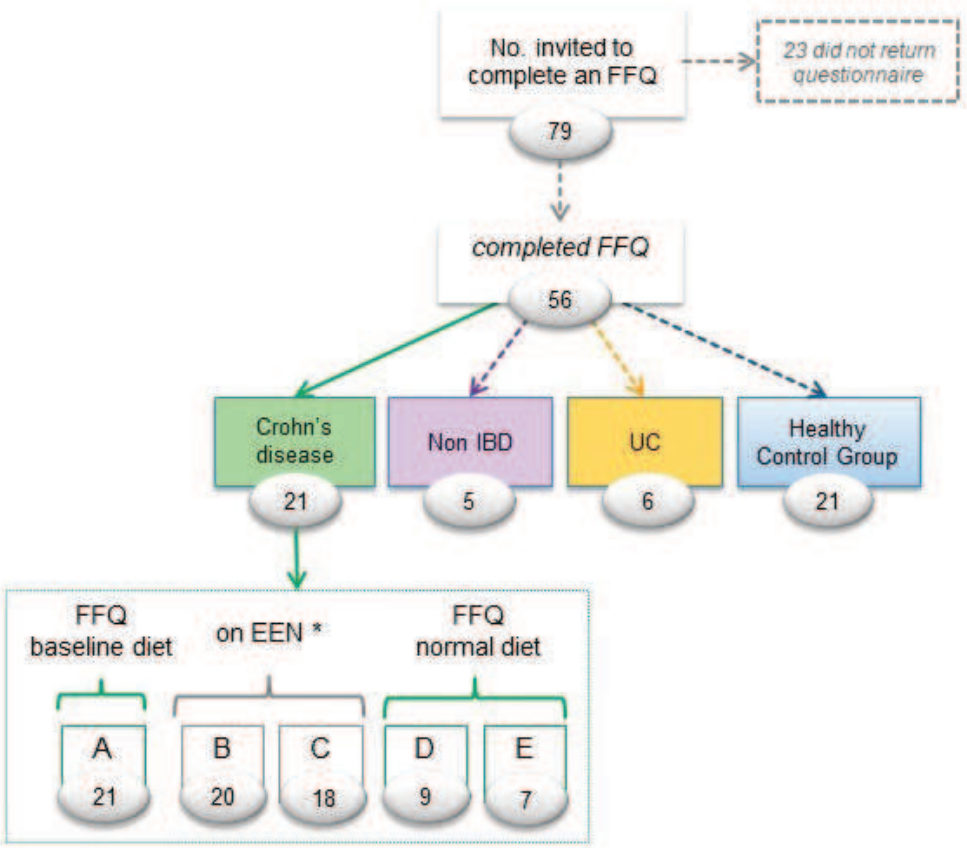


Figure 7.1: Number of FFQ questionnaires collected from each study group at baseline and children with Crohn's disease at: A -baseline; D -2wks and E -8wks normal habitual diet. * Composition of diet at B and C -4wks and 8wks EEN was calculated from the known amounts of enteral nutrition taken during dietary treatment.

An FFQ was given to each participant at baseline and subsequently at each time point for children with Crohn's disease when faecal samples were requested. Participants were prompted to think about hidden ingredients in composite foods such as milk and sugar in tea; or butter in sandwiches. They were asked to record the foods and drinks they had consumed in the previous 2-weeks. Participants were also requested to fill out a 3-day food diary shown in (Appendix 7.1.1). Although fourteen healthy children completed these, only three children with Crohn's disease completed the requested 3-day food diary at 2-weeks and 8-weeks post EEN. No children with non-IBD conditions or UC returned their 3-day diary. Compliance was so poor, that a decision was reached not to include 3-day diaries in the study. Perhaps because the study was about Crohn's disease, those with UC and non-IBD conditions often failed to complete and return FFQs after a reminder was sent, leaving the study with only six FFQs for children with UC and five for non-IBD conditions. At each subsequent time-point fewer children with Crohn's disease returned their FFQ (Fig. 7.1).

7.3 Results

7.3.1 Differences in dietary intake at baseline

Four children with Crohn's disease and two children with UC, reported they were struggling to eat their normal diet, reflected in their median estimated energy intake (Fig. 7.2). Although median energy intake (kcal) for children with Crohn's and UC was less than healthy children, the difference was not significant (p=0.24)(Table 7.3).

There was no difference between children with Crohn's disease and healthy controls or other patient groups for carbohydrates (including sugars), protein or fat (Table 7.3). Fibre intake was reduced compared with healthy children in all three patient groups, and was significant for both children with Crohn's and UC ($p<0.05$). Estimated intake of vitamin A (retinol), vitamin E, vitamin B_7 (biotin) and vitamin C were significantly reduced in children with Crohn's disease (Table 7.3; Fig. 7.4). There was a strong significant correlation between fibre and vitamin C intake in healthy children, however the same relationship was not seen in children with Crohn's disease (Fig. 7.3).

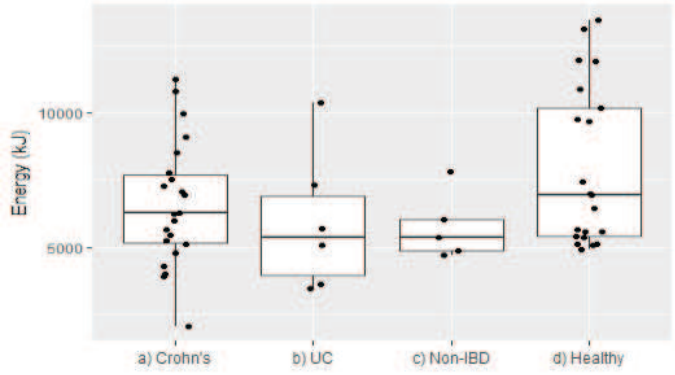


Figure 7.2: Estimated energy intake from FFQs for all groups of children at baseline. No significant difference seen between groups: Kruskal-Wallis p=0.24

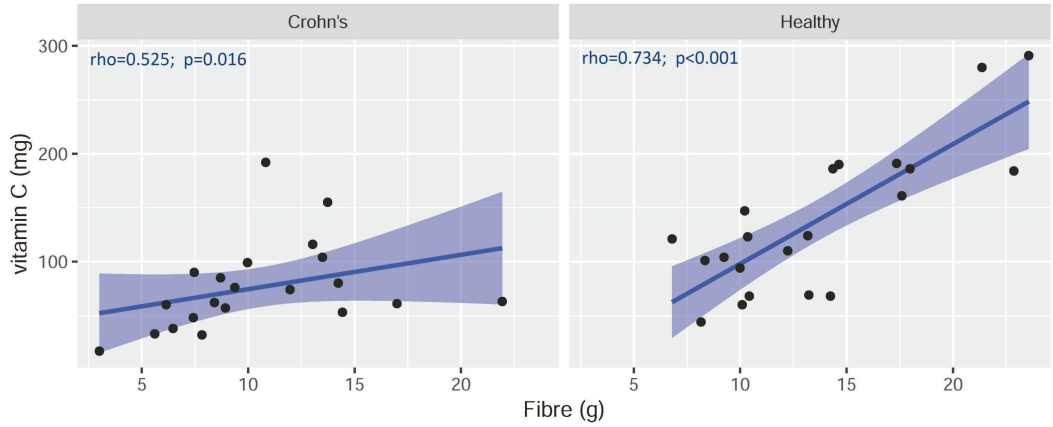


Figure 7.3: Relationship between fibre and vitamin C intake from FFQs in children with Crohn's disease and healthy children at baseline. Shaded area shows the 95% confidence interval Spearman rank.

Table 7.3: Estimated dietary intake (FFQ) at baseline

Dietary component	Crohn's (n=22)	UC (n=6)	non-IBD (n=5)	healthy (n=21)	*p-value* $KW \star$
Macro-nutrients					
Water	789 (305)	635.28 (272)	843 (155)	988 (430)	p=0.27
Energy (kcal)	1488 (480)	1276 (651)	1273 (231)	1643 (633)	p=0.24
Carbohydrate (g)	211 (80)	155 (53)	167 (16)	221 (67)	p=0.41
Protein (g)	56.8 (16.6)	51.3 (30.1)	46.9 (13.7)	59.1 (15.2)	p=0.32
Fat (g)	52.3 (16.3)	53.2 (21.4)	47.7 (13.5)	59.9 (20.1)	p=0.11
SFA (g)	23.0 (9.0)	23.2 (8.9)	21.4 (5.2)	27.8 (13.4)	p=0.16
MUFA (g)	17.8 (5.6)	18.3 (6.2)	16.1 (4.8)	20.5 (8.6)	p=0.13
PUFA (g)	8.2 (2.3)	6.9 (4.0)	6.18 (1.7)	9.0 (3.0)	p=0.06
Choline (mg)	155 (37.8)	161 (89.7)	153 (46.0)	161 (84.5)	p=0.73
Sugars (g)	86.0 (42.3)	70.7(38.6)	95.4 (10.9)	105.9 (48.6)	p=0.33
Starch (g)	108.5 (39.3)	103.5 (48.2)	104 (28.2)	101 (40.0)	p=0.68
Fibre (g)	***9.15** (4.08)	***7.93** (3.55)	8.18 (2.15)	13.18 (4.72)	**p=0.02**
Micro-nutrients					
Na (mg)	2131 (881)	2187 (755)	2004 (212.01)	2206 (1142)	p=0.44
Cl (mg)	3203 (1142)	3203 (1230)	3088 (426)	3499 (1401)	p=0.42
K (mg)	2176 (912)	1668 (338)	1771 (80)	2476 (1036)	p=0.06
Ca (mg)	932 (486)	577 (218)	822 (136)	926 (216)	p=0.50
Mg (mg)	207 (92)	150 (51)	169 (28)	227 (79)	p=0.12
P (mg)	1105 (504)	826 (432)	916 (227)	1140 (399)	p=0.23
Fe (mg)	8 (3)	7 (3)	7 (2)	10 (5)	p=0.31
Cu (mg)	0.9 (0.3)	0.6 (0.3)	0.8 (0.2)	0.8 (0.3)	p=0.20
Zn (mg)	6.7 (2.6)	5.7 (3.1)	5.6 (1.7)	7.0 (2.0)	p=0.30
Mn (mg)	2.0 (0.7)	1.5 (0.7)	1.7 (0.4)	2.4 (0.9)	p=0.08
Se (ug)	28 (9.6)	28 (11.1)	22 (1.5)	31 (11.9)	p=0.63
I (ug)	168 (75)	132 (79)	119 (13)	181 (39)	p=0.21
Vit A retinol (ug)	****212** (108)	256 (145)	193 (40)	309 (156)	**p=0.02**
Carotene (ug)	1271 (1086)	1300 (604)	1391 (589)	1600 (1130)	p=0.14
Vit D (ug)	1.4 (0.7)	1.2 (0.6)	1.2 (0.4)	1.6 (0.8)	p=0.25
Vit E (mg)	***5.5** (1.4)	4.8 (2.5)	*4.3 (1.2)	6.5 (2.3)	**p=0.01**
Vit B_1 thiamine (mg)	1.3 (0.5)	1.2 (0.6)	1.1 (0.1)	1.3 (0.6)	p=0.66
Vit B_2 riboflavin (mg)	2.0 (1.3)	1.4 (0.6)	1.6 (0.4)	1.9 (0.9)	p=0.76
Vit B_3 niacin (mg)	17.6 (8.9)	14.2 (5.9)	11.9 (1.5)	14.0 (5.3)	p=0.69
Vit B_5 pan. acid (mg)	5.3 (2.2)	3.5 (1.6)	3.7 (0.3)	5.2 (1.8)	p=0.42
Vit B_6 (mg)	1.7 (0.7)	1.2 (0.3)	1.3 (0.1)	1.4 (0.5)	p=0.79
Vit B_7 biotin (ug)	***24** (12)	14 (14)	21 (6)	30 (11)	**p=0.05**
Vit B_9 (ug)	238 (135)	237 (146)	200 (37)	244 (130)	p=0.62
Vit B_{12} (ug)	5.0 (1.4)	3.1 (1.9)	3.8 (1.3)	5.3 (2.1)	p=0.47
Vit C (ug)	*****62.5** (34.8)	80.5 (36.3)	96.0 (35.6)	123.0 (81.5)	**p<0.001**

Median (IQR) are shown. Dunn's test of multiple comparisons following a significant $\star$ Kruskal-Wallis (KW) test was used. Difference from healthy children is significant at * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$.

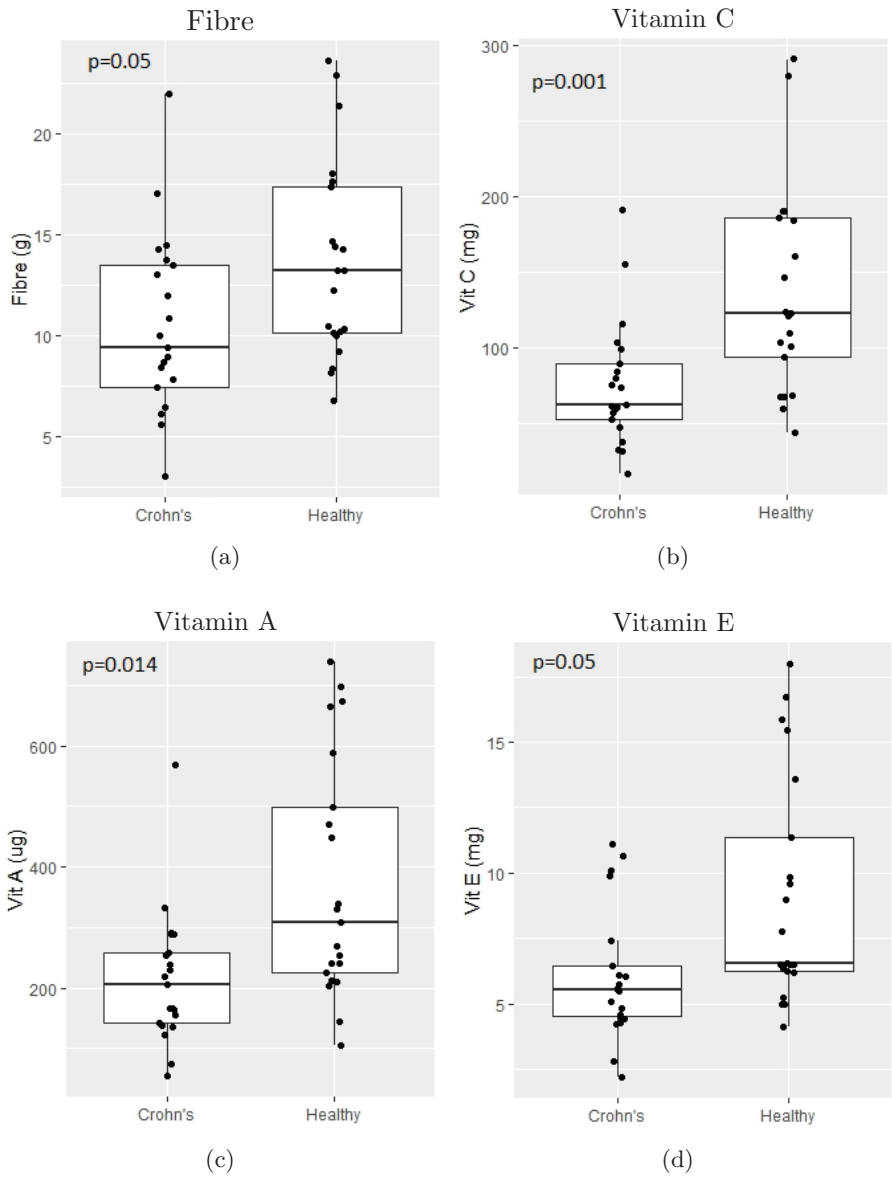


Figure 7.4: The key differences between children with Crohn's disease and healthy children, in estimated daily dietary intake based on FFQ questionnaires at baseline for (a) fibre; (b) vitamin C (ascorbic acid); (c) vitamin A (retinol) and (d) vitamin E. Dunn's test of multiple comparisons following a significant Kruskal-Wallis test was used.

7.3.2 Dietary intake and gender

A metric distance scaling plot (MDS) was used create profiles of macronutrient intake at baseline, based on estimated diet from FFQs, to look for any differences between boys and girls with Crohn's disease. No difference was seen between baseline dietary profiles based on gender (Fig. 7.5).

7.3.3 Diet at baseline as a predictor of response to EEN

No relationship was seen between energy intake at baseline in children with Crohn's disease and disease severity measured using the weighted paediatric Crohn's disease

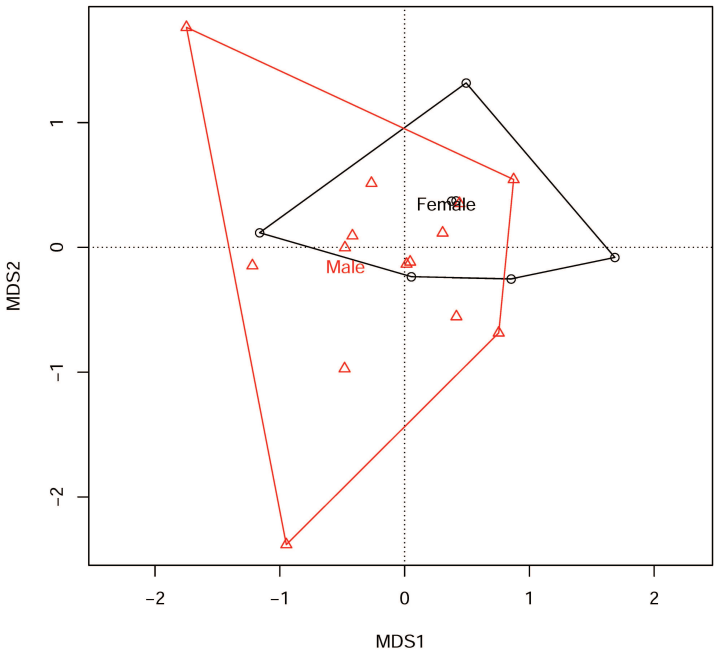
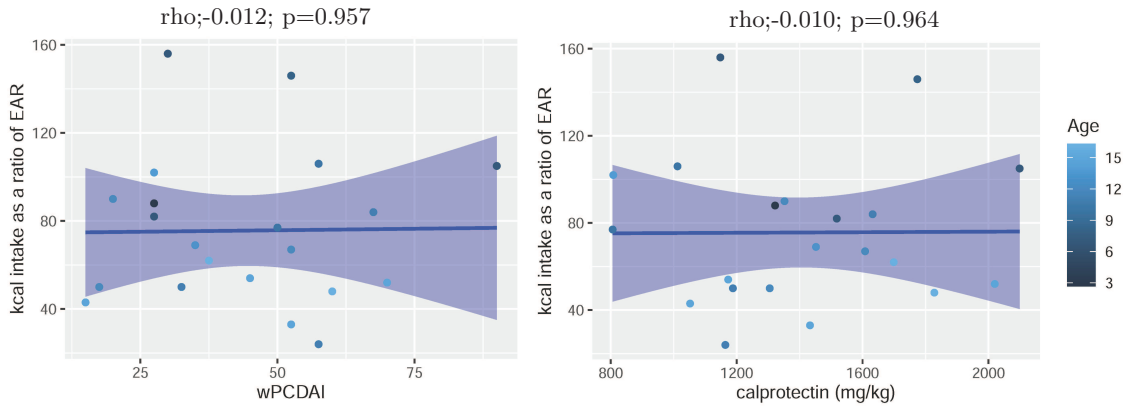


Figure 7.5: Estimated baseline macronutrients as estimated from FFQs, in children with Crohn's disease split by gender. No gender difference is seen between baseline dietary profiles. Metric distance scaling plot (MDS), PERMANOVA: R2=0.092; p=0.142

activity index (wPCDAI) score (Fig. 7.6). Thus reduced energy intake at baseline did not predict disease activity. The composition of diet at baseline did not predict which children would respond to treatment with EEN (Table 7.4; Fig. 7.7).



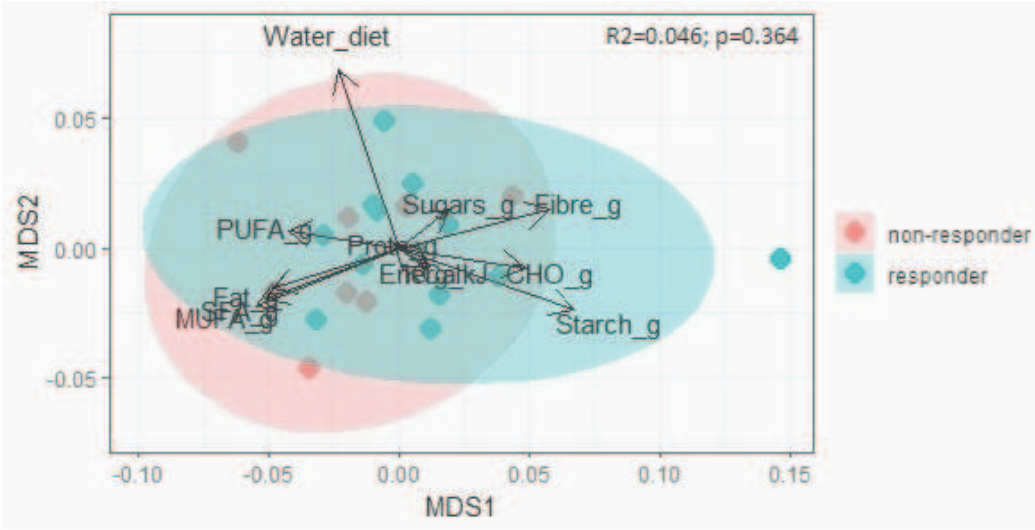
(a) (b)

Figure 7.6: Relationship between energy intake (kcal) at baseline, expressed as a percentage of estimated average requirement (EAR) to correct for age and gender, and disease activity measured using (a) the weighted paediatric Crohn's disease activity index (wPCDAI) and (b) faecal calprotectin in children with Crohn's disease. Spearman: no correlation seen.

Table 7.4: Estimated dietary intake from food frequency questionnaires (FFQ) at baseline in non-responders and responders to EEN, based on dietary components which were significantly reduced in children with Crohn's disease.

	Children with Crohn's disease		
Dietary component *	non-responder (n=8)	responder (n=13)	MWU test
Fibre (g)	9.2 (6.6)	9.4 (5.2)	p=0.915
Vitamin A retinol (ug)	193 (96.2)	206 (122)	p=0.456
Vitamin E (mg)	5.6 (2.5)	5.5 (1.9)	p=0.645
Vitamin B_7 biotin (ug)	22.7 (12.5)	24.5 (13.5)	p=0.972
Vitamin C (mg)	61.5 (34)	74 (37)	p=0.972

Results are shown as the median (IQR). Significance of difference at baseline between non-responders and responders to treatment with EEN calculated using Mann-Whitney. * Dietary components shown were all significantly reduced in children with Crohn's compared with healthy children.



(a)

Figure 7.7: Diet at baseline as a predictor of response to EEN in children with Crohn's disease: for estimated daily dietary intake based on FFQ questionnaires for macronutrients. No difference is seen in dietary profiles between responders and non-responders; Metric distance scaling plot (MDS) PERMANOVA.

7.3.4 Dietary intake during treatment with EEN

Twenty-three (72%) children with Crohn's disease took EEN as 5-6 ∼300ml shakes per day orally and nine (28%) children via nasogastric tube (NG-tube). Children opted to use a NG-tube if they felt unable to tolerate the taste after being given samples to try. Two of the nine children who took EEN via NG-tube began taking EEN orally, but switched to NG-tube within the first week. Although children who used an NG-tube were less likely to fail EEN, the difference was not significant (χ^2 p=0.18) (Chapter 3; Table 3.13) Of the thirty-two children with Crohn's disease who were treated with EEN there was no difference in time to relapse between children who took EEN via NG-tube and those who took it orally (Fig. 7.8)

Children who responded well to the treatment were kept on EEN for 8-weeks to induce

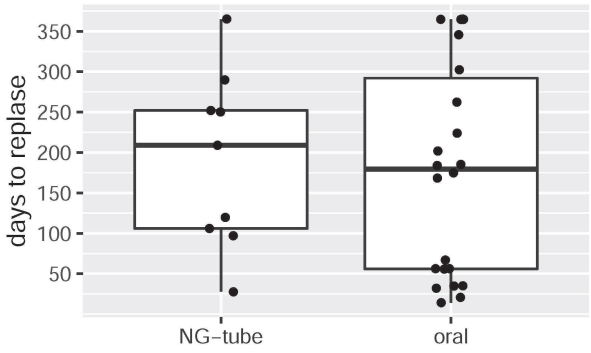


Figure 7.8: Number of days from start of treatment with EEN to disease relapse in children with Crohn's disease who took the feed orally (oral) and those who took EEN via nasogastric tube (NG-tube). (No difference between groups).

remission. Three children who did not respond to EEN managed to stay on EEN for the full 8-weeks. Two children who failed to respond were on EEN for 6-weeks and another two children stopped at around 4-weeks treatment. Two children, who were clear non-responders to treatment, stopped EEN after only 1-week. Children who failed treatment with EEN were treated with corticosteroids.

To get an understanding of how EEN is nutritionally different from the child's baseline diet, the estimated daily food intake (FFQ) for each child with Crohn's disease, was compared with their 8-week prescribed intake of EEN (composition of Modulen given in appendix 7.2). The first major difference was that Modulen as a liquid feed increased the water content of the child's diet (Table 7.5). The proportions of macronutrients as a ratio of total energy intake on EEN are similar to the estimated baseline diet of children with Crohn's disease, but with a reduced ratio of carbohydrate (57%) intake compared with baseline (66%), and a higher ratio of fat (24%) compared with baseline diet (16%). The composition of fatty acids was different on EEN, with a higher proportion of saturated fatty acid on EEN (67%) compared with estimated baseline diet (46%).

Modulen is deliberately given as a low residue diet and thus has no fibre content. The amount of sugar is also reduced on EEN suggesting the median reduced from 86g on baseline diet to 66g of sugar on EEN, although sugar at baseline was highly variable (Table 7.5). In terms of micronutrients sodium was greatly reduced on EEN from an estimate of 2,131mg (IQR 881) at baseline to 748mg (IQR 101) on EEN, while all other micronutrients were increased on EEN. All vitamins recorded were also increased on EEN when compared with the estimate of baseline diet from FFQs (Table 7.5).

Table 7.5: Differences between estimated dietary intake from food frequency questionnaires (FFQ) and known intake at 4-weeks EEN (Modulen) given as median (IQR)

Dietary component	wk-0 (baseline) (n=12)	wk-4 (EEN) (n=12)	Wilcoxon paired test
Macro-nutrients			
Water	749 (395)	1870 (340)	**p<0.001**
Energy (kcal)	1370 (698)	2200 (400)	**p=0.003**
Carbohydrate (g)	188 (95)	238 (43)	p=0.077
Protein (g)	55 (24)	79 (14)	**p=0.002**
Fat (g)	52 (20)	101 (18)	**p<0.001**
SFA (g)	20 (11)	57 (10)	**p<0.001**
MUFA (g)	18 (6.2)	17 (3.1)	p=0.970
PUFA (g)	8.0 (2.8)	11 (1.9)	**p=0.034**
Choline (mg)	150 (50)	154 (28)	p=0.388
Sugars (g)	70 (51)	66 (12)	p=0.380
Fibre (g)	8.8 (5.0)	0 (0)	**p<0.001**
Micro-nutrients			
Na (mg)	2177 (959)	748 (136)	**p<0.001**
Cl (mg)	3306 (1455)	1606 (292)	**p=0.002**
K (mg)	1926 (848)	2640 (480)	**p<0.001**
Ca (mg)	712 (568)	1958 (356)	**p<0.001**
Mg (mg)	181 (89)	440 (80)	**p<0.001**
P (mg)	933 (516)	1320 (240)	**p<0.001**
Fe (mg)	7.5 (4.0)	24 (4.3)	**p<0.001**
Cu (mg)	0.8 (0.3)	2.2 (0.4)	**p=0.003**
Zn (mg)	6.6 (3.2)	21 (3.8)	**p<0.001**
Mn (mg)	2.0 (1.0)	4.3 (0.8)	**p<0.001**
Se (ug)	29 (12)	75 (14)	**p<0.001**
I (ug)	149 (113)	216 (39)	**p=0.002**
Vitamin A retinol (ug)	164 (92)	1804 (328)	**p<0.001**
Vitamin D (ug)	1.5 (1.0)	22 (3.9)	**p<0.001**
Vitamin E (mg)	5.3 (1.5)	29 (5.2)	**p<0.001**
Vitamin B_1 thiamine (mg)	1.2 (0.5)	2.6 (0.5)	**p<0.001**
Vitamin B_2 riboflavin (mg)	1.7 (1.2)	2.8 (0.5)	**p<0.001**
Vitamin B_3 niacin (mg)	15.7 (8.1)	25.5 (4.6)	**p<0.001**
Vitamin B_5 pantothenic acid (mg)	4.3 (3.2)	10.6 (1.9)	**p<0.001**
Vitamin B_6 (mg)	1.4 (0.8)	3.6 (0.7)	**p<0.001**
Vitamin B_7 biotin (ug)	23 (14)	70 (13)	**p<0.001**
Vitamin B_9 (ug)	198 (133)	528 (96)	**p<0.001**
Vitamin B_{12} (ug)	3.9 (3.0)	7.0 (1.3)	**p<0.001**
Vitamin C (mg)	61 (30)	207 (38)	**p<0.001**

Significance of difference from baseline and 4-weeks EEN calculated using Wilcoxon paired test. Unpaired data from all time-points are shown in appendix 7.3.

7.3.5 Dietary intake post-EEN

Paired data showing the estimated dietary intake at baseline and again at 2-weeks post-EEN (study week-10) for patients with Crohn's disease, shows a median energy increase which was not statistically significant (Table 7.6).

Table 7.6: Differences between estimated dietary intake from food frequency questionnaires (FFQ) at baseline (week-0) and 2-weeks post-EEN (week-10)

Dietary component	wk-0 (n=9)	wk-10 (n=9)	Wilcoxon paired test
Macro-nutrients			
Water	739 (277)	800 (391)	p=0.203
Energy (kcal)	1210 (475)	1456 (385)	p=0.359
Carbohydrate (g)	172 (58)	191 (75)	p=0.426
Protein (g)	49.7 (20.7)	51.7 (26.7)	p=0.496
Fat (g)	46.6 (17.2)	54.6 (7.6)	p=0.496
SFA (g)	17.1 (9.3)	22.4 (4.5)	p=0.301
MUFA (g)	17.2 (4.8)	18.4 (2.0)	p=0.652
PUFA (g)	7.1 (2.1)	7.8 (1.0)	p=0.820
Choline (mg)	148 (58)	129 (113)	p=0.203
Sugars (g)	67.9 (39.2)	93.9 (30.1)	p=0.203
Fibre (g)	8.7 (2.0)	10.0 (4.4)	p=0.652
Micro-nutrients			
Na (mg)	1898 (1033)	1657 (607)	p=0.820
Cl (mg)	2886 (1636)	2554 (1017)	p=0.910
K (mg)	1607 (766)	2140 (856)	p=0.098
Ca (mg)	616 (456)	707 (271)	p=0.203
Mg (mg)	157 (73)	169 (80)	p=0.213
P (mg)	751 (430)	958 (415)	p=0.203
Fe (mg)	7.0 (2.4)	6.9 (1.8)	p=0.426
Cu (mg)	0.7 (0.2)	0.7 (0.2)	p=0.820
Zn (mg)	6.0 (2.7)	5.8 (2.6)	p=0.734
Mn (mg)	1.8 (1.0)	1.9 (1.1)	p=0.734
Se (ug)	26 (16)	28 (16)	p=0.779
I (ug)	115 (116)	168 (53)	p=0.098
Vitamin A retinol (ug)	163 (71)	214 (53)	p=0.039
Vitamin D (ug)	1.4 (0.9)	1.3 (0.7)	p=0.820
Vitamin E (mg)	5.1 (1.6)	6.2 (2.0)	p=0.496
Vitamin B_1 thiamine (mg)	1.1 (0.5)	1.1 (0.4)	p=0.820
Vitamin B_2 riboflavin (mg)	1.4 (1.3)	1.5 (0.2)	p=0.359
Vitamin B_3 niacin (mg)	13.7 (9.3)	12.9 (7.3)	p=0.820
Vitamin B_5 pantothenic acid (mg)	3.3 (2.9)	4.1 (2.0)	p=0.426
Vitamin B_6 (mg)	1.2 (0.8)	1.4 (0.6)	p=0.734
Vitamin B_7 biotin (ug)	21.6 (11.1)	20.2 (10.3)	p=0.129
Vitamin B_9 (ug)	195 (59)	209 (111)	p=0.250
Vitamin B_{12} (ug)	3.0 (2.3)	3.6 (1.0)	p=0.570
Vitamin C (mg)	74 (28)	68 (59)	p=0.440

Results are given as median and (IQR). Difference from baseline is significant at $p \leq 0.05$. Study week-10 represents 2-weeks of normal habitual diet post-EEN. Unpaired data from all time-points is shown in appendix 7.3.

Seven children who went into remission on EEN and completed an FFQ at both baseline and 2-weeks post EEN (study week-10) are shown in figure 7.9. At 2-months post EEN patient 036 had relapsed; by 8-months post EEN patients 031, 038 and 041 had also relapsed and gone onto treatment with steroids. Three patients 015, 016 and 055 were still in remission at the end of the study (11-months post-EEN).

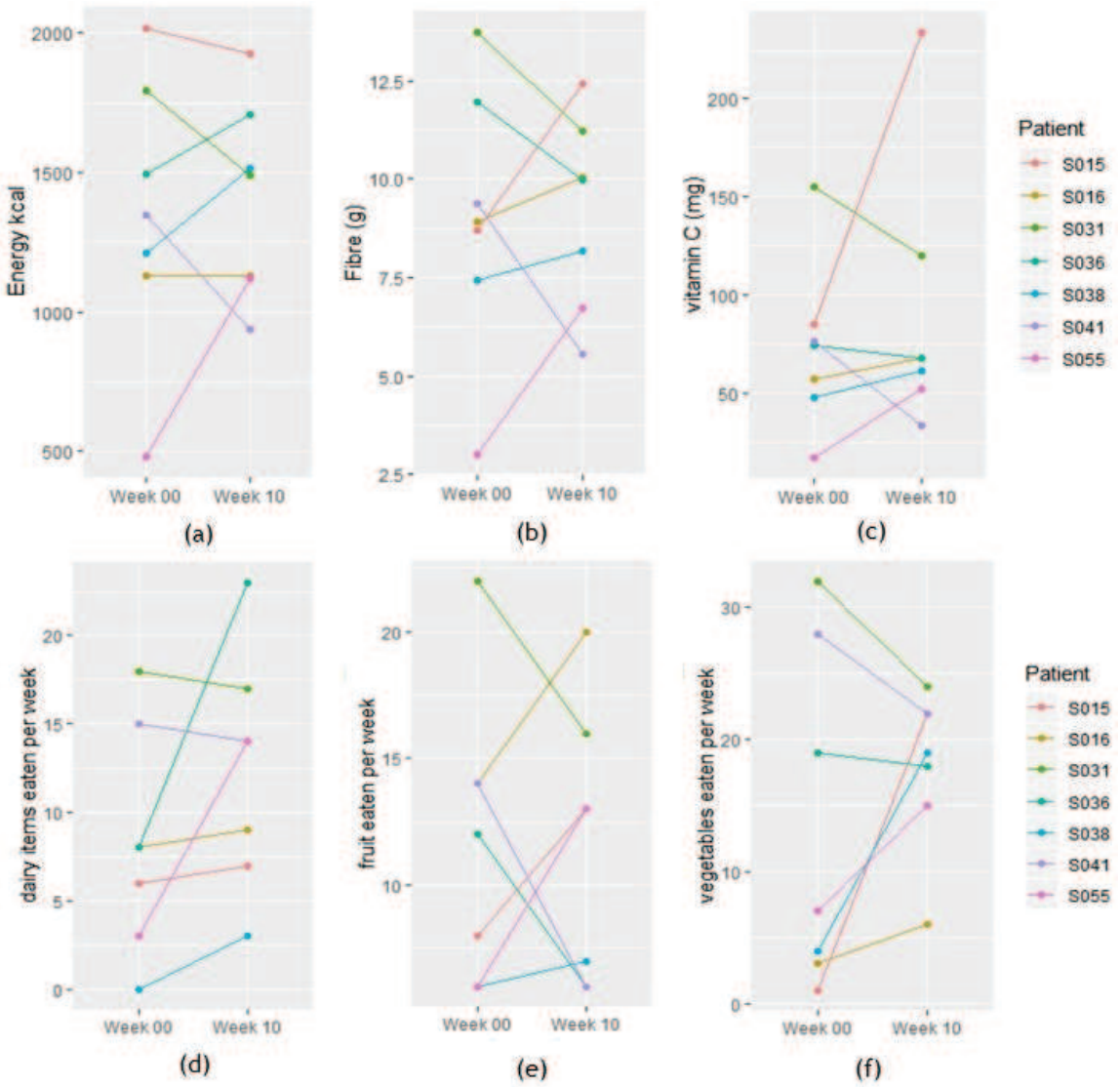


Figure 7.9: Changes in estimated diet at baseline (wk-0) and post-EEN (wk-10) for estimated dietary intake based on FFQ questionnaires for: (a) energy (kcal); (b) fibre intake; (c) vitamin C intake; (d) dairy intake; (e) fruit intake, and (f) vegetable intake.

7.3.6 Linking dietary intake with gut microbiota

At genus level taxonomy, differences in the correlation between children with Crohn's disease and healthy children were seen for macronutrient intake at baseline (Fig. 7.10): including fat (particularly PUFA); protein and fibre. Five genera, including three Firmicutes, a Proteobacteria and an Archaea genus were significant between children with Crohn's disease and healthy children ($p<0.05$).

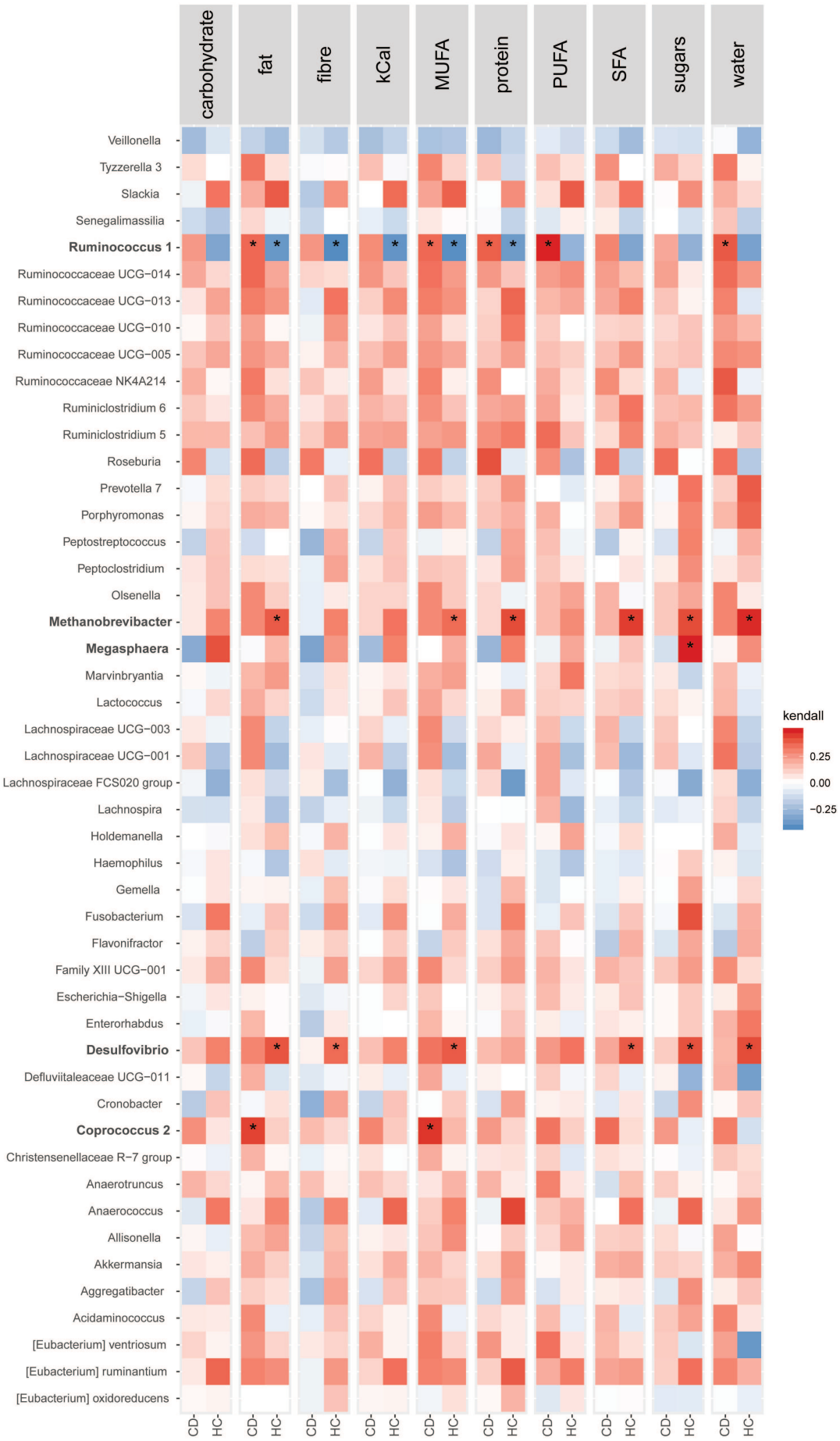


Figure 7.10: Correlation between macronutrient dietary intake (FFQ) and gut microbiota genera in children with Crohn's disease (CD) and healthy children (HC) at baseline. Kendall rank correlation significant at * $p \leq 0.05$ (Multiple comparison - Benjamini-Hochberg).

7.4 Discussion

7.4.1 Summary of results

Compared with healthy children, the composition of dietary intake at baseline in children with Crohn's disease had significantly less estimated intake of vitamin A, E, B_7 and C. Fibre intake was also significantly reduced in both children with Crohn's disease and UC. No differences in macronutrient intake were seen between boys and girls with Crohn's disease to explain the increased incidence in boys.

Neither energy intake or composition of diet at baseline predicted which children would respond to treatment with EEN. As known, fibre intake is reduce to zero on EEN. However due to EEN being fortified, all vitamins and micronutrients (except NaCl), along with energy and water intake were significantly increased during EEN, making many aspects of the diet compositionally different from their baseline diet. Although as a group no significant differences were seen in dietary intake between children with Crohn's disease at baseline and 2-weeks post EEN; three of these children decreased their fibre, fruit and vegetable intake at 2-weeks post EEN (one relapsing at 6-weeks and two relapsing at 8-months post-EEN). Four children increased their fibre, fruit and vegetable intake at 2-weeks post EEN (three of these were still in remission at 11-months post-EEN). Hence all children made alterations to their baseline diet at 2-weeks post-EEN.

In the current study, diet at baseline was significantly associated with five genera, including three Firmicutes, a Proteobacteria and an Archaea genus. *Ruminococcus 1* showed a positive correlation in healthy children but a negative correlation in children with Crohn's disease, which was significant for fat and protein. Of five *Ruminococcus 1* species (SNVs) which showed this correlation pattern, only two abundant species showed a significant correlation (data not shown). Isolating strains of *Ruminococcus* from Crohn's disease patients, to look at how nutrition *in vivo* affects behavioural changes in fermentation capacity in these strains, might help to explain these differences.

7.4.2 Differences in dietary intake between Crohn's and healthy children

A 2011 systematic review looking at dietary risk factors concluded that total fats, PUFAs, omega-6 fatty acids, and meat were linked with an increased risk of IBD.[68] The review concluded that increased fibre and fruit intake reduced the risk of Crohn's disease. Although the FFQ might be considered a blunt tool, the current study findings support the review, in that, children with Crohn's disease had significantly lower intake of fibre and vitamin C (Table 7.3). Data from the current study suggests children with IBD have a reduced intake of vitamins including vitamin A, E, B_7 and C, when compared with healthy children. Given malabsorption is a key feature

of Crohn's disease and UC, low intake of key vitamins could have negative effect on inflammation. Increased levels of vitamin E has been shown in mouse models, to modulate neutrophil migration in the mucosa of the lung, increasing polymorphonuclear leukocyte elastase activity, boosting antimicrobial defence against pneumococci.[589] Vitamin A (retinol) deficiency has been associated with an increase in infectious disease.[590] Retinol imprints the homing of leukocytes to the gut enhancing induction of T_{reg} cells,[591] and is a dominant mediator of $CD4^+$ T-cell immunity and homoeostasis. It is also known Th1 and Th17 cell immunity are dependant on metabolites of retinol.[592] It has been shown that vitamin deficiency for serum retinol and vitamin E is significantly more prevalent in Crohn's disease patients who had active disease.[593]

Vitamin D It has been proposed that increased incidence of Crohn's disease in northern Europe is due to lack of sunlight, resulting in loss of vitamin D_3.[594] Although in the current study there was no significant difference in estimated dietary vitamin D intake between groups, the estimated daily intake for all children including healthy children at around 1.6µg was well below the recommended daily intake of 10µg.

Vitamin D is a hormone which has a diverse range of effects such as increased dendritic cell function, autophagy and expression of the Crohn's disease associated gene NOD2 which is involved in bacterial sensing. Vitamin D deficiency is starting to be recognised as a potential driver of IBD.[595] *In vitro* studies have looked at the role of vitamin D in macrophages and mouse models of IBD.[596;597;598] A 2010 study found vitamin D deficiency left mice prone to colitis via dysregulation of colonic antimicrobial activity and impaired bacterial homoeostasis.[599] Deficiency also has an effect by reducing the expression of epithelial tight junction proteins,[600] and elevating Th1-driven inflammation in the gut[601] A 2015 meta-analysis showed that of 938 patients with IBD 64% were more likely to be vitamin D deficient compared to 953 healthy controls (OR=1.64; 95% CI: 1.30, 2.08; I^2=7%; p<0.0001). This study also found no association between IBD and vitamin D deficiency and latitude (p=0.34).[602] The study does have some limitations as the odds ratios came from dichotomous (categorical) data in many of the studies, and confounding factors such as diet were not recorded. It is therefore unclear whether vitamin D deficiency in patients with IBD is a result of disease related malabsorption caused by damage to the mucosa,[603] or whether it might be a contributing factor to disease onset and progression.[604;605]

Although supplementation with vitamin D_3 has a potential therapeutic impact on the course of Crohn's disease,[594] lack of sunlight and vitamin D_3 alone cannot explain the marked increase in incidence over time.[594] Therefore, large randomised control trials administering variable doses of vitamin D supplementation with data on IBD type, disease location and disease activity would help us understand the potential therapeutic significance of vitamin D, but further work using animal models are needed to uncover the mechanisms in terms of both immunology and the gut microbiota.

Vitamin C Although vitamin C does not play a direct role in immune function, it plays a role in redox recycling of other important antioxidants such as regenerating vitamin E from its oxidised form.[606] Vitamin C also increases the bioavailability of iron from ingested food by increasing the gut absorption of non-heme iron.[607] A negative correlation between vitamin C intake and risk of Crohn's[545] and risk of UC has been shown.[546] Since vitamin C intake is linked with the intake of fruit and vegetable fibre it is difficult to separate vitamin C from fibre as a risk factor. A recent Chinese study using a colitis mouse model has shown that vitamin C reduced inflammation, by blocking pro-inflammatory cytokines, and increasing tight-junction proteins occludin and ZO-1.[608] The study also reported that vitamin C promoted the production of SCFA in the gut, as well as playing a role in regulating the microbiota.[608] It is interesting in the current study that six parents of children with IBD were well aware of poor fruit and vegetable intake, with one family reporting their child refused to eat any type of fruit. Therefore it might be useful for future studies to explore any potential anti-inflammatory properties of vitamin C, in children with Crohn's.

Zinc undernutrition has also been shown to impair mediators of innate immunity including natural-killer cell activity.[609] Overall undernutrition could have implications in helping to maintain gut inflammation in children with Crohn's disease especially where malabsorption and diarrhoea are key symptoms.

Fibre With increasing evidence that IBD is linked to a dysbiosis of the gut microbiota, there is growing interest in whether diet can positively influence disease activity. Using dietary fibres as pre-biotics, such as inulin-type fructans and arabinoxylan-oligosaccharides, have been shown to increase numbers of *Bifidobacteria* in the gut.[610;523] *Bifidobacteria* have been shown to be important in the gut by degrading non-digestible carbohydrates; providing protection against pathogens; synthesising B vitamins; conjugating linoleic acids and stimulate the immune system.[611] Increasing dietary fibre has also been shown to increase butyrate in the human colon which as the preferred energy source for the colonocytes, maintains gut barrier functions as well as having immunomodulatory and anti-inflammatory effects. It has been shown the butyrogenic effects of dietary fibre such as fructans and oligosaccharides, are due to cross-feeding interactions between *Bifidobacteria* and butyrate-producing colon bacteria, such as *Faecalibacterium prausnitzii* (clostridial cluster IV); *Anaerostipes*, *Eubacterium* and *Roseburia* species (clostridial cluster XIVa).[523] These cross-feeding interactions could favour the co-existence of other beneficial bacterial strains and butyrate-producing bacteria in the colon, leading to increased diversity and a more stable microbiome.

It is therefore interesting to see a significantly reduced dietary fibre in children with IBD compared with healthy children in the current study. These findings support the evidence that dietary fibre is a risk factor for IBD.[547;540] Murine studies using soluble fibres and resistant starch;[561] pectin;[562] guar gum;[563] and a multi-fibre mix,[564] all

show fibre to have anti-inflammatory effects. There is a great deal of controversy around whether fibre is beneficial to patients with Crohn's disease, given EEN which is used to induce remission contains no fibre, and is proposed to work by depriving the colonic bacteria of a food source.

7.4.3 Gender and food choices

Although there is worldwide evidence showing variation in male/female ratios in Crohn's disease in Asia,[90;91;92;93] across Europe and North-America,[94] which might suggest that boys have a higher risk of developing Crohn's disease in childhood/adolescence than girls, it has not been demonstrated this is a true gender difference based on biological differences. In adult populations studies including North America and Western Europe suggest females have a higher incidence of Crohn's disease, where Asian studies mostly see higher rates in adult males.[612] Hence it is unlikely that incidence of Crohn's disease is linked with gender differences in genetic risk profiles. However, variations in gender risk could be linked with cultural differences in gender roles, resulting in exposure to different environmental risk factors such as dietary intake. The gender difference in Scotland, inline with other developed countries is rising.[84] The fact that gender ratio is changing over time suggests exposure to the risk factors is unequal between boys and girls, thus it makes sense to ask whether differences in diet could provide clues as to why boys seem to be at higher risk of IBD and particularly Crohn's disease.[84]

The current study is the first in Scotland to compare the dietary intake of boys and girls with Crohn's disease. No difference in macronutrient profile was seen between boys and girls (Fig. 7.5), however the current study had a low sample size, especially girls (n=7). The FFQ is also an estimate of diet and thus a more detailed dietary assessment such as the 5-step multi-pass dietary record conducted on a larger samples size may show differences not seen in the current study.

As discussed in Chapter 1; section 1.7, a gender bias in incidence could be due to behavioural differences in reporting symptoms, as well as differences in clinical referral and diagnosis.[95] However if this were the case, we would expect to see baseline gender differences in disease severity, as well as differences in SDS anthropometric measures at baseline. However the current study found no difference in disease activity or SDS-weight, SDS-height or SDS-BMI at baseline between boys and girls (Fig. 3.8). Identifying any gender-specific dietary differences between children with Crohn's disease and healthy children are important, because combined analysis has the potential to mask dietary associations which might be more distinct in one gender than the other.

7.4.4 Diet as a predictor of response to EEN

The current study showed compositional gut bacteria differences at baseline between responders and non-responders to treatment with EEN, and therefore it was predicted children who failed EEN would have a diet compositionally different from children who responded to treatment with EEN. However estimated dietary intake from FFQ data in the current study, showed no differences between responders and non-responders for fibre, vitamin A, vitamin B7 vitamin E, vitamin C, or energy intake (dietary components that were significantly different between Crohn's disease and healthy children). However only eight non-responders returned in an FFQ, and four of these were girls, hence low sample size and possible gender bias means further study is required before rejecting the hypothesis that dietary intake at diagnosis could predict response to EEN. Further studies which include higher numbers of patients, detailed dietary records such as the five-step multi-pass, as well as separate gender analysis, would help to answer this question.

7.4.5 The composition of EEN and its anti-inflammatory effects

The current study shows dietary intake at baseline in children with Crohn's disease is compositionally different from EEN (Modulen) in a number of ways (Table 7.5). EEN is the primary therapy used across Europe to induce disease remission as well as promote optimum growth,[613;125] and to date Crohn's disease studies into EEN have focused on the fact it contains no fibre, with an assumption that lack of fibre starves gut bacteria in the colon leading to mucosal healing.[567] There are a number of ways that different components of EEN could decrease inflammation in the gut (Table 7.7)

Table 7.7: EEN - Potential modes of action

Reduced exposure to gut microbiota
- lack of fibre leads to starvation of colonic bacteria[144]
Increased exposure to protective compounds
- caseins (binding with potential antigens)[614;615;616]
- micronutrients (especially iron, zinc)[617;609]
- vitamins (especially Vit A, D, C, E)[618]
Reduced exposure to deleterious compounds
- food triggers (wheat, gluten, dairy, lactose, emulsifiers, sulphites and others)[619]
- salt, high intake:
• stimulates gut TH17 response and exacerbates colitis in mice*[620;621;622;566]
• damages colonic mucosa via reduction in goblet calls in mice*
• increased gut permeability*[620]
• changed gut bacteria profile in mice[623]

* Indirect effect could be due to changes in gut bacteria.

Reduced exposure to gut microbiota Twenty-three dietary fibre intervention studies on IBD adult patients (6 single-arm & 17 placebo-controlled) have tested different fibres including: fructans; psyllium; oat bran; wheat bran and germinated barley fibres.[624] Studies looking at the effect of fructans concluded that supplementation could reduce symptoms; improve gut immune function leading to a reduction in gut inflammation via modulation of the gut microbiota in IBD patients.[624] However other types of fibre had mixed results, which is perhaps not surprising, since dietary fibre constitutes a varied group of chemicals which have different properties.[624] On one hand reduced fibre is linked with risk for IBD and fibre intervention studies support this finding by suggesting increased fibre may help to reduce gastrointestinal symptoms;[619] however contrary to this, EEN a zero fibre diet, is shown to induce remission in children with Crohn's disease. This is perhaps not surprising if the gut bacteria are the intermediary which drive increased inflammation. Low fibre diets could be altering both cross-feeding between bacteria as well as altering genetic expression of some bacteria causing them to switch to feeding on mucosal glycans which in turn damage the gut mucosa. A zero fibre diet reduces colonic bacteria to such low levels, that any negative effects from cross-feeding or mucosal degradation are prevented, until the normal low fibre diet is resumed. If the reduction in fermentation leads to a reduction in toxic metabolites such as 1-propanol, 1-butanol and methyl/ethyl esters of SCFA,[488] this might also explain why EEN leads to mucosal healing. The current study shows how quickly after return to normal habitual diet the inflammatory marker calprotectin increases (Fig. 6.5d),[625] suggesting benefits from EEN are short lived.

In the current study the number of bacterial species reduced on EEN, and hence it is important to know which groups of commensal bacteria are reduced during EEN in order to understand how these changes might lead to reduced inflammation. Future metagenomic studies could help identify which bacterial genes might be triggering the host inflammatory response.

A North American study[540] has shown a reduced risk of Crohn's disease in diets which included fruit-based fibre but no protective effect from grain, cereals and legume fibre. The protective effect is thought to come from the SCFA derived from the fermentation process by gut bacteria, inhibiting the transcription of pro-inflammatory mediators.[626] Fibre also helps to maintain gut barrier integrity as well as reduce the translocation of pathogens.[569] Depriving the gut bacteria of fibre in murine models has been shown to deplete mucosal thickness causing inflammation and tissue damage.[571]

Increased exposure to protective compounds Although the assumption that lack of fibre in EEN drives the reduction in inflammation, particularly as low-fibre diets are a risk factor for Crohn's disease,[540] the current study shows there are a number of other nutritional components in EEN, which are significantly different from habitual

diet. These could also play a role in regulating the inflammatory response, thus fibre which is associated with other nutrients could be a confounding factor. On EEN, mineral and vitamin intake is increased by 2-fold for most micronutrients; vitamin C is increased 3-fold; vitamin E 5-fold; while vitamins A and E are increased 10-fold. As mentioned previously vitamin D is a hormone which controls expression of the NOD2 gene, involved in bacterial sensing, hence an increase could have the potential to improve bacterial sensing in the gut. Increased vitamin D has also been shown to protect mice from colitis,[599] as well improve the integrity of tight-junctions in the gut.[600] Hence in the current study, it is possible the increase in vitamin D from ∼1.6µg pre-EEN to 21.6µg during EEN could play some role in mucosal healing.

Vitamin C plays a role in redox recycling of other important antioxidants such as regenerating vitamin E from its oxidised form[606] and increasing bioavailability of iron from ingested food.[607] Micronutrient deficiencies have a negative impact on patient health in Crohn's disease,[618] with iron deficiency being the most common.[627] Lack of iron can affect cell-mediated immune effector pathways and cytokine activity.[617] Under inflammatory conditions iron is diverted away from the circulation to storage within the reticuloendothelial system, to prevent pathogenic bacteria accessing it,[628] leading to anaemia in Crohn's patients. Zinc undernutrition has been shown to impair mediators of innate immunity including natural-killer cell activity;[609] therefore increased intake of iron and minerals such as zinc, in the presence of a depleted gut microbiota due to low fibre during EEN, could play an important role in reducing inflammation in children with Crohn's disease.

In the current study fat intake during EEN was increased 2-fold which does not seem to fit with the theory that increased fat intake is a risk factor for Crohn's disease.[68] However only the amount of saturated fatty acid (SFA) increased during EEN while medium and polyunsaturated fatty acids remained similar to normal diet at baseline. Evidence from a study of 182 children with Crohn's disease and 250 healthy children show a gene–diet interaction, in which variants in genes for fatty acid metabolism affect the relationship between Crohn's disease risk and the ratio of PUFA consumption.[541] Dietary fat has been suggested to determine the therapeutic effect of enteral diets in Crohn's disease, with one study comparing whole protein based EEN with different fat compositions in sixty-two children with Crohn's disease.[629] The linoleate rich formula (n6 polyunsaturated fatty acid) had higher remission rates (63% vs 27%; p=0.008) than an oleate formula (monounsaturated fatty acid).[629] Thus the type of dietary fat used in formulas could be important in the efficacy of EEN.

Milk caseins are immunomodulatory proteins, able to regulate leucocyte behaviour; control cell growth and reduce inflammation.[614;615;616] Interest in the immunomodulatory properties of caseins has focused on neonates,[630] and although Modulen, the formula most used for EEN, is advertised as '*100% casein based which contains a naturally occurring anti-inflammatory factor*', little research has looked at the role

caseins play in reducing inflammation in Crohn's disease. In an *in vitro* model, enterocytes exposed to inflammatory stimuli, decreased response when incubated with Modulen, suggesting the feed could be acting directly on enterocytes, altering intracellular signalling to lower production of pro-inflammatory IL-8.[631] A study looking at five milk based diets in a colitis mouse model found Modulen was the most effective at reducing inflammation and neutrophil infiltration. All of the milk based diets reduced cytokine expression and reduced inflammation to varying degrees.[632] However this study did not control for the role which lack of dietary fibre might play in reducing inflammation.[632]

Another study suggests glutamine, arginine and vitamin D_3 found in polymeric formulas, have the ability to block phosphorylation via the NF-κB pathway in an HT-29 colonic epithelial cell model, which led to a decrease in IL-8 production.[633] Thus further research is needed to assess whether EEN has a direct anti-inflammatory effect, independent of altering the gut microbiota.

Further research using murine and cell models, is needed to understand which components of polymeric formulas have a direct effect on host cells, and which have a secondary effect by driving changes in the gut microbiota. *In vitro* fermentation studies using gut bacteria from patients with Crohn's disease, with or without fibre, as well as different fat and micronutrient compositions, could help to explore what role different dietary components have on gut bacterial diversity during EEN.

Reduced exposure to deleterious compounds Taking EEN might help children avoid possible detrimental compounds present in their normal habitual diet. Large studies on children taking EEN show remission rates are in the region of 80%.[634;283;635] In one study patients who failed to fully comply with the exclusive diet had a 35% lower remission rate (NS).[634] This suggest reduced exposure to dietary components rather than exposure to EEN alone, is responsible reducing inflammation. Although some evidence suggests partial enteral nutrition (PEN) can induce remission, PEN still leads to a significant reduction in exposure to normal foods.

It is also noteworthy that salt (NaCl) intake is reduced by $\sim$1/3 during EEN compared with normal diet in children with Crohn's disease (Table 7.5). Studies have shown a high salt diet stimulates the immune response as well as increase gut permeability in mice (Table 7.7).[566] Further work needs to be done to assess the role high salt diets might play on the human gut mucosa and whether reducing salt intake could help to maintain remission in Crohn's patients.

7.4.6 Dietary choices post-EEN

In the current study participants and their parents were aware this was a study about diet, and that treatment with EEN is zero fibre diet. Therefore their awareness of

diet as a treatment, may have influenced their food choices post-EEN. Results from the current study show that with the exception of Vitamin A there were no significant differences between dietary intake pre-and 2-weeks post-EEN (Table 7.6). However the differences in seven individual children who went into remission on EEN suggest that those children who increased their fibre (g); vitamin C; fruit and vegetable intake were less likely to relapse than children who reduced intake in these food groups, regardless of treatment with MEN or an immunosuppressant (see summary Fig. 7.11). This might suggest that diet post EEN, particularly fibre and vitamin C from fruit and vegetables is more important than MEN or immunosuppressant treatment at preventing disease relapse.

Given that studies have found reduced risk for Crohn's disease in people who have increased fruit fibre in the diet,[540;547] it is possible that increasing fibre post-EEN could prolong time to relapse. The largest study which focused on using MEN post-EEN as maintenance therapy, has shown it does not appear to increase time to relapse.[153] The current study also saw no increased time to relapse in children using MEN (Fig. 3.6).[625] Given that MEN has zero fibre, if increased fibre can increase the length of time in disease remission then using MEN could potentially reduce daily fibre intake and thus reduce length of remission post-EEN. Studies using a murine model, followed by human clinical trials, are urgently needed to assess whether MEN, or paradoxically, increased dietary fibre would keep children in disease remission for longer.

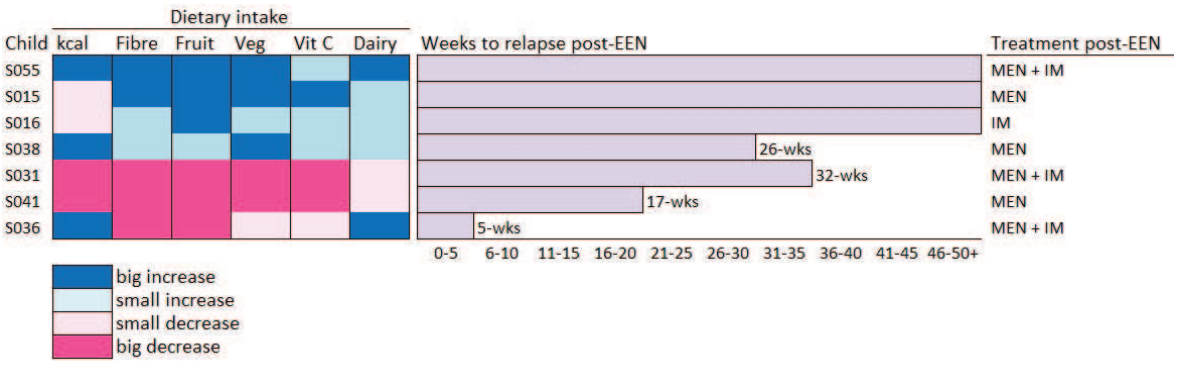


Figure 7.11: A summary of changes in dietary intake at 2-weeks post-EEN compared with baseline in seven children with Crohn's disease who went into remission on EEN. Estimated dietary intake (energy kcal, fibre, Vit C) and number of food items eaten (Fruit, Veg, Dairy) was calculated from paired FFQs. Days to relapse are shown along with treatment post EEN. IM -immunosuppressant; MEN -maintenance enteral nutrition. Summary of data shown in Fig. 7.9

7.4.7 Linking dietary intake with gut microbiota

Only a few studies have examined the role of diet on the gut microbiota of healthy children.[636;209] Research suggests the diet of people living in Western countries compared with developing countries, contain a higher proportion of fats and protein while being lower in foods such as fruit, vegetables and fibre. One of these studies which

compared the gut microbiota of children between 1-6 years old from rural Africa and Italy found in infants who were breastfed, the microbiota in both populations was similar being dominated by *Bifidobacterium*.[636] Yet, once African children were weaned onto their local diet, which contained a higher proportion of fibre than Italian children the African children demonstrated an enrichment of their Bacteroidetes; reflected by increased *Prevotella* and *Xylanibacter* genera, which contain genes for both cellulose and xylan hydrolysis. Children on Italian diets had less Bacteroidetes which were also from a different genus (*Bacteroides*); and had high abundance of Firmicutes relating to higher levels of protein, fat and simple carbohydrates in their diet. These results fitted with a previous proposal by Wu *et al.*[212] suggesting dietary patterns are correlated with specific gut microbiota 'enterotypes' in adults. A *Prevotella* enterotype being associated with an increased intake of carbohydrates and simple sugars; and a *Bacteroides* enterotype being linked to an intake of higher animal protein and saturated fats.[212]

Although useful, there are a number of limitations to the interpretation of data from these studies on children. The impact of differences in genetics, climate, socioeconomic status, sanitation, interaction with animals and other cultural practices which might have had an impact on the gut microbiota between these populations cannot be ruled out. Although it might be temping to consider the diet of rural African populations as a standard for good gut health, this may not in fact be true. However given that the UK is undergoing an obesity epidemic and that gut bacterial changes driven by overnutrition can be found in a large number of 'healthy' children, it might also be flawed to consider the healthy children in our study as an ideal.

In the current study five genera, three Firmicutes, a Proteobacteria and an Archaea genus were significantly different between children with Crohn's disease and healthy children (Fig. 7.10). Of these *Ruminococcus 1* was of particular interest as it has been shown when fibre is restricted, some *Ruminococcus* strains can switch to digesting mucosal proteins which in turn causes a loss of mucosal protection in turn allowing access to potential pathogen such as Proteobacteria species.[637] Mucin degrading *Ruminococcus* strains and commensals with similar genes, could play a key role in dysbiosis by increasing the amount of bacteria associated with the mucosa in Crohn's disease.[422]

7.4.8 Study limitations - collecting dietary questionnaires

The current study had a poor return rate of 3-day food diaries, and with several returned by young adults being incomplete. Therefore the study was unable to include these and had to rely on FFQ data for estimated nutrient intakes (Fig. 7.1). A list of issues which lead to changes in the way dietary data was collected as well as reasons for poor return rates are given in Table 7.8. Future studies should consider the diffi-

Table 7.8: Assessment of barriers to completing and returning food questionnaires

Person	Issue *
Child	- Difficult for young children to remember what they ate at school lunches, friends house, or clubs. - Found task boring because it took too long to complete. - Could not find time: five children with CD were competing in sport at competition level and were out most evenings and weekends training.
Parent	Finding time was impossible due to: - having young children or a baby in the house. - demands of a single parent family. - having a child with learning difficulty/autism. - feeling overwhelmed by the number of questionnaires. - feeling they had done this already at baseline, and did not see the need to complete the same questionnaire again.
Researcher	- Talking to child and parent on the phone at the same time was impractical. - Younger children lacked confidence to speak on the phone. - Parent lacked knowledge about what children ate outside the home. - Parents report what they offer children to eat, which might not be fully consumed.

CD - Crohn's disease. * Comments were recorded from verbal and written communications from participants and parents.

culties of collecting dietary information from children and young adults, and consider whether collecting this information in a clinical setting with the assistance of a dietitian would be more effective. This would potentially increase costs as the dietitian might need to spend extra time-with patients; however since patients usually see a dietitian at review appointments, and patients often sit in the waiting area between seeing clinicians/IBD nurse/dietitian/phlebotomists, there could be an opportunity to use this wait time, to complete dietary questionnaires. Asking patients to attend an appointment with a researcher to discuss diet, after visiting the clinician, the IBD nurse, the dietitian and then a phlebotomist was challenging, since most families were aiming to get back to work/school as quickly as possible. Understanding the practical dynamics of both the clinical setting, as well as patient/family needs are important in planning a successful dietary study.

7.4.9 Summary

As suggested in chapter 5, EEN may work either by altering specific bacterial groups involved in inflammation, or via a total reduction in bacterial load 'gut rest'. It has been proposed that the pathogenesis of IBD could be linked with a Western type diet which is high in fat, n-6 PUFA, processed meat and low in fibre rich green vegetables and fruit. Therefore it is possible EEN is either providing direct nutritional improvements or, some aspect of the diet such as casein proteins are having a direct anti-inflammatory effect on gut epithelial cells. However animal models suggest the presence of gut bacteria is necessary for inflammation to occur, and modulation of

bacteria using probiotics and antibiotics have been linked to inflammation in the gut. If diet is directly driving inflammation, antibiotics would have little or no effect in the induction of remission in patients with Crohn's disease. It is however possible dietary antigens directly affect apical tight junctions or degrade the mucosal layer creating a niche for some pathogenic bacteria which in turn drives the inflammatory response. For example sodium caproate, a medium chain fatty acid found in milk fat, has been shown to increase gut permeability in rat ileum by enlarging tight junctions.[553] as well as in ileal samples taken from patients with Crohn's disease.[554]

On going and future research The role of diet in Crohn's disease is of great interest to the scientific community, because not only might diet be driving the rise in disease in susceptible individuals, but diet also has the potential to become a successful nutritional therapy at disease onset, and maintenance of remission in Crohn's disease. A number of research studies have started or are being planned to assess the role of diet and the gut microbiota in relation to patients with IBD. The challenge for dietary intervention is relating the gut microbiota impact directly to health outcomes, to enable the exploration of food intake patterns in terms of carbohydrate, protein, or fat profiles. Multifactorial experiments will be needed to tease out a dietary profile, which leads to healthy gut bacterial and metabolite homoeostasis and is able to maintain a stable anti-inflammatory state in the gut.

- The Food and Resulting Microbial Metabolites (FARMM) study is being conducted by the Crohn's and Colitis Foundation of America (CCFA)[638]
- Study on the Genetic, Environmental and Microbial interactions that cause IBD (GEM Project) by the Crohn's and Colitis Canada[639]
- The Prognostic effect of Environmental factors in Crohn's and Colitis (PREdiCCt) study[640]

The FARMM study[638] is a controlled feeding study with healthy volunteers being fed a 'Western' diet, EEN, or a vegan diet for 2-weeks to see the impact on the gut microbiota and faecal metabolites. The GEM Project[639] is looking at healthy first-degree relatives of Crohn's disease patients to assess genetic, environmental factors and the gut microbiota, with the intention of identifying long term risk factors in the development of disease. The PREdiCCT study[640] aims to recruit IBD patients in remission, to assess the relationship between diet, lifestyle, genetics and gut microbiota to risk of disease relapse over 2-years. Although a huge amount of work has been done to understand the use of enteral nutrition in recent years there still lies ahead a great deal of research before EEN and MEN can be considered safe.

A focus of this research should aim to understand the interaction between dietary components, the gut microbiota and inflammation in Crohn's disease and UC. Work should also focus on how dietary therapies including EEN and MEN could be used

to manipulate the gut bacteria back to a non-dysbiotic state which protects the gut from inflammation. More work also needs to be done to understand malnutrition at the point of diagnosis and whether it is linked with disease outcomes. More work also needs to be done looking into the long term safety of using dietary interventions such as EEN and MEN as studies show these strategies seem to drive the gut microbiota into a more extreme dysbiosis which could have long term effects on health if used regularly. Therefore working out an optimal dietary regimen for maintenance of remission which is both effective and safe is important.

7.4.10 Key findings

The current study:

- Children with Crohn's disease have less intake of vitamins (A, E, B_7 and C) and fibre compared with healthy children.
- Energy intake and composition of diet at baseline did not predict response to treatment with EEN.
- EEN is compositionally different in micro-nutrient and vitamin status, as well as fibre from the normal habitual diet of patients with Crohn's disease.

New findings from this study:

- The use of MEN as a dietary supplement was not associated with prolonged remission time.
- Increased intake of fibre from fruit and vegetables post-EEN may increase remission times in children with Crohn's disease, and needs further investigation.
- Dietary intake at baseline highlights an altered relationship between children with Crohn's disease and healthy children for *Ruminococcus 1*; suggesting this genera is functionally different between the two groups.

8 General Discussion and Conclusions

Science of the microbiome is revolutionising not only how we think about gut disease but also how we see ourselves as human beings. We consist of approximately 30 trillion human cells and around 39 trillion microbial cells,[641] which translates to approximately 20,000 human genes versus over 3.3 million microbial genes.[642] It is also worth noting that our human genome does not change much over time whereas the microbiome changes not only from birth to old age but also on a daily basis, depending on environment and diet.

The commensal microbiota have co-evolved with the host to assist in digestion, providing energy for cellular metabolism; making compounds such as vitamins and SCFA; to prime and maintain the intestinal immunology; as well as provide a first line of defence against pathogens.

Research into the role of the microbiota in the development of IBD has reached a milestone. In recent years genome-wide association studies have been able to link IBD with genetic loci which point to an inappropriate immune response to the gut microbiota.[61] Also research into the microbiota community structure of IBD patients has linked pathogenesis with characteristic changes in composition, which reinforce the idea that IBD results from an altered interaction between gut bacteria and host mucosal immunity. We have employed 'omic' technologies in the current study with the aim of increasing our understanding of these interactions between host and the resident gut microbiota.

8.1 Practical issues around recruitment and study design

Prior to the start of the research phase of the project, there were a number of practical and legal hurdles to complete before recruitment could begin. Eight-months of administration was involved in setting up and gaining approval for this study. This could have been reduced substantially by: a) avoiding recruitment from community hospitals and clinics where the large amount of administration did not justify the small number of patients recruited from these centres; b) having earlier support from the University department of Research and Development who have a detailed working knowledge of the processes involved; c) applying for NHS R&D approval at the same time as ethics approval.

During the study it was apparent the researcher was asking patients questions and taking anthropometric measurements which were already being taken by the dietitian. Dietitians in one local hospital (Wishaw) shared a room with the researcher to avoid this duplication, thus reducing time the child had to spend in clinic. The researcher also did not have access to databases containing clinical appointments, and was thus dependent on clinical staff to keep them up to date. This did not always work well in

practice and a number of opportunities to meet patients for recruitment or to collect samples were missed, as clinical staff were very busy and sometimes failed to pass on details. A number of children with Crohn's disease had follow-up appointments in their local hospital and these clinic times often overlapped making it impossible for one person to attend all clinics to follow-up patients recruited at baseline. Therefore recruitment success could be more efficient and effective if it were carried out by a research nurse/dietitian based within the clinical setting. Reducing the time burden on patients would likely result in more patients completing the study. This study might also have benefited from a shared research database between the clinical team; the clinical research facility (CRF) team and the researcher; to improve communication about appointments, needs or concerns about the participant; and could duplicate as a log of recruitment and samples collected. This would have allowed members of the clinical team to be more involved with the study, and saved time on research paperwork.

Dietary questionnaires The current study had a poor return rate of detailed food diaries, and had to rely on FFQ data for estimated nutrient intakes. The key issues were that parents did not always know what their children had eaten at school and that young adults 12+ who self completed forms did not take time to do these thoroughly. Attempts to conduct the 5-step multi-pass questionnaire failed because it was not possible to speak with both parent and child simultaneously, and many families could not commit to 3-calls per week since they were involved in activities outside of the home. Several parents also reported that they felt the burden of filling out dietary questionnaires was too much.

Participants were asked to attend an appointment with a researcher to discuss diet, after visiting the clinician, the IBD nurse, the dietitian and then a phlebotomist, when most families had to return to work/school as quickly as possible, and therefore struggled to spend a 3^{rd} hour in clinic answering dietary questions. It would perhaps have been better to collect dietary data in the clinical setting with the assistance of a dietitian, where both child and parent are present. This would potentially increase costs as the dietitian would need to spend extra time-with patients; however since patients usually see a dietitian at review appointments, and patients often sit in the waiting area between seeing clinicians/IBD nurse/dietitian/phlebotomists, there could be an opportunity to use this time, to complete dietary questionnaires. Therefore understanding the practical dynamics of both the clinical setting, as well as patient/family needs are important in planning a successful dietary study.

One drawback of the current study and others is that baseline faecal samples may not fully represent the child's normal diet. Many children are unwell at the point of attending hospital pre-diagnosis, and not managing to consume their normal diet. Hence it is difficult to know if differences seen in the gut bacteria profiles at baseline

are temporary changes due to a limited diet, or are linked with long-term habitual diet. Future studies could use animal models to control for diet at baseline and assess the impact of enteral nutrition independent of dietary variations. Although human studies may seem the obvious choice to understand human disease, the inability to control variables such as diet or medication can make them poor models to understand which factors affect outcomes. However animal models of colitis may not fully represent inflammation in humans so also have limitations.

Treatment with MEN Since the current study was observational, it could not influence the numbers of children choosing to take maintenance enteral nutrition (MEN) post-EEN. ∼55% of children had refused MEN in previous years, however only 6/23 (26%) of children who went into remission on EEN opted not to take MEN, one then going onto MEN around 17-weeks post-EEN. It is unclear why uptake was so high, but it is possible after explaining the study, participants became more aware of possible benefits of MEN as a therapy. It is also conceivable that in discussions with clinical staff, IBD-nurses and dietitians the researcher influenced the likelihood they would recommend MEN as maintenance treatment. A combination of both of these may have influenced the uptake of MEN.

Although all children who opted not to take MEN were on an immunosuppressant, only 8/17 children taking MEN were taking an immunosuppressant, creating a third subgroup. Children taking an immunosuppressant as well as MEN creates a confounding factor, as it could have an effect on the gut microbiota profile and metabolites, making understanding differences between these three groups more challenging, due to the lower numbers in each group.

Variation in populations Bacterial activity has a huge influence on the availability and bio-activity of many compounds including SCFA derived from the diet, and it is becoming clear, although all healthy individuals have a common core microbiota, there are pathways which are found only in some populations or individuals. For example, a species of *Bacteroides* which can degrade the sulphated algal polysaccharide porphyran has only been detected in Japanese individuals;[643] and the ability to convert the soy isoflavone to equol is not present in all people.[644] Thus the potential for complex inter-individual variation in the effects of these derived molecules due to variations in microbiota diversity is substantial. It is therefore important to replicate studies across different populations, including gender and age, since functional dysbiosis in the form of SCFA may be the result of unique disrupted microbial pathways in different populations.

8.2 Crohn's disease at diagnosis

Malnutrition is common in children with IBD, particularly in active Crohn's disease where weight loss at diagnosis can be as high as 90%.[48;49] The fact that children with Crohn's disease have reduced age related growth, as supported by the current study, is important especially since it is associated with a delay in puberty.[10;11] The current study showed that albumin, CRP and ESR correlated with BMI z-scores at baseline, suggesting increased disease severity, led to an increased risk of poor growth outcomes in children with Crohn's disease. For this reason finding a dietary approach to reduce inflammation in children with Crohn's may help to achieve age appropriate growth targets.

Inflammatory markers including faecal calprotectin are increased in IBD patients. Not all children with Crohn's disease have raised CRP or ESR, however the current study shows that children with Crohn's disease have an overall blood inflammatory marker profile which is distinct from that of children with UC, non-IBD conditions and healthy children. Although individual clinical blood tests are examined in light of other test results, they have not been used to differentiate Crohn's, UC and non-IBD conditions. Using an overall profile which includes CRP, ESR, albumin, WBC and AST/ALT could be included as part of a larger study in future, to test whether Crohn's disease could be differentiated from UC; and at no additional cost since these blood tests are undertaken routinely.

By analysing the global microbiota community, the current study has shown, along with similar recent studies, that patients with Crohn's disease have reduced microbiota diversity compared with healthy children and non-IBD control patients. Many healthy commensals including butyrate producing species of Firmicutes are decreased in abundance, while a number of gram-negative potential pathobionts are increased in children with Crohn's disease (Fig. 8.1).

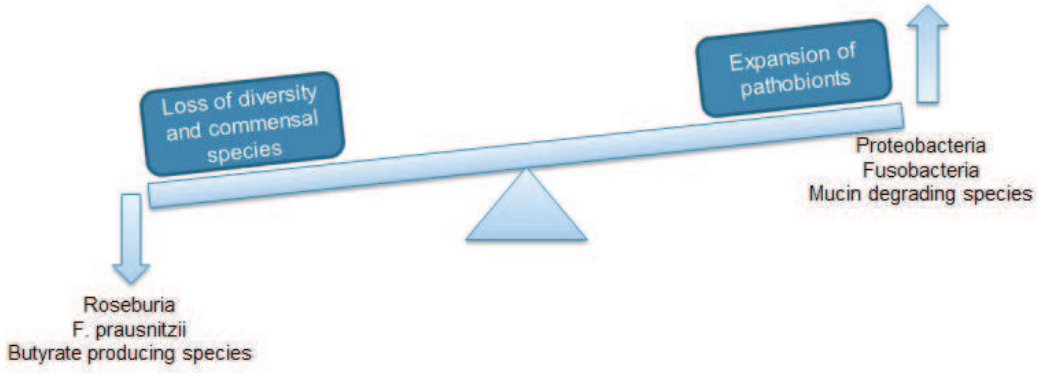


Figure 8.1: Inflammation in children with Crohn's disease is possibly due to reduced bacterial diversity of key commensal species along with increased potential pathogenic and mucin degrading species in the gut.

It has been suggested an increase in mucin-degrading bacteria, could be driving inflammation in Crohn's disease by damaging the protective layer and opening a niche

for pathogens.[645] Since some bacteria switch to mucin feeding when fibre is unavailable this theory holds weight. The current study has shown that children with Crohn's disease have a reduced intake of fibre particularly from fruit, which perhaps puts them at greater risk from mucin-degrading species.

The contribution of mucin-degrading bacteria towards microbial dysbiosis has still to be explored. To date only a few species have been identified as mucin-degrading[645] but they include commensal species of *Bacteroides*, which the current study and others have shown to be increased in Crohn's disease patients. *Bacteroides* and similar commensals could be present in high enough numbers to cause damage to the mucosa under low fibre conditions. However, known mucin-degraders such as *Akkermansia*,[204] are reduced in Crohn's disease patients[227] and have been associated with a healthy mucosa;[646] thus the relationship between mucin feeding bacteria and a healthy mucosa is complex and needs to be better understood.

It is important to see the gut microbiota as an ecosystem, and that a number of principles of microbial ecology, such as 'stability' and 'resistance', apply to these complex systems. A number of studies including the Human Microbiome project have shown, although there is a huge amount of bacterial diversity between healthy individuals, when we look functionally at the metabolic pathways a lot of this variability tends to disappear, and the functional genome is much more stable between individuals.[234]

The current study supports recent studies[465;470] which show reduced levels of the fatty acids valerate (C5), hexanoate (C6) and octanoate (C8) differentiate IBD from healthy individuals. It was shown in Chapter 6 that in children with Crohn's disease, hexanoate (C6) is positively associated with a number of bacterial genera, particularly commensal Firmicutes. This is of interest because studies have shown these medium chain fatty acids have antimicrobial properties which are commonly used in animal husbandry to control pathogens such as *Salmonella*.[273;274]It is therefore possible that reduced levels of these fatty acids in children with Crohn's results in loss of protection from pathogens, and may explain why Proteobacteria, especially the Enterobacteriaceae are increased in Crohn's disease patients.

Bacteria have the potential to induce both acute and persistent infection. The capacity of bacteria to cause chronic disease is dependant on their ability to influence or control their environment within a host. Some bacteria can establish a niche for themselves within intestinal epithelial cells or circulating immune cells. Regardless of host defences intracellular microbes can survive by hiding themselves from normal immunological pathways.[431;647] As a final defence the host cells can induce apoptosis, thus removing these intracellular microbes. However some bacterial pathogens can keep these infected cells alive for long periods, facilitating intracellular replication of bacteria, resulting in persistent inflammation, particularly where macrophages and dendritic cells are involved.[647;648]

A recent systematic review[68] looking at dietary intake and the risk of developing IBD concluded that increased fibre and fruit intake reduced the risk of Crohn's disease, and patients in the current study have significantly reduced fibre intake at baseline along with a lower intake of vitamins (A, E, B_7 and C) compared with healthy children. Given malabsorption is a feature of Crohn's disease, this reduced vitamin intake could have negative effects since vitamins A, E and C have direct and indirect immunoregulatory roles.[589;589;590;591;592] It has also been shown that vitamin A and E deficiency is more prevalent in Crohn's disease patients who have active disease.[593] Vitamin C intake is linked with the intake of fruit and vegetable fibre, hence it is difficult to separate these as risk factors. Thus future studies could further explore whether vitamin C intake in patients, is truly a risk factor in Crohn's disease.

It was shown in Chapter 5 that dietary intake at baseline had a positive association with *Ruminococcus 1* in children with Crohn's disease but a negative association in healthy children. This suggests that the strains of this genus have a functionally different relationship with nutritional status in children with Crohn's disease. It is not clear from the current study if it is the same strain which has altered behaviour due to a different environment or if children with Crohn's disease in fact have a different strain of *Ruminococcus 1*. Sequencing *Ruminococcus* strains to identify mucin-degrading genes might help to answer this question.

8.2.1 What is driving dysbiosis?

Malnutrition Reduced fibre intake (Chapter 7) in children with Crohn's disease may lead to a reduction in diversity of Firmicutes, particularly the Clostridiales. This could open a niche for Proteobacteria particularly Enterobacteriaceae to thrive. This change in microbiota also leads to changes in SCFA/MCFA which could lead to a loss of antimicrobial protection against gram-negative bacteria again opening a niche for Proteobacteria species. The loss of fibre may have an additional effect by activating some bacterial species to switch from fermenting fibre, to feeding on mucin glycans and damaging the protective layer of the gut mucosa (Fig. 8.2). This theory that low fibre intake under certain conditions, could be driving inflammation, is supported by recent findings which show increasing fibre intake post-EEN keeps children in remission and maintains low levels of calprotectin.[619]

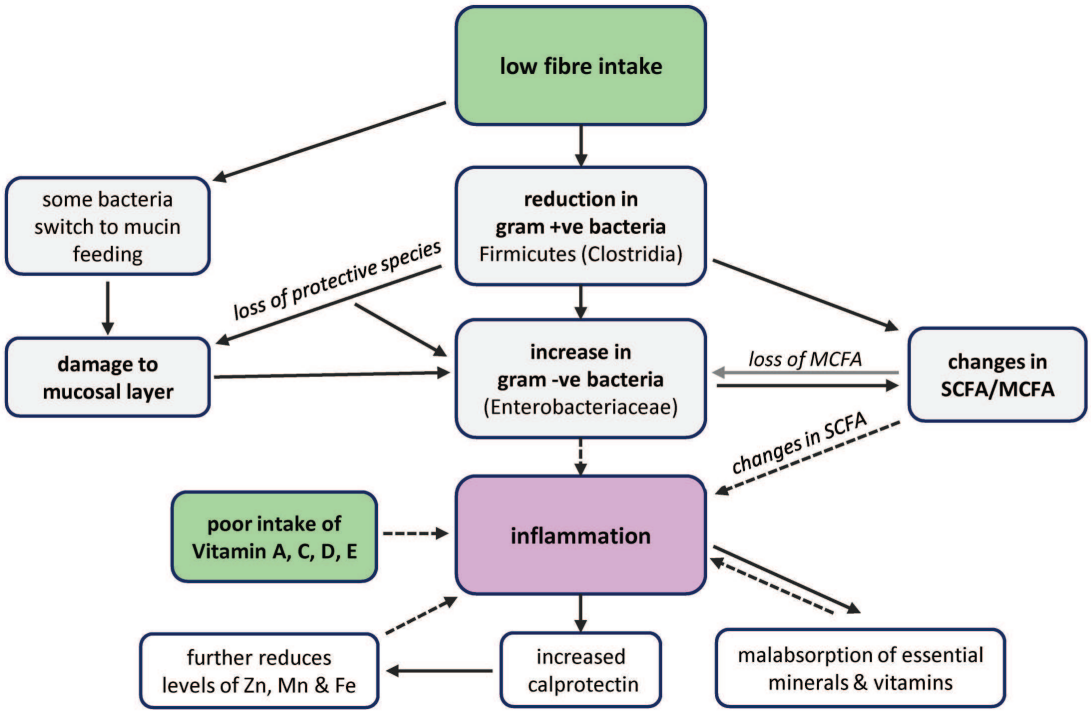


Figure 8.2: Proposed model of the potential factors which drive inflammation in Crohn's disease. Dashed arrows show the factors which could lead directly to inflammation in the gut.

The cuckoo effect In Chapter 5 it was shown that Crohn's disease patients often have individual species which dominate the luminal flora or are higher in abundance than would be expected. Dysbiosis in Crohn's disease could be driven and maintained by bacteriocin producing strains of bacteria which act like 'cuckoos' to push other bacteria into decline, opening a niche for themselves to dominate the ecosystem. Due to lack of crosstalk with host cells, this would lead to a loss of microbial and immunological homeostasis. Identifying and isolating bacteria which dominate the microbiota in individual patients, and characterising the effect bacteriocins have on other commensals, could help to eliminate this as a possible driver of dysbiosis in Crohn's disease.

8.3 Treatment with exclusive enteral nutrition (EEN)

EEN has been used to induce remission in children with Crohn's disease for many years, however only recently has research tried to uncover the mechanisms by which EEN leads to reduced inflammation. Although some studies have suggested that EEN might affect the gut barrier by increasing tight junction function or have direct anti-inflammatory effects,[631;649;650] there is now clear evidence that EEN leads to significant and meaningful changes in the gut microbiota. Furthermore, SCFA concentrations have been shown to decrease during treatment with EEN. Studies show that these changes are rapid, usually with 1-week, and are maintained through-

out the course of treatment with EEN. These alterations in microbial structure and metabolic function in the current study (Chapters 5 & 6) parallel both clinical improvements, and a decrease in faecal calprotectin; all of which return to pre-treatment levels shortly after cessation of treatment. In fact, the current study found that 1-year after the start of treatment with EEN, only 35% of respondents remained in disease remission.

Studies looking at changes in gut microbiota during EEN, including the current study, have highlighted the large amount of inter-patient variation in the way the gut microbiota change during EEN. This is surprising given that all patients are on an identical diet, and suggests that the baseline microbiota profile combined with host genetics determine changes during EEN; and may explain why EEN fails to induce remission in 1:5 children with Crohn's disease.

Three studies, including the current study,[217;144] reported a decrease in *F. prausnitzii* during EEN, a prominent bacterium known to provide positive metabolic functions in the healthy gut. Although this decrease along with other commensal Firmicutes is explained by the complete loss of fibre, there remains a paradox as to why this loss in healthy commensals, occurs alongside mucosal healing and reduced inflammation.

The current study is the first study to look at the multi-dimensional profile of SCFA/MCFA as they change during treatment with EEN. Unlike the gut microbiota which move further away from that of healthy children during EEN, the SCFA/MCFA profile actually moves towards that of healthy children (Fig. 8.3). This would suggest that, although fermentation is reduced during EEN, particularly butyrate, changes in fatty acid ratios might restore an anti-inflammatory state to the gut thus driving mucosal healing. Thus it is possibly the relationship between different SCFA/MCFA, rather that the amounts, which are key to the overall control of functional pathways involved with immune-regulation.

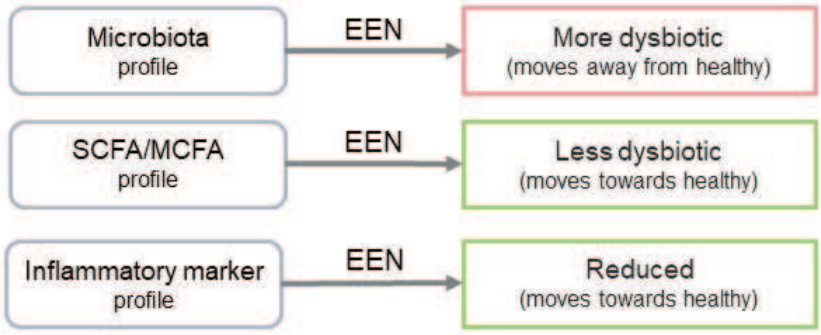


Figure 8.3: Results summary showing that treatment with exclusive enteral nutrition (EEN) unlike short and medium chain fatty acids (SCFA/MCFA) does not push microbiota profiles towards that of healthy controls.

The fact that EEN has been consistently shown to drive microbial dysbiosis away from that associated with healthy children however may be of concern. Around 10% of

patients on the 'RISK' study[72] were on antibiotics, thus the group were able to report that antibiotic use amplified microbial dysbiosis associated with Crohn's disease in a similar way to that seen during EEN, with a reduction in healthy commensals and an increase in pathobionts. Serious consideration needs to be given to the potential damage that EEN might have in further reducing species diversity in some individuals, and longer term monitoring of the gut microbiota is needed to ensure that healthy commensals return to pre-treatment levels in the longer term especially given that in the current study, 75% of children relapsed within 1-year.

An interesting add-on to the current study was that multidimensional scaling of routine clinical blood profiles suggests it might be possible, using selected features of routine bloods, to design a test which could not only identify IBD patients but distinguish Crohn's disease from UC patients. It was not within the scope of the current study to analyse the numbers needed to test the sensitivity or specificity of such a test, but it would be relativity easy to conduct such an analysis, perhaps including other inflammatory conditions such as idiopathic juvenile arthritis or coeliac disease, as these are routine clinical tests.

Weight gain on EEN In chapter 3 EEN was shown to be particularly effective in addressing growth velocity. Children with Crohn's disease who were underweight pre-EEN put on more weight during EEN than those who were normal weight at baseline. Although no child with Crohn's disease was overweight at the start of the study, two were overweight 8-weeks post-EEN and three children were overweight by the end of the study.

A recent study reported that at 3-months post-EEN 17% of children on MEN were overweight compared with 8% of the non-MEN group.[153] At 12-months this had reduced to 10% and 8% respectively. Therefore enteral nutrition on occasions might be given in quantities which move children towards an unhealthy weight. It would be useful for treatment centres to monitor not only weight-gain, but also SDS z-scores and re-evaluate the amounts of enteral nutrition given to individual children dependant on their BMI, so that children of a healthy weight do not become overweight on EEN or MEN.

8.4 Predicting response to EEN

Although it might be possible in future to characterise key aspects of the gut microbiota to predict the response to EEN, and also the risk of subsequent disease relapse, this will be challenging. The current study and others, have demonstrated a large inter-patient variability which would make the design of such markers difficult. Although the current study showed that twenty-five species (SNVs) could discriminate between responders from non-responders, a larger cohort including both children and

adults, as well as accounting for variability due to different cultures and diets, would be needed to see which bacteria discriminate responders from non-responders across these populations, before this could be developed and tested as a marker to predict response to EEN.

Blood-markers CRP, ESR, albumin and the faecal marker calprotectin have been suggested as possible markers of response to EEN, given the assumption that those with more severe disease might be less likely to respond. However the current study has shown that disease severity along with blood and faecal markers was not associated with response to EEN. This was supported by anthropometric measures which showed no differences in BMI or growth between responders and non-responders to EEN, either at baseline or at 28-weeks post-EEN. The current study also found that energy intake and composition of diet at baseline did not predict response to treatment.

Although a recent meta-analysis was unable to link disease location with efficacy of treatment with EEN due to lack of data,[26] the current study agrees with those studies which found patients with ileal involvement were more likely to respond to treatment with EEN.[27;28;146] However study sizes to date have not been large enough to say with certainty if children with isolated colonic disease are more likely to fail EEN than children with ileal involvement.

Collecting dietary data from FFQs allowed the current study to look at all the composition differences between EEN (Modulen) in terms of micro-nutrient and vitamin status, as well as fibre intake, and the baseline diet of patients with Crohn's disease. The lack of fibre in EEN and its effect in lowering the bacterial load has been well documented, however there are other key differences which could have an effect on gut inflammatory status. EEN may provide increased exposure to protective compounds such as caseins which bind with antigens.[614;615;616] Increased exposure to micronutrients, especially iron and zinc both of which are involved in immune pathways, may help to reduce inflammation.[617;609] Vitamin intake is also increased on EEN, including vitamin-D which is known to control expression of the NOD2 gene involved in gut bacterial sensing, as well as improving tight-junction integrity.[599;600] Vitamin D increased 10-fold during EEN and could play a role in mucosal healing.

8.5 Treatment with maintenance enteral nutrition (MEN)

Following successful treatment with EEN, studies have proposed that supplementing the normal post-EEN diet with enteral nutrition (MEN), could maintain long-term remission.[101] Several methods have been tested, including overnight nasogastric feeds combined with daytime diet and the addition of a daily supplement of around 20% energy intake.

The current study found that, although children with Crohn's disease had age appropriate growth during EEN, growth velocity was not maintained once children went

back onto normal diet, despite the diet being supplemented with 20% MEN. To date no study has reported on the combined use of immunosuppressants, meaning drug use as a confounding factor is ignored, hence the current study spilt patients up into those treated with MEN only; an immunosuppressant only; or both. No differences in growth were seen between the three types of maintenance therapy, however samples sizes were too small to reject the hypothesis.

The current study also showed that faecal calprotectin returns to pre-treatment levels more rapidly than previously recognised once children return to normal diet; and that MEN had no effect in maintaining the anti-inflammatory effect of treatment with EEN. The current study also demonstrated that MEN does not sustain the microbiota profiles achieved during EEN. The same was true of SCFA profiles which returned to pre-treatment levels once back onto normal diet; and treatment with MEN post-EEN did not help to maintain SCFA profiles achieved during EEN. The use of MEN as a dietary supplement was also not associated with prolonged remission time. Thus the current study does not support the hypothesis that 20% MEN can extend remission times in children with Crohn's disease.

Although not significant, results from the current study suggest intake of fibre from fruit and vegetables post-EEN may increase the length of remission times and therefore needs further investigation. It is probable that EEN, as a zero fibre diet, reduces colonic bacteria to low enough levels, that any negative effects from cross-feeding or mucosal degradation are prevented, until the normal low fibre diet is resumed. However as bacterial levels recover, the fibre intake in children with Crohn's disease, may not be high enough to provide nutrition to those genera, which under low fibre conditions are mucin-degraders. Although sample sizes were not high enough, there is some evidence from the current study that those children who increased their fibre intake post-EEN, particularly fruit, were more likely to maintain remission than children who decreased their fibre intake. Further work is needed to assess if increasing fruit intake post-EEN could help to extend remission times in children with Crohn's disease.

A recent Israeli study of seventy-eight children,[619] where half were trialled with an exclusion diet also containing increased fruit and vegetables (CDED) plus 50% (Modulen) for 6-weeks, followed by CDED with 25% Modulen for 6-weeks; and half which received EEN for 6-weeks followed by a free diet with 25% Modulen for 6-weeks. 75% reach remission on the CDED plus Modulen compared with 59% on EEN. During the first 6-week the CDED was based on either excluding or reducing the following: animal/dairy fat; high fat from other sources; wheat; red or processed meat and protein sources rich in taurine; emulsifiers; artificial sweeteners; carrageenan and sulphites.[619] Once children were in remission the next phase increased exposure to fruits, vegetables and legumes, thus increasing fibre intake with the aim of supporting commensal Firmicutes and healthy SCFA production. The most interesting finding from this

study is that calprotectin levels were not only maintained once children went back onto normal diet but continued to fall. In the CDED group Firmicutes also continued to increase while the decrease in Proteobacteria achieved during the induction period was sustained. The EEN group which were taking 50% MEN post-EEN were unable to maintain reduced Proteobacteria and Firmicutes started to decrease.[619] Another attractive feature of the CDED diet is that it did not reduce bacterial α-diversity in the way EEN does, thus patients do not face the risk of losing commensal species required for healthy metabolic function. Although the group put the focus on the exclusion part of the diet, it is just as likely that the increase in fibre is responsible for maintaining remission in these children.

8.6 Summary

The idea that alterations in the gut microbiota as a collective might be driving inflammation rather than a single causative agent is the largest development in IBD research. Research has entered a new era of 'omics' research, allowing us to generate high-throughput microbiota data which could help to change the way clinicians carry out diagnostic testing and treat IBD. Microbiota data combined with host genetics could also lead towards more personalised therapies, whereby we can identify which children will respond well to induction diets, possibly including personalised probiotic treatment, as well as which children may benefit from a more drugs based approach.

No specific diet has been clearly demonstrated to cause or prevent Crohn's disease, and it is vitally important to improve our understanding of the relationship between individual dietary intake and the gut microbiota before dietary manipulation can be used safely in the longer-term to induce remission or reduce flare-ups. The current study was conducted to try and fill a gap in our understanding of the role diet plays in Crohn's disease in children. Despite difficulties in obtaining dietary data the current study has shown that the diet of children with Crohn's disease is lacking in fibre particularly from fruit intake. This was reflected in the faecal microbiota data with reduced numbers of butyrate producing bacteria which rely on dietary fibre.

Crohn's disease has been shown to be a complex disease and will require a multifaceted research approach to properly understand the relationship between diet, microbiome and host genetics. Recent advances in sequencing and computational biology are continuing to create new methods which allow the study of more complex systems and how different factors within microbial communities relate to one another and the host.[72]

In the final analysis, this PhD provided several novel insights into the relationship between the gut microbiota, diet and Crohn's disease (Fig. 8.4). This was explored by studying three aspects: a) gut microbial diversity b) bacterial metabolites in the form of SCFA, and c) dietary intake.

Conclusion The gut microbiota and the metabolites they produce relate to disease activity as shown by inflammatory markers and therefore play a role in Crohn's disease aetiology. Treatment with EEN is linked with distinct changes in microbiota and SCFA profiles which move in opposite directions during EEN. The study concludes that since SCFA profiles move towards controls it is more likely that changes in SCFA drive the reduction in inflammation.

Treatment with MEN does not maintain the gut microbiota or SCFA profiles achieved during EEN, and is accompanied by a rapid increase in calprotectin post-EEN. Evidence also suggests that increasing fibre and possibly vitamin C, in the diet of children during EEN and post-EEN may help to improve both induction and maintenance of disease remission in children with Crohn's disease.

Future research There is a need to co-ordinate microbiota and metabolite research, particularly small studies, so they use comparable methods which will allow these studies to pool data. Future studies also need to include other inflammatory conditions such as JIA and coeliac disease, so that specificity against these conditions can be tested.

More detailed work using animal colitis models is urgently needed, which can control for diet and test a number factors including fibre and vitamin C, which may have an indirect role in gut immunoregulation. *In vivo* fermentation models could be used to explore the role that increased calprotectin plays in altering the gut microbiota by limiting Zn and Fe. It is also possible that mucin-degrading bacterial strains are involved in mucosal damage; hence identifying mucin-degrading genes, as well as growing these strains in a mucosal cell model to test the impact of these strains under different conditions (e.g. low/high fibre), would highlight any impact these strains play in Crohn's disease.

Although some bacteria discriminate responders from non-responders to EEN, wider population studies are needed before this could be used as a tool to identify which children might respond treatment with EEN or other treatment diets.

Further studies are needed in larger cohorts to examine whether increased fibre, exclusion diets, or EEN are more effective and safer to induce and maintain remission in children with Crohn's disease. Understanding which microbiota or SCFA/MCFA profiles reduce inflammation in Crohn's disease would lead to improved treatments.

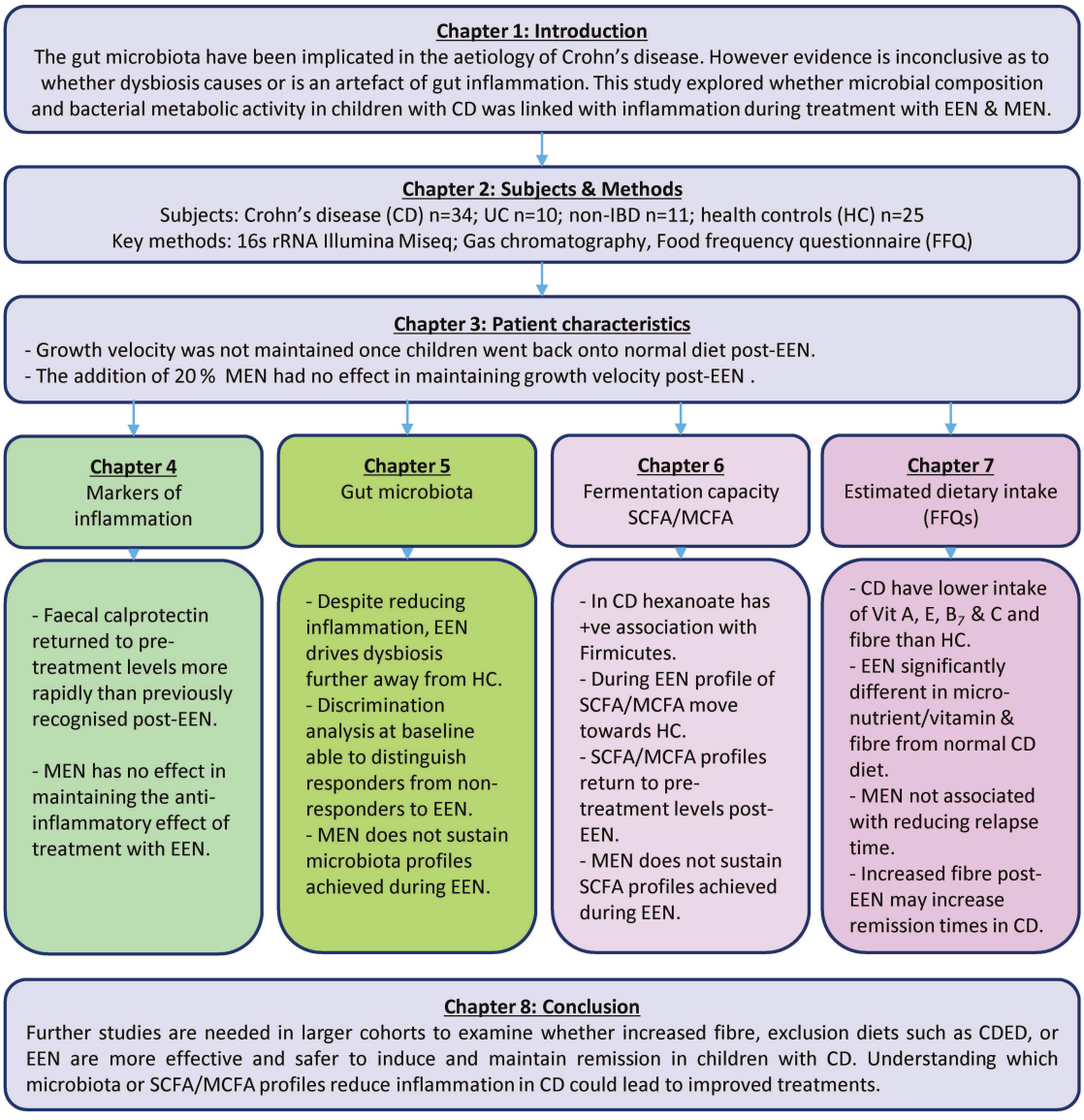


Figure 8.4: Flowchart of PhD showing chapters and main findings.

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Appendices

1 Appendix: West of Scotland Research Ethics approval

Appendix: Ethics approval p1-2

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Dear Dr Russell

Study title: **Gut Microbial Taxonomy and Metabolism in Paediatric Crohn's Disease during Exclusive and Supplementary Enteral Nutrition using OMICS Technologies.**

REC reference: **14/WS/1004**
IRAS project ID: **141190**

The Research Ethics Committee reviewed the above application at the meeting held on 18 June 2014. Thank you to Mrs Clark and Dr Hansen for attending to discuss the application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Mrs Sharon Macgregor, WoSREC5@ggc.scot.nhs.co.uk.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below. .

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on question 2 of the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Summary of discussion at the meeting

Ethical issues raised by the Committee in private discussion, together with responses given by the researchers when invited into the meeting

Recruitment arrangements and access to health information, and fair participant selection

It was noted that children from hospitals other than Yorkhill would be recruited but the process was unclear.

The researchers advised that all diagnostic scopes will be carried out at Yorkhill. All patients will have a named Gastroenterologist at each site and that clinician will also feed into the study.

Participant information sheet (PIS) [E Info leaflet for Crohn's Carer]	1	18 April 2014
Participant information sheet (PIS) [J Information Sheet 3-7 Healthy]	1	23 May 2014
Participant information sheet (PIS) [P When will samples be taken]	1	20 May 2014
Participant information sheet (PIS) [K Information Sheet 8-11 Healthy]	1	20 May 2014
Participant information sheet (PIS) [Q Sample Handling Instructions for Patients May 2014]	1	20 May 2014
REC Application Form [REC_Form_28052014]		28 May 2014
Research protocol or project proposal [Z EEN Proposal Final V4]	4	20 May 2014
Summary CV for Chief Investigator (CI) [IRAS CV 2013 Richard Russell]	1	18 July 2013
Summary CV for student [Clare Clark CV]	1	22 April 2014
Summary CV for supervisor (student research) [CV K Gerasimidis]	1	23 May 2014

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- *Notifying substantial amendments*
- *Adding new sites and investigators*
- *Notification of serious breaches of the protocol*
- *Progress and safety reports*
- *Notifying the end of the study*

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

14/WS/1004	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Care and protection of research participants; respect for potential and enrolled participants' welfare and dignity

It was unclear in the application what will happen to the biopsy tissue of the non-Crohn's Disease (CD) sufferers.

The researchers confirmed that it would be the same procedure for the CD samples and that they will be stored for future analysis.

Other general comments

The researchers were asked what "OMICS Technologies" are. It is stated in the title of the study but not mentioned anywhere in the submission.

The researchers confirmed that this is a broad term to cover a range of new techniques which all end in –omics (e.g. protenomics, genomics) etc.

The technology allows sampling for very many different things in a large number of samples, relatively cheaply and quickly and is a semi- automated technology.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [V HC Poster]	1	20 May 2014
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [W Insurance Client Info Letter - Clinical Trials]	1	20 May 2014
GP/consultant information sheets or letters [S GP Letter]	1	20 May 2014
Letter from funder [YCC Award Letter]	1	12 September 2013
Non-validated questionnaire [T Health Check Questionnaire]	1	20 May 2014
Non-validated questionnaire [U Table Compliance Questionnaire]	1	20 May 2014
Participant consent form [A Assent form]	1	20 May 2014
Participant consent form [B Patient Consent Form]	1	20 May 2014
Participant consent form [C Parent Consent From]	1	20 May 2014
Participant information sheet (PIS) [H Information Sheet 8-11 Colonoscopy]	1	18 May 2014
Participant information sheet (PIS) [N Information Sheet 8-11 Crohn's]	1	20 May 2014
Participant information sheet (PIS) [F Info leaflet for Carer of Healthy child]	1	18 April 2014
Participant information sheet (PIS) [L Information Sheet 12+ Healthy]	1	20 May 2014
Participant information sheet (PIS) [R Local Contacts]	1	23 May 2014
Participant information sheet (PIS) [D Info leaflet for Colonoscopy Carer]	1	18 April 2014
Participant information sheet (PIS) [I Information Sheet 12+ Colonoscopy]	1	20 May 2014
Participant information sheet (PIS) [O Information Sheet 12+ Crohn's]	1	20 May 2014
Participant information sheet (PIS) [G Information Sheet 3-7 Colonoscopy]	1	20 May 2014
Participant information sheet (PIS) [M Information Sheet 3-7 Crohn's]	1	20 May 2014

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Attendance at Committee meeting on 18 June 2014

Committee Members:

Name	*Profession*	*Present*	*Notes*
Professor Pauline Banks	Reader (Older Persons' Health)	No	
Dr Stewart Campbell	Consultant Physician & Gastroenterologist	Yes	
Dr James Curran	GP	Yes	
Dr Darryl Gunson	Lecturer	Yes	
Dr Gillian Harold	Consultant Radiologist	No	
Dr Ahmed Khan	Consultant Psychiatrist	No	
Professor Eddie McKenzie	Statistician	No	
Canon Matt McManus	Parish Priest	No	
Ms Janis Munro	Key Account Manager	Yes	
Dr Gregory Ofili (CHAIR)	Consultant Gynaecologist	Yes	
Mrs June Russell	Retired (Research Chemist)	Yes	
Mr Charles Sargent	Retired	Yes	
Dr Marcel Strauss	Consultant Radiologist	No	
Mrs Liz Tregonning	Retired (Special Needs Teacher)	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Dr Judith Godden	Scientific Officer/Manager
Mrs Sharon Macgregor	Co-ordinator

Written comments received from:

Name	*Position*
Professor Eddie McKenzie	Statistician
Canon Matt McManus	Parish Priest

Yours sincerely

SMacgregor

for
Dr Gregory Ofili
Chair

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

"After ethical review – guidance for researchers"

Copy to: Ms Emma-Jane Gault, University of Glasgow
Ms Joanne McGarry, NHS Greater Glasgow & Clyde

2 Appendix: BIG Study information leaflets

2.1 Appendix: Parent/guardian information leaflets

NHS Greater Glasgow and Clyde

Will my child's participation be kept confidential?

Yes. Participants who have given consent to take part in the study will be allocated an anonymous code which will be used to label all samples and data, in order to protect your child's identity during all lab work and analysis of sample data. Relevant medical history will be collected from medical notes in line with the NHS Code of Practice (Scotland) Version 1.0 and current data protection laws. Other than the direct research team, data may also be monitored by representatives of NHS GG&C to ensure compliance with regulations.

How will the information I provide be used?

The anonymous data generated from your child's samples will be used to produce a report and thesis on how diet, gut bacteria and other factors affect inflammation in children with gut inflammation. We will present the results at scientific conferences, publish in scientific journals and provide a report of our findings for children and parents who would like this information. There will be no personal information in any of these reports.

Who is organising & funding the study?

This research is co-organised by the University of Glasgow and clinical staff at the Royal Hospital for Sick Children in Glasgow. It is funded by The Yorkhill Children's Charity and has been reviewed by the West of Scotland Research Ethics Committee.

What if I have questions or concerns?

If you have any concerns or questions about the study you can contact the researcher, Clare Clark. If you would like to speak to someone other than the researcher please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

The study researcher

Clare Clark will always be happy to answer any questions you might have about the study.

Please contact Clare by email:

c.clark.1@research.gla.ac.uk

or by phone/text

07730 465230 / 0141 201 8688

University of Glasgow

NHS Greater Glasgow and Clyde

What do we need to do to take part?

A researcher will provide you with detailed information and if your child would like to take part in the study we will ask:

- questions about your child's health and what medications they use.
- to complete a questionnaire about your child's diet.
- to measure your child's height, weight, and hand grip strength.
- to permit us, with approval of your clinician, to access your child's medical notes for data on medication, disease activity, height, weight etc.
- your child to provide a stool and urine sample. This can be collected at a later date from your home by a researcher, or when you next visit the clinic. We will ask you for further samples if your child has gut inflammation, but this is up to you.
- your child's hospital doctor to provide us with an extra small tube (teaspoon) of blood when your child is having a blood sample taken for routine medical reasons, so no extra needle insertion.
- for permission to take some additional mucosal biopsies (tiny skin samples) during your child's colonoscopy.

Please note that your child can still take part even if they don't want to give additional skin samples. Your doctor will be happy to discuss this with you before your child has their colonoscopy

If after colonoscopy, your child is found not to have any gut inflammation, we will say thank you for taking part and will not ask you for any further samples.
If however your doctor tells us that your child has gut inflammation which is consistent with Crohn's disease and your doctor decides to put your child on a special diet, to try and improve their health, we will ask you and your child to continue on the study. We would then ask your child for 5 more stool & urine samples and 2 blood samples over the next year and also occasionally ask about your child's diet. We will provide you with a detailed sheet of how and when sample collections will happen, but this will always be at your convenience, and you can withdraw from the study at any time you like. We will keep these samples for future measurements as new tests become available.

Are there any benefits or risks if we take part?

Although there are no benefits from taking part in the study, we hope that you might find it a positive and rewarding experience, in helping us to better understand the role that gut bacteria play in causing or protecting from diseases that involve inflammation in the gut. During colonoscopy, additional biopsies will only be taken by your surgeon if they feel it is safe to do so, but you will still be able to take part in the study if no biopsies are taken. We do not anticipate any risks from this research study.

University of Glasgow

Invitation

We would like to invite you and your child to take part in a research study. Before you decide please read the following information leaflet and please contact us if you would like more information. This study is undertaken by Clare Clark (BSc. MRes) as part of her University of Glasgow, 3 year postgraduate research project. The study is supported jointly by the University of Glasgow Human Nutrition section and the gastroenterology team at the Royal Hospital for Sick Children, Yorkhill.

Why are we doing this study?

We are interested in the role that gut bacteria play in inflammatory conditions like Crohn's disease. 90% of the cells in a human body are bacteria, but very little is known about the role these bacteria play in both protecting and harming the human gut. New techniques have shown that the bacteria in guts of Crohn's disease patients are different. It is important for us to try and establish whether these differences in gut bacteria are the consequence or the cause of Crohn's disease. This study will aim to confirm which bacteria might be involved and identify bacterial markers which would help doctors to target the best individual treatment for children with gut inflammation.

Why has my child been chosen?

We are asking you because your child has Crohn's disease, and your doctor has asked him/her to undergo treatment with a special liquid diet called enteral nutrition to try and improve their symptoms. We want to find out how enteral nutrition works, by measuring changes in your child's gut bacteria while they are on enteral nutrition and compare this with your child's normal diet once they have finished their treatment.

Do we have to take part?

No. It is up to you and your child to decide whether or not to take part. If your child is happy to take part you will both be given an information sheet to keep and be asked to sign a consent form. If your child decides to take part they will be free to withdraw from the study at any point they wish, and without giving a reason. This study will run alongside your child's clinical care and is supervised by their gastrointestinal care team but will not impact on your child's care in any way.

NHS Greater Glasgow and Clyde

University of Glasgow

NHS Greater Glasgow and Clyde

Gut microbial taxonomy and metabolism in paediatric Crohn's disease during exclusive and supplementary enteral nutrition using OMICS technologies

An information leaflet for carers of young people interested in joining a research project about gut inflammation.

Bacteria & Inflammation in the Gut

University of Glasgow

yorkhill children's charity

Will my child's participation be kept confidential?

Yes. Participants who have given consent to take part in the study will be allocated an anonymous code which will be used to label all samples and data, in order to protect your child's identity during all lab work and analysis of sample data. Relevant medical history will be collected from medical notes in line with the NHS Code of Practice (Scotland) Version 1.0 and current data protection laws. Other than the direct research team, data may also be monitored by representatives of NHS GG&C to ensure compliance with regulations.

How will the information I provide be used?

The anonymous data generated from your child's samples will be used to produce a report and thesis on how diet, gut bacteria and other factors affect inflammation in children with gut inflammation. We will present the results at scientific conferences, publish in scientific journals and provide a report of our findings for children and parents who would like this information. There will be no personal information in any of these reports.

Who is organising & funding the study?

This research is co-organised by the University of Glasgow and clinical staff at the Royal Hospital for Sick Children in Glasgow. It is funded by The Yorkhill Children's Charity and has been reviewed by the West of Scotland Research Ethics Committee.

What if I have questions or concerns?

If you have any concerns or questions about the study you can contact the researcher, Clare Clark. If you would like to speak to someone other than the researcher please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

The study researcher

Clare Clark will always be happy to answer any questions you might have about the study.

Please contact Clare by email:

c.clark.1@research.gla.ac.uk

or by phone/text

07730 465230 / 0141 201 8688

What do we need to do to take part?

A researcher will provide you with detailed information and if your child would like to take part in the study we will ask:

- questions about your child's health and what medications they use.
- you to complete a questionnaire about your child's diet.
- to measure your child's height, weight, and hand grip strength.
- to permit us, with approval of your clinician, to access your child's medical notes for data on medication, disease activity, height, weight etc.
- your child to provide 6 stool and urine samples over the course of one year. These will be collected from your home at a time that is convenient for you, or when you visit the clinic.
- your child's hospital doctor to provide us with an extra small tube (teaspoon) of blood when you come into clinic, but only when your child is having a blood sample taken for routine medical reasons, so no extra needle insertion is required.

We will provide you with a detailed sheet of how and when sample collections will happen, but this will always be at your convenience, and you can withdraw from the study ay any time you like. We will keep these samples for future measurements as new tests become available.

Are there any benefits or risks if I take part?

There are no direct risks or benefits from taking part in the study. However we hope that you and your child might find it a positive and rewarding experience, in helping us to better understand the role that gut bacteria play in causing or protecting the gut in children with Crohn's disease .

NHS Greater Glasgow and Clyde

Invitation

We would like to invite you and your child to take part in a research study. Before you decide please read the following information leaflet and please contact us if you would like more information. This study is undertaken by Clare Clark (BSc. MRes) as part of her University of Glasgow, 3 year postgraduate research project. The study is supervised jointly by the University of Glasgow Human Nutrition section and the gastroenterology team at the Royal Hospital for Sick Children, Yorkhill.

Why are we doing this study?

We are interested in the role that gut bacteria play in autoimmune diseases like Crohn's disease. 90% of the cells in a human body are bacteria, but we don't know enough about the role that bacteria play in both protecting and harming the human gut. New techniques which identify bacteria in the gut, have shown that the bacteria in guts of Crohn's disease patients are different. We are trying to find out whether these bacterial differences are the consequence or the cause of Crohn's disease, and to identify bacterial markers which would help doctors improve treatment for children with Crohn's disease .

Why has my child been chosen?

Your child has been chosen because they are healthy and will be compared with children who have conditions that involve gut inflammation such as Crohn's Disease. We will be inviting about 40 other healthy children of different ages to take part in this study.

Do we have to take part?

No. It is up to you and your child to decide whether or not to take part. If you decide to take part you will be given an information sheet to keep and be asked to sign a consent form. If you are willing to take part you will be free to withdraw from the study at any point you wish and without giving a reason.

University of Glasgow

NHS Greater Glasgow and Clyde

Gut microbial taxonomy and metabolism in paediatric Crohn's disease during exclusive and supplementary enteral nutrition using OMICS technologies

An information leaflet for parents/carers of healthy children interested in helping a research project about gut bacteria.

Bacteria & Inflammation in the Gut

University of Glasgow

yorkhill children's charity

How will the information I provide be used?

The anonymous data from your child's stool, urine and dietary information, will be used to produce a report and thesis on how the diet, gut bacteria and other factors in healthy children is different from children who have Crohn's disease. We will present the results at scientific conferences, publish in scientific journals and provide a report of our findings for children and parents who would like this information.

Who is organising & funding the study?

This research is co-organised by the University of Glasgow and clinical staff at the Royal Hospital for Sick Children in Glasgow. It is funded by The Yorkhill Children's Charity and has been reviewed by the West of Scotland Research Ethics Committee.

What if I have further questions or concerns?

If you have any concerns or questions about the study you can contact the researcher, Clare Clark. If you would like to speak to someone other than the researcher please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

The study researcher

Clare Clark will always be happy to answer any questions you might have about the study.

Please contact Clare by email:

c.clark.1@research.gla.ac.uk

or by phone/text

07730 465230 / 01412018688

What do we need to do to take part?

A researcher will provide you and your child with detailed information and ask whether you would like to take part in the research study. If you and your child agree we will:

- ask questions about your child's health and any medication they are taking.
- ask you to complete a questionnaire about your child's diet.
- measure your child's height, weight, and hand grip strength to see how strong they are.
- ask your child to provide a stool and urine sample. This can be collected from your home at a date/time that is convenient for you.

We will keep these samples for future measurements as new tests become available.

Are there any benefits or risks if I take part?

There are no direct benefits or risks from taking part in the study. However we would hope that you might find it a positive and rewarding experience in helping us to better understand the role that gut bacteria play in causing or protecting us from disease.

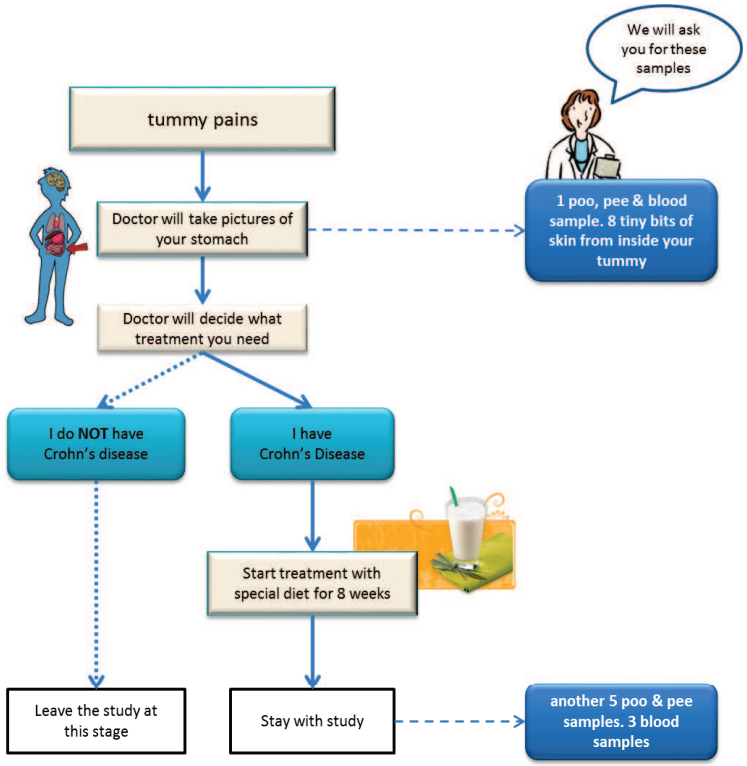
We will also be happy to provide you with a report which sums up the results of this study for you if you are interested.

Will my child's participation be kept confidential?

Yes. Participants who give consent to take part in the study will be allocated an alphanumeric code which will be used to label all samples and data, in order to protect your child's identity during all lab work and analysis of sample data, in line with the NHS Code of Practice (Scotland) Version 1.0 and current data protection laws. Other than the direct research team, data may also be monitored by representatives of NHS GG&C to ensure compliance with regulations.

Information Sheet

This will help you to understand what will happen during the study.



University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

BIG STUDY

Age 7 and under
For children under investigation for gut inflammation

Hi! We want to find out how bugs (bacteria) that normally live in our tummies keep us healthy and protect us from being ill.

What are bacteria?
Bacteria (bugs) are so small that we can't see them with our eye. Bacteria live everywhere. They are on your skin, in your nose, on your teeth and also living in your tummy. We can't live without them.

Why me?
We are asking you because you are coming into hospital so your doctor can have a look inside your tummy with a special camera (colonoscopy).

Do I have to take part?
No. It is up to you. If you agree to take part we will ask you to sign a form which says you are happy to take part. If you change your mind, that's fine. You can stop at any time by just telling your parent or us that you have changed your mind. We will still look after you just as well.

Hi, I'm a friendly bacteria. Nice to meet you

What if I say yes?
If you take part, a researcher will measure your height and weight and ask some questions about your health. We will ask you and your parent questions about what you eat and ask you to give us a poo (stool) and pee (urine) sample. We will ask your hospital doctor to gives us an extra teaspoonful of blood and some extra tiny skin samples from the samples your doctor is going to take from inside your tummy. These are things your hospital doctor will be doing anyway. Only if your doctor then decides you should go on a special diet, to try and make you feel well, we will collect 5 more poo & pee samples and 2 more blood samples over the next year (only one sample every 1 to 3 months). We will also sometimes record your diet.

How will it affect me?
It will not affect you in any bad way. You might enjoy taking part. We will give you a £10 shopping voucher for each poo (stool) sample you give to us, and put your name into a prize draw, just to say thank you.

What if I have questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

yorkhill children's charity

Information Sheet

Then, only if your doctor decides you should go on a special diet to try and make you feel well, we will collect 5 more stool & urine samples and 2 more blood samples over the next year (only one sample every 1 to 3 months). We will also occasionally ask you about your diet.

Are there any benefits or risks?
No, there are no risks and you will not directly benefit from the study. Extra samples from the lining of the gut (biopsies) are only taken by your hospital doctor when it is safe to do so and as part of your normal hospital care. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and put your name into a prize draw, just to say thank you. We will also not tell anyone except your GP (doctor) that you are taking part in the study. We hope to make the study enjoyable for you.

What will you do with my samples?
We will do some scientific tests on your samples to identify which bacteria are most common in your gut and also measure some of the chemicals that these bacteria produce (metabolites). This will help us to see which bacteria help to keep us healthy. We would also like to keep these samples for future measurements as new tests become available.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in stomach problems. The study is funded by Yorkhill Children's Charity and is being done with the approval and supervision of your care team at NHS Greater Glasgow & Clyde and a team of research scientists at University of Glasgow.

What if I have other concerns or questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

Clare will always be happy to answer any questions you might have about the study.

Please contact Clare by email:
c.clark.1@research.gla.ac.uk
or by phone/text
mobile **07730 465230**
(office - 0141 201 8688)

If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

BIG STUDY

Age 8-11
For children under investigation for gut inflammation

Hi! We are asking you to take part in our research project, which is about the role that gut bacteria (bugs) play in children with stomach problems.

What are bacteria?
Bacteria are tiny bugs that live everywhere. Although too small for us to see, they are on your skin right now, in your nose, all over your teeth and also living in your guts. In fact 90% of the cells in a human body are actually bacteria and we could not live without them.

Why are we doing this research?
Very little is known about how some bacteria protect and others harm the human gut. New scientific methods have shown that the bacteria are different in the guts of some children with long term gut problems and we want to find out why. This study will aim to answer some of these questions.

Why have I been chosen?
Because you are coming into hospital to have a colonoscopy test where a doctor will look inside your intestines (guts) with a tiny video camera. During this test, your doctor takes tiny pieces of the lining of your gut, each smaller than a grain of rice (biopsies) and views them under a microscope to find out what's been causing your medical problems. You will be asleep (under general anaesthetic) during this test and will not feel it or remember having it done. We would like to take a few extra biopsies from you, so that we can learn which bacteria (bugs) keep us healthy and which make us feel unwell.

Do I have to take part?
No. It is up to you. If you agree to take part you will be asked to sign a form which says you are happy to take part. If you change your mind you can stop at any time without giving a reason, by just telling your parent you have changed your mind. We will still look after you just as well.

What will happen if I take part?
If you take part a researcher will measure your height and weight and ask some questions about your health. We will also ask some questions about what you eat and will ask you to donate a stool (poo) and urine (pee) sample. We will also ask your doctor if we can have an extra teaspoon of blood when you come in for your colonoscopy, but only if you are having blood taken as part of your care, so no extra needle insertion is needed.

yorkhill children's charity

Information Sheet

What are the possible benefits and risks of taking part?
There are no direct benefits from taking part in the study. During gut examination (colonoscopy), additional gut lining samples (biopsies) will only be taken by your doctor when they feel it is safe to do so. The bowel wall has no pain nerves, so the biopsies will not hurt at all, and any slight bleeding usually stops very quickly. This is a safe procedure done on hundreds of young people in Scotland every year and hence there are no risks from taking part. We hope you might find it a positive and rewarding experience. You can still take part in the study if no biopsies are taken. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and also add your name into a prize draw, just to say thank you.

Will my participation be kept confidential?
Yes. When you give consent to take part in the study we will allocate you a code number which will be used to label all samples and data. This hides your identity during all following research. Only the direct research team will have access to your name and contact details, as well as the people from the health board who check that the study is going well. If you are happy we will inform your GP that you are participating in the study.

How will the information I provide be used?
The data will be used to produce a report on how diet, gut bacteria and other factors affect gut health in some gastrointestinal diseases.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in gut problems. The study is funded by Yorkhill Children's Charity and will be supervised by a team of doctors, dieticians and scientists based at Yorkhill and the University of Glasgow.

What if I have more questions?
If you have any questions you or your parent/carer can contact us. Clare will always be happy to answer any questions you might have about the study..

Please contact Clare by email:
c.clark.1@research.gla.ac.uk
or by phone/text
mobile **07730 465230**
(office - 0141 201 8688)

If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

yorkhill children's charity

University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

Age 12+
for young adults under investigation for gut inflammation

We are inviting you to take part in a research study. Before you decide please read the following information and ask us for more information if you have questions.

Why are we doing this research?
90% of the cells in a human body are bacteria, but very little is known about the role these bacteria play in both protecting and harming the human gut. New methods have shown that the bacteria in guts of some children may play a role in a number of gut diseases, including one called Crohn's disease. However, as yet, we do not know if these differences in gut bacteria are the result or the cause of this type of illness.

Why have I been chosen?
Because you are coming into hospital to have a colonoscopy test where a doctor will look inside your intestines (guts) with a tiny video camera.

Do I have to take part?
No. It is up to you and your carer/parent to decide whether you would like to take part. If you decide to help us by taking part you will be given an information sheet to keep and be asked to sign a consent form. At all times throughout the study you are free to withdraw and without giving a reason. This study will run alongside your clinical care and is supervised by your gastrointestinal care team but it will not impact on your care in any way.

What will happen if I agree to take part?
1. We will ask questions about your health and any medication you are taking.
2. We will ask questions about your diet.
3. We will ask to measure your height, weight, and hand grip strength to see how strong you are.
4. We will ask for you to provide a stool (poo) and urine (pee) sample. This can be collected when you visit the clinic or from your home using a prepaid taxi.
5. We will ask your hospital doctor to give us an extra small tube (teaspoon) of blood when you are having this done anyway by the nurse for routine medical reasons, so no extra needle insertion is needed.
6. We will ask for some extra tiny biopsy samples from the gut lining. You will be asleep (under general anaesthetic) during this test and will not feel it or remember having it done. Your doctor takes tiny pieces of the lining of the gut, each smaller than a grain of rice (biopsies) and views them under a microscope to find out what's been causing your medical problems.

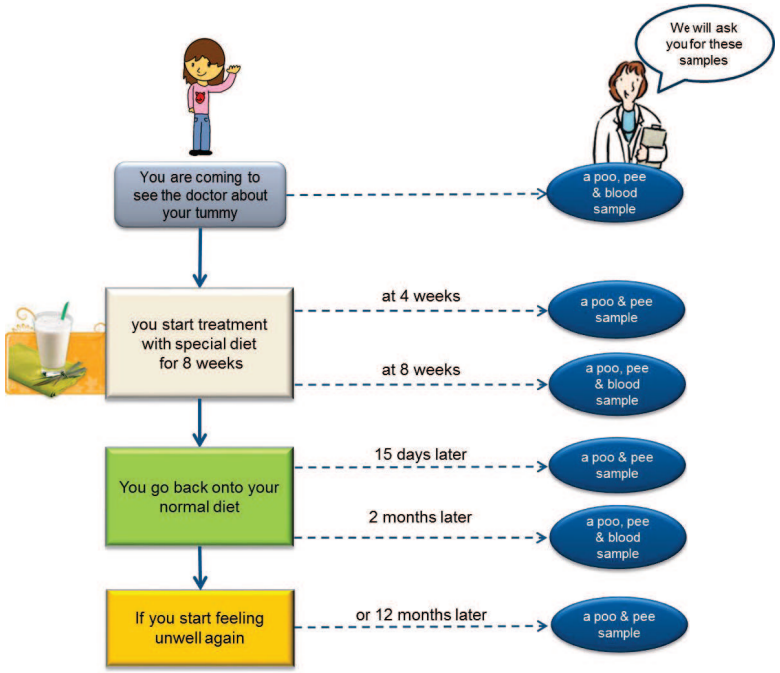
These are things your hospital doctor will be doing anyway, but you can still take part even if you don't want to give us tissue samples.

7. Only if your doctor then decides you should go on a special diet, to try and improve your health, we will collect 5 more stool & urine samples and 2 more blood samples over the next year. We will also occasionally ask about your diet.

We would like to keep all these samples for future measurements as new tests become available.

yorkhill children's charity

This is when we will take samples if you are happy to give them.



Information Sheet

University of Glasgow

NHS Greater Glasgow and Clyde

BIG STUDY

Age 7 and under
For children with Crohn's disease

Hi! We want to find out how bugs (bacteria) that normally live in our tummies keep us healthy and protect us from being ill.

What are bacteria?
Bacteria (bugs) are so small that we can't see them with our eye. Bacteria live everywhere. They are on your skin, in your nose, on your teeth and also living in your tummy. We can't live without them.

Why me?
We are asking you because you have Crohn's disease and your doctor has asked you to come into hospital for treatment on a special diet (which you may have been on before) to try and make you feel better. We want to find out how this diet helps you.

Do I have to take part?
No. It is up to you. If you agree to take part we will ask you to sign a form which says you are happy to take part. If you change your mind, that's fine. You can stop at any time by just telling your parent or us that you have changed your mind. We will still look after you just as well.

Hi, I'm a friendly bacteria. Nice to meet you

What if I say yes?
At different times over the next year (1 to 3 months apart) a researcher will measure your height and weight and ask some questions about your health. We will also ask you and your parent questions about what you eat and will ask you to give us 6 stool (poo) and urine (pee) samples. We will ask your hospital doctor to gives us an extra teaspoonful of blood but only at times when your doctor is doing this anyway. We will also sometimes record your diet.

How will it affect me?
It will not affect you in any bad way. You might enjoy taking part. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and put your name into a prize draw, just to say thank you.

What if I have other concerns or questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

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What will happen if I take part?
If you take part a researcher will measure your height and weight and ask some questions about your health. Over a year we will ask some questions about what you eat and ask you to donate 6 stool (poo) and urine (pee) samples. We will also ask your doctor if we can have an extra teaspoon of blood, but only if you are already having blood taken as part of your care. We will occasionally ask you about your diet. *See attached the sheet to see when we will do these.*

Are there any benefits or risks?
No, there are no risks or benefits from the taking part but we hope to make it enjoyable for you. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and put your name into a prize draw, just to say thank you. We will also not tell anyone except your GP (doctor) that you are taking part in the study.

What will you do with my samples?
We will do some scientific tests on your samples to identify which bacteria are most common in your gut and also measure some of the chemicals that these bacteria produce (metabolites). This will help us to see which bacteria might help to keep us healthy. We would also like to keep these samples for future measurements as new tests become available.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in stomach problems. The study is funded by Yorkhill Children's Charity and is being done with the approval and supervision of your care team at NHS Greater Glasgow & Clyde and a team of research scientists at University of Glasgow.

What if I have other concerns or questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

Clare will always be happy to answer any questions you might have about the study.

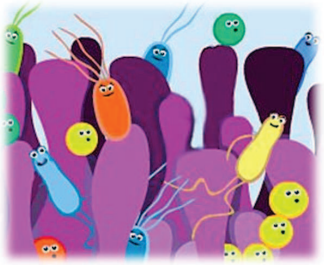
Please contact Clare by email:
c.clark.1@research.gla.ac.uk
or by phone/text
mobile **07730 465230**
(office - 0141 201 8688)

If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

Age 8-11
for children with Crohn's Disease

Hi! We are asking you to take part in our research project, which is about the role that gut bacteria (bugs) play in children with stomach problems.

What are bacteria?
Bacteria are tiny bugs that live everywhere. Although too small for us to see, they are on your skin right now, in your nose, all over your teeth and also living in your guts. In fact 90% of the cells in a human body are actually bacteria and we could not live without them.



Why are we doing this research?
Very little is known about how some bacteria protect and others harm the human gut. New scientific methods have shown that the bacteria are different in the guts of some children with long term gut problems and we want to find out why. This study will aim to answer some of these questions.

Why have I been chosen?
We are asking you because you have Crohn's disease, and your doctor has asked you to come into hospital for treatment on a special liquid diet called enteral nutrition to try and make you feel better. We want to find out how this diet helps you by measuring changes in your gut bacteria while you are on the diet.

Do I have to take part?
No, it is up to you. If you agree to take part you will be asked to sign a form which says you are happy to take part. If you change your mind you can stop at any time without giving a reason, by just telling your parent you have changed your mind. We will still look after you just as well.

Information Sheet

What are the possible benefits and risks of taking part?
There are no benefits or risks from taking part in the study. However we hope that you might find it a positive and rewarding experience. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and put your name into a prize draw, just to say thank you.

Will my participation be kept confidential?
Yes. When you give consent to take part in the study we will allocate you a code number which will be used to label all samples and data. This hides your identity during all following research. Only the direct research team will have access to your name and contact details. If you are happy we will inform your GP that you are participating in the study.

How will the information I provide be used?
The data will be used to produce a report on how diet, gut bacteria and other factors affect gut health in children with Crohn's disease.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in stomach problems. The study is funded by Yorkhill Children's Charity and is being done with the approval and supervision of your care team at NHS Greater Glasgow & Clyde and a team of research scientists at University of Glasgow.

What if I have more questions?
If you have any questions you or your parent/carer can contact us. Clare will always be happy to answer any questions you might have about the study.

Please contact Clare by email:
c.clark.1@research.gla.ac.uk
or by phone/text
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(office - 0141 201 8688)

If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

yorkhill children's charity

University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

BIG STUDY

Age 12+
for young adults with Crohn's disease

We are inviting you to take part in a research study. Before you decide please read the following information and ask us for more information if you have questions.

Why are we doing this research?
90% of the cells in a human body are bacteria, but very little is known about the role these bacteria play in both protecting and harming the human gut. New methods have shown that the bacteria in the guts of children with Crohn's disease tend to be different. However, as yet, we don't know if these differences in gut bacteria are the result or the cause of Crohn's disease. The study will try to help answer this question.

Why have I been chosen?
We are asking you because you have Crohn's disease, and your doctor has asked you to come into hospital for treatment on a special liquid diet called enteral nutrition (which you may have had before) to try and improve your symptoms. We want to find out how this diet helps you, by measuring changes in your gut bacteria while you are on the diet.

Do I have to take part?
No. It is up to you and your carer/parent to decide whether you would like to take part. If you decide to help us by taking part you will be given an information sheet to keep and be asked to sign a consent form. At all times throughout the study you are free to withdraw and without giving a reason. Although this study will run alongside your clinical care and is supervised by your gastrointestinal care team it will not impact on your care in any way.

What will happen if I take part?
If you agree to take part:

1. We will ask questions about your health and any medication you are taking.
2. We will ask questions about your diet.
3. We will ask to measure your height, weight, and hand grip strength to see how strong you are.
4. We will ask you to provide 6 stool (poo) & urine (pee) samples (only one sample every 1 to 3 months). This can be collected when you visit the clinic or from your home using a prepaid taxi.
5. We will ask your doctor to take an extra small tube (teaspoon) of blood on 3 occasions when you are having this done anyway by the nurse for routine medical reasons, so no extra needle insertion.

We would also like to keep these samples for future measurements as new tests become available.

yorkhill children's charity

University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

BIG STUDY

Age 7 and under
Healthy child

Hi! We want to find out how bugs (bacteria) that normally live in our tummies keep us healthy and protect us from being ill.

What are bacteria?
Bacteria (bugs) are so small that we can't see them with our eye. Bacteria live everywhere. They are on your skin, in your nose, on your teeth and also living in your tummy. We can't live without them.

Why me?
We are asking you because you are fit and healthy.

Do I have to take part?
No. It is up to you. If you agree to take part we will ask you to sign a form which says you are happy to take part. If you change your mind, that's fine. You can stop at any time by just telling your parent or us that you have changed your mind.

Hi, I'm a friendly bacteria. Nice to meet you

What if I say yes?
If you take part a researcher will measure your height and weight and ask some questions about your health. We will also ask some questions about what you eat and will ask you to donate a poo (stool) and pee (urine) sample if you are happy to do this. We will use this information to learn about which bugs help to keep us healthy.

How will it affect me?
It will not affect you in any bad way and you might enjoy taking part. We will give you a £10 shopping voucher for the poo (stool) sample you give to us and put your name into a prize draw, just to say thank you.

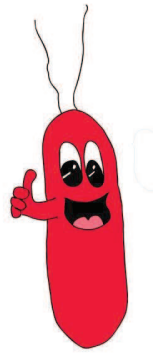
What if I have questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

yorkhill children's charity

Information Sheet

What will happen if I take part?
If you take part a researcher will measure your height and weight and ask some questions about your health. We will also ask some questions about what you eat and will ask you to donate a stool (poo) and urine (pee) sample.

Are there any benefits or risks?
No, there are no risks or benefits from the taking part but we hope to make it enjoyable for you. We will give you a £10 shopping voucher for each poo (stool) sample you give to us, and put your name into a prize draw, just to say thank you. We will also not tell anyone except your GP (doctor) that you are taking part in the study.



What will you do with my samples?
We will do some scientific tests on your samples to identify which bacteria are most common in your gut and also measure some of the chemicals that these bacteria produce (metabolites). This will help us to see which bacteria might help to keep us healthy. We would also like to keep these samples for future measurements as new tests become available.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in stomach problems. The study is funded by Yorkhill Children's Charity and is done with the supervision of leading doctors at Yorkhill, NHS Greater Glasgow & Clyde and a team of research scientists at University of Glasgow.

What if I have other concerns or questions?
If you have any questions tell your parent or carer and they will contact us so we can help answer them.

Clare will always be happy to answer any questions you might have about the study.

Please contact Clare by email:
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or by phone/text
mobile
(office - 0141 201 8688)

If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

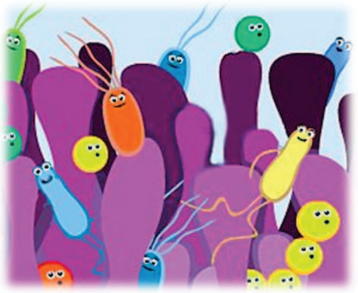
Information Sheet

BIG STUDY

Age 8-11
Healthy child

Hi! We are asking you to take part in our research project, which is about the role that gut bacteria (bugs) play in children with stomach problems.

What are bacteria?
Bacteria are tiny bugs that live everywhere. Although too small for us to see, they are on your skin right now, in your nose, all over your teeth and also living in your guts. In fact 90% of the cells in a human body are actually bacteria and we could not live without them.



Why are we doing this research?
Very little is known about how some bacteria protect and others harm the human gut. New scientific methods have shown that the bacteria are different in the guts of some children with long term gut problems and we want to find out why. This study will aim to answer some of these questions.

Why have I been chosen?
You have been chosen because you are healthy and we can compare you with children who are unwell. We will also be asking another 40 healthy children of different ages to take part in this study.

Do I have to take part?
No. It is up to you. If you agree to take part you will be asked to sign a form which says you are happy to take part. If you change your mind you can stop at any time without giving a reason, by just telling your parent you have changed your mind.

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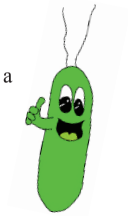
Information Sheet

Are there any benefits or risks if I take part?
There are no benefits or risks from taking part in the study. However we hope that you might find it a positive and rewarding experience. We will give you a £10 shopping voucher for each poo (stool) sample you give to us and add your name into a prize draw, just to say thank you.



Will my participation be kept confidential?
Yes. When you give consent to take part in the study we will allocate you a code number which will be used to label all samples and data, in order to protect your identity during all following lab work and analysis of sample data. Only the direct research team will have access to your name and contact details.

How will the information I provide be used?
The information we obtain from your samples will be used to produce a report on how diet, gut bacteria and other factors in the guts of children who have Crohn's disease compare with healthy children.

Who is doing the study?
Clare Clark, a postgraduate student researcher, is carrying out this study to increase our understanding of bacteria in stomach problems. The study is funded by Yorkhill Children's Charity and is being done is supervised by doctors at Yorkhill, NHS Greater Glasgow & Clyde and a team of research scientists at University of Glasgow.

What if I have more questions?
If you have any questions you or your parent/carer can contact us. Clare will always be happy to answer any questions you might have about the study.

Please contact Clare by email:
c.clark.1@research.gla.ac.uk
or by phone/text
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If you would like to speak to someone other than the researcher, please contact Dr Dalia Malkova, University of Glasgow Human Nutrition Section, Glasgow Royal Infirmary on 0141 201 8690.

yorkhill children's charity

University of Glasgow

Information Sheet

NHS Greater Glasgow and Clyde

BIG STUDY

Age 12+
Healthy young adults

As a healthy young person, we are inviting you to take part in a research study which will look at how gut bacteria might contribute to inflammation in children with gastrointestinal diseases such as Crohn's disease.

Why are we doing this research?
90% of the cells in a human body are bacteria, but very little is known about the role these bacteria play in both protecting and harming the human gut. New methods have shown that the bacteria in the guts of children with Crohn's disease are different. However, as yet, we don't know if these differences in gut bacteria are the result or the cause of Crohn's disease. The study will try to help answer this question.

Why have I been chosen?
We are interested in illnesses which affect the health of the gut. You have been chosen because you are healthy and so we can compare you with children who are unwell with conditions that involve the gut. We will be inviting other healthy children of different ages to take part in this study.

Do I have to take part?
No. It is up to you and your carer/parent to decide whether or not to take part. If you decide to help us by taking part you will be given an information sheet to keep and be asked to sign a consent form. If you do take part you will be free to withdraw from the study at any point you wish and without giving a reason.

What will happen if I agree to take part?
1. We will ask questions about your health and any medication you are taking.
2. We will ask questions about your diet.
3. We will ask to measure your height, weight, and hand grip strength to see how strong you are.
4. We will ask you to provide a stool (poo) and urine (pee) sample. This will be at a date that is convenient for you and can be collected from your home using a prepaid taxi.

We would like to keep these samples for future measurements as new tests become available.

yorkhill children's charity

INSTRUCTIONS FOR THE COLLECTION OF A FAECAL SAMPLE

1. Lift the toilet seat.
2. Place the bracket and the sample pot across the bowl. Lower the toilet seat.
3. Sit on the toilet seat. Check to make sure the sample pot is right below you.
4. If needed move the bracket and bucket around.

You do not have to use the bracket if you do not find it necessary.




5. Let your entire bowel movement fall into the pot *(please provide whole bowel movement if you can)*

Do NOT urinate or place toilet paper into the pot.

6. Stand up. Lift the pot out of the bracket and cover it with the lid.
7. Remove the bracket. Finish toilet activities.
    a. If you are in the hospital the researcher will collect the sample immediately.
    b. If you produce the sample at home then:

-Please turn over for instructions about sample handling at home

SAMPLE HANDLING AT HOME

Please follow these instructions below as carefully as possible after you have the sample;

1. Put on the plastic gloves provided. Cover the pot, containing the sample, with the lid. Seal it tight, and then place it inside the plastic bag.


2. Tear open the Anaerogen™ sachet and place the inner sachet on **top** of the lid inside the poly bag. DO NOT put it inside the pot. It will warm up and remove oxygen.

3. Immediately twist the neck of the bag really tight, trying to push out as much air as you can. Then make a firm double knot. It is very important to **tie the bag firmly** to keep air out.



4. **Place the bag with the sample into the cool bag with the ice pack from your freezer.**

IMPORTANT: call the researcher immediately after the sample is produced.

Please call the researcher Clare Clark on 07730 465230

If there is no reply please leave a text message or voicemail with your name if possible.

3 Appendix: Paediatric Crohn's disease activity index

weighted Pediatric Crohn's Disease Activity Index (wPCDAI)

History (Recall, 1 week)			
Abdominal Pain			**Score**
0 = None	**10** = Mild: Brief, does not interfere with activities	**20** = Moderate/Severe: Daily, longer lasting, affects activities, nocturnal	____
Patient Functioning, General Well-Being			**Score**
0 = No limitation of activities, well	**10** = Occasional difficulty in maintaining age appropriate activities, below par	**20** = Frequent limitation of activity, very poor	____
Stools (per day)			**Score**
0 = 0-1 liquid stools, no blood	**7.5** = Up to 2 semi-formed with small blood, or 2-5 liquid	**15** = Gross bleeding, or ≥ 6 liquid, or nocturnal diarrhea	____
Laboratory			
Erythrocyte Sedimentation Rate			**Score**
0 = < 20 mm/hr	**7.5** = 20-50 mm/hr	**15** = > 50 mm/hr	____
Albumin			**Score**
0 = ≥ 3.5 g/dL	**10**= 3.1-3.4 g/dL	**20** = ≤ 3.0 g/dL	____
Examination			
Weight			**Score**
0 = Weight gain or voluntary weight stable/loss	**5** = Involuntary weight stable, weight loss 1-9%	**10** = Weight loss ≥ 10%	____
Perirectal Disease			**Score**
0 = None, asymptomatic tags	**7.5** = 1-2 indolent fistula, scant drainage, no tenderness	**15** = Active fistula, drainage, tenderness, or abscess	____
Extra-intestinal Manifestations (fever ≥ 38.5 °C for 3 days over past week, definite arthritis, uveitis, E. nodosum, P. gangrenosum)			**Score**
0 = None		**10** = One or more	____
			Total Score (0-125):

Cutoff Values of wPCDAI (0-125 points)

Remission	Mild	Moderate	Severe Disease Activity
<12.5	12.5-40	>40-57,5	>57.5

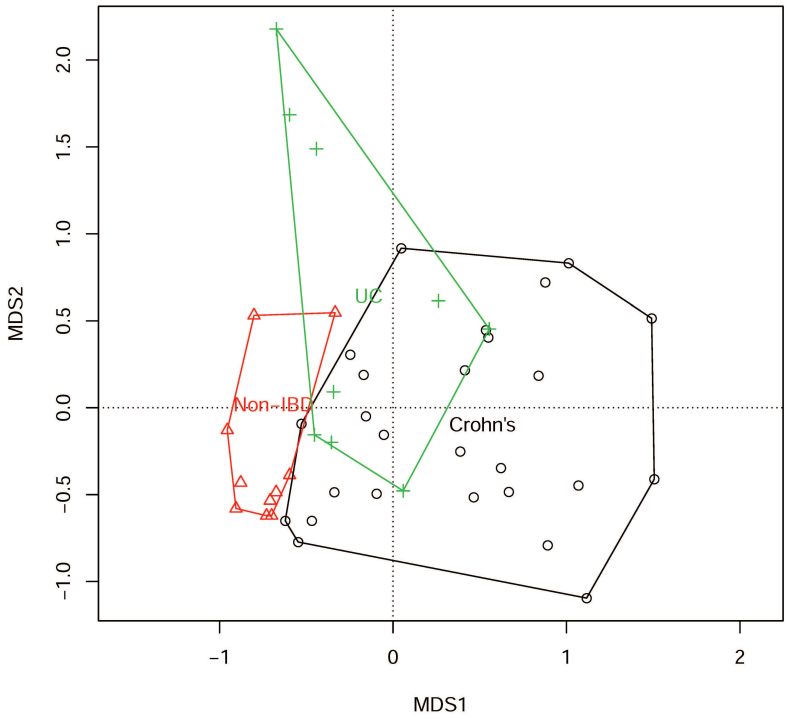
Simple Endoscopic Score for Crohn's Disease (SES-CD)

	Ileum	*Right Colon*	*Transverse colon*	*Left colon*	*Rectum*	***TOTAL***
Size of ulcers (0-3)						
Ulcerated surface (0-3)						
Affected surface (0-3)						
Narrowing (0-3)						
					SES-CD=	

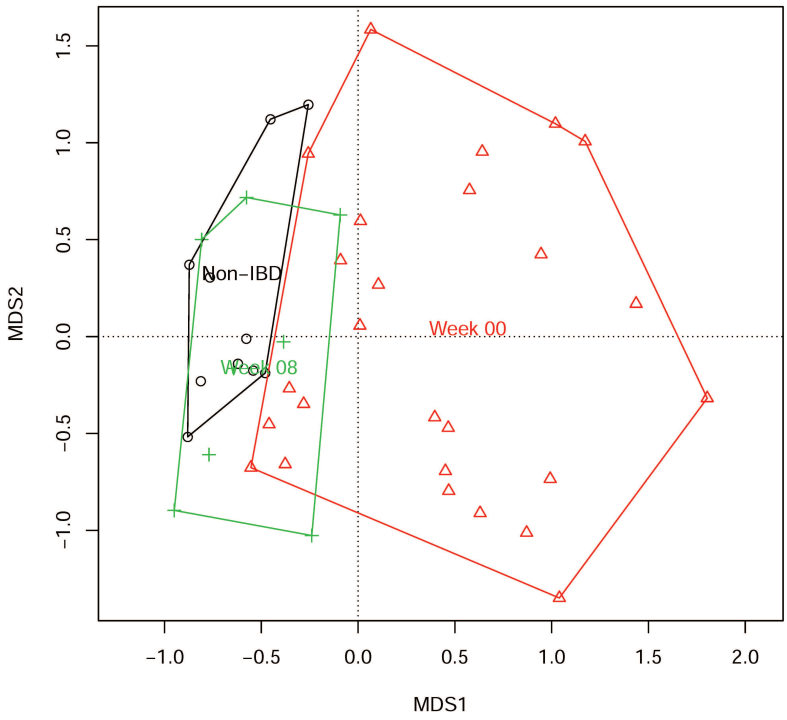
	0	***1***	***2***	***3***
Size of ulcers	*None*	*Aphthous ulcers (ø=0.1-0.5 cm)*	*Large ulcers (ø=0.5-2 cm)*	*Very large ulcers (ø> 2cm)*
Ulcerated surface	*None*	*<10%*	*10-30%*	*>30%*
Affected surface	*Unaffected*	*<50%*	*50-75%*	*>75%*
Narrowing	*None*	*Single, can be passed*	*Multiple, can be passed*	*Cannot be passed*
Global assessment	*Mucosal healing*	*Mild inflammation*	*Moderate inflammation*	*Severe ulcerating inflammation*

Paediatric Crohn's disease activity index (wPCDAI) form. Simple endoscopic score form for Crohn's disease shown below.

4 Appendix: Blood markers of inflammation in IBD



Difference in blood result profiles between children with Crohn's disease, UC and non-IBD conditions shown by multi-dimensional scaling (MDS) spider plot. PERMANOVA: R2=0.197; **p=0.0001** (*vegan; euclidean*).



Changes in blood result profiles between baseline (week 00) and after 8-week treatment with EEN (week 08) in children with Crohn's disease; shown by multi-dimensional scaling (MDS) plot. PERMANOVA: R2=0.225; **p=0.0001**; Children with non-IBD conditions (black) are shown for reference.

5 Appendix: Classification of bacteria associated with IBD

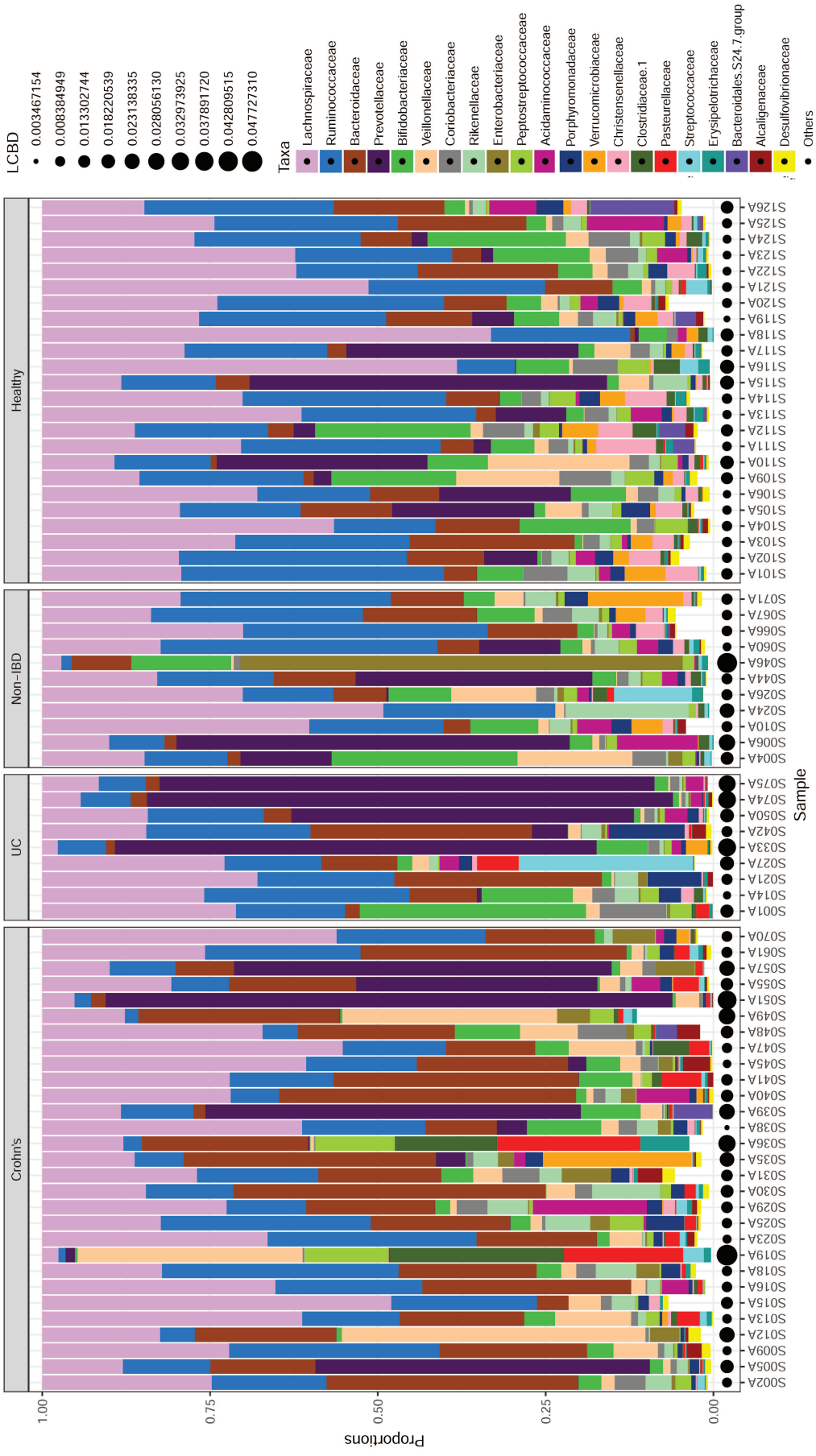
Classification of bacteria/archaea associated with IBD

Phylum	Class	Order	Family	Genus
Archaea (Euryarchaeota)	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Varibaculum
		Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium
		Corynebacteriales	Corynebacteriaceae	
	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella
				Eggerthella
				Slackia
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
			Porphyromonadaceae	Coprobacter
				Parabacteroides
			Prevotellaceae	Paraprevotella
				Prevotella
			Rikenellaceae	Alistipes
			S24-7	
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus
		Lactobacillales	Enterococcaceae	Enterococcus
			Lactobacillaceae	Lactobacillus
			Streptococcaceae	Streptococcus
		Erysipelotrichales	Erysipelotrichaceae	
				Turicibacter
	Clostridia	Clostridiales	Christensenellaceae	
			Clostridiaceae	Clostridium
				Hungatella
			Defluviitaleaceae	
			Eubacteriaceae	Eubacterium
			Family XI	
			Family XIII	
			Lachnospiraceae	Lachnoclostridium
				Anaerostipes
				Blautia
				Butyrivibrio
				Roseburia
				Tyzzerella
				Pseudobutyrivibrio
			Peptococcaceae	
			Ruminococcaceae	Anaerotruncus
				Faecalibacterium
				Ruminococcus
				Subdoligranulum
	Negativicutes	Acidaminococcales	Acidaminococcaceae	Acidaminococcus
		Selenomonadales	Veillonellaceae	Allisonella
				Anaeroglobus
				Dialister
				Megasphaera
				Selenomonas
				Veillonella
Tenericutes	Mollicutes			
Fusobacteria		Fusobacteriales	Fusobacteriaceae	
Proteobacteria	α-proteobacteria	Rhizobiales	Brucellaceae	Mycoplana
		Rhodospirillales	Rhodospirillaceae	
	β-proteobacteria	Neisseriales	Neisseriaceae	Eikenella

Classification of bacteria/archaea associated with IBD) (continued)

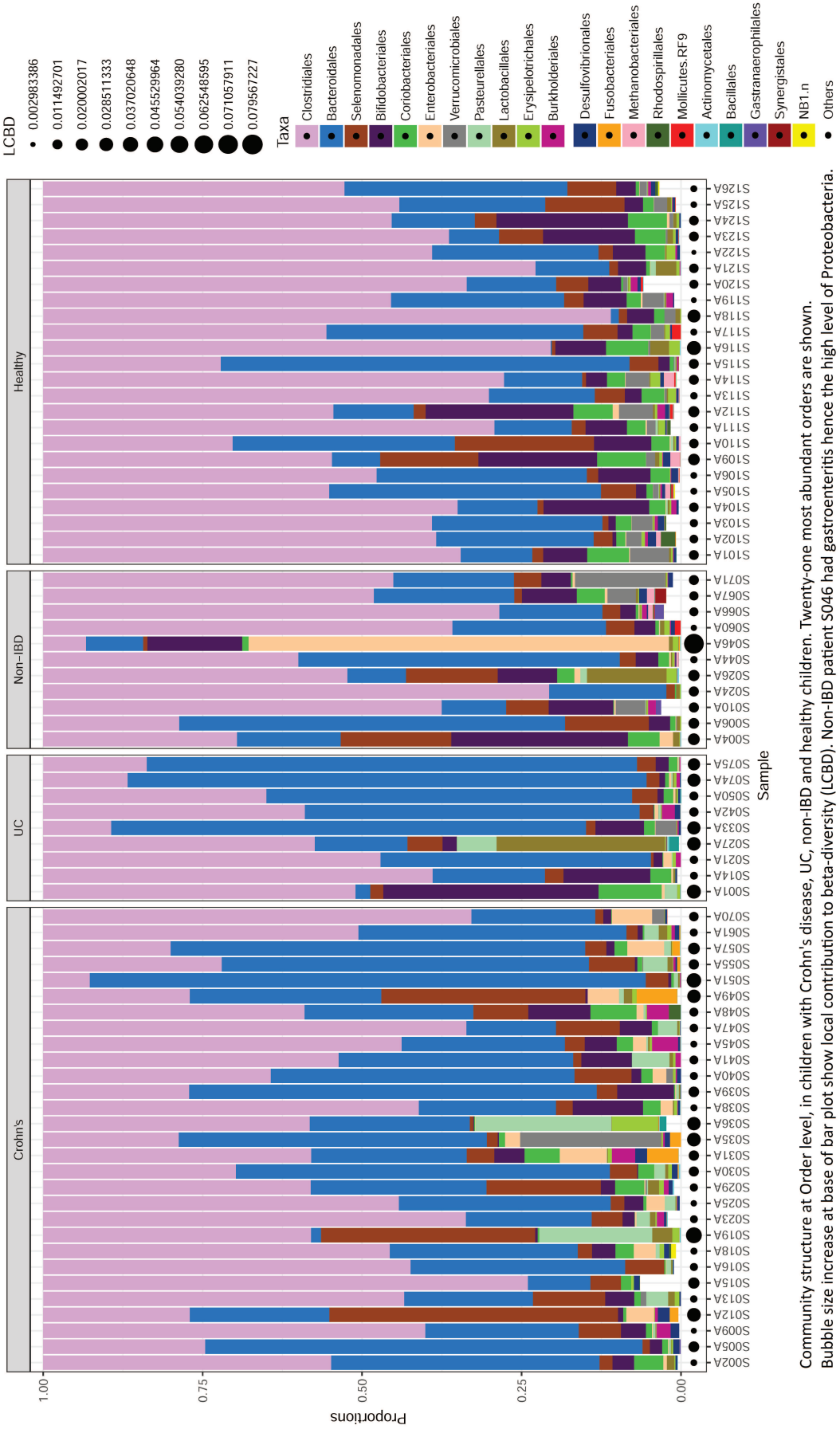
Phylum	Class	Order	Family	Genus
		Burkholderiales	Sutterellaceae	Sutterella
	δ-proteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
				Desulfovibrio
	ε-proteobacteria	Campylobacterales	Campylobacteraceae	Campylobacter
			Helicobacteraceae	Helicobacter
	γ-proteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
				Escherichia
				Cronobacter
				Citrobacter
				Pantoea
				Shigella
			Morganellaceae	Proteus
		Pasteurellales	Pasteurellaceae	Haemophilus
				Aggregatibacter
		Pseudomonadales	Pseudomonadaceae	Pseudomonas
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia

5.1 Appendix: Bacterial community structure at Family level taxa



Community structure at Family level, in children with Crohn's disease, UC, non-IBD and healthy children. Twenty-one most abundant families are shown. Bubble size increase at base of bar plot show local contribution to beta-diversity (LCBD). Non-IBD patient S046 had gastroenteritis hence the high level of Enterobacteriaceae, (Escherichia-Shigella)

5.2 Appendix: Bacterial community structure at Order level taxa



Community structure at Order level, in children with Crohn's disease, UC, non-IBD and healthy children. Twenty-one most abundant orders are shown.
Bubble size increase at base of bar plot show local contribution to beta-diversity (LCBD). Non-IBD patient S046 had gastroenteritis hence the high level of Proteobacteria.

5.3 Appendix: Taxa increased/decreased at baseline in children with Crohn's disease compared with healthy children

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease compared with healthy children (differential analysis using negative binomial)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
↗ **Phyla increased**					
Fusobacteria	52.05	5.90	0.67	9.71E-19	6.31E-18
Proteobacteria	1506.51	2.50	0.37	1.30E-11	5.62E-11
↗ **Families increased**					
Bacillales Family XI	20.31	2.81	0.57	8.52E-07	4.10E-06
Fusobacteriaceae	46.35	5.98	0.68	2.55E-18	6.77E-17
Enterobacteriaceae	354.06	3.60	0.61	4.50E-09	3.41E-08
Neisseriaceae	3.31	2.31	0.46	6.10E-07	3.23E-06
Pasteurellaceae	620.44	4.43	0.67	3.19E-11	3.38E-10
↗ **Species (SEQ variants) increased**					
SEQ 14 Bacteroides	639.34	2.71	0.74	2.40E-04	1.19E-03
SEQ 31 Bacteroides	410.18	2.05	0.84	1.41E-02	3.55E-02
SEQ 38 Bacteroides	309.78	2.19	0.75	3.51E-03	1.08E-02
SEQ 50 Bacteroides	127.14	2.36	0.87	6.32E-03	1.80E-02
SEQ 213 Bacteroides	9.23	3.92	0.63	4.11E-10	8.18E-09
SEQ 287 Clostridium sensu stricto 1	12.25	2.13	0.70	2.21E-03	7.49E-03
SEQ 284 Clostridium sensu stricto 1	9.73	2.23	0.67	9.12E-04	3.70E-03
SEQ 218 Collinsella	7.02	2.32	0.62	1.89E-04	9.74E-04
SEQ 385 Dialister pneumosintes	4.13	2.65	0.58	4.95E-06	4.03E-05
SEQ 195 Eggerthella	8.75	2.96	0.60	8.73E-07	8.70E-06
SEQ 15 Escherichia-Shigella	201.67	2.82	0.61	4.11E-06	3.49E-05
SEQ 162 Eubacterium hallii group	4.59	2.82	0.61	3.39E-06	2.98E-05
SEQ 71 Eubacterium oxidoreducens group	170.28	2.58	0.80	1.26E-03	4.75E-03
SEQ 94 Eubacterium oxidoreducens group	143.50	3.00	0.83	2.78E-04	1.34E-03
SEQ 79 Flavonifractor	48.73	3.49	0.65	8.79E-08	1.02E-06
SEQ 310 Fusobacterium	8.15	3.73	0.64	6.01E-09	8.93E-08
SEQ 321 Fusobacterium	8.07	3.53	0.63	2.48E-08	3.41E-07
SEQ 39 Haemophilus	536.81	4.25	0.68	3.20E-10	6.55E-09
SEQ 319 Haemophilus	5.86	2.12	0.62	6.40E-04	2.74E-03
SEQ 8 Lachnoclostridium	210.99	3.16	0.74	1.90E-05	1.25E-04
SEQ 113 Lachnoclostridium	20.73	2.25	0.65	5.22E-04	2.31E-03
SEQ 136 Lachnoclostridium	16.25	2.24	0.66	6.82E-04	2.89E-03
SEQ 149 Lachnoclostridium	6.78	3.44	0.62	2.98E-08	3.95E-07
SEQ 496 Lachnoclostridium	3.24	2.25	0.57	8.46E-05	4.85E-04
SEQ 392 Lachnoclostridium	2.83	2.02	0.57	3.67E-04	1.71E-03
SEQ 124 Lachnospiraceae	77.24	2.16	0.69	1.84E-03	6.43E-03
SEQ 184 Lachnospiraceae	21.23	5.16	0.72	5.40E-13	1.78E-11
SEQ 212 Lachnospiraceae UCG-008	25.72	3.03	0.71	1.89E-05	1.25E-04
SEQ 299 Megasphaera	12.56	4.38	0.66	3.34E-11	8.15E-10
SEQ 183 Paraprevotella	4.65	2.85	0.63	6.43E-06	5.12E-05
SEQ 227 Peptostreptococcus	31.73	5.53	0.71	7.25E-15	3.66E-13
SEQ 5 Prevotella 7	38.93	5.78	0.78	1.41E-13	5.08E-12

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease compared with healthy children (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 46 Veillonella	513.33	3.65	0.77	1.87E-06	1.75E-05
SEQ 58 Veillonella	103.03	3.50	0.70	5.69E-07	5.82E-06
SEQ 101 Veillonella	98.14	2.93	0.70	2.91E-05	1.81E-04
SEQ 144 Veillonella	18.46	3.52	0.66	1.07E-07	1.23E-06
SEQ 211 Veillonella	13.11	2.61	0.68	1.28E-04	6.98E-04
SEQ 325 Veillonella	5.82	3.20	0.64	6.16E-07	6.22E-06
SEQ 355 Veillonella	3.64	2.45	0.59	3.18E-05	1.96E-04
↘ **Phyla decreased**					
Euryarchaeota	32.05	-5.58	0.62	4.25E-19	5.53E-18
Tenericutes	31.98	-3.68	0.61	1.89E-09	6.16E-09
Verrucomicrobia	274.36	-3.01	0.73	3.58E-05	9.30E-05
↘ **Families decreased**					
Methanobacteriaceae	33.55	-5.90	0.62	1.79E-21	9.47E-20
Christensenellaceae	494.81	-4.07	0.57	7.15E-13	9.47E-12
Clostridiales vadinBB60 group	27.65	-4.25	0.53	1.98E-15	3.51E-14
Defluviitaleaceae	8.06	-2.59	0.48	5.12E-08	3.24E-07
Peptococcaceae	6.76	-2.68	0.44	1.00E-09	8.88E-09
Rhodospirillaceae	11.30	-3.21	0.59	5.50E-08	3.24E-07
Verrucomicrobiaceae	339.52	-3.29	0.75	1.29E-05	5.70E-05
↘ **Species (SEQ variants) decreased**					
SEQ 262 Acidaminococcus	3.10	-2.38	0.55	1.55E-05	1.07E-04
SEQ 11 Akkermansia	297.46	-3.24	0.83	9.05E-05	5.14E-04
SEQ 123 Akkermansia	5.52	-3.09	0.60	2.90E-07	3.10E-06
SEQ 166 Alistipes	96.81	-2.07	0.87	1.75E-02	4.28E-02
SEQ 188 Alistipes	6.48	-3.61	0.57	2.84E-10	5.97E-09
SEQ 366 Alistipes	6.28	-3.61	0.58	4.69E-10	9.11E-09
SEQ 887 Alistipes	2.89	-2.25	0.50	6.63E-06	5.22E-05
SEQ 254 Allisonella	8.45	-3.92	0.59	3.29E-11	8.15E-10
SEQ 527 Anaerostipes	6.67	-2.83	0.56	4.71E-07	4.88E-06
SEQ 289 Anaerotruncus	30.13	-3.74	0.70	7.49E-08	8.99E-07
SEQ 793 Anaerotruncus	4.96	-2.14	0.57	1.54E-04	8.20E-04
SEQ 725 Anaerotruncus	3.60	-2.02	0.52	9.86E-05	5.49E-04
SEQ 1034 Anaerotruncus	2.85	-2.23	0.50	6.87E-06	5.36E-05
SEQ 197 Bacteroidales S24-7 group	6.60	-3.63	0.61	3.22E-09	5.42E-08
SEQ 207 Bacteroidales S24-7 group	5.19	-3.25	0.59	3.85E-08	4.70E-07
SEQ 104 Bacteroides	10.92	-3.47	0.67	2.37E-07	2.56E-06
SEQ 141 Bacteroidetes VC2.1 Bac22	93.86	-3.26	0.69	2.17E-06	1.98E-05
SEQ 229 Barnesiella	22.30	-5.12	0.70	3.38E-13	1.16E-11
SEQ 210 Barnesiella	5.78	-3.42	0.58	3.89E-09	6.24E-08
SEQ 16 Bifidobacterium	948.13	-2.95	0.77	1.42E-04	7.60E-04
SEQ 142 Blautia	63.66	-2.48	0.76	1.19E-03	4.62E-03
SEQ 283 Blautia	29.07	-5.88	0.63	1.49E-20	1.88E-18
SEQ 839 Blautia	3.51	-2.60	0.54	1.31E-06	1.24E-05
SEQ 281 Butyrivibrio	4.46	-3.00	0.58	2.00E-07	2.20E-06
SEQ 683 Christensenellaceae	3.84	-2.23	0.52	1.64E-05	1.12E-04

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease compared with healthy children (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 57 Christensenellaceae R-7 group	316.48	-6.67	0.63	7.04E-26	2.66E-23
SEQ 121 Christensenellaceae R-7 group	46.57	-2.06	0.74	5.20E-03	1.50E-02
SEQ 202 Christensenellaceae R-7 group	44.39	-5.82	0.60	2.49E-22	4.72E-20
SEQ 252 Christensenellaceae R-7 group	43.22	-4.95	0.62	1.22E-15	7.12E-14
SEQ 308 Christensenellaceae R-7 group	12.37	-3.71	0.55	1.63E-11	4.57E-10
SEQ 435 Christensenellaceae R-7 group	5.39	-2.47	0.51	1.31E-06	1.24E-05
SEQ 501 Christensenellaceae R-7 group	4.17	-2.89	0.52	2.73E-08	3.69E-07
SEQ 576 Clostridiales vadinBB60 group	9.30	-4.17	0.61	9.75E-12	2.95E-10
SEQ 1041 Clostridiales vadinBB60 group	2.74	-2.15	0.47	3.86E-06	3.32E-05
SEQ 115 Clostridium sensu stricto 1	101.95	-2.36	0.65	2.99E-04	1.42E-03
SEQ 354 Collinsella	13.36	-4.31	0.57	4.25E-14	1.79E-12
SEQ 91 Coprococcus 2	207.88	-3.64	0.78	3.18E-06	2.83E-05
SEQ 110 Coprococcus 2	138.56	-2.63	0.76	5.06E-04	2.25E-03
SEQ 216 Coprococcus 2	55.11	-2.57	0.80	1.35E-03	5.01E-03
SEQ 454 Coriobacteriaceae	5.63	-2.09	0.47	8.29E-06	6.22E-05
SEQ 602 Coriobacteriaceae	3.34	-2.28	0.51	9.08E-06	6.74E-05
SEQ 647 Coriobacteriaceae	2.75	-2.16	0.49	1.13E-05	8.13E-05
SEQ 670 Defluviitaleaceae UCG-011	4.93	-2.46	0.54	4.52E-06	3.80E-05
SEQ 957 Defluviitaleaceae UCG-011	3.11	-2.39	0.50	1.90E-06	1.75E-05
SEQ 464 Enterorhabdus	7.64	-3.87	0.58	3.23E-11	8.15E-10
SEQ 688 Erysipelotrichaceae	5.21	-3.26	0.56	4.58E-09	7.07E-08
SEQ 40 Eubacterium coprostanoligenes group	265.24	-2.48	0.83	2.98E-03	9.32E-03
SEQ 164 Eubacterium coprostanoligenes group	22.81	-3.96	0.68	5.34E-09	8.08E-08
SEQ 443 Eubacterium coprostanoligenes group	7.58	-3.85	0.59	8.06E-11	1.85E-09
SEQ 667 Eubacterium coprostanoligenes group	5.71	-3.40	0.57	2.39E-09	4.21E-08
SEQ 156 Eubacterium coprostanoligenes group	2.98	-2.37	0.55	1.74E-05	1.18E-04
SEQ 696 Eubacterium oxidoreducens group	2.84	-2.22	0.50	8.05E-06	6.09E-05
SEQ 331 Eubacterium ruminantium group	21.26	-3.33	0.68	1.03E-06	1.01E-05
SEQ 208 Eubacterium ventriosum group	59.27	-3.17	0.61	1.72E-07	1.94E-06
SEQ 553 Family XIII UCG-001	6.22	-2.12	0.48	1.12E-05	8.13E-05
SEQ 429 Holdemanella	3.49	-2.59	0.57	4.84E-06	4.03E-05
SEQ 651 Holdemanella	2.94	-2.28	0.53	1.52E-05	1.06E-04
SEQ 382 Incertae Sedis	3.49	-2.58	0.57	4.92E-06	4.03E-05
SEQ 259 Lachnospira	24.52	-3.14	0.66	2.21E-06	1.99E-05
SEQ 440 Lachnospiraceae FCS020 group	9.23	-2.35	0.60	9.09E-05	5.14E-04
SEQ 750 Lachnospiraceae NC2004 group	3.48	-2.25	0.52	1.50E-05	1.05E-04
SEQ 452 Lachnospiraceae UCG-001	5.34	-2.38	0.55	1.34E-05	9.57E-05
SEQ 419 Lachnospiraceae UCG-003	8.81	-4.09	0.60	6.62E-12	2.09E-10
SEQ 311 Lachnospiraceae UCG-008	6.62	-2.82	0.54	1.80E-07	2.01E-06
SEQ 479 Lactococcus	7.06	-2.33	0.57	3.88E-05	2.37E-04
SEQ 421 Marvinbryantia	7.88	-2.57	0.57	7.30E-06	5.64E-05
SEQ 179 Methanobrevibacter	48.07	-6.62	0.66	1.06E-23	2.67E-21
SEQ 403 Mollicutes RF9	5.82	-3.43	0.59	6.68E-09	9.72E-08
SEQ 652 Olsenella	7.45	-3.83	0.60	1.37E-10	2.96E-09
SEQ 370 Paraprevotella	3.02	-2.33	0.54	1.41E-05	1.00E-04

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease compared with healthy children (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 55 Phascolarctobacterium	240.85	-5.17	0.87	2.94E-09	5.05E-08
SEQ 367 Pseudobutyrivibrio	24.40	-5.62	0.69	3.35E-16	2.11E-14
SEQ 98 Roseburia	120.88	-4.29	0.73	3.96E-09	6.24E-08
SEQ 131 Ruminiclostridium 5	39.31	-2.03	0.51	6.33E-05	3.69E-04
SEQ 397 Ruminiclostridium 5	10.66	-3.01	0.55	3.41E-08	4.38E-07
SEQ 444 Ruminiclostridium 5	7.88	-2.14	0.50	2.09E-05	1.34E-04
SEQ 618 Ruminiclostridium 5	4.35	-2.96	0.55	8.12E-08	9.60E-07
SEQ 1008 Ruminiclostridium 5	2.60	-2.06	0.49	2.30E-05	1.46E-04
SEQ 217 Ruminiclostridium 6	12.90	-4.34	0.57	3.25E-14	1.45E-12
SEQ 439 Ruminiclostridium 9	12.49	-4.62	0.56	1.28E-16	8.81E-15
SEQ 361 Ruminococcaceae	9.49	-2.89	0.47	5.25E-10	9.94E-09
SEQ 373 Ruminococcaceae	7.90	-3.92	0.60	9.30E-11	2.07E-09
SEQ 827 Ruminococcaceae	4.62	-3.06	0.55	3.03E-08	3.95E-07
SEQ 413 Ruminococcaceae	3.57	-2.63	0.52	4.50E-07	4.73E-06
SEQ 135 Ruminococcaceae NK4A214 group	68.04	-2.20	0.67	1.08E-03	4.24E-03
SEQ 379 Ruminococcaceae NK4A214 group	17.82	-3.35	0.69	1.16E-06	1.13E-05
SEQ 351 Ruminococcaceae NK4A214 group	17.55	-2.16	0.68	1.57E-03	5.72E-03
SEQ 48 Ruminococcaceae UCG-002	252.98	-3.35	0.76	9.98E-06	7.33E-05
SEQ 56 Ruminococcaceae UCG-002	150.08	-3.01	0.80	1.57E-04	8.32E-04
SEQ 187 Ruminococcaceae UCG-002	30.00	-5.98	0.70	1.44E-17	1.37E-15
SEQ 388 Ruminococcaceae UCG-002	18.66	-5.22	0.67	6.53E-15	3.53E-13
SEQ 327 Ruminococcaceae UCG-002	14.76	-4.87	0.65	9.33E-14	3.71E-12
SEQ 119 Ruminococcaceae UCG-005	119.94	-3.69	0.64	7.33E-09	1.05E-07
SEQ 161 Ruminococcaceae UCG-005	78.19	-5.29	0.60	1.54E-18	1.67E-16
SEQ 181 Ruminococcaceae UCG-005	36.10	-2.04	0.72	4.52E-03	1.35E-02
SEQ 295 Ruminococcaceae UCG-005	27.66	-4.52	0.59	1.21E-14	5.72E-13
SEQ 494 Ruminococcaceae UCG-010	9.82	-4.25	0.50	1.93E-17	1.63E-15
SEQ 662 Ruminococcaceae UCG-010	7.09	-3.75	0.57	3.99E-11	9.44E-10
SEQ 751 Ruminococcaceae UCG-010	4.37	-2.97	0.52	8.79E-09	1.23E-07
SEQ 622 Ruminococcaceae UCG-010	2.93	-2.27	0.53	1.81E-05	1.21E-04
SEQ 288 Ruminococcaceae UCG-013	11.80	-3.57	0.59	1.21E-09	2.23E-08
SEQ 190 Ruminococcaceae UCG-014	95.07	-7.61	0.66	1.68E-30	1.27E-27
SEQ 200 Ruminococcaceae UCG-014	20.00	-2.06	0.66	1.76E-03	6.23E-03
SEQ 209 Ruminococcaceae UCG-014	13.93	-4.78	0.64	9.80E-14	3.71E-12
SEQ 233 Ruminococcaceae UCG-014	10.96	-3.64	0.66	3.64E-08	4.55E-07
SEQ 418 Ruminococcaceae UCG-014	6.62	-3.64	0.60	1.39E-09	2.50E-08
SEQ 279 Ruminococcaceae UCG-014	3.75	-2.14	0.54	7.26E-05	4.20E-04
SEQ 574 Ruminococcaceae UCG-014	2.84	-2.22	0.52	2.07E-05	1.34E-04
SEQ 86 Ruminococcus 1	213.90	-2.36	0.74	1.37E-03	5.05E-03
SEQ 219 Ruminococcus 1	44.47	-6.07	0.64	2.20E-21	3.34E-19
SEQ 235 Ruminococcus 1	9.85	-4.26	0.63	1.14E-11	3.33E-10
SEQ 653 Ruminococcus 1	3.10	-2.38	0.51	3.72E-06	3.23E-05
SEQ 28 Ruminococcus 2	603.88	-3.50	0.78	7.69E-06	5.88E-05
SEQ 171 Ruminococcus 2	9.27	-2.99	0.65	5.02E-06	4.05E-05
SEQ 116 Ruminococcus gauvreauii group	28.30	-2.05	0.68	2.72E-03	8.78E-03

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease compared with healthy children (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 393 Ruminococcus gauvreauii group	4.33	-2.95	0.54	3.67E-08	4.55E-07
SEQ 342 Senegalimassilia	20.82	-3.81	0.65	3.80E-09	6.24E-08
SEQ 433 Slackia	13.80	-4.14	0.62	2.93E-11	7.91E-10
SEQ 372 Thalassospira	2.87	-2.24	0.52	2.03E-05	1.32E-04
SEQ 278 Turicibacter	9.45	-2.25	0.60	1.80E-04	9.33E-04
SEQ 81 Tyzzerella 4	38.54	-2.65	0.73	2.68E-04	1.32E-03
SEQ 365 unidentified	24.37	-5.62	0.67	7.35E-17	5.57E-15

5.4 Appendix: Changes in Taxa in children with Crohn's disease after 4-weeks treatment on EEN

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease after 4-weeks treatment on EEN (differential analysis using negative binomial)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
↗ **Phyla increased**					
Verrucomicrobia	310.00	4.53	0.96	2.57E-06	1.54E-05
Cyanobacteria	8.31	2.86	0.66	1.45E-05	5.78E-05
↗ **Families increased**					
Enterobacteriaceae	1225.08	2.58	0.64	5.69E-05	3.16E-04
Porphyromonadaceae	912.31	2.01	0.63	1.35E-03	5.50E-03
Verrucomicrobiaceae	284.16	4.31	0.91	2.28E-06	2.31E-05
Christensenellaceae	211.68	2.43	0.70	5.54E-04	2.60E-03
Family XIII	94.42	2.71	0.58	2.81E-06	2.45E-05
Rhodospirillaceae	21.61	4.02	0.71	1.43E-08	2.91E-07
Staphylococcaceae	8.68	2.66	0.61	1.46E-05	9.88E-05
Peptococcaceae	5.80	2.20	0.58	1.53E-04	7.79E-04
Corynebacteriaceae	3.76	2.19	0.46	2.06E-06	2.31E-05
↗ **Species (SEQ variants) increased**					
SEQ 7 Ruminococcus torques ATCC27756	2169.36	5.23	0.76	4.80E-12	1.51E-10
SEQ 8 Lachnoclostridium	918.91	2.46	0.72	6.35E-04	2.81E-03
SEQ 54 Cronobacter	481.73	7.13	0.81	8.90E-19	1.01E-16
SEQ 69 Ruminococcaceae	325.66	3.93	0.68	6.84E-09	1.62E-07
SEQ 79 Flavonifractor	235.28	2.56	0.68	1.67E-04	9.84E-04
SEQ 68 Ruminococcaceae UCG-002	231.74	2.17	0.84	9.93E-03	2.93E-02
SEQ 92 Clostridiales bacterium canine/085	201.66	3.61	0.69	1.60E-07	2.46E-06
SEQ 113 Lachnoclostridium	170.13	3.15	0.72	1.21E-05	1.11E-04
SEQ 145 Lachnoclostridium	120.04	6.21	0.73	1.75E-17	1.42E-15
SEQ 81 Tyzzerella 4	113.04	3.89	0.86	5.50E-06	5.38E-05
SEQ 136 Lachnoclostridium	100.68	2.92	0.74	7.19E-05	5.16E-04
SEQ 51 Bacteroidetes VC2.1 Bac22	96.91	3.21	0.92	5.27E-04	2.43E-03
SEQ 121 Christensenellaceae R-7 group	81.44	2.54	0.86	3.19E-03	1.12E-02
SEQ 84 Enterobacter	78.42	6.34	0.71	3.27E-19	4.64E-17

Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease after 4-weeks treatment on EEN (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 150 Faecalitalea	78.02	5.78	0.77	6.51E-14	2.84E-12
SEQ 159 Hungatella	63.35	3.99	0.77	2.41E-07	3.42E-06
SEQ 77 Blautia	52.59	6.67	0.88	4.24E-14	2.18E-12
SEQ 178 Lachnospiraceae	52.25	2.99	0.77	9.56E-05	6.57E-04
SEQ 116 [Ruminococcus] gauvreauii group	49.66	2.66	0.79	7.71E-04	3.34E-03
SEQ 250 Family XIII AD3011 group	46.50	5.12	0.74	3.40E-12	1.13E-10
SEQ 195 Eggerthella	44.90	3.04	0.72	2.65E-05	2.21E-04
SEQ 40 [Eubacterium] coprostanoligenes group	42.16	2.68	0.86	1.89E-03	7.28E-03
SEQ 242 Hungatella	36.76	3.55	0.64	3.80E-08	7.70E-07
SEQ 192 Ruminiclostridium 9	35.89	4.23	0.72	5.05E-09	1.25E-07
SEQ 177 Christensenellaceae R-7 group	34.48	2.22	0.75	3.14E-03	1.11E-02
SEQ 275 Lachnoclostridium	32.34	3.70	0.72	3.05E-07	4.22E-06
SEQ 230 Ruminiclostridium 5	29.78	2.58	0.63	4.82E-05	3.60E-04
SEQ 272 Eisenbergiella	28.11	5.68	0.69	2.32E-16	1.46E-14
SEQ 169 Lachnoclostridium	26.56	3.48	0.64	5.64E-08	1.07E-06
SEQ 146 Veillonellaceae bacterium canine/211	20.07	4.25	0.78	5.01E-08	9.80E-07
SEQ 341 Ruminococcaceae UCG-004	19.63	2.05	0.70	3.46E-03	1.20E-02
SEQ 104 Bacteroides	17.84	3.93	0.80	1.01E-06	1.22E-05
SEQ 244 Incertae Sedis	16.77	3.43	0.65	1.13E-07	1.83E-06
SEQ 149 Lachnoclostridium	16.73	3.06	0.69	8.98E-06	8.48E-05
SEQ 181 Ruminococcaceae UCG-005	13.87	3.19	0.70	5.63E-06	5.41E-05
SEQ 292 Blautia	13.33	3.33	0.70	2.04E-06	2.26E-05
SEQ 286 Erysipelatoclostridium	12.19	3.05	0.64	1.80E-06	2.08E-05
SEQ 126 [Ruminococcus] gauvreauii group	10.32	3.25	0.76	1.97E-05	1.67E-04
SEQ 361 Ruminococcaceae	9.90	2.61	0.60	1.24E-05	1.11E-04
SEQ 499 Ruminiclostridium 5	9.75	3.06	0.64	1.70E-06	2.01E-05
SEQ 491 Flavonifractor	9.36	2.65	0.65	4.09E-05	3.22E-04
SEQ 142 Blautia	8.21	3.84	0.70	3.56E-08	7.48E-07
SEQ 280 Gastranaerophilales	8.07	3.62	0.68	9.26E-08	1.64E-06
SEQ 413 Ruminococcaceae	7.95	3.79	0.60	3.00E-10	8.51E-09
SEQ 99 Staphylococcus	7.55	3.24	0.65	6.85E-07	8.83E-06
SEQ 488 Lachnoclostridium	7.03	2.31	0.58	7.80E-05	5.53E-04
SEQ 118 Anaerostipes caccae DSM14662	6.85	2.35	0.63	2.05E-04	1.16E-03
SEQ 226 Clostridium sp. NHT38	6.65	3.51	0.66	9.54E-08	1.64E-06
SEQ 263 Butyricimonas	6.64	2.24	0.68	1.01E-03	4.11E-03
SEQ 599 Christensenella minuta	6.32	2.26	0.59	1.28E-04	8.04E-04
SEQ 202 Christensenellaceae R-7 group	6.17	2.50	0.65	1.34E-04	8.34E-04
SEQ 297 Marvinbryantia	5.76	2.60	0.62	3.09E-05	2.54E-04
SEQ 402 Anaerotruncus sp. NML070203	5.75	2.30	0.65	4.40E-04	2.10E-03
SEQ 472 Intestinimonas butyriciproducens	5.42	2.23	0.61	2.39E-04	1.32E-03
SEQ 432 Actinomyces	5.05	2.23	0.61	2.32E-04	1.29E-03
SEQ 123 Akkermansia	4.99	2.74	0.66	3.42E-05	2.77E-04
SEQ 554 Dorea	4.90	2.14	0.60	3.78E-04	1.86E-03
SEQ 435 Christensenellaceae R-7 group	4.88	2.00	0.51	8.92E-05	6.24E-04

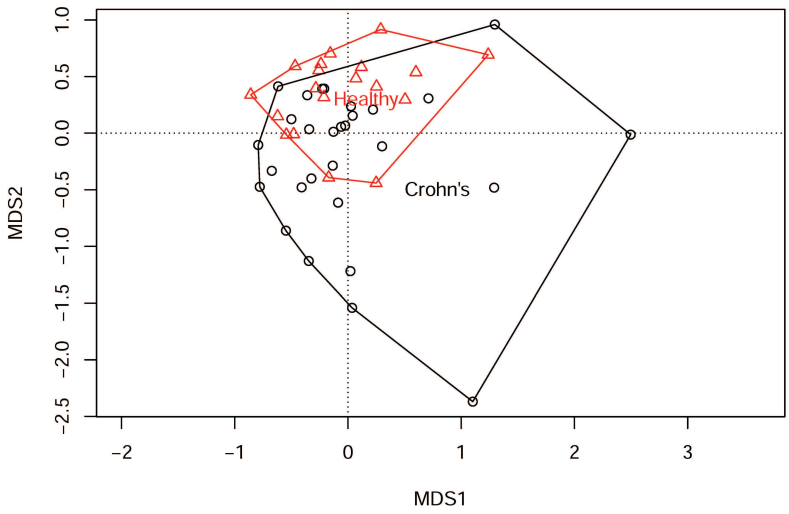
Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease after 4-weeks treatment on EEN (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 316 Lachnospiraceae UCG-008	4.47	2.88	0.63	5.34E-06	5.31E-05
SEQ 373 Ruminococcaceae	4.35	2.83	0.61	3.86E-06	3.90E-05
SEQ 593 Oscillibacter	3.99	2.13	0.56	1.50E-04	9.08E-04
SEQ 251 Lachnoclostridium	3.98	2.68	0.61	9.99E-06	9.29E-05
SEQ 344 Ruminococcaceae UCG-004	3.93	2.33	0.57	4.92E-05	3.62E-04
SEQ 243 Sutterella	3.42	2.43	0.61	6.95E-05	5.05E-04
SEQ 147 Peptoclostridium difficile QCD23m63	3.18	2.30	0.59	9.79E-05	6.57E-04
SEQ 278 Turicibacter	3.12	2.27	0.60	1.42E-04	8.77E-04
SEQ 387 Butyricimonas	3.08	2.24	0.58	9.85E-05	6.57E-04
SEQ 366 Alistipes	2.98	2.27	0.59	1.14E-04	7.41E-04
SEQ 343 Lachnoclostridium	2.92	2.15	0.59	2.86E-04	1.54E-03
SEQ 339 Pantoea	2.91	2.15	0.59	2.91E-04	1.56E-03
SEQ 209 Ruminococcaceae UCG-014	2.87	2.13	0.58	2.62E-04	1.43E-03
SEQ 1111 Actinomyces	2.84	2.10	0.55	1.24E-04	7.91E-04
SEQ 679 Lachnoclostridium	2.83	2.10	0.56	1.84E-04	1.07E-03
SEQ 148 Bacteroides salyersiae DSM18765	2.77	2.06	0.58	3.50E-04	1.78E-03
↘ **Phyla decreased**					
Fusobacteria	21.98	-4.51	0.67	1.98E-11	2.37E-10
↘ **Families decreased**					
Veillonellaceae	880.05	-3.28	0.71	3.32E-06	2.53E-05
Prevotellaceae	864.20	-3.84	0.89	1.69E-05	1.03E-04
Pasteurellaceae	257.17	-5.19	0.70	8.09E-14	4.94E-12
Bacteroidales S24-7 group	22.83	-3.94	0.75	1.72E-07	2.62E-06
Fusobacteriaceae	18.70	-4.35	0.68	1.95E-10	5.93E-09
↘ **Species (SEQ variants) decreased**					
SEQ 18 Pseudobutyrivibrio	426.66	-3.84	0.89	1.62E-05	1.41E-04
SEQ 20 Bifidobacterium pseudocatenulatum	389.26	-2.34	0.95	1.42E-02	3.82E-02
SEQ 24 Dialister	321.86	-5.13	0.77	2.33E-11	6.95E-10
SEQ 10 Bifidobacterium longum infantis	252.91	-3.51	0.68	2.16E-07	3.14E-06
SEQ 6 Prevotella 9	238.90	-8.80	0.90	2.18E-22	1.24E-19
SEQ 39 Haemophilus	236.29	-5.14	0.71	3.41E-13	1.29E-11
SEQ 46 Veillonella	177.23	-6.34	0.70	1.62E-19	4.59E-17
SEQ 16 Bifidobacterium	165.42	-2.23	0.88	1.14E-02	3.28E-02
SEQ 25 Subdoligranulum	148.02	-6.30	0.75	2.83E-17	2.01E-15
SEQ 3 Prevotella 9	122.27	-6.28	0.86	3.90E-13	1.38E-11
SEQ 58 Veillonella	88.08	-5.55	0.69	6.31E-16	3.58E-14
SEQ 139 Blautia	54.18	-5.43	0.73	8.59E-14	3.48E-12
SEQ 101 Veillonella	54.15	-5.96	0.66	2.77E-19	4.64E-17
SEQ 103 Lachnospira	51.07	-4.51	0.73	5.14E-10	1.39E-08
SEQ 5 Prevotella 7	50.22	-2.77	0.90	2.07E-03	7.84E-03
SEQ 111 Lachnospiraceae UCG-008	49.84	-3.88	0.79	7.87E-07	9.91E-06
SEQ 50 Bacteroides	46.05	-2.85	0.81	4.75E-04	2.21E-03
SEQ 128 Incertae Sedis	43.54	-6.33	0.73	3.05E-18	2.88E-16
SEQ 64 Clostridium sensu stricto1	37.33	-2.90	0.77	1.55E-04	9.24E-04

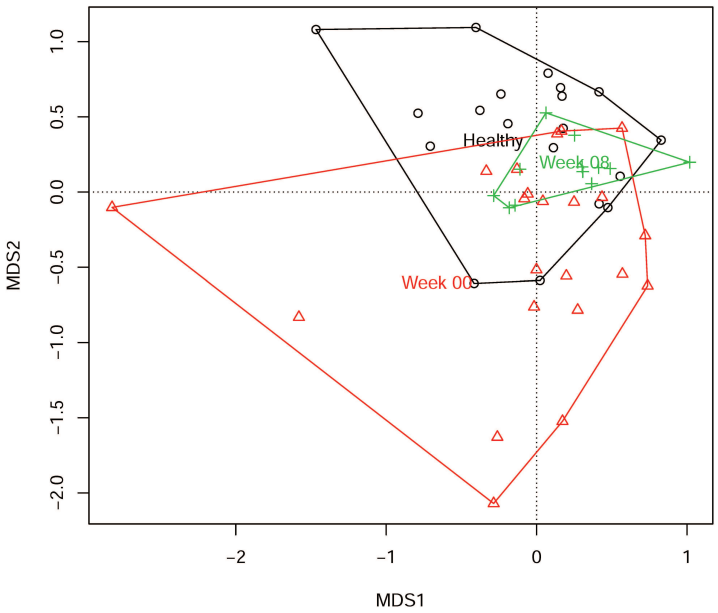
Bacterial taxa where abundance was significantly increased/decreased in children with Crohn's disease after 4-weeks treatment on EEN (differential analysis using NB) (continued)

Taxon	base mean	$\log_2$ fold change	log fold SE	p-value	p-adj
SEQ 70 Parasutterella	36.33	-3.63	0.77	2.36E-06	2.57E-05
SEQ 32 Roseburia	27.92	-5.68	0.75	4.85E-14	2.29E-12
SEQ 71 [Eubacterium] oxidoreducens group	25.50	-2.87	0.74	1.06E-04	7.00E-04
SEQ 36 Bacteroides	21.64	-2.20	0.83	8.21E-03	2.52E-02
SEQ 290 Lachnospiraceae	17.85	-2.31	0.67	5.57E-04	2.55E-03
SEQ 144 Veillonella	12.90	-3.61	0.68	9.05E-08	1.64E-06
SEQ 282 Ruminiclostridium 9	11.54	-2.31	0.64	3.16E-04	1.64E-03
SEQ 28 Ruminococcus 2	11.52	-3.52	0.67	1.72E-07	2.57E-06
SEQ 86 Ruminococcus 1	10.64	-4.24	0.69	9.30E-10	2.40E-08
SEQ 78 Roseburia	9.89	-2.45	0.69	3.52E-04	1.78E-03
SEQ 245 RuminococcaceaeUCG-003	9.56	-2.89	0.62	3.21E-06	3.30E-05
SEQ 115 Clostridium sensu stricto1	7.87	-3.12	0.61	3.19E-07	4.31E-06
SEQ 73 Bifidobacterium bifidum NCIMB41171	7.67	-3.74	0.67	2.22E-08	5.04E-07
SEQ 208 [Eubacterium] ventriosum group	7.47	-2.63	0.60	1.33E-05	1.18E-04
SEQ 167 Roseburia	7.33	-2.17	0.65	8.17E-04	3.44E-03
SEQ 98 Roseburia	7.22	-3.65	0.65	2.54E-08	5.54E-07
SEQ 310 Fusobacterium	7.12	-3.43	0.65	1.16E-07	1.83E-06
SEQ 151 Ruminococcus 1	6.60	-3.50	0.66	1.16E-07	1.83E-06
SEQ 319 Haemophilus	6.11	-2.61	0.67	9.84E-05	6.57E-04
SEQ 206 Lachnospiraceae NK4A136	5.64	-3.26	0.64	4.34E-07	5.72E-06
SEQ 184 Lachnospiraceae	5.49	-3.36	0.68	8.44E-07	1.04E-05
SEQ 227 Peptostreptococcus	5.31	-2.03	0.60	7.43E-04	3.27E-03
SEQ 183 Paraprevotella	5.22	-3.13	0.67	2.49E-06	2.62E-05
SEQ 185 Subdoligranulum	5.04	-3.08	0.64	1.85E-06	2.10E-05
SEQ 299 Megasphaera	4.74	-2.59	0.63	4.48E-05	3.48E-04
SEQ 186 [Eubacterium] hallii group	4.31	-2.82	0.60	2.42E-06	2.59E-05
SEQ 211 Veillonella	3.95	-2.67	0.62	1.72E-05	1.48E-04
SEQ 300 Ruminococcus 1	3.45	-2.45	0.59	3.80E-05	3.03E-04
SEQ 198 Ruminococcus 1	3.29	-2.37	0.58	4.76E-05	3.60E-04
SEQ 189 Incertae Sedis	2.94	-2.17	0.57	1.47E-04	8.94E-04
SEQ 507 Lachnospiraceae FCS020 group	2.83	-2.10	0.55	1.21E-04	7.78E-04
SEQ 332 Ruminiclostridium	2.72	-2.03	0.55	2.00E-04	1.14E-03
SEQ 218 Collinsella	2.64	-2.06	0.58	4.24E-04	2.04E-03

6 Appendix: Short chain fatty acids (MDS polygon plots)



Difference in faecal SCFA profiles between children with Crohn's disease (black) and healthy children (red) as shown by metric multi-dimensional scaling (MDS) polygon plot (*vegan*). PERMANOVA: R2=0.07; p=0.011



Changes in SCFA profiles in children with Crohn's disease after 8-weeks treatment with EEN (week 08; green) compared with baseline (week 00; red), shown by metric multi-dimensional scaling (MDS) polygon plot. PERMANOVA: R2=0.108; p=0.002; healthy children (black) are shown for comparison.

7 Appendix: Dietary intake

7.1 Appendix: Dietary questionnaires

7.1.1 Appendix: 3-day dietary record

BIG Study no. _____

3-Day Dietary Record-Example

Day of week: *Monday* **Date** *03/11/2014*

Time/Place	Description of food/drink	Amount of food/drink
8:30am	Cornflakes (Kelloggs)	½ cup
home	Milk (Sainsbury's full-fat)	½ cup
breakfast	Bread (Mothers Pride, large white sliced, toasted)	2 thin slices
	Flora margarine	2 teaspoons
	Robinsons lemon marmalade	2 teaspoons
	Coffee (instant)	1 cup
	Milk (whole pasteurised)	1 tablespoon
10am	Packet of walkers salt & vinegar crisps	Multipack size
school	Tesco orange juice	Small carton 200ml
1:00pm	Cheese (Cheddar)	2 slices
school	Bread (white, crusty)	2 thick slices
	Butter	1 teaspoon
3:30pm	Coffee (instant)	1 mug (medium)
home	Mars Bar	1 (51 gram)
	Apple	1 fist
6:30pm	Turkey Fillet (frozen, grilled)	2 palms
home	Potatoes, old, boiled	3 egg size potatoes
	butter	1 tablespoon
	Peas (Birds Eye, frozen, boiled)	½ cup
	Heinz tomato ketchup	1 tablespoon
	Muller Light Strawberry Yogurt	1 pot (175 grams)
	Coffee, filter	1 cup
	Milk (Sainsbury's virtually fat-free)	1 tablespoon
8pm	chips	2 fists
friend's house	Tomato sauce (Morrisons)	1 tablespoon
watching TV	Coca cola	500ml bottle
9.30pm	Hot chocolate Cadbury's	3 desertspoons
home	(made with full fat milk)	mug
before bed	sugar	3 teaspoons

BIG Study no. _____

3-Day Dietary Record

ClareClark, Postgraduate researcher, University of Glasgow.

Instructions

It is important that you record **everything** that you eat and drink including snacks. Snacks include the things we eat at school break time, on the way home from school or while at the computer or sitting watching TV. They might include things such as sweets, biscuits, tea or a juice we might have if we stop off at a friend or relative's house; or a drink and cake before bed. Think about the places you have been and this will help you to remember snacks as they make up an important part of most people's diet. We hope this information will help us to understand the relationship between diet and gut bacteria so please be as accurate as you can.

- Please record 2 normal week days *e.g. Mon, Tue, Wed or Thur*
- and 1 typical weekend day *e.g. Sat*

Hence a total of 3 days

Please (i) start a separate page for each day.
(ii) start a separate line for each item.

Column 1

Please record time and place of eating *e.g. home, school, café, restaurant etc.*

Column 2

Describe each item as accurately as possible, including:
(i) type and **brand** if possible *e.g. McVities, Tesco, Cadbury's, Walkers etc.*
(ii) whether food is fresh, dried, canned, frozen, salted, smoked, etc.
(iii) whether food is **cooked**, if so give method of cooking *e.g. fried, baked, etc.*

Column 3

Record the amount of each item as eaten *(describe the amount of food after cooking)* using household measures *(e.g. teaspoon, tablespoon, cup, palm, fist, etc.).*

If food consists of several items, please list each on a separate line where possible *(i.e. instead of writing 'one cheese sandwich', record separately bread, margarine, cheese, etc.)*

Please remember to record all **drinks**, as well as food, giving weights where possible, or volumes if these are known. Remember to record separately any added **milk** and **sugar**.

An example is shown overleaf.

How to complete the questionnaire

Please take a few minutes to read the instructions carefully.

Please use **black or blue pen** to complete the questionnaire: do not use pencil.

For **every line** in the questionnaire, you need to tick **one box** to say how many times you usually have this food or drink.

- If you do not usually have any of this food or drink, please tick the first box (rarely or never).
- If you have the food or drink more than once a month but less than once a week, please tick the next box (one or two per month).
- If you have the food or drink every week but not every day, please tick one of the weekly boxes to tell us how many measures of this food or drink you have in a normal week (1 per week, 2-3 per week or 4-6 per week).
- If you have the food or drink every day, please tick one of the daily choices (1 per day, 2-3 per day, 4-6 per day or 7 or more per day).

For dishes that are made up of more than one food you may have to split it up into its separate parts e.g. a ham sandwich (2 slices of white bread, 1 teaspoon of butter and 2 slices of ham).

For a few foods, you may have more than one measure on several days a week but not every day. For these foods please use the daily choices which give approximately the same total intake per week, e.g. for 8-10 measures per week please tick 1 per day (see example of white bread below).

Example:

If you have a small bowl of cornflakes every day, three medium glasses of regular blackcurrant diluting juice every day, two slices of white bread 5 days a week, an apple twice a week, but never have peanut butter, your answers should look like this:

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
Unsweetened cereals (e.g. Cornflakes, Shreddies, Weetabix, Rice Krispies)	1 small bowl, 3 tablespoons or 1 piece						√			
Regular blackcurrant diluting juice	1 medium glass							√		
White bread or rolls	1 slice or roll						√			
Apple	1 small apple				√					
Peanut butter	1 teaspoon	√								

If you want to change an answer, simply cross out your first tick and add another one in the right box.

If you have any foods or drinks which are not listed, or if you are not sure about where to add any foods or drinks, please use section 17 ('other foods') at the end of the questionnaire.

It is very important that you put a tick on every line.
If you rarely or never have the food, it is very important that you tick the box for rarely or never.

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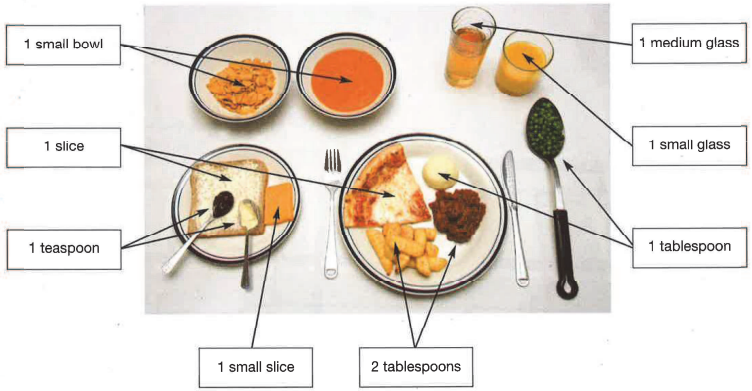
Scottish Collaborative Group Food Frequency Questionnaire version C3

Diet questionnaire for young people

We would like you to tell us what you usually have to eat and drink over the last 2-3 months. This should include all main meals, snacks, and drinks. You should also include any foods and drinks you eat outside your home, e.g. at school, at out of school clubs, at restaurants or cafes or with friends and other family members. You may want to ask your parent or guardian to help you with completing the questionnaire or to check some of the answers.

The questionnaire lists 146 types of foods and drinks. For each food or drink a measure is given which describes a small portion to help you estimate how much you usually have. The photograph below gives examples of some of these measures.



Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
5. Meat *(excluding Quorn and Soya)*										
Meat burgers or mince	1 small burger or 1 tablespoon									
Meat sauce (e.g. on pasta)	1 tablespoon									
Frankfurters	1 sausage									
Fried or grilled sausages	1 sausage									
Bacon or gammon	1 slice									
Cold ham or turkey	1 slice									
Salami or continental sausage	1 slice									
Stewed, fried, grilled or roast beef, pork or lamb	1 tablespoon or 1 slice									
Chicken nuggets	1 serving									
Casseroled, fried, grilled or roast chicken or turkey	1 tablespoon or 1 slice									
Meat or chicken pies, pasties or sausage rolls	1 individual pie or 1 roll									
6. Fish										
Fish fingers	1 finger									
Fish cakes or fish pie	1 fish cake or 1 tablespoon									
Grilled or poached white fish (cod, haddock, plaice)	1 small fillet									
White fish fried or cooked in batter or scampi	1 small fillet or 1 serving									
Grilled oily fish (fresh tuna, salmon, mackerel, herring)	1 small fillet									
Fried oily fish (fresh tuna, salmon, mackerel, herring)	1 small fillet or slice									
Smoked oily fish (kipper, mackerel, salmon)	1 small fillet									
Tinned tuna	1 tablespoon									
Tinned salmon, sardines, mackerel, pilchards	1 tablespoon or 1 small fillet									
Prawns	1 tablespoon									
7. Potatoes, rice and pasta										
Boiled, mashed or baked potatoes	1 tablespoon or 1 potato									
Potato croquettes or waffles	1 piece									
Roast or fried potatoes	1 potato or 2 tablespoons									
Oven chips	2 tablespoons									
Home-cooked chips	2 tablespoons									
Chips from a chip shop, café or restaurant	1 small bag									
Spaghetti and other pasta or couscous	2 tablespoons (cooked)									
Rice (all types)	2 tablespoons (cooked)									
Noodles (all types)	2 tablespoons (cooked)									

Please make sure that you have put a tick on every line before leaving this page

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
1. Breakfast cereals										
Unsweetened cereals (e.g. Cornflakes, Shreddies, Weetabix, Rice Krispies)	1 small bowl, 3 tablespoons or 1 piece									
Sweetened cereals (e.g. Frosties, Sugar Puffs, Coco Pops, Honey Nut Loops)	1 small bowl or 3 tablespoons									
Ready Brek or porridge	1 small bowl or 3 tablespoons									
Muesli (all types)	1 small bowl or 3 tablespoons									
2. Bread *(including sandwiches and toast)*										
White bread or rolls	1 slice or roll									
Brown or granary bread or rolls	1 slice or roll									
Wholemeal bread or rolls	1 slice or roll									
Croissants, garlic bread or Aberdeen rolls	1 roll or 2 slices									
Other breads (e.g. pitta, naan, tortilla, bagel)	1 piece									
3. Milk *(in drinks and on cereals)*										
Full fat cow's milk	1 small glass or 1/4 pint									
Semi-skimmed cow's milk	1 small glass or 1/4 pint									
Skimmed cow's milk	1 small glass or 1/4 pint									
Soya Milk	1 small glass or 1/4 pint									
Flavoured milk (e.g. chocolate, strawberry)	1 small glass or 1/4 pint									
4. Yogurt, cheese and eggs										
Drinking yogurts (Actimel, Yakult)	1 bottle									
Flavoured yogurts (e.g. all fruit yogurts, Crunch Corners, Crunchie)	1 small pot									
Fromage frais (all flavours)	1 small pot									
Natural, low fat or low calorie yogurt	1 small pot									
Cream (all types)	1 tablespoon									
Full fat cream cheese (e.g. Philadelphia)	1 tablespoon									
Cheddar-type cheese (including Cheese strings)	1 small slice or 1 stick									
Edam, Brie or cheese spreads (e.g. Dairylea)	1 slice, 1 piece or 1 tablespoon									
Low fat hard or soft cheese	1 slice or 1 tablespoon									
Eggs (boiled, fried, scrambled or omelette)	1 egg									

Please make sure that you have put a tick on every line before leaving this page

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
10. Fruit *(fresh, frozen and tinned)*										
Fresh fruit salad	1 tablespoon									
Tinned fruit (all kinds)	1 tablespoon									
Apples	1 small apple									
Oranges	1 small orange									
Bananas	1 small banana									
Grapes, melon, pear	1 small serving									
Kiwi	1 fruit									
Other fresh fruit (e.g. peaches, strawberries etc)	1 small serving									
Dried fruit (all kinds)	1 tablespoon									
11. Juice and other drinks										
Pure apple juice	1 small glass									
Other pure fruit juice (orange, pineapple etc.)	1 small glass									
High juice fruit drinks (Five Alive, Sunny Delight etc.)	1 small carton or medium glass									
Regular fruit juice drinks (e.g. Fruit Shoots, Capri Sun, Ribena cartons)	1 small bottle, pouch or carton									
Other fruit flavoured drinks including flavoured water (e.g. Calypso Cartoon)	1 carton, small bottle or medium glass									
Regular blackcurrant diluting juice	1 medium glass made-up									
No added sugar blackcurrant diluting juice	1 medium glass made-up									
Regular orange, lemon or other diluting juice	1 medium glass made-up									
No added sugar orange, lemon or other diluting juice	1 medium glass made-up									
Regular fizzy drinks (e.g. lemonade, Irn Bru, Cola)	1 medium glass or 1/2 can									
Low calorie or diet fizzy drinks	1 medium glass or 1/2 can									
Tap or mineral water (not in other drinks)	1 medium glass									
Smoothies (all kinds)	1 small bottle or carton									
Drinking chocolate powder	2 teaspoons or 1 sachet									
Tea (excluding fruit, herbal or green)	1 cup									
Coffee	1 cup									
Alcopops (e.g. Bacardi Breezer)	1 bottle									
Lager or beer	1 bottle or 1/2 pint									
Cider	1 bottle or 1/2 pint									
Wine	1 wine glass									
Spirits or liqueurs	1 pub measure									

Please make sure that you have put a tick on every line before leaving this page

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
8. Savoury dishes, soups and sauces										
Pizza	1 small (6 inch) pizza or 1 slice									
Quiche	1 slice									
Quorn, Soya or Tofu products (all types)	1 serving									
Nut roast, nut burgers or vegetable burgers	1 serving									
Baked beans	1 tablespoon									
Other beans or lentils (excluding soups)	1 tablespoon									
Canned or dried soup	1 small bowl									
Home-made soup	1 small bowl									
Bottled sauces (e.g. tomato ketchup)	1 teaspoon									
Tomato sauce (e.g. for pasta)	1 tablespoon									
Other sauces (e.g. cheese, white, curry, sweet & sour)	1 tablespoon									
Gravy	1 tablespoon									
Mayonnaise or salad cream	1 teaspoon									
Hummus	1 tablespoon									
9. Vegetables *(fresh, frozen and tinned)*										
Mixed vegetable dishes (e.g. stir-fry, curry)	1 tablespoon									
Peas or green beans	1 tablespoon									
Sweetcorn	1 tablespoon or 1 small cob									
Broccoli	1 tablespoon or 2 pieces									
Cabbage	1 tablespoon									
Spinach	1 tablespoon									
Other green vegetables (e.g. leeks, courgettes)	1 tablespoon									
Cauliflower, swede (neeps) or turnip	1 tablespoon									
Raw carrots	1/2 carrot									
Cooked carrots	1/2 carrot									
Onions	1/4 onion or 2 teaspoons									
Tomatoes	1 tomato									
Peppers	1/4 pepper									
Olives	4 olives									
Other salad vegetables (e.g. lettuce, cucumber, celery)	1 small serving									
Coleslaw	1 tablespoon									
Potato salad	1 tablespoon									

Please make sure that you have put a tick on every line before leaving this page

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
16. Sweets, chocolates and ice-creams										
Boiled, chewy or chocolate sweets (e.g. toffee, chews, fruit gums)	1 small packet									
Chocolate bars (e.g. Mars, Milky Way, Dairy Milk)	1 small bar									
Wrapped ice creams (e.g. Solero, Cornetto, choc ice)	1 ice-cream									
Other ice cream (all flavours)	1 scoop or 1 small tub									
Iced lollies	1 lolly									

17. Other foods

Please enter details of any foods or drinks which you have at least once a week which have not been included in the questionnaire above

Food or drink description	Amount usually consumed	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day

18. Brand details

Please give full details of the types (including brand name if possible) of any of the following foods which you usually have

Butter or Margarine (e.g. Flora Buttery)

..... Office code ☐☐

..... Office code ☐☐

Oil or fat used for home cooking (e.g. Tesco corn oil)

..... Office code ☐☐

..... Office code ☐☐

19. Dietary supplements

Please give as full details as possible (including brand name and amount used) of any supplements

	Brand name and strength	Amount usually taken per week (e.g. 7 tablets, 2 teaspoons)
Vitamins or multivitamins		
Cod liver oil or other oil		
Other supplement		

20. Any other information on your diet

Date of completing questionnaire .....

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
12. Sugar, jam and other spreads										
Sugar (on cereals and in drinks but not in cooking)	1 teaspoon									
Jam, honey or marmalade	1 teaspoon									
Peanut Butter	1 teaspoon									
Chocolate spread	1 teaspoon									
Marmite	1 serving									
Butter or margarine	1 teaspoon									
13. Crisps, nuts and savoury snacks										
Regular crisps (all types)	1 small bag									
Reduced fat crisps (all types)	1 small bag									
Other savoury snacks (Quavers, popcorn etc.)	1 small bag									
Peanuts and other nuts	1 small bag									
Savoury biscuits, crackers or breadsticks	1 biscuit or 2 sticks									
14. Biscuits and cakes										
Plain biscuits (e.g. Rich Tea, Digestive, ginger nuts)	1 biscuit									
Fancy biscuits (e.g. creams, iced biscuits)	1 biscuit									
Chocolate biscuits or cookies (all types)	1 biscuit									
Cereal bars or flapjacks	1 bar or slice									
Scones or pancakes	1 piece									
Doughnuts, muffins or pastries	1 piece									
Fruit cake or malt loaf	1 small slice									
Plain cakes	1 small slice									
Cakes with icing	1 small slice									
Cream cakes or gateaux	1 small slice									
15. Desserts										
Mousse, blancmange or trifle	1 small pot or 2 tablespoons									
Jelly	1 tablespoon									
Milk puddings (e.g. rice, semolina)	1 tablespoon									
Sponge puddings (jam, steamed, syrup etc.)	1 tablespoon									
Fruit tarts, crumbles or pies	1 small slice or 1 tablespoon									
Custard	1 tablespoon									
Cheesecake	1 small slice									

Please make sure that you have put a tick on every line before leaving this page

7.2 Appendix: Composition of Modulen IBD (enteral nutrition)

Appendix: Nutrient table for Modulen

NUTRIENT	per 250ml	per 1000ml
Energy (kJ)	1050	4200
Protein (g)	9	36
Carbohydrate (g)	27.5	110
Fat (g)	11.75	47
Fibre (g)	0	0
Vitamin A Retinol (IU)	700	2800
Vitamin D (IU)	100	400
Vitamin E (mg)	3.3	13.2
Vitamin K (mcg)	13.75	55
Vitamin C (mg)	24.25	97
Thiamine B_1 (mg)	0.3	1.2
Riboflavin B_2 (mg)	0.325	1.3
Niacin B_3(mg)	3	12
Pantothenic Acid B_5 (mg)	1.25	5
Vitamin B_6 (mg)	0.425	1.7
Biotin B_7 (mcg)	8	32
Folic Acid B_9 (mcg)	60	240
Cobalamin B_{12} (mcg)	0.8	3.2
Choline (mg)	18	72
Sodium (mg)	87.5	350
Potassium (mg)	300	1200
Chloride (mg)	187.5	750
Calcium (mg)	227.5	910
Phosphorous (mg)	152.5	610
Magnesium (mg)	50	200
Copper (mg)	0.25	1
Zinc (mg)	2.4	9.6
Iron (mg)	2.75	11
Selenium (mcg)	8.75	35
Chromium (mcg)	12.75	51
Molybdenum (mcg)	18.75	75
Manganese (mcg)	500	2000
Iodine (mcg)	25	100

7.3 Appendix: Estimated dietary intake from FFQs and known intake during EEN

Appendix: Estimated dietary intake from food frequency questionnaires (FFQ) and known intake during EEN given as median (IQR). Unpaired data is shown.

		EEN					
Dietary component	wk-0 (n=22)	wk-4 (n=20)	wk-8 (n=18)	wk-10 (n=12)	wk-16 (n=9)	wk-36 (n=7)	healthy (n=21)
Macro-nutrients							
Water	789 (305)	1870 (252)	1934 (268)	810 (233)	751 (191)	857 (305)	988 (430)
kCal	1488 (480)	2200 (267)	2275 (315)	1472 (431)	1430 (433)	1644 (560)	1643 (633)
CHO (g)	211 (80)	238 (32)	246 (34)	194 (59)	193 (91)	180 (24)	221 (67)
Protein (g)	56.8 (16.6)	79.2 (10.7)	81.9 (11.3)	51.8 (21.5)	51.2 (21.3)	53.8 (28.6)	59.1 (15.2)
Fat (g)	52.3 (16.3)	101.2 (13.6)	104.6 (14.5)	55.2 (5.0)	50.9 (9.9)	68.8 (33.5)	59.9 (20.1)
SFA (g)	23.0 (9.0)	57.2 (7.7)	59.1 (8.2)	24.1 (5.5)	21.4 (3.4)	29.4 (13.5)	27.8 (13.4)
MUFA (g)	17.8 (5.6)	17.2 (2.3)	17.7 (2.5)	18.4 (2.4)	17.7 (3.2)	22.4 (9.6)	20.5 (8.6)
PUFA (g)	8.2 (2.3)	10.6 (1.4)	10.9 (1.5)	8.0 (1.2)	8.1 (4.5)	9.7 (3.3)	9.0 (3.0)
Choline (mg)	155 (37.8)	154 (20.8)	159 (22.0)	134 (50.4)	142 (47.4)	136 (54.9)	161 (84.5)
Sugars (g)	86.0 (42.3)	66.0 (8.9)	68.2 (9.4)	95.2 (35.6)	69.8 (7.8)	86.4 (16.5)	105.9 (48.6)
Fibre (g)	***9.15** (4.08)	0 (0)	0 (0)	9.4 (2.6)	9.0 (4.1)	9.8 (3.3)	13.2 (4.7)

Dunn's test of multiple comparisons was used following a significant Kruskal-Wallis test. Difference from healthy children is significant at * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$.

Appendix: Estimated dietary intake from food frequency questionnaires (FFQ) and known intake during EEN given as median (IQR). Unpaired data is shown.

		EEN					
Dietary component	wk-0 (n=22)	wk-4 (n=20)	wk-8 (n=18)	wk-10 (n=12)	wk-16 (n=9)	wk-36 (n=7)	healthy (n=21)
Micro-nutrients							
Na (mg)	2131 (881)	748 (101)	773 (107)	1859 (506)	2334 (713)	2564 (977)	2206 (1142)
Cl (mg)	3203 (1230)	240 (216)	1661 (230)	2829 (801)	3579 (1302)	3961 (1404)	3499 (1401)
K (mg)	2176 (912)	2640 (356)	2730 (378)	2255 (703)	1938 (556)	2315 (1045)	2476 (1036)
Ca (mg)	932 (486)	1958 (264)	2025 (280)	818 (289)	787 (230)	811 (278)	926 (216)
Mg (mg)	207 (92)	440 (59)	455 (63)	179 (54)	191 (77)	202 (76)	227 (79)
P (mg)	1105 (504)	1320 (178)	1365 (189)	1005 (363)	1070 (314)	1132 (420)	1140 (399)
Fe (mg)	8.3 (3.1)	23.8 (3.2)	24.6 (3.4)	7.3 (1.7)	8.4 (4.1)	7.4 (3.2)	9.7 (4.8)
Cu (mg)	0.8 (0.3)	2.2 (0.3)	2.2 (0.3)	0.7 (0.2)	0.9 (0.3)	0.9 (0.4)	0.8 (0.3)
Zn (mg)	6.7 (2.6)	20.7 (2.8)	3.6 (3.0)	5.8 (2.1)	6.3 (2.0)	6.2 (2.8)	7.0 (2.0)
Mn (mg)	2.0 (0.7)	4.3 (0.6)	4.5 (0.6)	2.0 (0.6)	2.1 (1.3)	1.7 (0.4)	2.4 (0.9)
Se (ug)	28 (9.6)	75 (10.1)	77 (10.7)	28 (8.9)	29 (10.4)	31 (10.4)	31 (11.9)
I (ug)	168 (75)	215 (29)	223 (31)	168 (59)	153 (27)	199 (89)	181 (39)
Vitamins							
Retinol (ug)	**212 (108)	1804 (243)	1865 (258)	234 (82)	210 (25)	229 (145)	309 (156)
Vitamin D (ug)	1.4 (0.7)	21.6 (2.9)	22.3 (3.1)	1.2 (0.6)	1.4 (1.0)	1.4 (0.8)	1.6 (0.8)
Vitamin E (mg)	*5.5 (1.4)	28.6 (3.9)	29.6 (4.1)	6.3 (1.5)	5.7 (3.5)	5.6 (1.8)	6.5 (2.3)
Vitamin B_1 (mg)	1.3 (0.5)	2.6 (0.3)	2.7 (0.4)	1.2 (0.4)	1.2 (0.6)	1.4 (0.7)	1.3 (0.6)
Vitamin B_2 (mg)	2.0 (1.3)	2.8 (0.4)	2.9 (0.4)	1.6 (0.8)	1.6 (0.9)	1.8 (0.9)	1.9 (0.9)
Vitamin B_3 (mg)	17.6 (8.9)	25.5 (3.4)	26.4 (3.6)	13.7 (4.4)	15.4 (7.3)	14.9 (6.6)	14.0 (5.3)
Vitamin B_5 (mg)	5.3 (2.2)	10.6 (1.4)	10.9 (1.5)	4.3 (1.9)	4.7 (0.6)	4.8 (2.9)	5.2 (1.8)
Vitamin B_6 (mg)	1.7 (0.7)	3.6 (0.5)	3.8 (0.5)	1.5 (0.4)	1.4 (0.5)	1.6 (1.0)	1.4 (0.5)
Vitamin B_7 (ug)	24 (12)	70 (9)	73 (10)	25 (7)	21 (11)	25 (10)	30 (11)
Vitamin B_9 (ug)	238 (135)	528 (71)	546 (76)	226 (113)	245 (154)	183 (71)	244 (130)
Vitamin B_{12} (ug)	5.0 (1.4)	7.0 (0.9)	7.3 (1.0)	4.1 (1.7)	4.1 (0.8)	4.3 (2.3)	5.3 (2.1)
Vitamin C (mg)	***62.5 (34.8)	206.5 (27.9)	213.8 (29.6)	68.0 (29.6)	64.0 (17.8)	76.0 (65.2)	123.0 (81.5)

Dunn's test of multiple comparisons was used following a significant Kruskal-Wallis test. Difference from healthy children is significant at * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$.