Nutritional Aspects and Gut Microbiota in Paediatric Inflammatory Bowel Disease

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Author’s declaration

I declare that the work contained within this thesis is original and is the work of the author Konstantinos Gerasimidis. I have been solely responsible for the organisation and day to day running of this study as well as clinical measurements, laboratory analysis, and data processing, unless otherwise referenced

Mr Konstantinos Gerasimidis MSc, BSc, APHNutr

I certify that the work reported in this thesis has been performed by Konstantinos Gerasimidis and that during the period of study he has fulfilled the conditions of the ordinances and regulations governing the Degree of Doctor of Philosophy, University of Glasgow

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Dedicated to my parents

Eirini & Christos

Ithaca

“When you set out on your journey to Ithaca,
pray that the road is long,
full of adventure, full of knowledge.
The Lestrygonians and the Cyclops,
the angry Poseidon, do not fear them.
You will never find such as these on your path,
if your thoughts remain lofty, if a fine
emotion touches your spirit and your body.
The Lestrygonians and the Cyclops,
the fierce Poseidon you will never encounter,
if you do not carry them within your soul,
if your soul does not set them up before you.

Pray that the road is long.
That the summer mornings are many, when,
with such pleasure, with such joy
you will enter ports seen for the first time;
stop at Phoenician markets,
and purchase fine merchandise,
mother-of-pearl and coral, amber and ebony,
and sensual perfumes of all kinds,
as many sensual perfumes as you can;
visit many Egyptian cities,
to learn and learn from scholars.

Always keep Ithaca in your mind.
To arrive there is your ultimate goal.
But do not hurry the voyage at all.
It is better to let it last for many years;
and to anchor at the island when you are old,
rich with all you have gained on the way,
not expecting that Ithaca will offer you riches.

Ithaca has given you the beautiful voyage.
Without her you would have never set out on the road.
She has nothing more to give you.

And if you find her poor, Ithaca has not deceived you.
Wise as you have become, with so much experience,
you must already have understood what Ithacas mean.

Konstantinos P. Cavafy (1911)

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ABBREVIATION LIST

IBD	**Inflammatory bowel disease**
CD	**Crohn's disease**
UC	**Ulcerative colitis**
IC	**Indeterminate colitis**
IBDU	**IBD unspecified**
d	**Day**
mth	**Month**
wk	**Week**
y	**Year**
M	**Male**
F	**Female**
EEN	**Exclusive enteral nutrition**
EN	**Enteral nutrition**
ACD	**Anaemia of chronic disease**
IDA	**Iron deficient anaemia**
SCFA	**Short chain fatty acids**
BCFA	**Branched chain fatty acids**
CAM	**Complementary and alternative medicine**
GI	**Gastrointestinal**
Hb	**Haemoglobin**
FM	**Fat mass**
FFM	**Fat free mass**
DXA	**Dual x-ray absorptiometry**
BIA	**Bioelectrical impedance analysis**
ROS	**Reactive oxygen species**
RBC	**Red blood cells**
ESR	**Erythrocyte sedimentation rate**
SRB	**Sulphate reducing bacteria**
GALT	**Gut associated lymphoid tissue**

SUMMARY

As Crohn's disease (CD) is a disease of the gastrointestinal tract, nutrition is very important and is implicated in several aspects of the disease, from aetiology, management, and the long-term health of the patient. Nutritional therapy with EEN is the mainstream approach in the management of active paediatric CD and has a dual effect, inducing clinical remission and providing nutritional support and rehabilitation. Although its efficacy is well established by human trials and clinical experience, the mode of action remains unknown. Initial speculations for a mechanism of action mediated through gut rest, and protein/energy reconstitution have not been established. On the other hand the strong evidence for the role of the indigenous microbiota and micronutrients in disease aetiology and mucosal injury, challenged the researcher to explore whether the action of EEN is mediated through changes in these parameters.

This study aimed to assess aspects of the nutritional status of paediatric patients with inflammatory bowel disease (IBD), to appraise the use of nutritional remedies in the same population, and to explore putative mechanisms of action of EEN, the nutritional therapy, with the most robust evidence of clinically efficacy.

The first of these studies was a questionnaire survey which assessed the use of special diets, nutritional supplements, herbals, and alternative medicine in a representative sample of paediatric patients with IBD. Use of these treatments was declared by two thirds of the patients with probiotic use and dairy free diet being the commonest forms used.

Prevalence of anaemia and predictors of its progress at six and 12 months were assessed in a large retrospective case review study. Anaemia was as high as 73% of patients at diagnosis and haemoglobin concentration improvement at six and 12 post diagnosis was associated with the use of oral iron supplementation, improvement of nutritional status markers, growth, and systemic markers of disease activity.

In a mechanistic study the validity of a bedside method, used to assess the body composition of children with CD, was compared against a reference method. The agreement of the two methods was low and the inter-individual bias between them was substantial.

The aim of this thesis was to study the effect of EEN on gut microbiota diversity, bacterial metabolic activity, inflammatory response and nutritional status in paediatric patients with active CD.

Newly diagnosed children with active CD, and patients with longstanding disease, on clinical relapse, who started treatment with EEN as part of their standard clinical management, were recruited. Four stool samples were collected while on treatment with EEN and one when the patient returned to their normal diet. Bacterial diversity was assessed with molecular microbiology techniques, and bacterial metabolites were measured in serial

stool samples and correlated with faecal calprotectin levels, systemic inflammatory markers and clinical activity. A single stool sample was collected from their first-degree relatives and two serial samples from healthy children with no family history of IBD.

Significant changes were observed for the metabolic activity of the commensal microbiota during the course of treatment. In particular, faecal butyrate significantly decreased by more than 100%, faecal pH moved into the alkaline range, and a five-fold increase was observed in total sulphide but only in those patients who achieved clinical remission, or in whom faecal calprotectin levels decreased at the end of treatment. No such changes were observed in children who did not achieve complete clinical remission or when the treatment failed. Gut bacterial diversity did not change significantly during treatment, but was significantly lower than in healthy children, who also presented a higher degree of similarity between the two serial samples. Interestingly the gut microbiota of the healthy first degree relatives of CD children had significantly lower bacterial diversity compared with the healthy children but no such difference was observed compared with their CD relatives. The majority of the healthy relatives had also significantly high levels of calprotectin suggesting intestinal inflammation but no clinical presentation of gastrointestinal disease.

A secondary outcome of this study was to measure changes in the systemic and gut specific markers of disease activity during treatment. Although systemic markers of disease activity improved to normal levels in the majority of the patients, intestinal inflammation was still ongoing and only one of the 16 children had normal calprotectin levels at the end of EEN. Moreover, only in those patients who achieved clinically complete remission, did calprotectin levels decrease significantly at the end of treatment.

From the same cohort of patients three blood samples were also collected before, at the end of treatment and on normal diet. Changes in the concentration of 19 different micronutrients were measured in the serum and some in erythrocytes and correlated with systemic markers of disease activity. Serial changes in anthropometry and body composition were assessed during EEN and correlated with disease activity markers.

Body weight increased in all patients at the end of treatment but fat free mass significantly increased only in those patients who entered in clinical remission. Several micronutrients and mainly antioxidants were below the reference range for the majority of patients at the time of treatment initiation. Most of them improved by the end of treatment but the serum concentration of carotenoids further deteriorated with more than 90% of the patients presenting concentration lower than the lower sensitivity level of the assay. A strong association was found between systemic markers of disease activity and antioxidant vitamins, but not with intestinal inflammation.

In conclusion, paediatric patients with IBD, and mainly those with CD present with suboptimal protein/energy status, anaemia, and low circulating levels for many antioxidants.

Treatment with EEN corrected the majority of these markers of nutritional status but the carotenoid levels deteriorated. Although the improvement in the serum levels of some micronutrients can be an epiphenomenon of the acute phase response, it does not explain the depleted levels of carotenoids at the end of EEN. This may be attributed to the lack of carotenoids in the feeds used in conjunction with excessive utilisation during the active course of the disease.

On the other hand, the unhealthy intestinal microenvironment observed, in only those patients who clinically improved at the end of treatment; do not support a prebiotic mode of EEN action, as was proposed by a recent Italian study. These changes may be a secondary phenomenon, associated with changes in gut motility, better absorption of butyrate with disease improvement, or changes in the availability of colonic bacterial substrates. Alternatively these changes may be associated with changes in the microbiota composition and production of so far unknown bacterial bioproducts which may have a causative association with the onset and propagation of intestinal inflammation in CD.

These results may have significant implications in manufacturers of clinical nutrition products. Addition of carotenoids may improve the provision of antioxidant micronutrients and a dual nutritional therapy combining EEN with prebiotics, butyrate supplementation, or probiotics may increase the efficacy of the existing formulae. This should be addressed in future studies.

CHAPTER ONE

Nutritional aspects, enteral nutrition and gut microbiota in paediatric Crohn's disease

1.1. Inflammatory bowel disease

Crohn's disease (CD) and ulcerative colitis (UC), collectively known as inflammatory bowel disease (IBD) are idiopathic, lifelong, inflammatory, destructive conditions of the gastrointestinal (GI) tract. The disease relapses and remits throughout its course (1-4).

Crohn's disease and UC, although part of the same constellation present distinct clinical, extraintestinal and pathological characteristics that constitute the basis for their differential diagnosis (Table 1.1). Ulcerative colitis is characterized by continuous diffuse mucosal inflammation limited to the colon and rectum whereas CD is characterized by patchy, transmural inflammation, which may affect any part of the GI tract from the mouth to the anus (1-4).

Table 1.1: Differential diagnosis of UC and CD (adapted from (Baumgart & Sandborn 2007 (5))

	UC	CD
Clinical features		
Haematochezia	Common	Uncommon
Passage of mucus or pus	Common	Rare
Small bowel disease	No	Yes
Can affect upper GI tract	No (except backwash ileitis)	Yes
Abdominal mass	Rare	Sometimes on right side
Extraintestinal manifestations	Common	Common
Small bowel obstruction	Rarely	Common
Colonic obstruction	Rarely	Common
Fistulae and perianal disease	No	Common
Biochemical features		
Anti-neutrophil cytoplasmic antibodies	Common	Rarely
Anti-saccharomyces cerevisiae antibodies	Rarely	Common
Pathological features		
Transmural mucosal inflammation	No	Yes
Distorted crypt architecture	Yes	Uncommon
Cryptitis and crypt abscesses	Yes	Yes
Granulomas	No	Yes but rarely in mucosal biopsies
Fissures and skip lesions	Rarely	Common

A small proportion of patients with chronic inflammatory colitis that cannot be classified into either of the two types of disease by endoscopic and histology findings are characterized as indeterminate colitis (IC) or IBD unspecified (IBDU) using new phenotypic classification systems (6). The disease is more extreme in children with IBD but there is no difference in the spatial distribution (7). Recently a new consensus classification system has been proposed to characterise the diverse phenotypic presentation of IBD considering a) age of

disease onset b) disease location and c) disease behavior as the predominant phenotypic elements (Table 1.2) (6).

Table 1.2: Montreal phenotypic classification of IBD

Phenotype	Classification	Clinical features
Crohn's disease		
Age at diagnosis	A1	Below 16 y
	A2	Between 17 and 40 y
	A3	Above 40 y
Location	L1	Ileal
	L2	Colonic
	L3	Ileocolonic
	L4	Isolated upper disease
Behaviour	B1	Non structuring, non penetrating
	B2	Stricturing
	B3	Penetrating
		*p perianal disease modifier
Ulcerative colitis		
Ulcerative proctitis	E1	Involvement limited to the rectum (rectosigmoid junction)
Left side colitis	E2	Involvement limited to a proportion of the colorectum distal to the splenic flexure
Extensive colitis	E3	Involvement extends proximal to the splenic flexure
Inflammatory bowel disease unspecified	IBDU	IBD affecting the colon without small bowel involvement and no definitive histology or other evidence to suggest CD or UC

1.1.1. Epidemiology of IBD

Understanding the epidemiology of IBD may offer clues to the aetiology and pathogenesis of IBD and identify areas for further research (8). However defining the exact epidemiology of IBD is difficult mainly due to the insidious onset of the disease, the frequent marked delay in diagnosis, and the occasional misclassification of patients. In paediatrics the small number of cases hampers this further.

Ulcerative colitis incidence varies from 1-5/10^5 cases per year for Latin America, Africa and Asia to values up to 10-20/10^5 for the Northern America and Northern Europe. For CD the respective values are 5-10/10^5 for Northern America and Europe and 0.5-4/10^5 for Asia, Africa, Latin America and Southern Europe (9). CD and UC are most commonly diagnosed in early adulthood but the diagnosis may occur at any age (9) with approximately 15%-25% of IBD being diagnosed during childhood or adolescence (10).

Juvenile UC and CD occur with an average incidence of 5.2/10^5 cases per year in the UK and Republic of Ireland (11). Scotland presents the highest prevalence for CD and is

second after Ireland for UC (Table 1.3). The highest incidence occurs between 12-16 years (11;12) with almost 2/3 of the patients diagnosed with CD.

Epidemiology in IBD is a dynamic process and recent data show that IBD incidence and prevalence is increasing with time (8;11-15) in both developing and developed countries (9). Armitage and her colleagues (16) using the Scottish hospital discharge linked database, showed an increasing trend of both juvenile-onset (< 16 y old) CD and UC in Scotland. Combining their results with those of Barton et al (17) they estimated a steady increase for the crude incidence of CD from 1 per 10^5 between 1971-1975 to 3.1 per 10^5 for 1991-1995. In UC the results from Barton's study showed a marginal fall that reversed according to the results of Armitage (16). A higher incidence for juvenile CD but not UC onset was observed in the northern regions compared with the southern regions of Scotland (12).

Table 1.3: Incidence (x10^5/y) of IBD by country (adapted from Sawczenko, 2001 (11))

	All IBD	CD	UC	IC
UK	5.2	3.1	1.4	0.6
England	5.2	3.1	1.4	0.7
Scotland	6.5	4.2	1.8	0.6
Wales	5.2	3.2	1.7	0.3
Northern Ireland	3.6	2.4	1.0	0.2
Republic of Ireland	4.4	2.3	2.0	-

1.1.2. Pathogenesis of IBD

While IBD has been studied for decades it remains mysterious in its pathogenesis (18). Various factors keep researchers guessing why some individuals develop IBD while others are never afflicted. It is now speculated that a single and simple cause and effect relation probably does not account for most cases of IBD.

The most consensual model of disease pathogenesis suggests that disease occurrence is an interaction of four elements: genetic susceptibility, environment, abnormal immune host response and commensal gut microbiota (18) as discussed later in this thesis (Section 1.7)

Although the exact interaction of these elements remains unknown, IBD pathogenesis is believed to occur from errors in the interpretation or regulation of immune perception and responsiveness to endogenous microbiota, and thus disruption in the mucosal homeostasis, with initiation of immune response in genetically predisposed individuals (18).

1.1.2.1. Genetic factors

Clinical observations suggest that genetic factors are implicated in the pathogenesis of IBD. Family aggregation has long been recognized. First-degree relatives of an affected patient have an increased risk of IBD (13;14;19;20) whereas a substantially higher rate of disease concordance has been observed in monozygotic twins than in dizygotic ones, especially in

those with CD (21-24). Over the past 20 years a wide variety of candidate genes have been implicated in the pathogenesis of IBD (21). Many of these putative associations have not been reproducible whereas there is substantial consensus evidence for others (21). The region on chromosome 16 known as (IBD1) was first identified in 1996 by French researchers (25). Detailed mapping of this chromosome, resulted in the identification of the NOD2/CARD15 gene that encodes a cytoplasmic protein designated NOD2 or CARD15 which serves as a pattern recognition receptor for bacterial lipopolysaccharide, and regulates activation of nuclear factor-kβ and secretion of a-defensins by ileal paneth cells (26). People who are homozygous for variant NOD2 may have 20-fold or more increased susceptibility to CD (27;28). However fewer than 20% of patients with CD are homozygous for NOD2 variants so it does not fully explain the disease pathogenesis. Since these first observations a number of other candidate genes have been identified with the strongest evidence for the HLA region, MDR-1, as well as the recently implicated IL23R and ATG16L1 genes (29).

1.1.2.2. Environmental factors

Despite the strong evidence implicating genetic factors in IBD, environmental factors may also be important. Approximately 40%-50% of individuals with identical genetic makeup are discordant for CD, indicating that environmental influences play an important role particularly when the rapid increase in the incidence of IBD cannot be fully explained by slow long-term changes in the human genome.

Several epidemiological studies have implicated diet, anti-inflammatory drugs, vaccination history, smoking, seasonal variation, appedicectomy, use of swimming pools, and water supply to the occurrence and increasing incidence of IBD (9).

1.1.2.3. Gut microbiota

There is substantial evidence to incriminate the commensal gut microbiota in the pathogenesis of IBD (30). This is discussed in detail in Section 1.7.2.

1.1.3. Symptoms and clinical manifestations of IBD

For many patients, IBD imposes a substantial personal burden, with unpredictable fluctuating symptoms, time off work, intestinal and extra-intestinal complications (31) (Table 1.4), need for drugs with substantial side effects, possible surgical resection of part of the gut, and reduced quality of life (32).

There is no feature, characteristic of disease presentation, which can differentiate IBD from other diseases. Furthermore intestinal symptoms of IBD can be insidious and subtle delaying early diagnosis. Common presenting symptoms and clinical manifestations are

abdominal pain, diarrhoea, perirectal bleeding, lethargy, anaemia, and weight loss that are often misdiagnosed as infectious gastroenteritis, iron deficiency anaemia (IDA), thyroid disorders and eating disorders (15;33). In a British and Irish collaboration (33) 25% of CD children presented with the triad of abdominal pain, diarrhea and weight loss (Table 1.4). The variability in symptoms reflects and correlates with the different sites of GI involvement (34).

Common extra-intestinal manifestations in IBD patients are orofacial granulomatosis, angular stomatitis, erythema nodosum, hepatobiliary complications, anaemia, weight loss and, particularly in children, growth retardation and sexual immaturity (31). The impact of IBD on nutritional status and body composition of the patients is presented in detail in Section 1.3.2. Beyond the health related consequences of IBD, acute active disease may necessitate frequent hospital admittance, causing major disruptions in social, family life, professional career, and academic attendance in children that subsequently lead to low quality of life (32) and psychological concerns (35). Psychological support is now considered an integral part of the multidisciplinary approach to IBD management.

Table 1.4: Presenting symptoms and signs of paediatric IBD in UK (adapted from Sawzcenko & Sandhu 2003 (33))

	CD (n=379)		IC (n=72)		UC (n=172)	
Common symptoms						
Abdominal pain	274	(72%)	54	(75%)	106	(62%)
Diarrhoea	214	(56%)	56	(78%)	127	(74%)
Bleeding	84	(22%)	49	(68%)	145	(84%)
Weight loss	220	(58%)	25	(35%)	53	(31%)
Lethargy	103	(27%)	10	(14%)	20	(12%)
Anorexia	94	(25%)	9	(13%)	11	(6%)
Other symptoms						
Arthropathy	28		3		11	
Nausea vomiting	22		1		1	
Constipation/soiling	4					
Psychiatric symptoms	3					
Secondary amenorrhoea	1				1	
Signs						
Anal fistula	17					
Growth failure/delayed puberty	14		1			
Anal abscess, ulcer	8					
Erythema nodosum/rash	6				1	
Liver disease	3		2		5	
Appendicectomy	2					
Toxic megacolon						1

1.1.4. Management of IBD

Despite the progress in overall management of IBD over recent decades (36), no innovative curative treatment has been developed and contemporary treatment achieves mainly symptom control. Recent therapeutic goals for pediatric patients with IBD were established

and were described in the workgroup report of the First World Congress of Pediatric Gastroenterology, Hepatology and Nutrition (37).

The conventional treatment strategies in IBD are classified into inductive, to achieve disease remission, and maintenance, to prolong disease remission and prevent a subsequent flare up. In paediatric patients in particular, linear growth and pubertal development are two distinct characteristics that differentiate the options, and choice of management compared to adults.

Due to the chronicity of the disease and the lack of effective curative treatment patients with IBD are committed to stay on daily lifelong treatment. The choice of medication is determined by the type of disease (CD or UC), the severity, and the extent of disease involvement (Table 1.5) and therefore it is important to tailor treatment according to the particular individual needs of each patient (36;38-40).

The medical therapy of IBD has improved considerably in recent years (36;38-40). Recently the British Society of Gastroenterology (41) published practice guidelines for the management of IBD in adults, with however, negligible reference to paediatric patients. Medical treatment in paediatric IBD is extensively reviewed elsewhere (38) and the mainstream options are presented in Table 1.5.

Table 1.5: Common medical treatment options and indications of use in paediatric IBD (compiled from (36;38;39;41-44))

Drug	Disease	Role in IBD	Action	Examples	Side effects
Aminosalicylates (5-ASA)	Mild/moderate UC & CD	Induction and maintenance of remission	Control the release of lipid mediators, cytokines and oxygen species	Mesalazine Sulfasalazine	Nephritis Nephropathy Hepatotoxicity Pancreatitis, Pulmonitis Pericarditis
Corticosteroids	Moderate/severe UC & CD	Induction of remission	Potent immunologic and anti-inflammatory agents	Prednisolone Prednisone Hydrocortisone Budesonide	Infection Hypertension Weight gain Acne Myopathy Fluid retention Bone demineralization
Immunosuppresants	UC & CD	Maintenance of remission	Inducing T cell apoptosis by modulating cell signaling	Azathioprine (AZA) 6-mercaptopurine	Pancreatitis Fever Leukopenia, Hepatotoxicity Nausea
Antibiotics	CD	Induction of remission in mild/moderate peri-anal disease	Alteration of the bacterial flora	Metronidazole Ciprofloxacin	Nausea, Headache Glossitis Peripheral neuropathy
Methotrexate	CD	Induction of remission in moderate/severe, refractory, intolerant of standard therapies	Immunosuppression	Methotrexate	Myelosuppression Hepatotoxicity
Biological agents	CD & UC	Induction of remission in moderate/ severe, alternative induction therapy to steroids or as a first-line agent for severe perianal fistulizing disease	Antibody against proinflammatory cytokines	Infliximab	Nausea Headache Upper respiratory infections Fatigue Fever

With the treatment approach described in Table 1.5, most patients with active IBD will enter into a remission phase. However those who fail to respond to medical therapy can usually be operated and enter surgically induced remission. It is estimated that between 30% to 40% of patients with UC will need surgical intervention in the course of their disease, whereas between 70% and 80% of CD patients will have resection of a part of their GI tract (45). Indications for surgery include perforation, strictures, fistulae and toxic megacolon.

In a retrospective study in Scotland by Watson et al (15) 48% of paediatric patients with CD underwent surgery compared with 23% of patients with UC. It is noteworthy that the same study reports that the incidence of surgical intervention appeared to be decreasing. Fifty-four percent of paediatric IBD patients underwent surgery in the 1980 to 1989 cohort compared with 34% in the 1990 to 1999 group although the mean length of follow-up in the latter group was shorter, and this figure may have risen with time.

The use of nutritional therapy is a promising field in the treatment of IBD. A number of studies using in-vitro models, experimental animal models of colitis and human trials support the use of a range of potential nutritional remedies in the management of IBD including exclusive enteral nutrition (EEN), administration of probiotics and/or prebiotics, butyrate enemas or oral supplements, and the use of omega 3 fatty acids. These approaches are quite new and the evidence so far is controversial and inconclusive. The mostly studied and well established nutritional remedy in clinical practice is the administration of EEN in adult and paediatric patients with CD. This is described below (Section 1.5)

When conventional treatment fails to cure, the use of unconventional treatment purchased over the counter and provided by alternative health professionals are often used. This is discussed below in Chapter 1.2.

1.2. Complementary and alternative medicine in IBD

It is probably due to the serious and relapsing nature of the symptoms of the disease that there is increasing interest in the use of unconventional remedies by IBD patients. Complementary and alternative medicine (CAM) encompass a group of diverse medical and health systems, therapies, and products that are not presently considered to be part of conventional medicine. Within this definition, there is no firm description which specific methods and remedies comprise CAM and which do not (Table 1.2.1) particularly as new branches of established disciplines are continually being developed. Thus some studies have considered prayer and exercise as a form of CAM (46) whereas others restricted definition solely to consultations with alternative therapists (47). Moreover therapies that are included in the definition of CAM in some countries are part of integrated conventional medicine in others (i.e. traditional Chinese medicine in China).

Table 1.2.1: Common types of CAM

Special diets	Nutritional supplements	Herbals	Alternative therapists
Dairy/Milk free	Probiotics	Aloe	Acupuncture
Gluten/Wheat free	Prebiotics	Cat's Claw	Chiropractic
Low residue/Fibre	Fish/Omega 3 oils	Evening primrose	Hypnosis
Low carbohydrate	Glutamine	Echinacea	Massage
Gottschall diet	Vitamin/Minerals	Garlic	Relaxation therapy
Vegeterian/Vegan		Ginseng	Chinese therapies
		Grapeseed extract	Aromatherapy
		Milk thistle	Reflexology
		Slippery elm	Yoga
		Gingko biloba	Homeopathy
		Chinese herbs	

1.2.1. Prevalence of CAM in the general population

The use of CAM is common in the general population (48;49) and has increased substantially over time (50;51). Data from the National Health Interview Survey in 2002, in 31044 adults, showed that 62% of adults in the USA used some form of CAM therapy over the past two months (52). CAM is also becoming popular in the UK general adult and paediatric population (53;54). Results from the National Omnibus survey in Great Britain estimated that 10% of the general population had received some form of 23 named unconventional therapies from a CAM practitioner in the last year without assessing the usage of self-prescribed CAM therapies (55). Estimates of CAM use were similar in England, Scotland and Wales. Likewise a paediatric survey in Bath, England, estimated that 17.9% of children in the general population had used CAM at least once, and 6.9% had a consultation with a complementary medicine practitioner (53).

In parallel to that, the proportion of patients that are recommended to access a complementary practitioner through their primary care health providers is increasing. Thomas et al (53;56) interviewed general practitioners in England throughout 1995-2001 and observed that the proportion of practitioners that provided some access to CAM increased from 39% in 1995 to 49.4% in 2001.

The most common factor associated with the use of CAM is poor health esteem and increased morbidity (55). Ong et al (57) using a postal questionnaire in four English counties found that 60% of CAM users reported having a chronic illness or disability. Patients with increased mortality and morbidity risk, like cancer (58) and HIV infection (59) were among the common users of CAM. Use of CAM is similarly high in patients with functional and organic GI disorders. Ganguli and his colleagues (60) estimated use of CAM, over the last year to be as high as 52.5% in gastroenterology patients and Kong et al (61) in a study of 932 subjects from a gastroenterology clinic and 477 healthy controls found the incidence of

CAM use to be 49.5% for IBD patients, 50.9% for irritable bowel disease, 20% for general GI disorders and 27% for the healthy control group.

1.2.2. Literature review of studies on CAM use in IBD patients

An extensive electronic literature search was conducted to retrieve relevant studies on the use of CAM in IBD using subject specific keywords. Nineteen adult and four paediatric studies were identified. As it is outside the scope of this thesis to describe each of these studies separately, only well-designed studies with adequate sample size, reports from the UK, and all those conducted in pediatric IBD patients will be presented (Table 1.2.2).

Overall the studies were heterogeneous in design, responding populations, definitions of CAM (Table 1.2.2) and did not allow direct comparison or to draw conclusive results. Some studies were restricted to the use of CAM provided by specialized therapists (47;62), whereas others included oral remedies and nutritional supplements (multivitamins, probiotics) (61;63) bought over the counter. Dietary manipulations and special diets were included in most studies (63;64) but not all (65). Some studies assessed current use of CAM (66), whereas others looked into the overall past use since IBD diagnosis (67) or defined use within a specific timeframe (64). Methodology also varied between studies. Most studies used postal surveys (64;65), others used personal interviews or questionnaires administered at the patients' routine clinical appointments (61;67) and others a combination (62;65). The average prevalence of CAM use varied considerably between studies from as low as 4% (62) to up 72% of the responding population (66) subject to the CAM definition used (Table 1.2.2). Disease onset seemed to be the main trigger of CAM use, as the latter was significantly lower prior to disease diagnosis (63;68). Moreover the use of CAM in IBD is significantly higher compared with healthy people (61;67) or patients with other clinical conditions (47).

Herbal medicine, nutritional remedies and homeopathy were reported among the common types of CAM. Probiotics were used predominantly by IBD patients in some studies (64;66;69) but were uncommon in others (65). Differences in the prevalence and predominant types of CAM among studies could be attributed to the different study design and responding populations (Table 1.2.2). In particular patients recruited from tertiary hospitals may be patients with severe IBD or perhaps those that have benefited more from the conventional medicine. On the other hand geographical differences in the type of CAM may denote cultural differences and beliefs in the utility of CAM as well as different approaches to CAM by the medical staff and different promotion of CAM in countries that are traditional users of them.

Disease severity (63), high education level (68), high socio-economic status, female gender (61), young age (68), internet usage (64), prior or parental CAM usage, longterm use

of steroids (68) low quality of life and increased psychological stress (70), have all been reported as predictors of CAM use by IBD patients (65;68).

The main reasons given by IBD patients for use of CAM included the need for an optimum treatment (68), the wish to be actively involved in and personally responsible for their disease management (68), wish for a holistic therapeutic approach and, frustration with the ineffectiveness and side effects of conventional medicine (66;68). Common sources of information on CAM were magazines, internet search, friends, family and their medical doctors (66). Many patients did not reveal the use of CAM to their primary health providers and actually a few CAM users discontinued or reduced their conventional treatment on their own or were advised to do so by their CAM practitioners (47). In a German survey, 31% IBD participants reported reduction or discontinuation of conventional medicine on CAM use (68). This is of great concern as some of these therapies exert only a placebo effect, or may interfere with the action of conventional treatments. In the study by Hilsden et al (63) 53% of the patients who tried CAM reported some form of harm ascribed to CAM use.

Whatever the true action of CAM, many patients found CAM at least effective (68;70) and the majority of them would consider CAM use in the future (66;68).

1.2.3. CAM effectiveness in IBD. How good is the evidence?

Unlike CAM conventional medical treatment in IBD is supported by randomized control trials (RCT) evidence of their efficacy. On the contrary use of CAM is based mostly on theoretical foundations with a lack of well-designed clinical trials. The few studies that are reported in the literature are usually open label studies of poor design, although recent lines of evidence support the use of some of them (71). Probiotics, prebiotics, SCFA and omega 3 fatty acids have demonstrated some clinical efficacy in some studies in IBD whereas for other remedies such as acupuncture, wheat grass juice, aloe vera, and special diets more evidence is accumulating (72-74).

Ben Arye et al (72) in a double blind RCT showed that wheat grass juice appeared effective and safe as a single or adjuvant treatment of active distal UC. Treatment with wheat grass juice was associated with significant reduction in the overall disease activity and severity of rectal bleeding. Recently Joos et al (73) in a single blind RCT showed that patients with mild to moderate active CD treated with acupuncture for four weeks, experienced a decrease in their clinical activity index, inflammatory markers, and a better general well being compared with a control group. Finally, Langmead et al (74) found that oral aloe vera taken for four weeks produced a clinical response more often than placebo treatment and it also reduced the histological disease activity without presenting side effects.

1.2.4. Conclusion

In conclusion the use of CAM in adult IBD patients is well studied, but in children there is a lack of substantial evidence. Most of the paediatric surveys have been conducted in the US (64) and Australia (66) with only one multinational study which included only a small sample of UK patients from a single hospital in London, UK (65).

Extrapolation of the results of adult studies is inappropriate and can be misleading as there are considerable differences in disease management regimes between adult and paediatric patients (34). Furthermore, the use of CAM by paediatric patients reflects the choice of their guardians and how they cope with their child's disease.

Further studies should be undertaken to address the use of new non-medicinal products, like probiotics and prebiotics, with emerging evidence of efficacy in IBD (75;76). Characteristics of patients who may be more amenable to CAM use should be identified with a study. On the other hand health professionals that care for IBD children should be aware of CAM use by their patients as the latter can interfere with conventional medical treatment or may have serious health related side effects (77). This was explored in the study described in Chapter 2 of this thesis.

Table 1.2.2: Major studies on the prevalence of use of CAM in adult and paediatric patients with IBD

Study	Country; Setting	Sample; Methodology	CAM definition	Prevalence of CAM use	Predominant CAM	Predictors of CAM use	Comments
ADULT STUDIES							
Langhorst et al 2005 (68)	Germany; National IBD database	N:864; Mailed questionnaire	Self prescribed & CAM therapists	51.3% overall; 26.8% current users	Homeopathy, herbals, ayurvedic medicine; traditional Chinese medicine; probiotics used by 54.2%	More UC users than CD; high education; young age; steroids intake more than 10 g was the strongest predictor	6.8% used exclusive CAM permanently; 11.5% experienced side effects
Kong et al 2005 (61)	Sheffield UK; Outpatient GI clinic	N:1409:(311 IBD), healthy control group; Doctor-administered questionnaire	Oral supplements	49.5% IBD; 51% IBS; 20% general GI; 27% healthy controls	Multivitamins	More women than men	Most participants did not find CAM beneficial
Hilsden et al 2003 (63)	Calgary Canada; National IBD database	N:2847; Mailed questionnaire	Vitamins were not included; Prayers and exercise excluded	47.2%; 23.6% current users	Herbals; 27.5% used dietary modifications	Severe disease	15% used CAM before IBD diagnosis; Median number of therapies currently used:3; 53% reported harm of CAM use
Langmead et al 2002 (70)	London, UK; GI & general medicine outpatient clinic	N:239; Questionnaire completed in the clinic		28%	Herbals	Low quality of life scores for the emotional and social factors; Fatigue	No difference in use with non IBD patients; over 50% found it beneficial
Moody et al 1998 (47)	Leicestershire, UK; Database of chronic GI disorders	N:572 (219 IBD, 145 coeliac); Mailed questionnaire	CAM therapists	23% of Asians & 18% of Europeans; 8% for coeliac	Herbalists; homeopathists	No difference between ethnicities	Some patients were advised to reduce or stop conventional medication
Smart et al 1986 (62)	Nottingham, UK; Outpatient GI clinic	N=461 (96 IBS, 222 CD, 143 general GI); Questionnaire completed in the clinic; postal survey for CD patients	CAM Therapists	16% of IBS, 6% of CD, 2% of general GI; 11% of IBS and 4% of CD current users	Herbalists; homeopathists	Failure of conventional therapy	

PAEDIATRIC STUDIES							
McCann et al 2006 (67)	Leeds, UK; Outpatient clinic	N=75 with chronic illnesses (25 IBD) & 25 healthy control; Face to face Interviews	All types of CAM; Ever use of CAM		Only summary results for all GI patients		
Day et al 2004 (66)	Sydney, Australia; Outpatient GI clinic	N=46; Mailed questionnaire	All types of CAM; Ever use of CAM	72% current users; 2.4 agents in average	Probiotics, fish oil, herbals		Reasons of use: To avoid medication side effects, dissatisfaction with medication, experience by others; 12% found CAM partially effective; 4% found it expensive; the majority would use CAM again
Markowitz et al 2004 (64)	Greater Philadelphia area, US; IBD database	N=335; Mailed questionnaire	CAM use last 12 mth; No multivitamin only megavitamins	50.8%	Nutritional therapies (22% probiotics, 16.4% fish oils, megavitamins), special diets (dairy free diet)	Internet use, caloric supplementation, IBD surgery, school absences, low quality of life	
Heuschkel et al 2002 (65)	Multinational; Boston, Detroit US, London UK; Outpatient clinic & database	N=208 (51 from London); Questionnaire completed in the clinic or mailed	CAM use last 12 mth; No dietary modifications	41% excluding dietary modifications; 35% dietary modifications	19% megavitamins, 17% dietary supplements, 9% herbals, 6% homeopathy only 2% probiotics in London and 6% in total	No of adverse effects from conventional medicine, parental use	Reasons of use: feel better, hoping for a cure, disappointment with conventional treatment

IBS: Irritable bowel syndrome

1.3. Nutrition considerations in IBD

Beyond the GI manifestations of IBD, extraintestinal complications accompany the natural history of the disease and often appear before diagnosis. Amongst these, nutritional complications are present in the majority of patients with IBD and comprise an additional burden for the health professionals and the patients. Intervention through dietary counseling, nutritional supplements, EN or parenteral nutrition may become necessary at some point to reconstitute nutritional balance (35;78). In 2004 the North American Society for Paediatric Gastroenterology, Hepatology and Nutrition, published a consensus report on nutrition support for paediatric patients with IBD, highlighting the major nutritional problems, and proposing guidelines for assessment and monitoring of the nutritional status (35).

In IBD malnutrition can occur with all its different facets and can affect clinical outcomes, immune system function, gut mucosa barrier (79), antioxidant mechanisms, body growth, bone health and quality of life (35). Protein-energy undernutrition, micronutrient deficiencies and poor bone health are commonly reported in IBD, whereas in paediatric patients growth failure, and pubertal delay are additional features. Multiple factors are involved in the manifestation of malnutrition in IBD with suboptimal dietary intake and excessive losses being the main contributors (Table 1.3.1).

Table 1.3.1: Causes of malnutrition in IBD

Factor	Reason
Suboptimal intake	Reduced appetite
	Food aversions-fear of worsening symptoms
Malabsorption	Intestinal inflammation
	Intestinal resection
Excessive losses	Protein loosing enteropathy
	GI bleeding
	Excessive GI mucosa sloughing
	Fistula output
	Diarrhoea-steatorrhoea
Increased requirements	Increased metabolic needs
	Increased GI mucosa turnover
	Increased consumption of antioxidants
	Altered lipid and muscle oxidation
	Fever
Disease activity	Pro-inflammatory cytokines-growth
Drug nutrient interactions	Steroids-Inhibition of IGF-1
	Steroids-Poor bone mineralization
	Steroids-Impair fat and muscle metabolism
	Methotrexate-Impair folate metabolism
	Sulfasalazine-Inhibits folate absorption

1.3.1. Protein-energy malnutrition

IBD patients often suffer from protein energy malnutrition by the time of diagnosis, and their status fluctuates during the disease course (80;81). Recent weight loss is a common feature in the newly presented IBD patient, particularly in CD, and accompanies almost every

relapse (82;83). Around 60% of newly diagnosed children with CD, compared to 35% in UC, present at diagnosis with a history of recent weight loss (33;82).

As a subsequent result of excessive weight loss and undernutrition, basic anthropometry is affected. IBD patients and particularly those with CD, present with low BMI and are underweight compared with the national reference range or their healthy peers (33;84-88). Recent data, however, suggest that fewer patients at risk of undernutrition are now seen compared with previous studies. In fact a large proportion of patients are overweight at diagnosis, particularly UC patients, and this has been linked to the obesity epidemic observed in the general population (82;87). In a North American study of 783 newly diagnosed IBD children, low BMI was seen in 22%-24% of the children with CD, and 7%-9% of children with UC. Ten percent of the CD children and 20%-30% of the UC had high BMI consistent with the definition of overnutrition (82). Low risk of undernutrition has been described recently (87) in a cross-sectional study of adult IBD patients, and an alternative explanation suggested was the progress in medical treatment with the evolution of new drugs that spare the use of steroids, and achieve not only symptomatic relief but also mucosal healing (89;90).

Other than differences between the two types of IBD there is no consistent evidence to link specific disease characteristics with undernutrition. Some studies found an association with small bowel disease and risk of underweight (91) but others did not (82). A higher prevalence of undernutrition was also reported in patients with active (60%) compared with inactive disease (13.7%) in a recent study using the Subjective Global Assessment to screen for undernutrition (80).

1.3.2. Body composition

Body size dimensions and BMI should best be seen as an index of nutritional status and body composition (92). Although BMI correlates well with obesity (93) and defines risk of undernutrition (94) in the general population, its diagnostic accuracy to distinguish between fat and muscle stores in chronic illness, like IBD, is unknown.

There are several reasons that body composition in IBD may differ from that of healthy people. Inflammation and the secretion of pro-inflammatory cytokines may alter energy metabolism, protein turnover and substrate utilization (91), whereas the use of corticosteroids increases body fat and exerts negative catabolic effects on lean mass (95). Physical activity on the other hand was reported to be low in IBD patients and correlated inversely with fat mass (FM) in one recent report (87).

Several studies assessed the body composition of IBD patients and compared it with reference norms or healthy subjects, but results were inconsistent. A lower FFM compared

with healthy subjects and preservation of FM was found in some adult studies (85;87;91;96) but not all(97;98). A series of studies in newly diagnosed adult IBD patients (99) and other patients with longstanding disease in remission (100), failed to identify an overall difference in body composition between the two types of IBD and healthy control subjects, although gender specific differences were evident (101).

Strong associations between body composition and disease characteristics, like phenotypic characterization, duration of disease (85), and history of small bowel resection (87;96) have not been described extensively in adult studies. Sex differences have been reported occasionally in some adult studies (85;101) but not others (87) and one study found an inverse association between disease activity and body fat (100).

There are only a few studies that assessed body composition in IBD children. Among these studies, body lean mass is reported as significantly lower than healthy control groups (84;88;100;102;103) whereas gender specific associations with FM were observed in some (84;88;100;103). Sentogo et al., in a cross-sectional study (103), found that boys and girls presented lower FFM by 3.5 and 2.9 kg respectively, compared with the control sample, whereas FM, although not different from the control group in CD boys was 3.7 kg higher in CD girls. Similarly Burnham et al. in a cross-sectional study (84) found that children with longstanding CD had a 6% reduction in lean mass compared with healthy children after correcting for stature, age, pubertal stage and race (84). No similar deficits were found for FM that conforms to the definition of nutritional cachexia. Recently Thayu et al. in a well-designed study (88) of a cohort of newly diagnosed children with CD, also reported gender associated differences with body composition. Fat mass and lean mass for height (adjusted for age, race and pubertal stage) were lower in female than in male patients. Compared with a cohort of healthy controls, body composition in girls was more consistent with the definition of wasting (low lean and FM) whereas in boys there was mostly preservation of FM and deficits in lean mass consistent with the definition of cachexia. No associations were observed between body composition, clinical activity, disease location or delay of diagnosis.

Different characteristics of patients, and the recruitment of new (88) compared with longstanding patients (84), may explain some of the discrepancies found in adult and paediatric studies. Although many authors compared the body composition of IBD patients with a cohort of healthy controls, of similar age and gender, they did not to match for body weight, height or BMI and this might explain differences found in the absolute fat or lean mass but not in their relative body proportions (85;87;96;99). In IBD children in particular, impaired growth and pubertal delay are frequently seen and a proper comparison of body composition between CD and healthy children needs to account for these confounders (84;85;87;88;96;99).

On the other hand as there is no method to measure body composition accurately (92), the use of different techniques makes a direct comparison between studies difficult. Indeed the results of each study are inherent to the limitations and theoretical assumptions of the method used, that may additionally not be valid in illness (92). Most in vivo body composition methods have been tested and validated in healthy individuals or animal cadavers and their applicability in chronic illness is questionable given the changes that occur in the hydration level and distribution of water fluids in the body compartments (104). Using IBD as a prime example, studies showed that the hydration level of lean mass is not the same as with healthy subjects (104) and in fact depends on gender (101). Therefore application of body composition methods that consider a constant hydration of FFM (DXA, bioimpedance, double labelled water) is inaccurate and may produce erroneous results. Comparison and validation of the commonly used body composition methods in disease are needed. This thesis is presenting a relevant study between bioimpedance measurements of body composition and DXA in Chapter 4.

1.3.3. Growth

Faltering linear growth is commonly encountered in children with IBD, and often precedes disease diagnosis (102;103;105-107). Approximately 23%-25% of the children present deviations from their growth velocity and height for age centiles and are significantly shorter than their healthy peers (103). Sentogo et al. (103) found significant growth deficits in children with CD, compared with healthy children, and failure to attain their genetic potential for linear growth when their heights deficits were compared with their estimated mid-parental target height. This effect was more profound in boys. Girls did not differ from the healthy controls. Nevertheless most children with IBD will eventually achieve final heights within the normal range for the general population or close or their genetic potential (108-110).

The exact mechanisms by which growth impairment occurs in IBD are unclear. Undernutrition, the effect of circulating proinflammatory cytokines on growth promoting factors and growth plate (111), long-term use of steroids (84;111-113) and genetic make up (114) are all implicated (115). On the other hand skeletal or bone age is negatively affected in CD particularly in boys, and may explain to some extent the degree of growth deficits seen in IBD (103). This coincides with the fact that most of the patients will eventually achieve final adult heights within their normal or slightly lower than their genetic potential (110;112).

1.3.4. Bone health

Bone mineralization is another important aspect in the care of the growing child with IBD, particularly as peak bone mass, attained during adolescence, is the most important

determinant of lifelong skeletal health (116;117). Osteopenia and osteoporosis are important extraintestinal manifestations in IBD that may be related to increased risk of fractures (116;118-120) although adequate evidence in children is missing. Semeao et al (121) reported five cases of vertebral compression fractures in paediatric patients with CD, but Persad et al. (122) found no difference in the prevalence of long bone fractures between paediatric IBD patients and their siblings.

A large number of studies reported osteopenia and osteoporosis in adult and paediatric patients with IBD (85;96;102;113;123-127). Total body or femoral neck and lumbar spine bone mineral content or density were significantly lower than the reference cut offs or compared with healthy individuals (101-103;117;128). A disease associated effect has been reported with poor bone health seen more often in CD than UC (85;102;129;130). Disease location, disease duration, and history of disease activity were risk factors in some studies but not others (103;120;131-133). In a study by Burnham (113) the difference in bone mineral content between CD children and healthy control was eliminated when the authors accounted for the differences in lean mass in a regression model. This suggests that decreased mechanical stress is an important factor for reduced bone health in children with CD (117) and opens the window of opportunity to improve bone mass by optimising lean tissue gain with nutrition and weight bearing exercise in patients with IBD.

Poor nutrition and nutritional status, vitamin D deficiency, the effect of pro-inflammatory cytokines on bone resorption, and the longterm use of high steroid doses (85;102;132;134-139) are the main contributors that can negatively affect bone mineralization in IBD.

1.3.5. Delayed puberty

Delayed puberty frequently complicates the clinical course of young patients with IBD, more often in CD than UC and in boys than in girls (84;102;103). In a study of young patients with CD, menarche occurred at age 16 years or later in 73% of female patients in whom disease onset preceded puberty. In a few patients, menarche was delayed until the early twenties. In contrast, menarche occurred at 14 years or younger in all patients with juvenile-onset UC (108). Likewise Boot et al. (102) found a mean delay of puberty of 0.7 y in Dutch children with IBD and Burnham (84) 1.5 y in CD children in the USA.

Delayed pubertal onset may influence linear growth and final adult height. Moreover delayed puberty can affect quality of life and self-esteem although this has not been addressed in a study to date. Undernutrition has always been thought to be the main reason for delayed puberty in IBD patients. However, puberty may be delayed despite a normal nutritional status. Observations in animal models of experimental colitis suggest that

inflammatory mediators may have a direct adverse influence, independent of undernutrition, on the onset and progression of puberty (140).

1.3.6. Micronutrient status in IBD

Vitamins, minerals and trace elements, collectively known as micronutrients, are essential for the normal function and development of the human body (Table 1.3.2). In IBD some micronutrients may have additional importance or play additional roles in the pathogenesis and clinical course of the disease (Table 1.3.2). Micronutrient deficiencies can influence clinical outcomes and disease progression in IBD, by affecting the immune system, tissue repair, antioxidant defence system, body growth and bone mineralization. A consensus report of the North American Society of Paediatric Gastroenterology, Hepatology and Nutrition suggested routine screening for micronutrients status in paediatric patients with IBD (35).

Of particular interest is the evidence that supports an association between the body's antioxidant defense system, and the pathogenesis and gut injury of IBD (141;142). IBD is characterised by aggravation of inflammatory cells (granulocytes, monocytes and neutrophils) at the site of the intestinal lesion and production of reactive oxygen species (ROS) as part of the normal immune properties of these cells. Reactive oxygen species are free radicals which include superoxide anion, hydrogen peroxide, nitric oxide, and hypochloride. In the human body the oxidative action of the free radicals is counteracted by antioxidant micronutrients and uncontrolled production coupled with reduced removal from an impaired endogenous antioxidant mechanism may cause tissue damage (143;144).

There is good evidence to suggest that IBD patients are oxidatively stressed (145;146). This is believed to be the result of an imbalance between increased ROS production (142) and a suppressed antioxidant defence mechanism to compensate adequately, causing damage to biological macromolecules (142;146-148) and possibly intestinal lesions. Wenland et al (146) found that lipid peroxidation, a marker of ROS production, was higher in CD patients, compared with healthy controls. Indeed the plasma antioxidant vitamins were low despite no profound differences in the dietary intake of antioxidants between CD patients and healthy controls. A putative association between ROS and gut injury in IBD is also supported by the use of the aminosalicylates in IBD which are believed to work through an antioxidant property and scavenging of oxidative radicals (149).

Table 1.3.2: Micronutrient basic body functions and importance in IBD

Micronutrient	Main functions	Main deficiency disease	Importance in IBD
Retinol/b-carotene (A)	Visual pigments in the retina; antioxidant; normal development; differentiation of tissues	Night blindness; xerophthalmia; keratinization;	1. Antioxidant 2. Deficiency exacerbates colitis (150)
Calciferol (D)	Calcium balance	Rickets, osteomalakia; osteoporosis	3. Bone health (151) 4. Fracture 5. Deficiency in IBD (136;152) 6. Antiinflammatory properties (153)
Tocopherol/tocotrienols (E)	Antioxidant	Neurological dysfunction	1. Antioxidant 2. Treatment of colitis (154)
Phylloquinone/Manaquinones (K)	Blood clotting; bone formation	Impaired blood clotting; haemorrhagic disease	1. Bone health (155;156)
Thiamin (B_1)	Enzyme co-factors; carbohydrate alcohol metabolism	Peripheral nerve damage (Beri-Beri); central nerve lesions (Wernicke-Korsakoff syndrome)	No data
Riboflavin (B_2)	Enzyme co-factors in oxidation and reduction reactions; nutrient metabolism	Lesions of corner mouth, lips, tongue; seborrhoeic skin lesions	No data
Nicotinic acid/Nicotinamide (Niacin)	Enzyme co-factors in oxidation and reduction reactions; part of NAD and NADP	Pellagra: photosensitive dermatitis, depressive psychosis	No data
Pyridoxine/Pyridoxal/Pyridoxamine (B_6)	Coenzyme in transamination, decarboxylation of amino acids, glycogen metabolism; role in steroid hormone	Disorders of amino acid metabolism, convulsions	1. Hyperhomocysteinemia more common in IBD (157) 2. Thromboembolic Complications (158)
Folic Acid	Coenzyme in transfer of one carbon fragments; nucleic acids synthesis	Megaloblastic anaemia	1. Risk of deficiency in methotrexate & sulphasalazine users 2. Colorectal cancer (159-161) 3. Megalobastic anaemia Hyperhomocysteinemia (162)
Cobalamin (B_{12})	Coenzyme in transfer of one carbon fragments; nucleic acids	Percinious anaemia (megaloblastic anaemia with degeneration of the	1. Megalobalstic anaemia 2. Malabsorption in patients with ileal disease or

	synthesis; folate metabolism	spinal cord)	resection (163) 3. Hyperhomocysteinemia; Thrombosis risk (164)
Pantothenic	Functional part of coenzyme A and acyl carrier protein	Peripheral nerve damage (burning foot syndrome)	No data
Biotin (H)	Coenzyme in carboxylation reactions in gluconeogenesis and fatty acid synthesis	Impaired fat carbohydrate metabolism, dermatitis	No data
Ascorbic Acid	Coenzyme in hydroxylation of proline and lysine in collagen synthesis; antioxidant, iron absorption	Scurvy; impaired wound healing; loss of dental cement; subcutaneous hemorrhage	1. Antioxidant
Carotenoids	Antioxidant system		1. Decrease oxidative stress (150) 2. Deficient in CD (165)
Calcium (Ca)	Growth and skeleton formation; muscle function; blood clotting	Poor bone health; Osteoporosis; Fractures	1. Bone health (166) 2. Fractures
Magnesium (Mg)	ATP stabilization	Abnormal muscle function; disruption of Ca metabolism	1. Malabsorption
Phosphorus (P)	Growth and skeleton formation; blood buffering capacity; energy production	Anorexia, bone pain, rickets; osteomalacia; muscle weakness, ataxia, susceptibility to infections	No data
Sodium (Na)	Osmotic and electrolyte homeostasis; water balance; nerve conduction; active cellular transportation; mineral bone of the apatite		1. Electrolyte disturbances in severe diarrhoea
Chloride (Cl)	Osmotic and electrolyte homeostasis; hydrochloric acid in the stomach		No data
Potassium (P)	Osmotic and electrolyte homeostasis; enzyme cofactor in energy metabolism and cellular growth and division, nerve and muscle function	Muscle and neural dysfunction; cardiac arrest;	1. Electrolyte disturbances in severe diarrhoea
Iron (Fe)	Reaction catalyst, blood synthesis; oxygen transportation; energy production;	Anaemia, fatigue, restlessness; impaired work performance; abnormal thermoregulation; suppressed immune system; adverse effect on psychomotor and mental development	1. Impaired absorption in IBD (167) 2. Anaemia 3. Oral iron exacerbate colitis and increase oxidative stress (159)
Zinc (Zn)	Enzyme cofactor; antioxidant	Growth retardation; sexual and	1. Decreased absorption (168)

		skeletal immaturity; neuropsychiatric disturbances; dermatitis, alopecia; diarrhea; loss of appetite; susceptibility to infections	2. Mucosal permeability (144;169) 3. Antiinflammatory properties (170) 4. Antioxidant
Copper (Cu)	Enzyme cofactor; function of immune, nervous and cardiovascular system; bone health; iron metabolism; haemopoiesis; mitochondrial function; energy production; antioxidant	Anaemia; neutropaenia; bone fractures; hypopigmentatio; impaired growth; abnormalities of glucose and cholesterol metabolism	1. Antioxidant
Selenium (Se)	Enzyme cofactor; antioxidant; catalyst	Keshan disease; immune system dysfunction;	1. Deficient in IBD (171;172) 2. Antioxidant
Iodine (I)	Thyroid hormone synthesis	Goiter; cretinism;	No data
Manganese (Mn)	Enzyme co factor	Impaired growth; skeletal abnormalities; impaired reproduction; nutrient metabolism; weight loss; dermatitis	No data

Following these observations clinical trials found a reduction of markers of oxidative stress and improved antioxidant status on supplementation with antioxidant micronutrients (171;173;174). Nevertheless a positive association between improvement of disease activity and restoration of oxidative status has not been shown (174).

Although clinical presentation of frank micronutrient deficiencies in IBD is very rare (175) and largely limited to case reports (176), suboptimal circulating levels for virtually every vitamin, mineral and trace element have been reported previously primarily in adult patients and a few paediatric IBD patients (Table 1.3.3). Antioxidant trace elements (eg. Zn, Se, Cu) and vitamins (vitamins A, E, C, carotenoids) were the main nutrients consistently reported in lower circulating concentrations in IBD patients compared with healthy controls or the normal reference range (Table 1.3.3).

Suboptimal dietary intake, increased utilization, malabsorption and increased enteric losses have all been postulated. Some studies have linked nutritional deficiencies with clinical disease activity and inflammatory markers (Table 1.3.3) but whether micronutrient depletion plays an important role in the pathogenesis and perpetuation of the mucosal lesions or is the result of it needs to be studied further.

On the other hand the changes in the serum levels of many antioxidants and their association with systemic and clinical activity indexes (175-177) can be an epiphenomenon of the acute phase response that accompanies chronic inflammatory conditions, malignancy and infection. Reduced serum levels of micronutrients are often used to define deficiency states, but these levels may correlate better with markers of disease activity and inflammation rather than being reflective of body tissue stores or functional deficits (178). A prime example of this is the perturbation in trace elements and vitamin concentrations in patients with cancer of the colon which can be partially reversed by anti-inflammatory treatment, without supplementation (179).

1.3.7. Principal causes of malnutrition in IBD

The aetiology of malnutrition in IBD is multifactorial as are its manifestations. Poor diet, increased energy needs, altered metabolism, malabsorption, excessive GI losses and nutrient-drug interactions are among the major causes (Table 1.3.1).

Table 1.3.3: Major studies assessed systemic levels of micronutrients in patients with IBD.

Study	Age	Micronutrients	Results	Comments
		ADULTS STUDIES		
Hengstermann et al 2008 (80)	132 quiescent IBD; 35 active; 45 controls	Vit C, Vit E, carotenoids, Se, Zn, Cu,	Low to control: Vit C, carotenoids	No major differences with disease activity
Filippi et al 2006 (97)	54 CD remission	Vit C, Vit A, Vit D, Vit E, Vit B_1, Vit B_6, Vit B_{12}, Vit E, Folate, Niacin, β-carotene, Fe, Cu, Ca, P, Mg, Zn,	Low to reference: Vit C, Cu, Niacin, Zn, Fe, B_6, B_1, B_{12}, Folate, b-carotene, Vit E	
D'Odorico et al 2001 (147)	83 IBD; 386 controls	Vit A, Vit E, carotenoids	Low to control: Vit A, Vit E, some carotenoids, no disease type effect, Difference between active & inactive; disease activity was negatively associated with carotenoids	Oxidative DNA damage was increased in IBD; no association between impaired oxidative status & disease activity; Malnourished lower antioxidants than well nourished
Wendland et al 2001 (146)	37 CD; 37 controls	Vit C, Vit E, carotenoids, GSHPx, Se	Low to control: Vit C, Carotenoids	No association with disease activity; Some inversely correlated with inflammatory markers; lipid peroxidation markers did not correlated with disease activity; Some carotenoids associated with lipid peroxidation
Geerling et al 2000 (99)	69 new IBD; 69 controls	Vit A, Vit E, Vit C, β-carotene, Mg, B_1, B_{12}, Folate, Cu, Zn, Se, GSHPx	Low to control: UC: β-carotene, Mg, Se, Zn lower in UC; CD low B_{12}	
Geerling et al 1998 (100)	32 CD in remission; 32 controls	Vit A, Vit E, Vit C, β-carotene, Mg, B_1, B_{12}, Folate, Cu, Zn, Se, GSHPx	Low to control: β-carotene, Vit C, Vit E, Se, Mg, GSHPx, Zn, Vit D	No difference for Vit E/cholesterol; Se associated with % body fat; A high proportion at risk of deficiencies
Kuroki et al 1993 (175)	24 (plus adolescents) CD; 24 controls	A, E, B_1, B_2, B_6, B_{12}, C, Niacin, Biotin, Pantothenic acid, Folate,	Low to control: Vit A, Vit E, B_1, B_2, B_6; Folate; High to control: pantothenic acid	No disease location or activity effect; B_2 and niacin inversely correlated with CDAI
Fernandez-Banares et al 1989 (180)	23 IBD active ; 89 controls	Vit A, β-carotenoid, Vit E, B_1, B_2, B_6, B_{12}, Folate, Biotin, Vit C	Low to control: Vit A, β-carotenoid, biotin, folate, Vit C, Vit B_1	Vit E lower in colitis but Vit E: Cholesterol normal; B_2 lower but B_6 higher in colitis; Vit B_{12} lower in upper GI disease
		PAEDIATRIC STUDIES		
Ojuawo & Keith 2002 (181)	74 new IBD; 40 controls	Zn, Se, Cu	Low to control: Se, Zn only for CD; Cu higher than UC and control	

Levy et al 2002 (145)	22 CD & 10 control	Retinol, β-carotene, α-tocopherol, γ-tocopherol	Low to control: Retinol	No difference between active and inactive disease
Bousvaros et al 1998 (177)	97 (plus young adults) IBD; 23 control	Vit A, Vit E	Low to reference: 14.4% low Vit A; 6.2% low Vit E	Deficiencies more prevalent in active CD
Hoffenberg et al 1997 (182)	24 IBD; 23 controls	Vit C, Vit A, Vit E β-carotenoid, γ-tocopherol, retinol binding protein, glutathione, GSHPx, Se	Low to control: Vit C; High to control: GSHPx, Vit E & Vit E/ Cholesterol	CD compared to control: high glutathione, GSHPx, Vit E; UC compared to CD lower Vit A; antioxidants inverse correlation with anthropometry
Thomas et al 1994 (183)	39 active CD; 86 controls	Se, GSHPx in erythrocytes and serum	Low to control: 10% low Se, GSHPx high in plasma but low in erythrocytes	

1.3.7.1. Dietary intake

Poor diet, with suboptimal intake in energy and nutrients, has always been considered the key contributor to undernutrition seen in IBD (184). Food aversion to avoid exacerbation of GI symptoms and anorexia mediated by the interaction of proinflammatory cytokines with appetite hormones (185-187) reduces intake in IBD patients, particularly during the active phase of the disease course (184;188). Nevertheless, results from studies that assessed dietary intake in IBD patients are conflicting. Some studies reported energy intakes close to the national guidelines or to healthy subjects (97;99;100;189) but others did not (87;190). In the only paediatric study Thomas et al. (190) showed that children with active CD consumed on average 420 kcals less than the intake of their siblings (matched for height, sex and weight) whereas for 21%, the energy intake was lower than national recommendations compared with 10% in the control group.

Apart from the adequacy of energy intake there are also good reasons to assume that the micronutrient intake of CD patients is impaired despite no obvious differences in the energy intake with compared wit the healthy population. Food aversion and elimination diets with avoidance of high fibre foods and dairy products (87) may compromise a balanced micronutrient intake. Data on micronutrient intake are contradictory and no conclusions can be drawn. Some authors reported suboptimal intakes of micronutrients in IBD patients, compared with the healthy control and the national recommendations, whereas others failed to confirm these findings (Table 1.3.4).

1.3.7.2. Energy requirements and metabolism

Dietary intake, along with resting metabolic rate, physical activity expenditure and diet-induced thermogenesis are major components of the energy balance equilibrium. Failure to find clear differences in the caloric intake of IBD patients in some studies, and the differences in body composition observed in some others, as discussed above (Section 1.3.2), led to investigation of the other components of the energy balance equation.

Results of studies on the basal metabolic needs of patients with IBD are inconsistent and no obvious differences have been found with healthy controls (91;97;104). However when normalisation of the basic metabolic rate for the amount of FFM was considered, a higher basic metabolic rate/FFM ratio was commonly found in CD compared with UC patients and healthy controls. This may show that IBD patients may have increased energy expenditure per unit of FFM but low lean body mass.

Several studies on the energy metabolism of patients with IBD found that the non-protein respiratory quotient was significantly lower in CD compared with UC patients or healthy controls suggesting an increased lipid oxidation rate in CD patients that

Table 1.3.4: Major studies assessed dietary intake of micronutrients in patients with IBD

Study	Subjects	Design	Results
		ADULT STUDIES	
Aghdassi et al 2007 (189)	74 CD	7d diet records	Low to recommendations: Vit A, Vit E, Vit D, Vit B_1, Vit B_2, Vit B_6, Vit C, β-carotenoid, Niacin, Folate, Mg, Ca, Fe, Zn; No difference between active & inactive disease
Guerreiro et al 2007 (87)	78 CD mild disease; 80 controls	Semi-quant FFQ	Lower than controls: Ca, Vit C, Vit D, Vit E, Vit K; Lower than controls for recommendations: Ca, Vit A, Vit C, Vit D, Vit E
Filippi et al 2006 (97)	54 CD in remission; 25 controls	3 d diet records	Lower than controls: Females: β-carotene, Vit B_1, B_6, C, Mg; Higher for Zn Males lower β-carotene, Vit C Low to controls for recommendations: Zn, β-carotene, Vit B_1, Vit B_6, Vit C, Vit E, Mg
Geerling et al 2000 (99)	69 recent IBD; 69 controls	Diet history & FFQ	Lower than control: Ca, B_2, P in UC; No differences in CD
Geerling et al 1998 (100)	32 CD in remission; 32 controls	Diet history & FFQ	Lower than control: P Lower than controls for recommendations: Vit A
		PAEDIATRIC STUDIES	
Thomas et al 1993 (190)	24 CD; 17 control siblings	5 d dietary records	Lower than Control: Fe, Cu, Zn, Folate, Vit C

paralleled the lower FM seen in the former group of patients (91;98;191;192). Al-Jaouni et al (193) also found increased fat oxidation in CD patients that correlated positively with disease activity.

Higher diet induced thermogenesis, another small component in the energy balance equation, was found in one study (98) which could explain the lower weight and higher risk of undernutrition in IBD. Although there was no difference in the resting metabolic rate between healthy controls and CD patients, diet induced thermogenesis was higher (6% vs 10% respectively). However diametrically opposite results were presented by Al-Jaouni et al (193) who additionally found that diet induced thermogenesis was lower in patients with active compared with inactive disease.

1.3.7.3. Malabsorption

Malabsorption and energy loss due to non-digestion of food or malabsorption of nutrients could potentially influence energy balance, and partially explain undernutrition in patients with IBD. However, apart from some reports on specific micronutrients (194-197) in patients with ileal resection or with bile acid malabsorption, (198), rigorous evidence is lacking to support loss of dietary energy or other micronutrients due to malabsorption. Recently a small study in Israel (199) found that malabsorption is a major contributor to underweight in adult CD patients in remission. The authors found that GI energy excretion was higher in an underweight group with CD than in a normal weight group despite no differences between the two groups for dietary energy intake or resting metabolic rate. Similarly in another study malabsorption and increased faecal fat were observed in severely undernourished patients with CD (200). Interestingly, gastric acid and pancreatic enzyme secretion were severely impaired in 80% of these patients, which may be linked to the associated malabsorption (200). In fact following nutritional rehabilitation, stool fat output and malabsorption reduced with concomitant improvements in pancreatic enzyme synthesis, stores and secretion.

In theory the absorption of specific nutrients should be impaired when the disease is located at the site of specific nutrient absorption or the area has been resected. CD patients with ileal disease or resection are traditionally susceptible to vitamin B_{12} deficiency due to inadequate absorption (201). Iron absorption may also be diminished in active paediatric CD due to the excessive production of hepcidin, a hepatic peptide mediating the absorption of iron at the level of the enterocyte (167).

1.3.7.4. Gut losses

Apart from malabsorption in IBD, loss of nutrients can occur as a result of excessive intestinal mucosal sloughing, and through protein enteropathy from the ruptured gut in the

acute phase of the disease. Studies using whole gut lavage have shown that disease activity was closely paralleled by GI protein loss (202).

1.3.7.5. Drug-nutrient interaction

Several drugs used in IBD can interfere with the absorption, metabolism and excretion of nutrients. Two examples are the antagonistic interaction of methotrexate with folate metabolism and the long-term effect of steroids on calcium excretion and bone resorption.

1.4. Anaemia in IBD

Anaemia is one of the potential major nutritional issues in IBD. The term anaemia originates from the Greek word 'Αναιμία' meaning without blood and is a deficiency of erythrocytes or functional haemoglobin (Hb). This results in a reduced ability of the blood to transfer oxygen to the tissues, causing tissue hypoxia.

There are no universal established guidelines to define anaemia or a lower concentration of haemoglobin below which a person is characterised as anaemic. As a result the use of different reference range data often classifies the same subject into different states (anaemic or non anaemic) (203). The World Health Organization defines anaemia as a haemoglobin concentration less than 12 g/dl for non-pregnant women and <13 g/dl for adult men, whereas for children different age adjusted cut-offs are proposed (204) (Table 1.4.1). A haemoglobin concentration below 10 g/dl is commonly described as severe anaemia independent of sex and age.

Table 1.4.1: Age and sex adjusted cut-offs for the definition of anaemia according to WHO (204) and Dallman et al (205).

Age or gender group	Haemoglobin (g/dl)	Age or gender group		Haemoglobin (g/dl)
	WHO	Dallman et al 1979		
6 to 59 mth	11	0.5-1.9 y		11
5-11 y	11.5	2-4 y		11
12-14 y	12	5-7 y		11.5
Non-pregnant women (>15 y)	12	8-11		12
Pregnant women	11	12-14	Female	12
			Male	12.5
Men (>15 y)	13	15-17	Female	12
			Male	13
		18-49	Female	12
			Male	14

As the characterisation of the morphology (size, colour, shape) of erythrocytes is done with quick and cheap laboratory tests, it offers a starting point for the description of anaemia type. RBC can be characterized as microcytic (RBC too small), macrocytic (RBC too large), normocytic (normal size), hypochromic (Hb concentration too low), hyperchromic (Hb concentration too high), normochromic (Hb concentration normal), and combinations of RBC size and Hb concentration (206;207).

Surrogates for the investigation of suspected anaemia and differentiation of different types are: serum ferritin, serum iron, serum transferrin, folate, and vitamin B_{12}. Difficulties in diagnosis of anaemia arise when more than one type of anaemia with opposite effects on the haematological profile coexist (208). Moreover, as for many haematologically indices (serum iron, transferrin, ferritin) serum concentrations can be affected by the acute phase response, in various clinical conditions (IBD, malignancies, infection), their diagnostic value and interpretation can be ambiguous (209). A prime example of this is when active disease may result in a spuriously raised serum ferritin concentration masking an underlying iron deficiency caused by GI bleeding and poor dietary intake in IBD. When the diagnosis remains obscure, a bone marrow sample allows direct examination of the precursors to red cells.

1.4.1. Anaemia in IBD

Compared with the average awareness of other extraintestinal disease complications such as arthritis and nutritional status, the topic of anaemia in IBD receives little attention from clinicians. Anaemia is one of the most frequent comorbid conditions in IBD-related mortality (210). Overt or occult intestinal bleeding is a major symptom and a drop in haemoglobin occurs with each flare up in CD and UC. Anaemia alone or in conjunction with abnormal ESR levels demonstrates high positive predictive value for IBD diagnosis and in previous studies performed better than serologic antibodies (pANCA and ASCA) (211;212).

Two predominant types of anaemia have been identified in the context of IBD. Iron deficient anaemia (IDA) and the anaemia of chronic disease (ACD), which account for the majority of the cases. Anaemias associated with vitamin B_{12} and folate deficiency, or drug associated anaemia due to the long-term use of medication to manage IBD, have occasionally been reported but these are uncommon (Table 1.4.2) (213;214).

Table 1.4.2: Aetiology of IBD associated anaemia (adapted by Gasche et al. 2007 (215))

Common	Iron deficient anaemia Anaemia of chronic disease
Occasional	Cobalamin (B_{12}) deficiency Folate deficiency Drug induced anaemia
Exceptional	Haemolysis Myelodysplastic syndrome Aplastic anaemia (often drug induced) Inborn haemoglobinopathies or disorders of erythropoiesis

1.4.1.1. Iron deficient anaemia in IBD

Iron filings dissolved in vinegar were consumed by the ancient Greeks eager to acquire strength, and in 1600 AD iron was noted for its ability to restore “young girls when pallid, sickly and lacking colour to health and beauty” (216). Iron has a pivotal role in many metabolic processes including haemopoiesis, immunity and energy production, and the average adult contains 3-5 g of iron, of which two thirds are incorporated in haemoglobin (217). A normal balanced Western diet provides about 12-15 mg of iron daily, of which 5-10% is absorbed, principally in the duodenum and upper jejunum, where the acidic conditions help the absorption of iron in the ferrous form. Absorption is facilitated by the presence of other reducing substances, such as HCl and ascorbic acid and inhibited by reduction of gastric acidity (eg use of anti-acid) (207;218;219). The body has the capacity to increase its iron absorption in the face of increased demands like pregnancy, growth spurt, and iron deficiency status and reduce it when iron stores are excessively filled.

Once absorbed from the bowel, iron is transported across the mucosal cell to the bloodstream, where it is carried by the protein transferrin to developing red cells in the bone marrow and also to immune and liver cells. Hepatic iron stores comprise ferritin, a labile and readily accessible source of iron, and haemosiderin, an insoluble form found predominantly in macrophages. About 1 mg of iron a day is shed from the body in urine, faeces, sweat, and cells turnover from the skin and the GI tract (220). Menstrual losses of an additional 20 mg a month happen in women of reproductive age (208).

Iron deficient anaemia is classified morphologically as hypochromic and microcytic. In chronic iron deficiency the mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) are all reduced compared to the age and gender specific reference range. Serum iron, ferritin levels and transferrin saturation are reduced too whereas the total iron binding capacity is elevated (218;221) (Table 1.4.3). Bone marrow aspirates or biopsies demonstrate micronormoblastic hyperplasia of erythroid elements and decrease or absence of stainable iron particularly in macrophages (220;222).

Iron deficient anaemia is the major cause of anaemia in IBD, and this is ascribed to a negative iron balance from excessive iron loss through GI bleeding, increased epithelial sloughing in chronic GI inflammation, reduced dietary intake (223), as well as impairment of iron absorption, particularly during the active phase of the disease (167) or when the disease is located at the major sites of iron absorption (224).

As many of the systemic biological markers of the iron body stores are affected by the acute phase response, their diagnostic value in IBD is poor and can be misleading (Table 1.4.3). Decrease in serum iron and downregulation of transferrin is part of the acute phase response, causing functional iron deficiency, a state that can be misinterpreted as iron deficiency in a patient with active inflammation (209). On the other hand inflammation can cause false elevation of ferritin levels bringing ferritin levels of patients with true iron deficiency into the normal range (225). As a result higher cut offs have been proposed for ferritin in active IBD and evaluation in conjunction with other haematological parameters to detect iron depleted anaemic patients (215;226). Determination of serum transferrin receptor has been proposed as a diagnostic marker to distinguish IDA from other types of anaemia in IBD (227;228). This soluble transferrin receptor is elevated in iron deficiency and high rate erythropoiesis, whereas it is slightly low to normal in ACD providing a reliable marker to distinguish between IDA and other types of anaemia in IBD such as ACD (228).

1.4.1.2. Anaemia of chronic disease

The production of inflammatory cytokines in chronic inflammation, and malignancy has significant systemic effects including impaired haemopoiesis, and manifestation of ACD (216). Anaemia of chronic disease is second only to IDA in IBD (215;229).

Anaemia of chronic disease is refractory to the use of iron supplementation and is usually characterized by a moderate reduction in haemoglobin whereas the erythrocytes morphology is normal although slight hypochromia and moderate microcytosis can also be present (216). In contrast to IDA the total iron binding capacity is reduced or unchanged and the serum ferritin level is increased (Table 1.4.3). Bone marrow aspirates often show micronormoblastic hyperplasia with increased iron depots and a decrease in the number of iron containing erythroblasts (222). The measurement of the soluble form of transferrin receptor in serum may be an alternative to differentiate ACD from other types of anaemia and particularly IDA as described above (216;230).

The pathogenesis of ACD is not fully understood but recent evidence suggests this is immune driven and mediated by the action of inflammatory cytokines (231;232). Cytokines (IFN-γ, IL-10, TNF-□) and cells of the reticuloendothelial system induce changes in iron absorption (167), iron metabolism (insufficient iron release from reticuloendothelial cells), the proliferation of erythroid progenitor cells, the production of erythropoietin, and the life span of

red cells, all of which contribute to the pathogenesis of anaemia (231;232). Treatment of ACD is controversial and there is no universally accepted approach. The primary aim includes resolution of the inflammatory process, and downregulation of the expression of inflammatory cytokines. Use of human recombinant erythropoietin in conjunction with iron supplementation has been tested successfully in IBD studies (233-235).

Table 1.4.3: Characteristics and differences of the haematological presentation of IDA and ACD in IBD (adapted from Weiss 2005 (231))

	Iron deficient anaemia	Anaemia of chronic disease
RBC colour (MCH)	Hypochromic	Normo or slightly hypochromic
RBC size (MCV)	Microcytic	Norma or slightly microcytic
Plasma iron	Very Low	Low
Transferrin	High	Low
Transferrin saturation	Low	Low
Total iron binding capacity	High (decrease in active inflammation)	Low
Ferritin	Low (increase in active inflammation)	Normal to High
Erythrocyte protoporphyrin	High	High
Serum transferrin receptor	High	Normal

1.4.1.3. Megaloblastic anaemias (Vit B_{12} and folate deficiency)

Folate and vitamin B_{12} deficiency cause megaloblastic anaemia and the latter pernicious anaemia. Morphologically megaloblastic anaemias are characterized by large immature dysfunctional RBC (macrocytic) and are confirmed diagnostically by low serum levels of B_{12} and folate. Raised homocysteine concentrations are observed in folate and B_{12} deficiency and can also be used diagnostically. A bone marrow examination is usually performed to confirm megaloblastic hyperplasia.

Vitamin B_{12} deficient anaemia

Vitamin B_{12} is involved as an enzyme cofactor in a number of biochemical reactions and the synthesis of certain nucleic acids. In a state of B_{12} deficiency DNA replication is disturbed and nuclear maturation is arrested resulting in abnormally large dysfunctional red blood cells (megaloblasts) and anaemia onset. Reticulocyte counts are low and aspiration of bone marrow shows upregulated cell proliferation with characteristic megaloblastic abnormalities such as enlarged erythroid lineage cells. An increased MCV in conjunction with decreased vitamin B_{12} levels are characteristic of B_{12} associated megaloblastic anaemia. With a daily requirement for vitamin B_{12} of 1-3 µg and body stores of 5 mg clinical manifestation of B_{12} deficiency occurs late.

Formation of a protective complex with intrinsic factor produced by the gastric parietal cells is necessary for the absorption of vitamin in the terminal ileum. Any condition that alters

intrinsic factor production, such as inflammation of the gastric mucosa or malignancy, may interfere with the supply of vitamin B_{12}. Similarly as B_{12} absorption takes place in the terminal ileum, patients with active disease at this site or those who had their terminal ileum resected may have increased risk of developing B_{12} deficiency (236). Regular monitoring of the B_{12} status of these patients has been suggested (215).

Folate deficient anaemia

Natural folates (237) are largely found in the polyglutamate form, and these are absorbed in the duodenum and jejunum after deconjugation and conversion to the monoglutamate 5-methyl tetrahydrofolate. On average 50-60% of dietary folate is absorbed. Similar to B_{12}, folate participates in the synthesis of certain nucleic acids, and a deficit status presents the same hematologic findings as vitamin B_{12} with macrocytic anaemia. An inadequate diet, impaired absorption, or both usually cause folate deficiency. Of great concern are patients on medication with antagonists of folate metabolism or absorption. A prime example in gastroenterology is the patients on sulfasalazine, and the effect of the latter on the absorption of folate, as well as those on methrotrexate, an immunosuppressive agent, which interferes with the normal metabolism of folate. Diagnosis is based on macrocytic red blood cell maturation, as well as decreased serum or red blood cell folate levels. In combined iron and folate deficiency, the red blood cell folate level can be normal and the megaloblastic changes in erythroid cells may be obscured.

1.4.1.4. Drug induced anaemia in IBD

Long-term use of specific medication in chronic illness can interfere with normal haemopoiesis and subsequently lead to anaemia. Drugs used in the treatment of IBD, such as sulphasalazine, and methotrexate, can be associated with development of macrocytic anaemia attributed to secondary deficiency of folic acid. Prophylactic folic acid supplementation in these patients is recommended although as sulfasalazine has been commonly replaced by mesalazine, any drug interaction with folate lacks clinical relevance.

Azathioprine can cause myelosuppresion although nowadays monitoring of the bone marrow function by continuous blood monitoring protocols and the exclusion of use in patients with thiopurine methyltransferase deficiency has limited its incidence. Increase of MCV accompanies long term therapy with azathioprine (238) and is commonly used as marker of drug efficacy and compliance although it may also obstruct the early recognition of IDA (236).

1.4.2. Clinical consequences of anaemia

Since all human cells, tissues and organs depend on oxygen supply, anaemia onset is accompanied by a wide variety of clinical consequences. Significant determinants of the clinical picture of an anaemic patient are the type of anaemia and its degree. Commonly patients with mild anaemia develop pallor, headaches, dizziness, weakness or fatigue and poor concentration, whereas in more severe conditions shortness of breath and tachycardia are often reported. Angular stomatitis, finger clubbing, pica (consumption of non-food such as dirt, paper, wax, grass and hair) are signs of severe iron deficiency. Glossitis, neural tube defects and neuropathy are signs of folate and B_{12} deficiency. In megaloblastic anaemia due to folate deficiency central nervous system symptoms are not present in contrast to B_{12} deficiency. Folate supplementation may improve the (reversible) haematologic symptoms of megaloblastic anaemia even if they are caused by vitamin B_{12} deficiency, but does not stop progression of the (partially irreversible) neurologic manifestations of vitamin B_{12} deficiency. Therefore it is indicated that folate supplementation in deficient patients be accompanied by B_{12} substitution to avoid masking of B_{12} deficiency by improvement of the haematologic presentation on folate replenishment.

Anaemia impairs cognitive function and reduces quality of life (239) confirmed in studies where resolution of anaemia was accompanied by improvement in quality of life (240). For a long time it was thought that the clinical symptoms of anaemia occurred only when the haemoglobin level dropped abruptly as it had been suggested that patients would adapt to low haemoglobin levels if anaemia developed slowly. This has led to the concept of asymptomatic anaemia. However it was later shown that the process of adaptation to chronic anaemia was in fact adaptation to lower quality of life and that this could be reversed.

Table 1.4.4: Main therapeutic approaches in the management of anaemia in IBD

Type of anaemia	Therapy
Iron deficient anaemia	Diet rich in iron Oral iron supplementation Intravenous iron infusion Recombinant erythropoietin
Nutritional anaemias	Diet rich in micronutrients (B_{12} & folate) Oral folate supplementation Intramuscular B_{12} injection
Anaemia of chronic disease	Recombinant erythropoetin & oral/intravenous iron
Excessive bleeding/Very low haemoglobin	Blood donor transfusion

1.4.3. Treatment of anaemia with particular reference to IBD

Guidelines on the diagnosis and management of iron deficiency and anaemia in IBD have recently been published, without however distinguishing between adults and paediatric

patients (215). For the treatment of anaemia it is not only important to identify the type and the severity of the anaemia but also the nature of the anaemia so that therapy can be targeted at the underlying mechanism and be adjusted to the patient's needs.

With the advent of new more effective medication, that makes mucosal healing possible (90) or affects the inflammatory cascade at the initial step, a lower prevalence of anaemia may be anticipated. However such a theoretical approach has not been tested in clinical trials. In an anaemic patient in the active phase of disease the treatment of the underlying condition should be the primary target before any attempt to correct anaemia. This is particularly important in the management of ACD where the inflammatory response is the major contributor to the aetiology of ACD. The choice of treatment depends on the degree of anaemia. Gasche et al proposed a therapeutic algorithm in IBD associated anaemia together with consensus guidelines without however distinguishing between adult and paediatric patients (215). The main therapeutic approaches in the management of anaemia in IBD are outlined in Table 1.4.4.

1.4.4. Review of the studies on prevalence and predictors of anaemia in IBD

An extensive literature search was carried out, using Pubmed and searching the reference lists of original papers and reviews providing information on the prevalence of anaemia in IBD. Adult studies were excluded, as the primary scope of this review was to evaluate the prevalence of anaemia only in children with IBD.

Overall nine original articles and one study published in abstract form reported the prevalence of anaemia in paediatric IBD. Two systematic reviews including mainly adult studies (229;236) described in total four of these paediatric studies. In two other studies, measurements of haemoglobin were carried out and mean values were reported without reference to the overall prevalence of anaemia (20;83).

All but two studies were conducted in North America (Table 1.4.5); one study with 32 UC children in Europe (237), and one from Israel (228). Most studies measured anaemia in children younger than 18 years. Three studies expanded their inclusion criteria to young adults (228;241;242). The sample size varied from 11-526 children with only one reporting prevalence of anaemia in over 100 patients (243).

The definition of anaemia within the studies varied remarkably. All but two (241;242) used haemoglobin measurements to define anaemia, two used haematocrit cut offs (241;242), and in another (244), no definition of anaemia was given. The lower haemoglobin cut offs to distinguish between anaemic and non anaemic patients ranged from 10.5 to 13 mg /dl. All but three studies used generic cut offs to define anaemia in both girls and boys

whereas three studies used age adapted thresholds, as age related changes in haematologic indices are well recognized (221). All but two studies assessed anaemia close to the time of diagnosis. One study enrolled only patients with severe colitis (Table 1.4.5).

On the whole the prevalence of anaemia in paediatric IBD ranged between 41% and 88%. A mean estimate cannot be drawn. Neither can temporal changes be evaluated due to differences in the definition of anaemia between studies (245). Compared with the two systematic reviews which reported studies in mainly adult patients, prevalence of anaemia in children overall is reported to be higher. Wilson et al (229) estimated the prevalence of anaemia to be between 6-74% numerically lower than the range reported in the paediatric studies reviewed.

As documentation of anaemia was usually a secondary outcome in most of the paediatric studies only a few authors linked anaemia with disease and sociodemographic characteristics. Mack and his colleagues (243) showed that anaemia was associated with severity of disease activity. They observed that anaemia was more common in patients with severe disease than those with mild disease activity. The same group showed that in young patients (6-11 y) with severe disease, haemoglobin concentration was higher in CD than in UC ($p< 0.006$). No such difference was observed for the older group of children (≥ 12 y) or for children with mild or moderate disease. A negative association between haematocrit levels and disease activity has been described previously for CD patients particularly those who had isolated disease in the small intestine (246). Although Weinstein et al. (83) did not report the prevalence of anaemia, they found that mean haemoglobin concentration was lower in CD than UC, a finding that was more marked in the older group of patients (≥ 11 y). No such difference in the mean haemoglobin concentration was found between UC and CD in a state population based study in the United States (20). In a study published in abstract form only, Thayu et al (247) measured prevalence of anaemia in paediatric CD and tried to identify disease predictors associated with it. Anaemic patients had higher values of PCDAI and ESR than non-anaemic patients and the presence of upper GI tract disease was significantly associated with anaemia. Gender, duration of symptoms, body composition, or growth parameters were not predictors of anaemia.

Similar data from adult studies suggested an association between the presence of anaemia and high scores on the disease activity indices (214;235). Schreiber (235) found that anaemia was more common in CD than in UC patients whereas in a study from Israel, that accounted for the prevalence of anaemia by sex, the prevalence of anaemia was higher in women than in men (66.6% vs. 27.5% respectively) (248). Reilly (249) in a study of the impact of parenteral nutrition on clinical outcome found that the prevalence of anaemia was lower in CD patients with only small bowel involvement (33.3%) than in patients with disease involving the large bowel with or without small bowel. In contrast, RBC, haemoglobin and

anaemia were lower in patients with ileocolitis than in patients with small intestine involvement in a Japanese cohort of CD patients (250). Undernourished patients were more likely to be anaemic than well-nourished CD patients in another study (251).

Table 1.4.5: Studies that measured the prevalence of anaemia in paediatric IBD

Study	Country	Age	n (Male)	Disease	Definition of anaemia	Prevalence of anaemia	Predictors of anaemia	Comments
Mack et al 2007 (243)	US & Canada	<16	526 (310)	392 CD	< 6 y; Hb<11 6-11 y; Hb<11.5 >11 y; F: <12 M: <13	68%	Severe disease; In 6-11 y group with severe disease Hb was lower in UC than CD	
Howarth et al 2007 (252)	UK	<16	32 (11)	32 UC	Hb < 12	62.5%	N/A	
Khan et al 2002 (211)	US	≤18	90 (45)	51 UC	1-10 y; Hb<10.5 >10 y; Hb <11.5	41%	N/A	
Thomas & Sinatra 1989 (253)	US	≤18	24 (17)	24 CD	Hb < 12	71%	N/A	
Beeken 1979 (254)	US	8-21	11 (8)	11 CD	Hct < 36	72.7%	N/A	Did not measure anaemia at diagnosis
Burbige et al 1975 (213)	US	<16	58 (37)	58 CD	Hb < 11	51.7%	N/A	
Revel-Vilk et al 2000 (228)	Israel	9-22	63 (34)	5 CD	MCV < 77 fl F: Hb < 12; Ferritin < 6 µg/l M: Hb < 12.5; Ferritin < 23 µg/l	41.3% 11 IDA 15 ACD	N/A	Did not measure anaemia at diagnosis
Werlin & Grand 1977 (242)	US	6-20	19 (8)	5 CD	Hct ≤ 30	73.7%	N/A	New and patients with long-standing disease; Only with severe colitis
Thayu et al 2005 (247)	US	5-18	78 (44)	78 CD	<5 centile for age & sex	77%	High PCDAI and ESR; Upper GI disease involvement	
Gryboski 1994 (244)	US	≤10	N/A	40 CD	N/A	75% CD; 88.4% UC	Upper GI disease involvement	

1.4.5. Conclusion

The prevalence of anaemia in paediatric IBD is not well documented and the evidence comes from a limited number of studies with methodological flaws. Most studies included small sample sizes with only one study documenting the prevalence of anaemia in more than 100 patients. The definition of anaemia between the studies is heterogeneous and does not allow a direct comparison between the studies or assessment of secular trends in anaemia prevalence. Indeed the prevalence of anaemia in most of the current studies was a secondary outcome rather than a primary aim, and may have resulted in unrepresentative selective groups of patients that does not allow the extrapolation and generalization of findings. Thus the need of population based studies measuring anaemia at diagnosis before medical treatment has been applied is necessary to appropriately characterise prevalence and determinants of anaemia in paediatric IBD.

There is a lack of evidence on factors that might predispose to anaemia. Knowledge of this could help tackle the development of anaemia at an early stage. In light of new consensus guidelines on the categorization of disease location and behaviour new studies should be conducted to address whether disease phenotype favours anaemia. Undernutrition, growth faltering or demographics may also differ between anaemic and non anaemic IBD patients and should be investigated further.

To the best of our knowledge no study has measured the outcome and prognosis of anaemia during the natural history of the disease. As new treatments are now available which achieve mucosal healing (90) and tackle inflammatory cascade activation at the initial stages, temporal changes in the prevalence of anaemia and lower incidence at follow up should be expected. In particular possible association with specific treatment modalities deserves to be studied further. Such a study is described in Chapter 6 of this thesis.

1.5. Nutritional therapy in IBD

> *"Now to perform a true physician's part, and show I am perfect master of my art, I will prescribe what diet you should use, what food you ought to take and what refuse. –Ovid 43 BC"*

It is very attractive for the health professional to propose that he/she can improve the patient's condition by dietary manipulation. In contrast to coeliac disease, where the mucosal

lesion resolves with restriction of dietary gluten, the role of dietary treatment in IBD management is more complicated and controversial.

In paediatric CD the main focus of the dietetic intervention is on the artificial nutritional therapy which is defined as the provision of nutrients and energy in an artificial liquid food supplement. These can be administered orally, or when this is not possible intranasally through a nasogastric tube, or a stoma in the GI tract, for long-term nutritional support.

Artificial nutritional therapies can be classified by the nitrogen source; derived from either amino acids or intact protein component. Elemental feeds are developed by mixing single amino acids with other simple nutrient sources, are entirely free of dietary antigens and are commonly used in patients with food allergies. Semi-elemental feeds contain small oligopeptides (protein hydrosylates) and present less antigenic load than polymeric feeds, which contain whole protein. The protein comes predominantly from milk but also from egg and soya. Polymeric feeds are more palatable and cheaper than the other two forms and reduce the need for NG administration (255). In this thesis, wherever nutritional therapy is referred to it implies only artificial nutritional support unless otherwise stated.

This section is a concise review of the role of nutritional therapy in the management of IBD with a particular focus on the evidence in paediatric CD. The role of EEN in the induction of disease remission and nutritional rehabilitation in active paediatric CD is discussed in detail as is the evidence for mechanisms of action. Using subject specific keywords in Pubmed, more than 200 primary sources of evidence were retrieved on the nutritional management of CD. All the paediatric studies were reviewed and appropriate reference will be made to adult studies when evidence specific to children is scarce. Studies on the effect of EN on micronutrient status, and intestinal microbiota in IBD are described separately in the text in more detail. A summary of the paediatric studies on nutritional management of IBD is displayed in the evidence Table 1.5.1.

1.5.1. Nutritional therapy and disease activity in IBD

Nutritional support in the form of EEN, or rarely as total parenteral nutrition, is the mainstream treatment for induction of clinical remission in active paediatric CD in the UK, although different patterns of use are reported worldwide (256). In a survey of practice patterns 62% of western European paediatric gastroenterologists reported use of nutritional therapy compared with only 4% of their North American colleagues, who preferred corticosteroid use (256). It is noteworthy that EEN was not even mentioned in a recent state of the art North American review on treatment options in paediatric CD (257).

Table 1.5.1: Evidence table of all paediatric studies on the nutritional therapy in paediatric IBD

Study	Aim	Treatment	Patients	Outcomes	Results	Comments
Rodrigues et al 2007 (255)	Comparison between ED & PD on compliance, remission effect, use of NG tube, use of EEN on subsequent relapse	Retrospective; EEN (PD or ED); 6 wk	98 with active disease newly diagnosed	% remission rate; compliance rate; use of NG tube; reuse of EEN for subsequent relapse	No difference in any of the outcome apart from the use of NG that was lower in PD	Remission rate: 64% for ED and 51% for PD (ns)
Day et al 2006 (258)	Effect of EEN on disease activity	Retrospective; EEN (PD); 6-8 wk, some continued on supplementary feeds	27 with active disease; newly diagnosed and with longstanding disease	CA; inflammatory markers	89% completed their EEN course; no association between efficacy with disease location; most children improved within the first 4 wk; wt increased but in less degree in non responders	Remission rate better in new than long-standing disease; Cs sparing effect from EEN; Long remission on follow up; CA improved; Inflammatory markers improved
Borrelli et al 2006 (259)	Comparison between EEN & Cs on mucosal healing	RCT; EEN (PD); 10 wk vs Cs	37 children (19 EEN)	CA; histology; endoscopy; mucosal healing; albumin, CRP, ESR; anthropometry; side effects	No difference in clinical remission between EEN & Cs (79% vs 67%); CRP, ESR and albumin improved towards reference; No difference for Ht; Wt gain higher in EEN; less side effects in EEN (23%) vs Cs (67%)	14/19 in EEN vs 6/18 in the Cs showed mucosal healing; Only children on EEN presented improvement in histologic and endoscopic healing; good response for both colonic and ileal; well designed study; no concomitant treatment
Johnson et al 2006 (260)	Effect or EEN and partial EN on disease activity	RCT; EEN (ED or partial EN (50% ED & normal diet)); 6 wk	50 with active disease	CA; anthropometry; skinfolds; CRP; ESR; Hb; platelets; albumin	Remission in partial EN was 15% vs 42% in EEN; Only in EEN improvement of serum inflammatory markers, albumin, ESR and diarrhoea	Skinfolds improved; no difference between formulas
Berni-Canani et al 2006 (261)	Effect of EEN on disease activity (short and long term efficacy)	Retrospective, EEN (PD, SED, ED) vs Cs; 8 wk	47 newly diagnosed with active disease (37 on EEN & 10 on Cs)	CA; endoscopy; albumin; iron; histology; % relapse in 1y follow up	86.5% achieved remission; CA reduced in all groups; Endoscopic and histology improvement more pronounced in EEN (no difference between type of	Duration of clinical remission longer in EEN than in Cs; wt increased by 12% but no difference between EEN and Cs; Ht gain higher in EEN than in

					EEN); 7 children on EEN achieved histological remission but none in Cs; albumin and iron status improved in EEN	Cs; 90% reported adverse effects on Cs whereas 32.4% in EEN
Gavin et al 2005 (262)	Energy intake in EEN	EEN (PD-Modulen); 6-8 wk	40 with active disease	CA; anthropometry; % of recommended dietary intake	CRP decreased (78% less than 2mg/l); median wt increase 11%; energy intake in 82% of patients was around 118% of EAR; low BMI SDS at start was correlated with more wt gain	No disease site effect on remission rate
Knight et al 2005 (263)	Effect of EEN on short and long term outcome	(ED and 4 PD); 8 wk EEN	44 with active disease	CA; albumin; CRP; time to relapse	90% achieved remission; 62% relapsed within a y; EEN postponed Cs use for 68 wk;	No disease site effect on remission rate but colonic a trend to relapse earlier
Afzal et al 2005 (264)	Efficacy of EEN on disease activity; Association with disease location	EEN (PD); 8 wk	65 with active disease	CA; histology; endoscopy	Remission rate lower in colonic than disease involving ileum; CA improvement was better in disease involving the ileum; endoscopy and histology improved only in disease involving ileum	
Afzal et al 2004 (265)	Effect of EEN on disease activity; Association between quality of life and mucosal healing	EEN (PD); 8 wk	26 with active disease	CA; quality of life ; endoscopy; histology;	88.5% in remission; quality of life improved; histology and endoscopy improved; endoscopic or histological findings did not correlate with quality of life	
Ludvigsson et al 2004 (266)	Comparison of ED vs PD on disease activity	Multicenter RCT; 6 wk	31 with active disease	CA; remission rates	No difference in remission rates; 69% in ED vs 82% PD achieved remission; no difference in disease improvement between new and old CD patients; no	Higher wt gain in PD; patients had either quick (within 2 wk) or delayed (up to 6 wk) response

					association with disease location	
Bannerjee et al 2004 (267)	Effect of EEN on inflammatory markers, growth stimulating markers and nutritional restitution	EEN (PD); 6 wk	12 with active disease	CA; anthropometry; skinfolds; CRP; ESR; IL-6; IGF-1; IGFBP-3; leptin,	ESR, IL-6 improved to normal by d 3; CA, CRP, IGF-1 improved by d 7; wt, skinfolds, leptin, improved later after 14-21 d	Body composition markers measured not so sensitive to acute change as inflammatory and growth markers; anti-inflammatory and growth effects preceded nutritional status markers
Cameron & Middleton 2003 (268)	Effect of ED on orofacial CD	Case report; ED	Case report	Disease improved within 2 d		
Akobeng et al 2002 (269)	Effect of EEN (low or high in glutamine) on IGF-1	DBRCT; EEN (ED); 2 types of ED (high & low in glutamine); 4 wk	15 with active disease	IGF-1; anthropometry; MAC	IGF-1 was low at baseline and did not differ at the end of the study; proportionally more children on low glutamine presented increase in IGF-1 compared with the high glutamine feeds	MAC, and anthropometry improved only in the low glutamine group
Akobeng et al 2000 (270)	Effect of EEN (low or high in glutamine) on disease activity	DBRCT; EEN (ED high and low in glutamine) 4 wk	18 with active disease	CA; orosomucoid; platelets; ESR; abumin	Not difference in remission rates (55.5% in normal vs 44.4% in glutamine enriched); Improvement of CA was better in low than in high glutamine EEN	
Fell et al 2000 (90)	Effect of EEN on mucosal healing and mucosal inflammatory markers	EEN (PD); 8 wk	29 with active disease	CA; anthropometry; CRP; TNF-α; mucosal levels of IL-1β, IL-8, IL-10, INF-γ, TGF-β1; endoscopy; histology	79% in remission; CA, CRP, TNF-α decreased; wt increased; histology and endoscopy improved; IL-1β, IL-8, INF-γ decreased after treatment; TGF-β1 increased	In TI, histologic improvement was observed in 13/20; colonic improvement was observed in 12/25; Complete healing occurred in 8 and 2 cases with TI and colonic disease respectively.
Beattie et al 1998	Effect of EEN on growth	EEN; PD; 8 wk plus two mth on	14 with active disease	CA; IGF-1; IGFBP-3; anthropometry;	Wt increased, but not ht; skinfolds unchanged; CRP	

(271)		supplementary feeds		skinfolds, CRP	decreased within two wk; IGF-1 & IGFBP-3 increased	
Azcue et al 1997 (104)	Effect on EEN on body composition and energy expenditure	EEN (ED) vs Cs; 4 wk	24 children with active CD, 19 anorexic, 22 healthy controls	Body composition (double labelled water, BIA, total body K, extra and intracellular water with bromide dilution); REE with indirect calorimetric	All body composition parameters improved in EEN; REE increased also in EEN; ht increased only in EEN	Intracellular water and lean mass accretion were significantly higher in the EEN than in the prednisolone group; patients on Cs trend for higher FM at the end of treatment
Khoshoo et al 1996 (272)	Comparison between low and high fat EEN on disease activity	Crossover RCT; EEN; SED (high fat vs low fat); 6wks	14 adolescents	CA (PCDAI); skinfolds; body composition with bioimpedance	No difference between the formulas; improvement on body composition & skinfolds; improvement in CA	Patients on concomitant medication (Cs, antibiotics, 5-ASAs); Cs requirements decreased
Wilschanski et al 1996 (273)	Effect of supplementary feeds following EEN on maintenance of remission	EEN; (ED or SED) followed by nocturnal or not supplementary feeds	65 with active disease	CA (PCDAI); Hb, ESR; albumin; anthropometry	CA, ESR reduced; haemoglobin and albumin improved; less patients relapsed in supplementary feeds; growth velocity improved with EEN compared with previous y and ht velocity higher in supplemented group	72% got in remission; 60% relapsed within 12 mth
Papadopoulou et al 1995 (274)	Comparison between Cs & EEN on disease activity	EEN; ED vs Cs (historical control); 6 wk	58 (30 on EEN)	CA; anthropometry	CA improved in both treatments; Disease improvement better on ED; patients with proximal disease had longer remission period; ht improvement was better in EEN than Cs; albumin and Hb improved in EEN but not on Cs	
Breese et al 1995 (275)	Effect of EEN on immunological response	EEN (ED&PD) vs Cs vs cyclosporine; 8 wk	18 (6 on EEN) with small intestine involvement	% lymphokine secreting cells that produce IL-2 & INF-γ; histology	Mucosal healing improved in EEN but not in Cs; % lymphokine secreting cells that produce IL-2 & INF-γ	

					reduced	
Beattie et al 1994 (276)	Effect of EEN on disease activity	Case series; EEN; PD (Nestle TGF-beta2); 8 wk	7 with small bowel; newly diagnosed	CA; CRP, ESR, anthropometry; histology	CRP, ESR decreased; wt increased; CA decreased; ht velocity improved; histology improved in 6/7; 2/7 relapsed in 1 y	
Ruuska et al 1994 (277)	Comparison between Cs & EEN on disease activity	RCT; EEN (PD) vs Cs; 8 wk exclusively and PEN for following 3 wk	19 with active disease	CA; CRP; ESR; Hb; IgG; IgA; Prealbumin; Iron	No difference in remission rates, CRP, IgG, IgA, prealbumin, iron; more patients on EEN remained in remission at follow up	CA reduced, improved within two wk of treatment initiation; Wt increased the first 4 wk and remained stable then after
Thomas et al 1993 (190)	Comparison of EEN vs Cs	RCT; EEN; ED; 4 wk	24 with active disease (12 EEN)	CA; anthropometry	CA reduced in both treatments; no difference between treatments; ht velocity improved only in EEN; no difference with disease location	
Thomas et al 1993 (278)	Effect of EEN & Cs on IGF-1 and IGFBP-1	RCT; EEN (ED) vs Cs & sulphasalazine; 4 wk	29 (13 on EEN) with active disease	IGF-1; IGFBP-1; anthropometry	IGF-1 increased in both treatments; higher increase in Cs; IGFBP-1 decreased only in Cs; ht velocity was greater in EEN than in Cs	EEN improved growth despite higher increase of dietary intake in Cs; Perhaps Cs over-ride the benefits from increased energy intake, IGF-1 and reduced IGFBP-1
Polk et al 1992 (279)	Effect of intermittent EEN on growth and disease activity	One mth of EEN (ED) followed by 2 wk of exclusion and low fibre diet and then 2 mth on free diet	6 with growth failure (only one patient with active disease)	CA; anthropometry; IGF-1; albumin; skinfolds	CA reduced; ht, wt, IGF-1, albumin improved; no changes in skinfolds	Concomitant treatment (Cs, antibiotics, 5-ASA, mercaptopurine)
Aiges et al 1989 (280)	Effect of nocturnal supplementation on growth	Nocturnal partial supplementation with PD; 1 y	8 adolescents with growth failure and quiescent or mild disease & 4 controls	CA; anthropometry	Wt gain and ht velocity increased compared with last y and controls; CA improved compared with last y in subjects but not in controls	On concomitant treatment with Cs
Belli et al 1988 (281)	Effect of intermittent EEN on	EEN; 1 wk on ED followed by	8 with growth failure and 4 controls	CA; anthropometry; skinfolds; ESR; Hb;	Wt, ht, skinfolds, urinary creatinine increased	On concomitant treatment with Cs and 5-ASAs

	growth	3 wk on normal diet for 3 mth		TIBC; folic acid; urinary creatinine	compared with previous y and controls; CA decreased and use of Cs decreased compared with previous y and controls	
Sanderson et al 1987 (282)	Comparison between Cs & EEN on disease activity	RCT; EEN; SED; 6 wk	17 longstanding CD; (8 on EEN) with active disease; small bowel disease	CA; ESR; CRP; albumin; anthropometry	Improvement in CA, ESR, CRP, albumin, wt; No difference in CA between EEN and Cs; growth velocity improved only in EEN	
Sanderson et al 1987 (283)	Effect of EEN on gut permeability	EEN; ED; 6 wk	14 with active disease with small bowel involvement & 7 controls with no small bowel involvement	CA; gut permeability (lactulose/rhamnose); ESR; anthropometry	Gut permeability improved; 50% had permeability levels within normal range after treatment; wt increased; CA & ESR decreased in parallel with permeability improvement	
Blair et al 1986 (284)	Preoperative nutritional support with EEN	EEN; ED; 5 wk to 4.5 mth	11 with small bowel involvement, preoperative	CA; ESR; Hb; albumin; anthropometry, steroid reduction	ESR reduced; Hb, albumin, anthropometry increased; steroid use reduced, fistulas improved; postoperative complication reduced, reduced rates of postoperative relapses	
O'Morain et al 1983 (285)	Effect of EEN on disease activity	EEN; ED; 4 wk	15 with active disease and small bowel involvement	ESR; Hb; albumin; anthropometry	Inflammatory markers improved within 1-2 wk; ESR, decreased, Hb and albumin increased, some children had wt and ht improvement	Patients on concomitant treatments
Navarro et al 1982 (286)	Effect of EEN on disease activity	EEN; 2 to 7 mth; followed by supplementary feeds from 12-22 mth	17 underweight children; 10/17 growth retarded	CA	No difference between Cs and EEN; Improvement of patients with stenotic disease; reduced steroids dependency	Patients on 5-ASA
Morin et al 1982 (287)	Effect of EEN on disease activity,	EEN; ED; 21 d	10 newly diagnosed with active disease	CA; bacterial counts; folate;	CA reduced; wt, skinfolds improved; No changes in	

	body composition, gut flora, vitamin levels			carotene; anthropometry; skinfolds	bacteria; folate increased carotene decreased
Morin 1980 (288)	Effect of EEN on growth	EEN; ED; 6 wk	4 with growth failure; some but not all active	CA; anthropometry; skinfolds	CA improved; ht (1.8 cm) and wt (3.8 kg) improved; skinfolds improved; children continued growing after end of EEN

ED: Elemental diet; PD: polymeric diet; CA: clinical activity; Cs: corticosteroids; wt: weight; ht: height; TI terminal ileum; DBRCT: double blind randomized control trial

As it is unethical not to treat patients with active disease, no placebo controlled trial has been conducted so far to test the efficacy of EEN in IBD. Instead the efficacy of EEN to induce disease remission in active CD has been compared with that of other treatments, mainly steroids, in several investigations (Table 1.5.1). These have been reviewed in a few review articles and recently in an updated Cochrane meta-analysis of RCT (289) as presented below. With regard to UC there is little information available, although overall it seems that nutritional support does not possess a particular role in the management of active UC.

1.5.1.1. Nutritional therapy in adults with active CD

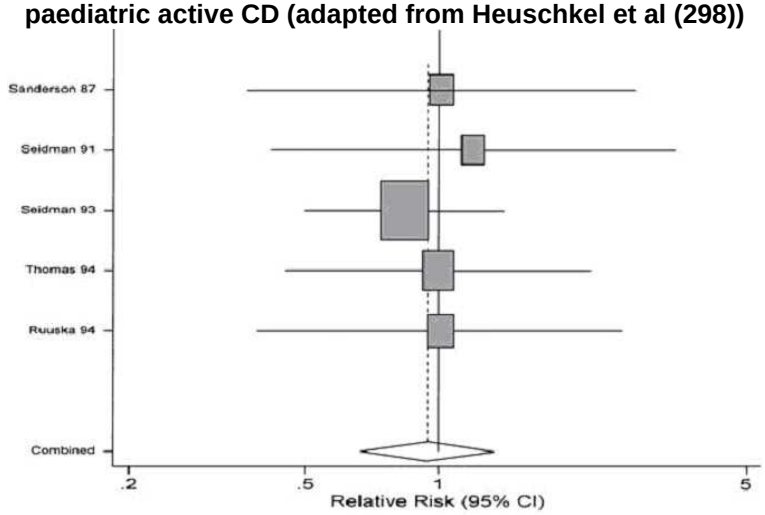
It is now more than 30 years since a group of surgeons reported the benefits of EEN in adults with active CD. Patients awaiting surgery for management of treatment-resistant disease experienced disease remission while receiving nutritional support for preoperative nutritional rehabilitation and bowel rest (290). Following this observation, O'Morain et al (285) conducted the first clinical trial in adult CD patients and found that elemental EEN was comparable in inducing clinical remission and correcting serum inflammatory markers compared with corticosteroids treatment. Subsequent adult studies confirmed these observations (291;292) although a European multicentre study, involving 107 adults CD patients showed that combined treatment of steroids and sulphasalazine was more effective than semi-elemental feeds in the induction of clinical remission (293). Three meta-analyses between 1995-1996 pooling the results of individual clinical trials concluded that although enteral feeding has a role in the management of CD it is inferior to steroids in achieving disease remission (294-296). Similar conclusions were recently reached by a Cochrane review in 2007 (289) which included eight trials with 212 patients treated with EEN and 179 treated with steroids, and yielded a pooled OR of 0.33 (95% CI 0.21 to 0.53) favouring steroid therapy. Remission rates varied considerably (20% to 84.2%) in the individual studies which could be due to differences in the study populations, route of administration, outcome measurements and methodology (289).

1.5.1.2. Nutritional therapy in children with active CD

Despite the conclusions of the three metanalyses, reporting a clear superiority of steroids to induce clinical remission in active CD, two subsequent metanalyses were conducted, including RCTs in paediatric only CD patients (297;298). Pooling the results of five paediatric studies with a total number of 147 children Heuschkel et al (298) found no difference (RR = 0.95 [95% CI: 0.67-1.34]) between EEN and corticosteroid treatment to achieve clinical remission (Fig 1) which was confirmed in an updated meta-analysis (297) including two

subsequent trials, (259;299). The overall reported remission rates on treatment with EEN varied from 42% (260) to 90% of cases (263) among the individual studies (Table 1.5.1).

Figure 1: Forrest plot of RCT on the effect of EEN on induction of clinical remission in paediatric active CD (adapted from Heuschkel et al (298))



1.5.1.3. Effect of nutritional therapy in remission maintenance

As IBD is chronic in nature, and there is no effective curative treatment, a large number of patients are anticipated to relapse within a year of remission induction (300;301) with some relapsing soon after food re-introduction (302). Patients with complicated disease (ie fistulae, strictures, perianal disease) are more susceptible to early relapse (303). In a large (n=113) adult retrospective analysis, 22% of patients relapsed in the first six months after EEN treatment whereas the probability of maintaining remission at three years was 38% (303). Likewise 2/3 of the patients in paediatric studies are estimated to relapse within a year of EEN induction of remission (263;273). No difference in the time to first relapse has been found between clinical remission induced with EEN or steroids in both adult and paediatric reviews (289;298) although a recent paediatric trial found higher relapsing rates in the steroid, compared with the EEN group, which was ascribed by the authors to the better mucosal healing achieved in the latter group of patients (261).

Following establishment of disease remission, second line treatments are commonly applied to prevent a subsequent relapse. Retaining a patient on EEN to prolong remission is impractical, if not unethical, and therefore a few studies tested the efficacy of supplementary nutritional support to maintain clinical remission and tackle a subsequent clinical flare up. In a Japanese adult study (304), adult patients who received half their total energy requirements as elemental diet and the remaining half by normal diet had a significantly lower relapse rate (34.6%) than patients who received unrestricted normal diet (64%) (OR: 0.4 [95% CI 0.16 to 0.98]). Similar results were presented recently by Yamamoto (305;306) and Verma (307;308) in two adult Japanese and two adult British studies respectively. In the Japanese studies

supplementary EN in parallel with a low fat diet prolonged disease remission, prevented increase in mucosal inflammation and inflammatory markers (306) and prevented post-surgical clinical recurrence compared with an unsupplemented group of patients (305). Likewise partial supplementation of an unrestricted diet was more likely to prolong remission (307) compared with an unsupplemented control group, and moreover gradually tapered steroid usage in British patients with steroid dependent disease (308).

1.5.1.4. Exclusive vs partial nutritional therapy in induction of disease remission

The origin of the exclusivity aspect of EN treatment is unknown and is possibly based on a putative mechanism of action through bowel rest. This theoretical concept was only recently tested in a randomized controlled trial of 50 paediatric patients with active CD who responded better on an exclusive course of nutritional therapy than to a partial regime (50% of dietary intake) in conjunction with free diet (260). The EEN group had a remission rate of 42%, which was significantly higher than that of 15% observed in the partial EN group.

1.5.2. Nutritional therapy and mechanisms of action in CD

Although there is extensive clinical experience and literature supporting the use of nutritional therapy in CD, the mechanisms of action and ideal formulation remain unknown and are still under investigation. There is still much debate about putative mechanisms of action mainly because the gut is a difficult organ to study directly. Therefore most of the evidence derives from in-vitro models and other surrogate indices of intestinal events that may not always represent events occurring at the mucosal site. Some of these hypothetical mechanisms are presented in Table 1.5.2 and are discussed below in detail.

Table 1.5.2: Proposed mechanisms of induction of remission with EEN in active CD

Bowel rest	Low antigenic load	Anti-inflammatory effect	Mucosal trophic effect	Nutritional effect
Reduces intestinal secretions	Low in microbial antigens	Reduction in systemic markers of inflammation	Improves intestinal permeability	Improves energy/protein status
Reduces gut motility	Low in dietary antigens/allergens	Direct mucosal anti-inflammatory effect	Accelerates intestinal healing	Replenishes micronutrient deficiencies

1.5.2.1. Effect of nutritional therapy on inflammatory markers

In parallel with the resolution of clinical GI symptoms (stool frequency, abdominal pain, perirectal bleeding), systemic inflammatory markers improve and return to normal levels within a few days of treatment with EEN (267). Several studies have shown that the clinical

activity response to EEN coincides with reduction in ESR, circulating CRP and other pro-inflammatory cytokines (Table 1.5.1).

Although the fall of systemic inflammatory markers is essentially a secondary response to GI inflammation and disease improvement, there is now strong evidence to suggest direct anti-inflammatory and immunomodulatory properties of EEN at the mucosal level (275;309) such as downregulation of mucosal proinflammatory cytokine mRNA (IL-1, IFN-γ, IL-8) in both terminal ileum and colon of paediatric patients with CD. Indeed these changes coincided with improvement in clinical activity, mucosal healing and circulating inflammatory markers (ESR, TNF-α, CRP) (90). Meister et al (310) in an in vitro experiment observed that incubation of mucosal biopsies from adult CD patients with elemental diet resulted in an increase in the ratio of anti-inflammatory/pro-inflammatory cytokines (IL-1 receptor antagonist/IL-1β). However incubation of biopsies from UC patients with elemental feeds did not improve the anti-inflammatory/pro-inflammatory ratio, in accordance with the lack of clinical effectiveness of nutritional therapy to suppress inflammation and induce remission in patients with active UC. Likewise Yamamoto showed return of mucosal pro-inflammatory cytokines (IL-1β, IL-1ra, IL-6, IL-8, TNF-a) to normal healthy levels in Japanese adult patients with active CD at the end of EEN (309), and prevention of increase in patients on long-term nutritional support (306). In fact the endoscopic and histologic improvement in the first of these studies was associated with a parallel decline of the mucosal cytokines and an increase of the IL-1ra/IL-1β ratio (309).

As assessing mucosal inflammation with blood markers may not be sensitive or specific to intestinal inflammation, and repeat endoscopies are impractical, expensive and require general anaesthesia in children, surrogate indices of local intestinal inflammation are required particularly for routine monitoring of disease activity. Nowadays new faecal markers of intestinal inflammation have recently become available that allow assessment of the disease at the site of presentation. Calprotectin and lactoferrin are two potential candidate markers with excellent diagnostic value to differentiate between intestinal inflammation and other functional digestive disorders (311). Both are non-invasive, cheap and could offer a suitable means to monitor intestinal inflammation and response to treatment. No study has yet assessed changes of these markers during nutritional therapy. On the contrary a study of the use of steroids in active CD failed to find changes in calprotectin levels despite improvement in clinical activity, suggesting that steroids offer symptomatic relief without however resolving intestinal inflammation and achieving mucosal healing (312). The effect of EEN on calprotectin is investigated in Chapter 6.

1.5.2.2. Effect of nutritional therapy on endoscopic, histological improvement and mucosal healing

There is a growing view that healing of the intestinal mucosa in IBD patients should be the principal therapeutic objective because only agents promoting mucosal healing may modify the longterm course of the disease. The primary objective of nutritional therapy is to induce clinical remission with minimal side effects. Because the inflammatory process is located in the intestinal mucosa, clinical remission should ideally be associated with a reduction of mucosal inflammation and mucosal healing. Various paediatric (90;264;276) and adult studies (300;309) have shown that mucosal healing is achieved with EEN more often than treatment with steroids whereas endoscopic and histological lesions repair does not coincide with clinical improvement when using steroids (259;261;275;313;314). A recent Italian study (259) showed improvement in endoscopic and histological findings at the end of treatment only in the group of patients on EEN. The proportion of children showing mucosal healing was significantly higher in the EEN (14/19) than the steroid group (6/18). Failure to achieve mucosal healing on steroid treatment might account for the higher relapse rate observed compared with a EEN treated control group of children, in another recent Italian study (261). Similar results were presented by Yamamoto et al (309) in adult patients with CD. Endocopic healing and improvement rates were 40% and 77% whereas histological healing and improvement were 20% and 55% for steroids versus EEN respectively. The authors speculated that clinical remission occurs first, preceding endoscopic improvement, whereas histological improvement takes place last.

1.5.2.3. Effect of nutritional therapy on intestinal permeability

In active CD, intestinal permeability is increased and its extent is positively associated with disease activity (315) and is a strong predictor of clinical relapse (316). Impaired intestinal barrier function may facilitate contact of mucosal immune-competent cells with antigenic substances from food and bacterial products from the host's intestinal flora activating the mucosal immune system. Some studies have shown that improvement in clinical disease activity coincides with reduction in intestinal permeability (283;317;318).

1.5.2.4. Nutritional therapy and antigenic effect

The initial speculation that the effectiveness of nutritional therapy in CD is due to a reduced antigenic load in the elemental feeds compared to the whole protein was revised in light of evidence suggesting no difference in the clinical efficacy of polymeric, semi-elemental and elemental feeds (300;302;319;320). This was recently reconfirmed by a Cochrane meta-analysis in adult studies (289). Similarly in children a recent retrospective analysis of case

series (261) and a randomized multicenter controlled trial (266) found no difference in disease activity improvement and remission rate between polymeric and elemental feeds. Remission rates were 69% on elemental diet compared to 82% in the polymeric feeding group.

1.5.2.5. Nutritional therapy and gut rest

One of the first indications and proposed modes of action of EEN in CD was the property of being liquid and readily absorbable in the proximal part of the GI tract, minimizing participation of the digestive system in the chemical and mechanical process of digestion and absorption of the nutrients. This was postulated to provide a degree of bowel rest and subsequently to accelerate healing in the already traumatized intestinal mucosa. However subsequent evidence (321;322) and a key study by Greenberg et al (323) found no difference in clinical remission between patients treated with TPN and bowel rest, a polymeric enteral feed, or peripheral nutrition with oral diet. This demonstrated that bowel rest was not the mechanism responsible for diet-induced remission in CD. Indeed, as colonocytes depend on exogenous nutrient provision through the diet, there are valid reasons to recommend the addition of fibre, and prebiotics in nutritional therapy feeds as these could promote intestinal health either by producing SCFA or by modulating an imbalanced commensal intestinal flora, towards an optimal healthier one (324).

1.5.2.6. Amount and type of fat

Long chain fatty acids may modulate eicosanoid synthesis and other immunomodulatory mechanisms thereby influencing disease outcome. It was therefore speculated that the amount and type of fat may be a determinant of the efficacy of EEN. In particular, ingestion of ω-3 fatty acids might suppress mucosal inflammation whereas ingestion of ω-6 fatty acids might stimulate the inflammatory cascade (325). A few studies compared nutritional formulations of different types and amounts of fat without however clear-cut results (261;320;326). The topic was recently reviewed by Zachos et al (289) in a Cochrane review that included only RCTs, which concluded that there is no evidence to suggest a difference in the efficacy to induce disease remission between low and high fat EEN. Moreover the equal therapeutic effect of polymeric and elemental feeds with high and low concentration of fat respectively, suggests a mechanism of action independent of the fat content in the feeds (319;320;327). On the other hand the type of fat may be a clue to the efficacy of EEN. Bamba et al observed decreased rates of remission with increasing amount of ώ-6 fatty acids in the elemental feeds. In contrast, a well designed double blind RCT European

multicenter study had to stop early because of low remission rates achieved in the low linolenic/high oleic compared with the high linolenic/low oleic formula (328).

1.5.2.7. Other aspects of nutritional therapy in the management of active CD

Nutritional therapy efficacy and disease location

At the moment there is limited data to suggest a better efficacy of nutritional therapies in CD patients with a specific disease phenotype (289;303) despite scarce evidence suggesting that patients with small bowel involvement respond better to EEN (329). A recent paediatric study showed that children with active CD do not respond well to EEN when the ileum is not involved although these results were not replicated by a Italian paediatric (259) and a Japanese adult study (309). Indeed there are case reports that EEN was even beneficial in orofacial presentation of CD (268).

As far as maintenance of disease remission is concerned, Teahon et al (303) in a retrospective analysis of more than 100 case series found that adult patients with isolated ileal and small bowel disease were more likely to remain in long-term remission compared with patients with colonic, or ileocolonic disease after induction of clinical remission with nutritional therapy. Similarly Gavin et al (262) in a paediatric retrospective study, found that patients with colonic disease tended to relapse earlier despite no obvious effect of the disease phenotype on induction of clinical remission with EEN.

Effect on nutritional therapy on disease activity in new and longstanding disease

Whether new patients respond better to EEN than those with long-standing disease, is quite controversial. Some studies showed that newly diagnosed patients are more able to respond to EEN than patients with long-standing disease (329) whereas others found no difference (266;330).

1.5.2.8. Effect of EN on nutritional status

1.5.2.8.1. Effect of nutritional therapy on weight and body composition in IBD

Regardless of whether EEN therapy is equal to or slightly inferior to steroids in inducing remission, its use is advocated by its nutrition promoting properties contrary to the detrimental side effects of long-term steroid usage on growth, body composition, bone health and body image. Improvement of the patients' protein-energy nutritional status has always been considered as one of the mechanisms of EEN action, although recent studies have

shown that anti-inflammatory properties preceded any changes in nutritional status (267;331). Moreover no difference in remission rates was found between malnourished and normal nourished adult CD patients on treatment with EEN.

In both adult (292;300;301;320) and paediatric studies (Table 1.5.1) nutritional therapy is accompanied by significant improvement of pre-treatment anthropometry. Weight gain accompanies disease improvement (276) and is component of the PCDAI and other disease activity indices (332).

A few authors have also used body composition techniques to assess changes in fat and lean mass during nutritional therapy in adults (300;301) and paediatric patients (Table 1.5.1). Improvement in surrogate measures of body composition, like skinfolds thickness and upper arm circumference, were commonly reported by some (260;267;272;281;287;288) but not all the studies (271;279). However none of these studies appropriately expressed/converted raw measurements of body composition to age and sex reference standardized values, as z-scores, and therefore contradictory results could be attributed to differences in the age and gender characteristics between the comparison groups.

Two paediatric studies assessed changes in the body composition of children with CD undergoing treatment with nutritional therapy. Khoshoo et al (272) used bioimpedance and skinfold measurements to assess changes in body composition in adolescents with active CD on treatment with EEN. Although their results may have been flawed by the concomitant use of steroids, they observed significant improvement in FFM assessed by both methods. In the second study Azcue et al (104) using highly sophisticated techniques (isotope dilution, extracellular bromide dilution, bioimpedance, total body potassium) assessed body composition changes and body fluid distribution and observed increments in all body compartments after treatment with EEN. Noteworthy differences in fat and lean mass accretion were observed between patients on EEN and corticosteroids. Steroid patients tended to accumulate more fat whereas intracellular water and lean mass accretion were significantly higher in the EEN group. This observation coincides with recent evidence that EN promotes protein anabolism by suppressing proteolysis, and increasing protein synthesis in adolescents with CD in remission (333), an effect that contrasts to the documented catabolic effects of steroids.

1.5.2.8.2. Effect of nutritional therapy on growth in CD

Linear growth retardation is commonly seen in children with IBD, predominantly CD (Section 1.3.3). Both suboptimal dietary intake and an inhibitory effect of inflammatory response cytokines are implicated (334). Substantial evidence suggests that nutritional therapy, both elemental and polymeric formulation, has growth-promoting effects (297). Nutritional therapy either exclusively (271;276), supplementary (273;280) or with intermittent course

administration (279) improved height velocity (271;276;278) and accelerated linear development in growth retarded children (280;281;288). This is an important advantage over the use of steroids and their well-documented growth inhibiting effects. In particular growth velocity improved in patients on EEN but remained unchanged (282) or improved to a lesser extent in patients on steroids (261;271;274).

The exact mechanism of the growth promoting effect of nutritional therapy is unknown and is speculated to be due to a composite effect from both nutritional replenishment and reversal of the inhibitory effect of inflammatory cytokines on growth hormonal mediators of growth like IGF-1 (267;271;278;279). It is noteworthy that a recent small study observed that the growth promoting effects of EEN preceded nutritional rehabilitation (267).

1.5.2.8.3. Effect on nutritional therapy on micronutrient status

Although a recent study showed that disease improvement precedes nutritional rehabilitation and may suggest that nutrition rehabilitation is not implicated as a mechanism of action of EEN, the authors of this study assessed only indices of energy-protein status (267). Whether disease improvement is a direct effect of improvement and replenishment of micronutrient status from EEN has not been investigated. Micronutrients play a vital role in metabolism, antioxidant status and the integrity of the colonic epithelium. Reactive oxygen species exhibit deleterious effects on epithelial cells in CD and studies have shown decreased levels of antioxidant enzymes and vitamins in the intestinal mucosa and in the plasma of patients with CD (Section 1.3.6). Replenishment of micronutrient status during EEN is one of the possible modes of action mediating the efficacy of EEN in CD where micronutrient deficiencies and malnutrition are common as opposed to its inefficacy in UC were deficiencies in minerals and vitamins are unusual.

Moreover, there are additional reasons why replenishment of micronutrient in EEN is important. Conventional EEN regimes, intended for use in active CD, provide age and gender specific energy requirements, as reflected by the increase in body weight and anabolic effects, but there is no evidence to ensure that the micronutrient content of these feeds is adequate to replenish deficiencies or supply the essential requirements of these patients. The dietary adequacy of micronutrients in these feeds has been ascribed to the provision of national reference daily intakes, originally established to cover the needs of the healthy population, but whether the same requirements apply to patients with CD is questionable. A study (335) 20 years ago failed to show improvement of vitamin status of IBD adults on EEN despite provision of higher amounts of micronutrients than the RDA.

Moreover EEN feeds are “artificial food” made of composite simple food ingredients and nutrients for which recommended allowances have been established and therefore may lack

‘non-nutrient compounds’ that occur naturally in food (e.g. polypenols), ave important role in the human health, but whose supplementation in artificial feeds is uncommon.

An extensive literature search was carried out by the researcher in 2004 and updated in 2008 to review all studies on the effect of EEN therapy on blood micronutrient status in IBD patients. Studies using total parenteral nutrition were excluded. Overall seven studies were retrieved. The studies were heterogeneous in design, subject characteristics, duration or type of feeds used, and the mode of nutritional support (Table 1.5.3). Four studies were conducted in CD adults, whereas two included UC patients. All but one study used EEN. One used partial supplementation of normal diet. The duration of nutritional therapy varied from 12 days to one year. Only one adult study including UC patients assessed a wide range of micronutrients. The rest assessed a limited number of micronutrients, focusing mainly on antioxidant trace elements and vitamins. Some studies assessed changes of the enzymatic antioxidant systems and one study measured changes in oxidative stress (Table 1.5.3).

The results were inconsistent and no overall conclusions could be drawn. Nutrients like Se increased in some studies but decreased in others (Table 1.5.3). Similar results were found for other nutrients like vitamin A. The use of different feed formulae and duration of administration among studies could explain some of these differences. No study in children as investigated changes in a wide range of vitamins. Thomas et al (183) assessed only changes in Se, whereas Akobeng et al (336) studied additionally vitamins A, E and C.

The as yet limited evidence does not support a mechanism for the therapeutic action of EEN that is mediated by micronutrient replenishment. Phylactos et al (148) showed that paediatric CD is characterized by reduced activity of enzymatic antioxidant systems in erythrocytes, but this did not improve with eight weeks treatment with EEN despite improvement in disease activity and systemic markers of inflammation (CRP and TNF-α). Although these results imply that the anti-inflammatory action of EEN in CD is caused by some mechanism other than restitution of specific antioxidants it still remains to be addressed whether disease improvement is associated with changes in other micronutrients. Akobeng et al (336) did not find significant changes in the antioxidant mechanisms or markers of oxidative stress of children with CD on four weeks of EEN. However the authors did not report changes of clinical activity or inflammatory markers and association.

There is limited evidence to suggest any micronutrient changes during therapy with EEN and how these correlate with the disease activity and inflammatory markers. Extrapolation of the results from adult studies to paediatric patients is inappropriate given the differences in the nutrient requirements between adults and children. The most comprehensive study in children included only four antioxidant nutrients and the duration of the EEN course was limited to one month. The recommended duration of EEN is now between six to eight weeks and different results might be expected. More evidence is needed

as to whether EEN fully meets the nutritional needs of children with CD and whether or not it replenishes any deficiencies. Failure of EEN to cover the nutritional needs of children with CD will have implications for both the feed manufacturers and clinical practice.

Changes in micronutrient status with EEN and correlation with disease activity need to be further studied although the interpretation of the results is difficult as other factors beyond nutritional status and actual nutrient stores can affect serum circulating levels. As discussed in Section 1.3.6 the acute phase response during inflammation can affect serum nutrient circulating levels irrespective of body stores and therefore measurements in other tissues or cells might be more appropriate measures of body micronutrient status (179). The effect of EEN on micronutrient status in investigated in Chapter 5

Table 1.5.3: Studies on the effect of EN therapy on the systemic micronutrient status in adult and paediatric IBD.

Study	Subjects	Nutritional therapy	Micronutrient	Micronutrient change
ADULT STUDIES				
Johtatsu et al 2007 (337)	8 CD	Partial EN; 1y; Elental low in Se and Zn	Se, Zn, Cu. Serum selenoprotein, GSHPx	Se and Zn depleted; Improved by supplementation with Se and Zn
Abad-Lacruz et al 1988 (335)	8 malnourished IBD	EEN; 12-28 d	Vit A, β-carotene, Vit E, Tocopherol:Cholesterol, B_1, B_2, B_6, B_{12}, Folate, Biotin, Vit C	Folate, biotin, b-carotene, vit C, tocopherol/chol unchanged; B_1, B_2 decreased; vit A and E increased
Teahon et al 1995 (331)	19 CD	EEN 5 wk; E028 & Vivonex	Fe, Mg, Cu, Zn	Fe increased, Cu decreased
Fernandez-Banares et al 1990 (294)	7 malnourished IBD	EEN; 20 d	Zn, Se, Cu	Zn, Cu did not improve, Cu:Zn ratio decreased; Se increased
PAEDIATRIC STUDIES				
Akobeng et al 2007 (336)	15 CD	EEN; 4 wk; 2 formulas with different glutamine content	Vit A, Vit E, Vit C; Malondialdehyde, Se, urate, glutathione	Se improved; Vit E and Vit C reduced; No effect on Vit A, glutathione, urate, MHA
Phylactos et al 2001 (148)	14 CD	EEN; 8 wk; Nestle (CT3211)	Antioxidants enzymatic system (Se-GPx, Cu/Zn-SOD)	No change
Thomas et al 1994 (183)	11 CD	EEN E028	Se, GSHPx	Se decreased; Se increased on the steroid group

1.6. Intestinal microbiota in IBD

1.6.1. Introduction to human intestinal microbiota

It has been known from early in the history of microbiology that the gut of people and other animals is inhabited by microbial species, mostly bacteria. Louis Pasteur first expressed his views to the French Academy of Sciences in 1885 on the importance of bacteria in the digestion of food believing that life in the absence of microbes would be impossible (338).

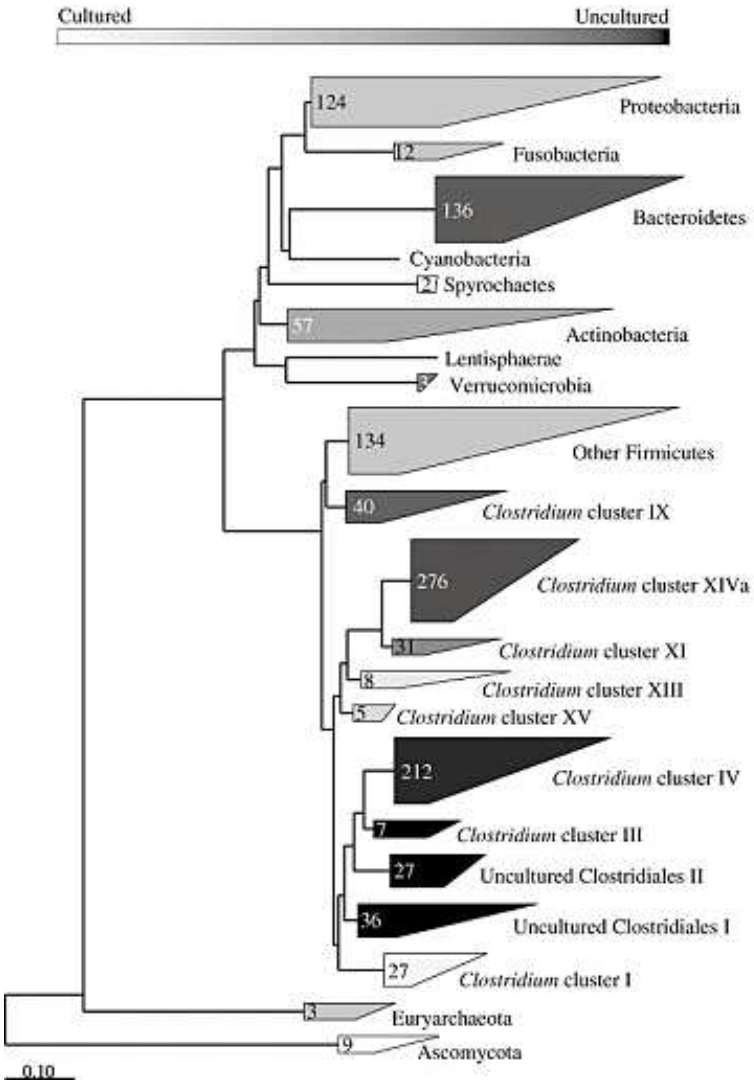
Many species of bacteria have evolved and adapted to live and grow in the human gut. The human intestinal habitat contains 300–1000 different species of bacteria, and the number of microbial cells within the large bowel lumen is tenfold the number of eukaryotic cells in the human body (339). The stomach and small intestine contain only a few species of bacteria adhering to the epithelia and some other bacteria in transit. The scarcity of bacteria in the upper tract seems to be primarily because of the composition of the luminal milieu (acid, bile, pancreatic secretion), which kills most ingested microorganisms, and secondly due to the propulsive GI motor activity towards the ileum. In contrast, the large intestine contains a complex and dynamic microbial ecosystem with high densities of living bacteria (around 60% of faecal mass), which achieve concentrations of up to 10^{11} or 10^{12} cells/g of luminal contents (340).

Members of nine bacterial phyla were found to inhabit the human GI tract of which *Firmicutes, Bacteroidetes*, and *Actinobacteria* are dominant. Members of *Proteobacteria* are also common and diverse, but they are usually secondary to the above (Fig 2). Anaerobic bacteria outnumber aerobic bacteria by a factor of 100-1000 (339). The genera *bacteroides, bifidobacterium, eubacterium, clostridium, peptococcus, peptostreptococcus*, and *ruminococcus* are predominant in the human gut, whereas facultative anaerobes such as *escherichia, enterobacter, enterococcus, klebsiella, lactobacillus, and proteus* are among the subdominant genera. Although the human gut harbors several hundreds of species belonging to common genera, within individuals an idiosyncratic combination of predominant species is present (341).

Many factors determine the microbial composition of the intestinal milieu (Fig 3). Colonization of the human GI tract starts immediately after birth and occurs within a few days of life. Mode of delivery and feeding method are early determinants of bacterial colonization (342). Although stable throughout adulthood the composition of the individual's flora changes in old age (343) and can also fluctuate due to circumstances, for instance in antibiotic treatment (344), or after dietary intervention with probiotics or fibre administration (345). Host genotype is also a determinant of gut microbiota diversity with a higher degree of bacterial similarity existing between relatives, particularly twins, than between people who are not related but live in the same environment (318).

The characterization of intestinal microbiota *in vivo,* and its spatial distribution is limited by restrictions in the accessibility of the GI tract. Thus most of our knowledge on intestinal microbiota composition and function comes from analysis of colonic luminal material, mainly faeces. Although faecal samples are easy to obtain, they represent only an approximate representation of the intestinal microbial environment, and metabolic activity, mainly that of the rectosigmoid region, which differs considerably from the microbiota in other segments of the GI tract (Fig 4) (346). Marteau collected caecal and faecal samples from healthy adults and found that the caecum harbored 100 times fewer anaerobes and facultative anaerobes represented a substantial fraction compared with the faecal microbiota (346). Faecal microbiota differs considerably from the bacteria adherent to the mucosa lining (347) and considerable research is now undertaken on the importance of the latter and its interaction with the intestinal epithelium in health and disease (348).

Figure 2: Small subunit rRNA-based phylogenetic tree of the distinct phylotypes that have been found in the human GI tract (adapted from Rajilic-Stojanovic et al 2007 (349))



The relative proportion of phylotypes that correspond to cultured representatives is indicated by different darkness of filling. Black fills indicate phylotypes detected in cultivation independent studies, while white indicates species detected in cultivation-base studies. The reference bar indicates 10% sequence divergence. Numbers of distinct phylotypes are given for each phylogenetic group.

Figure 3: Determinants of intestinal microbiota diversity and metabolic activity

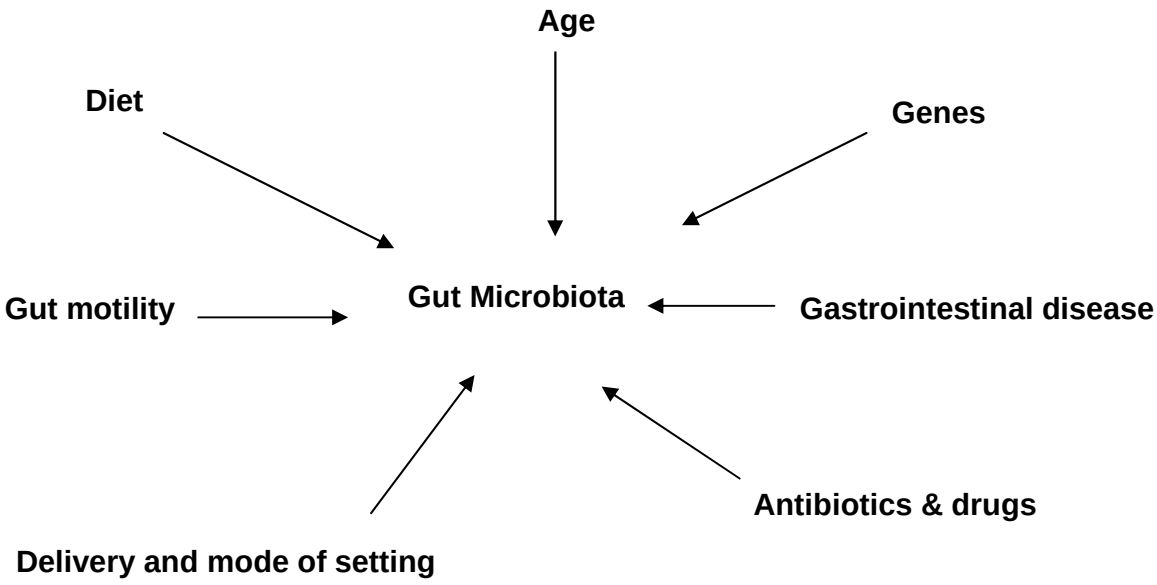
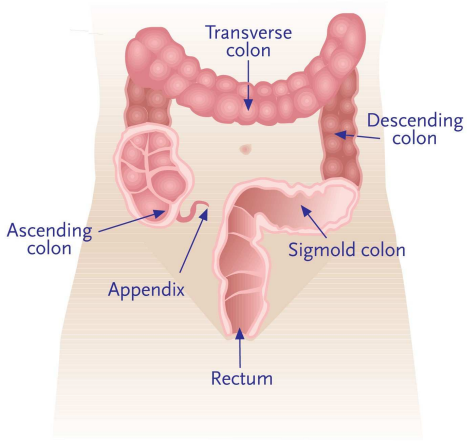


Figure 4: Regional differences in large bowel function in the human (adapted from Cummings 1997 (350))

Right Side

- Carbohydrate rich
- High moisture
- SCFAs rich
- Residence 6-16 h
- pH acidic
- High bacterial growth
- Main gases H_2 & CO_2



Left Side

- Protein rich
- Low moisture
- SCFAs low
- Residence 12-36 h
- pH neutral
- Slow bacterial growth
- Main gases H_2 & CO_2 & CH_4
- Ammonia, phenols

1.6.2. Methods for characterization of the intestinal microbiota

Much of our knowledge on the composition of the human gut microbiota is derived from bacteriological analyses that used traditional techniques of cultures on growth media, microscopy, and the determination of the fermentation properties and other biochemical specificities of bacterial isolates (340). Although these techniques are still useful in research and in clinical practice they are unable to culture all bacteria even under the most robust techniques and conditions (338). Even in the 1970 researchers had observed that the total counts of bacteria measured in faecal smears were higher than the total viable colony-forming units obtained by traditional culture methods on growth media. A recent approximate

estimate is that 60% to 80% of the bacteria in the digestive tract remain uncultured with conventional microbiology techniques.

This problem was overcome in the last decade by the emergence of molecular bacteriology techniques (351). Use of culture independent analytical approaches that utilize information from bacterial genetic material were the way forward. These techniques utilise the small subunit RNA (16S rRNA most commonly in the case of bacteria) that contains regions of nucleotide base sequence that are highly conserved and are interspersed with hypervariable regions, known as V regions. These regions contain signatures of phylogenetic groups even species and allow the characterization of bacteria from division, down to species level (338).

The combination of polymerase chain reaction (PCR) with denaturating or temperature gradient gel electrophoresis (TGGE) is a relatively new approach to monitor the diversity of complex bacterial ecosystems (352;353) and was firstly used by Zoetendal (341) for colonic contents. Bacterial DNA is extracted from the faecal sample and a variable region of the 16S rRNA gene is amplified by PCR from the DNA. The PCR product obtained from a sample contains 16S fragments of the same size but different sequence from all bacterial types present in that sample. Thereafter the various types are separated from each other by gel electrophoresis. The double stranded 16S fragments migrate through a polyacrylamide gel of homogenous consistency or containing a gradient of urea and formamide until they are partially denaturated by the temperature of the running buffer or the chemical conditions of the gel. Because of the variation in the 16S sequences of the different bacterial species, “DNA melting temperatures” are also different and therefore the different 16S species will stop “running” at different points across the gel. The separation of 16S fragments produces a profile of the dominant bacterial community “fingerprint” in which each bacterium is represented by a single band (352;353).

Another robust method to explore the intestinal microbiota is fluorescent in situ hybridization (FISH). Oligonucleotide probes, labeled with fluorescent dyes target 16S rRNA sequences inside the bacterial cells. Although an array of different specific probes can be used to recognize from single strains up to whole bacterial groups the use of large numbers of probes is impractical and the technique is restricted to the identification of bacteria for which specific probes exist. The method is best for enumeration of the numerically predominant members of the microbiota with a detection limit of 10^6 cells per g. A big advantage of this method is that bacteria can be detected in situ, which allows determination of the spatial organization of communities in the gut (354).

As a detailed description of other molecular techniques of probing the gut microbiota is beyond the scope of this thesis the reader is referred to relevant review articles (340;355;356).

1.6.3. Intestinal microbiota in health and disease

This abundant endogenous microbial load plays an important role in the homeostasis of the colon and the health of the host. Evidence from a plethora of studies, showed that this "neglected organ" exerts important metabolic, trophic and protective functions under a continual "cross-talk" between the commensal bacteria and intestinal epithelial cells (339;357;358) (Table 1.6.1).

The tremendous impact of indigenous luminal flora on the development and maturation of gut homeostasis is clearly illustrated by comparative studies of germ-free and conventionalized animals. Studies in gnotobiotic animals have shown that experimentally colonizing the intestine of germ-free rodents with single species selected from the indigenous flora has profound impact on the anatomical, physiological, and immunological development of the host, including effects on the epithelial cell functions and the composition of the gut-associated lymphoid tissue (357).

On the other hand some commensal bacteria can be opportunistic pathogens and a source of infection and sepsis under certain circumstances, as when the gut barrier is physically or functionally breached or when the bacterial profile is disturbed by the use of broad-spectrum antibiotics (359). Similarly a breakdown of tolerance to the indigenous microbiota is thought to be the key step in the development of intestinal inflammatory disorders like CD and UC. This is presented in detail in the Section 1.7.2.3.4.

Much current research aims to improve or sustain colonic health by modulating the profile and the metabolic activity of gut microbiota to a "healthier pattern" with the use of live beneficial food grade bacteria (probiotics) and the ingestion of food non-nutrient compounds that promote the growth and metabolic activity or the beneficial indigenous microbiota (prebiotics) (345).

Table 1.6.1: Main functions of commensal intestinal microbiota in health (adapted from O'Hara & Shahanan (360))

Protective functions	Structural functions	Metabolic functions
Pathogen displacement	Barrier fortification	Salvage of energy
Nutrient competition with pathogens	Epithelial cells differentiation and proliferation	Ion absorption and promotion of water absorption
Receptor competition with pathogens	Apical tightening of tight junctions	Vitamin K, folate, biotin production
Production of antimicrobial factors	Angiogenesis	Production of SCFA
Immune system development		Production of harmful metabolites (NH_3, H_2S, amines)

Perhaps the most interesting topic in GI physiology and microbiology is the delegate ability of the gut associated immune system (GALT) to interpret the intestinal ecosystem and

to distinguish between episodic pathogens and commensal bacteria and whether or not to induce a subsequent immune response.

The epithelium provides the first line of defence to the intestinal luminal microenvironment and plays an important role in sampling of commensal bacteria, pathogens or other antigens. Specialised types of immunosensory cells located at the intestinal epithelium are responsible and for the preservation of homeostasis or induction of immune response. Apart from the surface epithelial cells, M cells and dendritic cells have the ability to discriminate pathogenic from commensal bacteria in part, by using two major host pattern recognition receptor systems the family of Toll-like receptors and the nucleotide-binding oligomerization domain/caspase recruitment domain isoforms (NOD/CARD) (357;361). These receptors exert a fundamental role via immune-cell activation in response to specific microbial-associated molecular patterns like peptidoglycan, lipotechoic acids, lipopolysaccharide and flagellin.

1.6.4. Main functions of intestinal microbiota

How elephants grow and maintain their enormous body on an apparently poor diet consisting of leaves is noteworthy, particularly when they are actually monogastric and lack a rumen to maximise energy absorption. The answer lies in the structure of the digestive tract and like in many other monogastric animals, they host a highly developed caecum and large intestine with an abundant microbiota that salvage what the digestive enzymes cannot digest in the upper GI tract (350). With more bacterial cells in the gut than eukaryotic cells in the human body the collective metabolic activity of the normal flora represents a virtual hidden organ that would rival the activity of the liver (362).

1.6.4.1. Fermentation

The major metabolic function of the colonic microbiota is the fermentation of food matrices that escape digestion or absorption in the upper digestive tract, along with endogenous mucus or sloughed epithelial cells. The substrate availability in the human colon is estimated to be 20-60 g of carbohydrates and 5-20 g of protein per day in healthy individuals (350). Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial milieu of the GI tract yielding metabolizable energy for microbial growth and maintenance, and a variety of metabolic products for use by the host (363). The major end products of bacterial fermentation of carbohydrates in the large bowel are organic acids such as lactate, and short chain fatty acids (SCFA) together with hydrogen and carbon dioxide and methane. Protein fermentation also leads to SCFA, hydrogen and carbon dioxide. In addition branched chain fatty acids (BCFA) such as isobutyrate, isovalerate and 2-methylbutyrate are

produced by the fermentation of the respective amino acids valine, leucine, and isoleucine. Ammonia, amines and phenols are also produced from protein fermentation (350).

Fermentation is not the same throughout the large intestine (Fig 4) (364;365). In the caecum and right colon, fermentation is very intense with high production of SCFA, and a drop of the luminal pH to as low as 4.5 (364;365). In contrast as available substrate is limited in the distal colon fermentation of proteins occurs, with subsequent production of ammonia, branched chain amino acids and phenolic compounds, and increase of pH towards neutral. The average concentration of SCFA is as high as 70 to 140 mM in the proximal colon and falls to 20 to 70 mM in the distal colon (365;366).

1.6.4.2. Short chain fatty acids

Short chain fatty acids are organic fatty acids with a carbon chain of one to eight atoms. They are the primary end products of bacterial fermentation of carbohydrates, particularly indigestible polysaccharides and oligosaccharides, and to a lesser extent from proteins and amino acids (364;365). The predominant SCFA is acetate followed by propionate and butyrate in a molar ratio of around 60:20:20 respectively in healthy people (364;365) but this may be modified by dietary intervention (350).

SCFA production is regulated and depends on a number of factors such as the amount and the type of microbiota and fermentable material present in the lumen, and the gut transit time (364;367). Different non-starch polysaccharides (fibre) yield different qualities and quantities of SCFA. For example resistant starch is more fermentable than oat bran or cellulose and produces more butyrate than pectin (366;368). Disease, mainly GI disorders, can also determine the qualitative and quantitative characteristics of SCFA production.

The absorption of SCFA in the caecum and the colon is a very efficient process with only a small amount excreted in faeces (369). Passive diffusion of protonated SCFA and anion $Na^{+/}K^{+}$ exchange with parallel transport of water are two major mechanisms of absorption. Once absorbed, SCFA are metabolized at three major sites (369).

1) *Colonic epithelial cells use butyrate and to a lesser extent propionate and acetate for energy production and maintenance*
2) *Liver cells metabolize propionate eg for gluconeogenesis and acetate for lipogenesis*
3) *Muscle cells and other tissues produce energy from the oxidation of residual acetate*

Acetate

Acetate is the principal SCFA produced in the colon by fermentation. In humans acetate is not metabolized locally at the colon but is absorbed and transferred in the circulation (369;370). Acetate is mainly a fuel source for the host and participates in the de novo lipid and cholesterol synthesis sparing fatty acid oxidation.

Propionate

Propionate, the second major fermentation product, is readily absorbed and taken up by the liver where it constitutes a precursor of glucose production in gluconeogenesis (364;371). It can be used as metabolic fuel by colonocytes but with less efficiency than butyrate. Propionate may also be a mediator in the regulation of cholesterol synthesis most likely by inhibition of the cholesterol synthesis enzyme 3-hydroxy-3-methylglutaryl-CoA sythetase and reductase (372). A few studies examining the use of propionate supplementation as a potential cholesterol lowering agent gave contradictory results (373).

Butyrate

Butyrate has attracted the most attention. Compared with acetate and propionate, it is mostly taken up and used by the colonocytes although a small amount is absorbed and circulates in the bloodstream. Butyrate is the preferred fuel of the colonic epithelial cells and is used preferentially over propionate and acetate in a ratio of 90:30:50 and it is preferred over glucose or glutamine supplied by the blood stream (374-376). The percentage oxygen consumption attributable to butyrate, when this is the only colonic substrate, is around 70%. There is good evidence from animal models and in vitro experiments that butyrate has cell proliferative properties triggering differentiation in normal mucosa and in cancer cells induces growth arrest and apoptosis (377-379).

1.6.4.2.1. Function of SCFA in health and disease

The role of SCFA has been the topic of extensive research over the last 30 years. SCFA play an important role in the homeostasis and function of the large bowel (Table 1.6.2).

Table 1.6.2: Biological role and health benefits of SCFA in human

SCFA	Health benefits
All SCFA	Absorption of Na, K, Ca and water (380)
	Gut motility (381-383)
	Energy fuel for gut epithelium (375;376)
	Gut mucosa blood flow (384;385)
	Inhibit growth of pathogens and formation of carcinogenic compounds
Acetate	Energy substrate for the host
	Substrate for lipogenesis
Propionate	Substrate for gluconeogenesis
	Cholesterol metabolism
Butyrate	Gut epithelial proliferation and differentiation (386;387)
	Trophic effect (388;389)
	Induces apoptosis to cancinogenic cells (379)
	Anti-inflammatory properties (390)

Appraisal of the important biological role of SCFA in colonic health was the spark for scientists to investigate SCFA metabolism and production as determinants of the pathogenesis of several digestive disorders.

Harig et al (391) proposed that diversion colitis was secondary to a nutritional deficiency of the colonic epithelium due to the absence of SCFA and Kiely et al presented five cases studies of children with diversion colitis that improved in all cases with treatment on SCFA. However these results were not replicated (391) in a prospective double blind placebo control trial.

It is suggested that dietary fibre will provide prophylaxis from colonic cancer although the exact mechanism by which this happens is unclear. This protective effect may be mediated by the production of SCFA and particularly butyrate (392). Butyrate exerts two contradictory effects on normal host and cancer cells. It inhibits proliferation and stimulates differentiation on human cells lines whilst inducing apoptosis in carcinoma cells lines (379).

However, increased concentrations of butyrate may have detrimental effects in some GI conditions and this has been implicated in the mucosal injury seen in necrotizing enterocolitis (393;394). Data from preterm infants suggested that excessive production of butyrate during the third week of life may be involved in the pathogenesis of necrotizing enterocolitis commonly seen in this group of patients (395). Nevertheless excessive concentrations of butyrate may be an indication of alterations in the composition of microbiota, rather than a cause of enterocolitis, as full term children produce predominantly acetate and lactate during the first weeks of life (396).

In a recent in-vitro experiment on cancer cell lines, butyrate at low concentration promoted intestinal barrier function whereas the opposite effect was observed at high concentrations (397). The role of SCFA in IBD is discussed later in Section (1.7.1.1).

1.6.4.3. Lactate

Lactic acid is another product of fermentative action of the colonic microbiota on polysaccharides, predominately produced by lactobacilli and bifidobacteria, in the caecum (398). Lactate is found in D and L isomer form in the intestinal lumen and does not normally occur in high concentrations as it is further metabolized to other SCFA, mainly butyrate by colonic bacteria (399;400). D-lactate is produced solely by the colonic microbiota. L-lactate is also produced by the intestinal epithelium making the discrimination of origin difficult in colonic contents.

Of particular interest is D-lactate as it has been implicated in clinical GI conditions involving short segments of small intestine, such in necrotizing enterocolitis, and short bowel syndrome (398). Delivery of unabsorbed carbohydrates in these conditions increases the colonic supply of fermentative material and subsequently increases production of lactic acid and SCFA. This lowers the colonic pH and favors the growth of acid-resistant lactic acid bacteria that further ferment sugars to lactic acid and further reduce pH. In fact a lowering of

pH in acidic values although does not affect the production of lactate it inhibits its utilization by other lactate-utilizing bacteria (401). All these events subsequently result in the excessive accumulation of lactic acid in the colon and transmission to the circulation resulting to acidemia and metabolic acidosis (398).

In addition, similar to the other bacterial metabolites in the colon, lactate concentration in the large intestine may depend on a balance between production, intestinal absorption, and utilization by the commensal microbiota.

1.6.4.4. Other bacterial metabolites

Anaerobic metabolism of peptides and proteins by the intestinal microbiota along with the production of SCFA, generates a series of other metabolites such as, ammonia, amines, phenols, thiols, methane, and sulphide. Although the exact role of these molecules is not fully understood, in vitro and animal studies have implicated them in the aetiology of GI disorders such as colonic cancer and IBD (Section 1.7.1).

1.6.4.4.1. Hydrogen

Hydrogen gas production is an integral part of bacterial fermentation and it is excreted in breath and flatus. In addition, a large proportion is disposed by three bacterial reactions (350).

1) *Methanogenic bacteria reduce CO_2 to CH_4 consuming H_2 in this process*
2) *Sulphate reducing bacteria (SRB) and protein fermenting bacteria reduce sulphate and sulphur from amino acids to sulfide and hydrogen sulphide*
3) *Variable amounts of hydrogen are also consumed by acetogenic bacteria which reduce CO_2 to acetic acid.*

1.6.4.4.2. Sulphide

The large intestine harbours a class of gram-negative anaerobes that reduce sulphate and sulphites to sulphide. Sulphate reducing bacteria inhabit the distal colon and compete with methanogenic bacteria for utilization of the colonic hydrogen pool (402;403). Their predominant genus, *Desulfovibrios*, use gaseous hydrogen as an electron donor in sulphate reduction reactions and account for substantial deposit of hydrogen in the colon. Sulphide is also produced by protein-fermenting bacteria via the metabolism of unabsorbed sulphur containing amino acids. Thus dietary protein and inorganic sulphur can increase faecal excretion of sulphide in human studies in a dose response fashion (404).

Faecal sulphide concentrations represent a small amount of the actual production in the gut. Levitt et al (405) estimated that more than 95% of the produced sulphide leaves the

colonic content and is absorbed by the mucosa. In the colonic lumen, sulphide occurs in two chemical forms; free as hydrogen sulphide, and bound to divalent metal ions (Fe, Cu, Zn). In healthy subjects bound sulphide constitutes about 75-95% of total sulphide and its metabolic toxicity, although unknown, is believed to be negligible (406).

Sulphide's biological role, beyond the disposal of colonic hydrogen, is not well understood. In vitro experiments suggested toxic properties and inhibition of butyrate oxidation at the colonocyte (407) whereas recent evidence attributed potential beneficial anti-inflammatory effects to sulphide in GI health (408).

1.6.4.4.3. Ammonia

Ammonia is produced by the action of bacteria on dietary peptides, endogenous cellular debris and intestinal secretions (409) in the distal colon, particularly in the absence of fermentable fibre. Faecal ammonia may be associated with colon carcinogenesis, whereas excess accumulation in the circulation in liver disease is responsible for hepatic encephalopathy in these disorders.

1.6.4.4.4. Faecal pH

Colonic pH is used as an index of bacterial activity and the colonic microenvironment, but it is also important for the delivery of pH controlled-release drugs (410). Inaccessibility of the digestive tract does not allow direct pH measurements at the different segments of the colon and therefore faecal samples are used as a rough estimate of the colonic conditions and metabolic activity although on the whole it better reflects bacteria activity and conditions in the rectum.

Most studies that measured pH values at the different parts of the digestive tract, in health and disease, are derived from gut autopsy of cadavers, or in vivo investigations with the use of radiotelemetry capsules (411) that are not practical for routine continuous surveillance. Measurements of luminal pH in the normal GI tract have shown a progressive increase from pH 6.6 at the jejunum to 7.5 at the terminal ileum, a decrease to pH 6.4 on passage to the caecum and then a further rise to pH 7 at the left colon (412).

1.7. Intestinal microbiota in IBD

The enormous bacterial concentration and its close contact to the GI mucosa, the largest surface of the body, equipped with a highly sophisticated gut associated lymphoid tissue (GALT) immune system has always been incriminated luminal bacteria in the pathogenesis of IBD. This in parallel with the lesson learned with the discovery of the role of *Helicobacter pylori* in peptic ulcer disease, gastritis and gastric cancer triggered a considerable amount of research into the interaction between the intestinal microbiota and the mucosal epithelium in the pathogenesis of IBD. In IBD the most widely held view is that the development of disease reflects an immune response to the normal microbiota, rather than to a pathogen. The exact mechanism remains unknown and may be multifactorial which could explain the different phenotypic presentations of the disease. There is good evidence to suggest intestinal microbiota composition and its metabolic activity in the pathogenesis of IBD and perpetuation of intestinal injury. The following section (Section 1.7.1) is an outline of the evidence published thus far.

1.7.1. Metabolic activity of intestinal microbiota in IBD

1.7.1.1. Short chain fatty acids and IBD

A considerable amount of research has been undertaken on the metabolic activity of commensal bacteria in IBD, on the basis of evidence suggesting a central role of SCFA in colonic health. A potential association between bacterial metabolites and IBD pathogenesis was further intensified in light of evidence suggesting that butyrate modulates mucosal inflammation and proinflammatory cytokine expression via a mechanism that involves inhibition of activation of the nuclear factor-kappaB (NfκB) pathway (390;413-416).

For the purposes of this study, an extensive review of all studies on SCFA in IBD was carried out using Pubmed. The retrieved studies were classified into four subject categories.

1. *Colonic concentration of SCFA in IBD*
2. *Metabolism of SCFA in IBD*
3. *Animal experiments on the effect of SCFA in IBD*
4. *Clinical trials on SCFA use in IBD*

Colonic concentration of SCFA in IBD

In total six studies measured the colonic concentration of SCFA in IBD patients and all but one (417) were done in adults (Table 1.7.1). A recent study by van Nuenen et al (418) described the metabolic activity of the faecal microbiota of eight IBD patients, in an *in-vitro*

model of the colon, and found significantly increased production of SCFA and BCFA compared with stool samples of six healthy controls.

Different results were found among the studies, which measured SCFA concentrations in colonic contents of IBD patients, and therefore it is not possible to draw firm conclusions. Vernia et al (419) found significantly lower concentrations of SCFA and butyrate in 24 hour stool collections of 18 UC patients (87±11 mmol/kg) compared with 16 healthy controls (133±12 mmol/kg) and 20 CD patients (132±13 mmol/kg). These results are in contrast to those by Roediger (420) in faecal dialysates of UC adults and Treem et al (417) in spot stool samples of IBD children, who found significant higher levels of butyrate compared with healthy controls.

Inconsistent results have been reported for an association linking disease activity with the concentration of colonic SCFA in IBD patients (Table 1.7.1). Hove et al found no association with the faecal concentration of SCFA, whereas Roediger et al (420) observed high levels of butyrate increasing with disease severity in faecal dialysates of UC patients and Treem et al (417) found high levels of butyrate in active and low levels of acetate in quiescent UC children but no effect in spot stool samples of CD children.

Indeed Roediger et al (420) found that increased levels of butyrate in faecal dialysate correlated positively with the histological disease activity scores in mucosal biopsies whereas in a subsequent paper by Vernia (421) SCFA were found to be high in quiescent and mild UC (163±47 mmol/kg) and (148±63 mmol/kg) respectively, but were significantly decreased in severe disease (65±47 mmol/kg). In particular butyrate levels were low in severe UC (4.3±5 mmol/kg) compared to controls (13.7±8.4 mmol/kg) with the inference being that in severe disease there is relative butyrate deficiency and energy deficits to colonocytes.

The use of different methodologies among the studies with the use of different types of material for analysis (faecal water, spot samples, 24hrs collection) may partially explain these contradictory results (Table 1.7.1).

Metabolism of SCFA in IBD

The failure to confirm the hypothesis that IBD pathogenesis is the result of low SCFA production and on the contrary the increased concentrations observed in some of the studies (417;420) redirected subsequent research towards the colonocyte metabolism of SCFA.

In 1980, Roediger was the first to report that butyrate oxidation, was significantly impaired in UC, both during the active phase of the disease and in remission (422). Enhanced glucose and glutamine oxidation resulted as a compensatory mechanism. Their hypothesis, that IBD pathogenesis was the result of impaired butyrate metabolism was supported by the same group which showed that inhibition of beta-oxidation of fatty acids induced experimental colitis in rats and hence may be primarily associated with the

pathogenesis of UC (423). Nevertheless a causative association between butyrate and colitis was disputed by others who although observing diminished oxidation of butyrate in experimental models of colitis, reported that histological abnormalities preceded the defects in butyrate oxidation (424). Indeed the fact that disease remission was associated with normal oxidation and active disease with decreased oxidation in another study (425), suggests that UC mucosa is not intrinsically altered in butyrate oxidation, making this unlikely to be a primary causal defect leading to inflammation in UC.

Recently two studies attracted interest in the defects of butyrate metabolism in IBD. Santhana et al (426) suggested that the impaired oxidation of butyrate in UC was the result of impairment in the last enzyme (mitochondrial acetoacetylCoA thiolase) that participates in butyrate oxidation inside mitochondria. Suboptimal enzymatic activity was found both in normal and inflamed mucosa of UC patients. This was the result of increased oxidative stress at the mucosal level, which modified protein structure, and functionality of the enzyme. Interestingly similar defect was not found in CD patients. In the second study Thibault et al. (427) proposed that impaired butyrate oxidation was not a primary defect but the result of a decreased intracellular concentration of butyrate, due to low cellular uptake of butyrate in IBD patients. This was due to a defect in the production of one of the butyrate transporters caused by the action of pro-inflammatory cytokines. Thus there is some evidence suggesting impaired butyrate metabolism in IBD mainly UC. Evidence in CD is limited and non supportive thus far.

Use of SCFA for the treatment of IBD

The suggestion that diminished butyrate metabolism may cause energy deficiency in UC colonocytes (422), coupled with some observations of low colonic SCFA in patients with UC prompted the use of SCFA in animal models and in clinical trials for the management of UC primarily of the distal colon.

Animal model of colitis, have been used to study the efficacy of exogenous administration of SCFA on disease activity and mechanisms of action (Table 1.7.2). All of the studies reviewed used rectal enemas of butyrate, alone or in conjunction with 5-ASAs and compared the results against a placebo control group (eg saline). Butyrate improved disease activity, colonic macroscopic and microscopic histological findings in most studies (428-430) and improved mucosal barrier function and colonocytes viability in one (415).

The mechanisms that might be involved in the amelioration of colitis by butyrate administration include decreased expression and activation of the NFκB proinflammatory cascade with subsequent reduction in the secretion of pro-inflammatory cytokines (415;430). Moreover butyrate administration improved the expression of trefoil factor 3 that is implicated in epithelium integrity, protection and wound healing (430).

The first clinical report of the use of SCFA to treat IBD was an open label trial of a mixture of the three basic SCFA in 12 patients with refractory distal colitis. Of the ten patients who completed a six week trial, nine improved (431). Since then 12 additional clinical trials were identified that used SCFA in IBD (Table 1.7.3). All apart from one study (432) were done in UC patients mainly with distal colitis. The most common vehicle for butyrate administration was a sodium butyrate enema. Two studies used oral derivatives of butyrate (432;433). Five studies had a randomized double blind placebo controlled group although actual blinding was difficult, if not impossible, due to the strong odor of butyrate in rectal enemas. The number of patients in the studies varied between nine and 51. None of the studies was performed in paediatric patients.

Table 1.7.1: Colonic concentration of SCFA in IBD patients

Study	Design	Results
Van Nuenen et al 2004 (418)	In-vitro colonic model; Production of bacterial metabolites in faecal samples; 8 IBD & 6 healthy controls;	SCFA & BCFA production was higher in IBD than control samples
Hove & Mortensen 1995 (434)	Spot stool samples (103 UC, 127 CD, 70 other GI), 20 healthy controls; including patients with gut resection	No difference with controls; No effect of disease activity or length of small or large bowel on faecal SCFA concentration.
Treem et al 1994 (417)	Spot stool samples **children** (17 UC, 22 CD, 12 healthy)	No difference between CD & UC for SCFA; Acetate lower & butyrate higher in CD & UC compared with healthy controls; No difference between active & inactive for CD; In UC changes with activity: SCFA low in moderate-severe UC; Butyrate high in quiescent/mild UC
Vernia et al 1998° (421)	Faecal water (62 UC)	SCF A high in quiescent/mild but decreased in severe disease
Vernia et al 1988b (419)	24 hours faecal collections (18 UC & 20 CD), 16 controls	SCFA and particularly butyrate lower in UC than CD and healthy controls
Roediger et al 1982 (420)	Faecal dialysates (65 UC), 16 control	SCFA and mainly butyrate increased in severe disease; raised butyrate correlated with severity of mucosa

Table 1.7.2: Effect of SCFA administration in animal models of colitis

Study	Intervention	Outcome	Results
Song et al 2006 (430)	Sodium butyrate enema, 5-ASAs, combination, saline	Colonic damage; Tissue Myeloperoxidase; Trefoil factor 3 mRNA expression; Serum IL-1β production ; NFκB expression	All apart from saline reduced diarrhoea, improved colonic damage, myeloperoxidase activity, increased trefoil factor 3 mRNA expression, decreased serum IL-1β production and NFκB expression; Better effect with combination
Venkatraman et al 2003 (415)	Butyrate enema, saline	Intestinal permeability; Cell viability	Improved cell viability & mucosal permeability & PMN neutrophil infiltration; Inhibited heat shock protein expression & activation of NFκB
Butzner et al 1996 (428)	Butyrate enema, saline, no treatment	Colonic damage; Tissue Myeloperoxidase; Sodium absorption	In butyrate treated group diarrheoa stopped; Colonic damage and sodium absorption improved; Myeloperoxidase activity decreased
D'Argenio et al 1994 (429)	Sodium butyrate, mesalamine, combination or saline enema	Throboxane B2	Sodium butyrate alone or plus mesalamine reduced histological activity; Transglutaminase increased, in butyrate and in combination; Combination reduced throboxane B2 synthesis

Table 1.7.3: Clinical trials of the effect of SCFA in IBD patients

Study	Subjects	Intervention	Outcome	Results
Di Sabatino et al 2005 (432)	13 mild/moderate CD	Open label; 4 g/d butyrate for 8 wk	Endo, Hist, CA, cytokines, NFκB before and after treatment	69% clinical improvement; Endo, Hist improved in ileocaecum, ESR, NFκB and IL-1β decreased
Vernia et al 2003 (435)	51 distal UC, refractory to topical 5-ASA/steroids	RDBPC multicenter; 2 g 5-ASA and 160mM sodium butyrate enema or placebo for 6 wk	Endo; Hist; CA	Remission rate and disease improved, bowel movements & urgency better in intervention
Luhrs et al 2002 (414)	11 distal UC	Open label; butyrate enema 100 mM or placebo for 8 wk	Endo; Hist; CA	Hist, CA improved; less mucosal macrophages positive for NFκB translocation
Vernia et al 2000 (433)	30 mild/moderate UC	RDBPC; Oral butyrate 4g/d plus mesalazine 2.4g/d or placebo	Endo; Hist; CA	Endo, Hist, CA improved in both arms; better improvement vs baseline for intervention
Scheppach et al 1996 (436)	47 active distal UC	RDBPC; SCFA or butyrate enema vs placebo	Endo; Hist; clinical, inflammatory markers activity at 4 and 8 wk	Fewer segments were endoscopically affected on butyrate subjects than placebo
Steinhart et al 1996 (437)	38 active distal UC	RDBPC; Butyrate enema vs placebo for 3 wk	Endo; Hist; CA at baseline 3 and 6 wk	No difference between intervention and placebo
Patz et al 1996 (438)	10 distal UC refractory to 5-ASA & steroids	Open label; SCFA enema for 6 wk	Endo; Hist; CA at baseline and 6 wk	50% had clinical and endoscopic improvement; no histological improvement
Vernia et al 1995a (439)	40 mild/moderate distal UC	RDBPC; SCFA enema vs placebo for 6 wk	Endo; Hist; CA at baseline and 6 wk	Endo; Hist; CA improved
Vernia et al 1995b (440)	9 refractory to therapy	Open label; Sodium butyrate and 5-ASA for 4 wk	Endo; Hist; CA	Endo; Hist; CA improved in 7 out of 9 patients
Steinhart et al 1994 (441)	10 distal UC; refractory to rectal therapy/ oral 5-ASA	Open label; Butyrate enemas for 6 wk	Endo; CA at 3 & 6 wk	6 out of 10 improved; disease score decreased
Senagore et al 1992 (442)	45 proctosigmoiditis	Open label; Steroid enema vs 5-ASA enema vs SCFA	Endo; Hist; CA at 6 wk	Same proportion of patients improved in the three groups
Scheppach et al 1992 (443)	10 distal UC refractory to therapy	Single blind crossover; Butyrate enema and placebo for 2 wk	Endo; Hist; CA	Stool frequency ⇓; PR blood decreased; Endo and Hist improved
Breuer et al 1991 (444)	12 distal UC reftractory to therapy	Open label; SCFA enema	Endo; Hist; CA	CA and Hist improved

RDBPC: Randomised double blind placebo control trial; Endoscopy: endo; Histology: hist: Clinical activity: CA

All the open label studies and the majority of the randomized control trials favored the use of butyrate enemas in distal refractory UC although those with relatively large sample size did not find any difference in histological and endoscopic outcomes between treatment and placebo groups (Table 1.7.3). In most studies with a positive outcome, improvement of clinical disease activity was accompanied by improvement of inflammatory markers, histology and endoscopic findings. In two studies that tried to clarify mechanisms of action, use of butyrate was associated with decreased expression and activation of NFκB pathway (414;432).

In summary considering the available evidence, the cost of treatment, and drug associated side effects, butyrate could be a promising adjuvant in the management of distal colitis. On the other hand, considering the limited evidence in CD, in conjunction with current evidence linking commensal intestinal microbiota with CD aetiology (see Section 1.7), more studies should be anticipated. In particular use of butyrate enemas should be considered in the context of paediatric trials as a possible agent for sparing the use of other medication with severe side effects on bone health and linear growth. In addition other vehicles of butyrate administration should be developed for extensive disease as the use of butyrate enemas is practically limited to distal colitis only.

1.7.1.2. Lactate in IBD

Previous studies reported high colonic concentrations of lactic acid and particularly of the L-isomer in active distal UC (421;434;445). This may suggest increased mucosal production or impaired metabolism of lactic acid by lactate utilizing bacteria in the low luminal pH that may be encountered in IBD patients (410). A recent in vitro study in faecal samples, showed that although lactate production was sustained at pH less than 5.2, the utilization of lactate was reduced resulting in its excessive accumulation (401). On the other hand lactic acid concentration is positively associated with stool volume, steatorrhoea, and malabsorption (446) and might explain the increased levels seen in patients with active disease and severe diarrhoea.

In the only paediatric study that measured faecal lactate in IBD, no statistical significant differences were found between the two types of IBD diseases or compared with a healthy control group (417). Van Nuenen et al (418) in an in-vitro model of colonic fermentation found comparable results in lactate production rates in faecal microbiota of healthy and IBD patients.

1.7.1.3. Ammonia in IBD

Two studies measured colonic ammonia in IBD and in both cases this was higher than the healthy control group. Roediger et al (420) measured ammonia among other bacterial metabolites in the colon of patients with UC and healthy controls and found that UC patients, particularly those with histological findings of severe disease, had higher levels of ammonia compared with non-inflammatory conditions. Similarly Van Nuenen et al (418) in an in-vitro model of colonic fermentation found higher production of ammonia by the faecal microbiota of eight IBD (5 CD) patients compared with healthy subjects. Increased concentration and production of ammonia could infer increased proteolytic action or impaired utilization by the intestinal microbiota in IBD patients.

1.7.1.4. Colonic pH in IBD

In IBD patients different and contradictory patterns of luminal pH have been reported in the literature (410). Ewe et al (447) did not find any difference in luminal pH values across the GI tract between patients with active IBD and healthy controls. Similar results were presented by Press et al (448) although higher luminal pH was measured in the terminal ileum, caecum, and ascending colon of UC patients compared with healthy controls. In the same study comparable results were found between active and inactive disease.

In contrast very low pH values (2.3-3.4) were recorded in the caecum and right side of the colon in severely active UC (449) compared with healthy subjects but not in CD patients with ileocaecal resection (450). Luminal pH in the right colon of the patients with ileocaecal resection was higher by 0.5 units compared with healthy controls (450). Sasaki et al (411) measured comparable luminal pH in the stomach and small intestine of CD and healthy controls, but the colonic pH profiles differed with lower values observed in active or quiescent CD than seen in controls. The overall mean luminal pH in the right colon was 5.3±0.3 for the patients versus 6.8±0.2 for the healthy controls ($p < 0.01$) and that in the left colon was 5.3±0.7 versus 7.2± ($p < 0.01$).

Many factors can determine colonic pH including the amount and type of fermentable material in the colon, the type and number of the bacterial species, colonic motility, production, absorption, or utilisation of the fermentation products by other colonic bacterial, as well as abnormalities in the secretion of bicarbonate and the buffering effect of mucus and blood in active colonic disease (410). Alterations in these factors may explain the different results observed in the studies above.

1.7.1.5. Sulphide in IBD

Several lines of evidence pointed to sulphur metabolism, being implicated in the aetiology of mucosal injury in IBD. A prime example is the use of sulphated polysaccharides, to induce features of colitis in animal models (451).

First Roediger and colleagues found that fatty acid oxidation at the epithelium lining could be hampered by a range of sulphur containing compounds. Among those tested, sodium hydrogen sulphide selectively reduced fatty acid beta oxidation at the level of short chain acyl dehydrogenation (407;452) and caused greatest mucosal injury. Based on these observations a hypothesis was proposed that cellular inhibition of butyrate by sulphides can induce energy deficient state in colonocytes resulting in mucosal inflammation and injury.

An extensive literature review was carried out to identify studies linking IBD with sulphide metabolism. The retrieved studies in IBD were categorized into the following four categories.

1. *Prevalence of Sulphate Reducing Bacteria (SRB) in UC and comparison with healthy subjects.*
2. *Luminal concentrations and production rate of sulphide in UC and comparison with healthy controls.*
3. *Functionality of sulphide detoxification pathways in IBD.*
4. *Pharmaceutical intervention and sulphide metabolism.*

Prevalence of SRB in UC and comparison with healthy subjects

Increased numbers of SRB bacteria in faecal samples of UC patients have been reported in some studies. Loubinoux et al (453) using cultivation techniques on selective growth media linked with multiplex PCR found that SRB were more frequently observed in IBD patients (68%) than in healthy controls (24%) or patients with other GI disorders (37%). Moreover different SRB strains were observed between healthy and IBD subjects suggesting strain specific differences between IBD and healthy controls. In particular the prevalence of *Desulfovibrio Piger* was significantly higher in IBD (55%) compared with healthy individuals (12%) or patients with other GI disorders (25%). Association of SBR counts with disease activity was reported by one study (454), which found that total viable counts of SRB were increased in patients with clinical and endoscopic active disease activity compared with patients with quiescent disease (454).

On the other hand recent studies using robust molecular biology techniques of bacterial community analysis failed to confirm the results from bacteriological studies of increased growth or number of SRB counts. For example Fite et al detected SRB in all

mucosal samples of IBD patients and healthy individuals using molecular microbiology techniques (455).

Sulphide concentration and production

There is inadequate evidence to suggest that luminal sulphide concentrations are different in IBD patients. A group from the University of Dundee, Scotland, showed that in untreated UC patients faecal sulphide excretion was higher than in healthy controls and treatment with 5-ASAs lowered both concentration and production of sulphide (454;456). On the contrary comparable free and total faecal sulphide concentrations in IBD and healthy controls were presented by others (406;457). No association between luminal sulphide concentration and disease activity or location have been found (457).

Compared with healthy subjects, a two to threefold increase in sulphide production rate has been found in faecal and mucosal samples of UC patients (458;459). Addition of various organic sulphur containing substrates (mucin and taurocholate) increased hydrogen sulphide release much more readily than sulphate intake implicating the action of other protein fermenting bacteria in the production of sulphide over and above the SRB. Increased production of sulphide has been reported also in pouchitis, an inflammatory condition of the ileal-anal pouch of UC patients (460).

Different form of sulphide, different effect?

Only a few studies have tried to account for the origin of the measured concentrations of faecal sulphide in samples from IBD patients. Sulphide occurs in two forms in the intestinal tract, free as hydrogen sulphide and bound to divalent metal ions. Differences in the toxicity of the two forms and failure to distinguish between them in the studies might explain some of the discrepancies reported above. Jorgensen & Mortensen (406) hypothesized that the binding ability of faecal material to sulphide was low in UC compared with healthy controls but they failed to prove this.

Functionality of sulphide detoxification pathways in IBD

Faecal sulphide concentrations represent a balance between production and removal of this toxic metabolite. Epithelial enzymes react with this and produce other sulphur containing compounds which are less harmful for the colonic mucosa (461;462). Thus elevated concentrations of sulphide could be a secondary result of defective detoxification mechanisms in the large bowel (463) although the evidence so far is circumstantial and not supportive (462).

Pharmaceutical intervention and sulphide metabolism

In view of a possible causative role of sulphide in IBD mucosal injury, a few studies investigated whether the action of mainstream IBD drugs is mediated through changes in the metabolism of sulphide in the gut. Among them, 5-ASAs such as sulphasalazine reduced the production of sulphide but not the growth of SRB in in-vitro experiments (454;456) and subsequently differences in the concentration of faecal sulphide were observed between patients on treatment with 5-ASAs and those not. Moore et al (457) however did not confirm these results and indeed found comparable sulphide concentrations between patients on 5-ASAs and others on different medical regimes. Difference in disease activity between these studies may explain this discrepancy.

The results of the effect of metronidazole and ciprofloxacin, two commonly used antibiotics, on the production of sulphide and SRB counts have been contradictory (460;464). Recently prebiotic ingestion reduced hydrogen sulphide concentrations but not SRB counts in healthy volunteers and may thus have a putative place in the treatment of patients with UC (464). Other new agents, like bismouth based derivatives, have also been proposed for use in IBD to inhibit the growth of SRB and suppress production of hydrogen sulphide (460).

Hydrogen Sulphide in IBD. History Revised?

Despite the evidence that hydrogen sulphide can be a toxic mediator in gut health, recent studies found paradoxical actions depending on its concentration and the circumstances in which it is generated. For example, hydrogen sulphide may play a role in protecting gastric mucosal tissue from injury and exerts anti-inflammatory actions, and potentially inhibits leukocyte adherence to the vascular endothelium (465). Fiorucci and his colleagues (466) in a recent study compared the regular form of mesalamine with a new hydrogen sulphide releasing derivative and found that the latter was more effective than mesalamine in reducing the severity of animal colitis. In particular the new derivative was more effective in reducing granulocyte infiltration into the colonic tissue, and furthermore reduced the expression of mRNA for several key proinflammatory cytokines and chemokines.

Effect of dietary sulphate and sulphur containing nutrients on sulphide production

Diet is a strong determinant of faecal sulphide concentration (467). A diet rich in sulphur containing amino acids increases the production and excretion of sulphide in a dose dependent manner (404) by the action of protein fermenting bacteria. Similarly ingestion of sulphate products increased generation of sulphide by the action of SRB in the colon in one study (468) but not in a rat model given sulphate supplemented drinking water for one year

(469). An association with dietary sources of sulphur may justify discrepancies found between studies, such as the elevated levels of sulphide previously reported by Pitcher (454) in active UC but not by Moore. The high sulphide concentrations observed in the first study could actually be an epiphenomenon, related to sulphur amino acid fermentation of the mucus and excessive sloughed cells in active disease or increased dietary intake of sulphur containing food that has been shown to precede disease exacerbation (470).

Effect of transit time on sulphide production

Gut transit time can also affect sulphide production and comprise a confounding factor in the interpretation of sulphide's role in health and disease. Concentration of faecal sulphide and reduction rates of faecal sulphate are increased in faster intestinal transit times and are decreased in slower transit (468) and may explain any differences between active and inactive disease.

1.7.2. Gut microbiota composition and IBD

An imbalanced enteric microenvironment may provide a constant stimulus that activates the inflammatory cascade in patients with genetic defects in gut barrier function, innate and adaptive immune system. In the following section (Section 1.7.2.1) the topic will be reviewed and the essence of the enormous amount of evidence that implicates the enteric microbiota in the pathogenesis of IBD will be presented. For an in depth review of the state of the art the reader is referred to the recent report by Sartor (30).

1.7.2.1. Clinical human evidence associating the gut microbiota in IBD

There is both clinical and experimental evidence to implicate the intestinal microbiota in the onset and perturbation of IBD microbiota. Further to the localization of disease to intestinal segments with the highest bacteria concentration, clinical evidence linking the intestinal microbiota to IBD, comes from the observation that faecal stream diversion prevents and treats CD, whereas exacerbation occurs upon restoration of the faecal flow (471).

Therapeutic intervention with selective antibiotics to decrease postoperative recurrence of ileal disease (472), along with the beneficial use of probiotic preparations in maintenance of remission in UC, and prevention of pouchitis, implicates the endogenous intestinal microbiota as a key player in the intestinal inflammation seen in IBD (75;76;473). Moreover there is persuasive evidence for loss of immunologic tolerance to components of the commensal flora in patients with IBD as this is reflected in serologic and cellular immune reactivity to enteric microbes (474;475).

1.7.2.2. Experimental evidence from animal models of colitis

Studies in animal models of colitis have made a major contribution to our understanding of the importance of dysregulation of interactions between host mucosal cells and the resident luminal bacteria in the pathogenesis of IBD. Several genetically engineered rodents develop chronic intestinal inflammation under conventional conditions but fail to develop colitis in a germ-free state (476). Administration of a normal colonic microbiota induces inflammation, although different bacteria exhibit different effects (477) or lead to different phenotypic disease characteristics. For example in interleukin-10 deficient mice, *E. coli* induced proximal colitis whereas *Enterococcus faecalis* led to the development of distal colitis (478). Similar to human studies, the involvement of intestinal microbiota in disease aetiology is supported by the administration of probiotic bacterial strains in animal models of colitis which modify disease expression by favorably altering bacterial composition, immune status, and inflammatory response (479).

1.7.2.3. Putative mechanisms to implicate intestinal microbiota in IBD

Compared with the scientific progress from epidemiological evidence in identification of genes implicated in IBD onset, the exact mechanisms by which gut bacteria are involved in IBD pathogenesis remain largely elusive. Four mechanistic theories have been proposed and are described in the following section (30;474).

a. *Microbial pathogens or functional changes in commensal bacteria trigger intestinal inflammation.*
b. *Defective intestinal barrier function allows bacteria or their components/products to contact and activate the gut-associated immune system.*
c. *Intolerance and defective host immune response to commensal microbiota initiates and perpetuates inflammation.*
d. *IBD is the result of disruption of a harmonic microbial symbiosis in the intestinal ecosystem.*

1.7.2.3.1. Microbial pathogens or functional changes in commensal bacteria

Since the first descriptions by Samuel Wilks in 1859 and Burrel Crohn in 1932, extensive work has been directed to the identification of a specific pathogen that could be of aetiologic importance in IBD. Among the microbes of historical interest that have been postulated to play a role in the pathogenesis of IBD are: *Mycobacterium avium subspecies paratuberculosis, Listeria, Pseudomonas species, and Helicobacter pylori* (474). Although no definite bacterium has yet been identified a few have been suggested. However identifying a

pathogen as a cause of IBD is rather difficult, as many of the bacteria in the human intestinal ecosystem are not yet culturable or a potential pathogen may be part of the subdominant intestinal microbiota that current molecular methods of analysis are not able to identify.

Mycobacterium avium subspecies paratuberculosis has been most intensely investigated but there are still arguments regarding its role in the causation of IBD, mainly CD (30). In ruminants infection with Mycobacterium causes spontaneous granulomatous enterocolitis similar to CD and it was first cultured from resected CD tissues in 1984 (480). Since this first observation a few studies have reported detection of *Mycobacterium avium subspecies paratuberculosis* in IBD patients using slow growing culture (481), polymerase chain reaction, FISH (482), or a metagenomic approach (483) but the detection rates varied between 0% and 100% (456;484) suggesting other or additional causes of IBD. Recently a metagenomic analysis of the gut microbiota (483), did not detect *Mycobacterium avium subspecies paratuberculosis* 16S rRNA in mucosal specimens of a large number or patients with IBD. Likewise a large well-designed double-blinded placebo controlled RCT using an antibiotic cocktail failed to sustain remission in CD (485).

Among the non-mycobacterial species, *E. coli* warranted considerable attention. Prevalence of *E. coli* has been reported as 3-4 times higher in the mucosal microbiota of patients with IBD (486) whereas novel adherent-invasive *E.coli* have also been described to colonize the ileal epithelium of patients with CD (487;488). In ileal specimens, adherent-invasive *E. coli* were found in 22% of CD chronic lesions compared with 6% of controls. Similar findings were found by Baumgart et al (487) who observed adherent-invasive E. coli in more than a third of CD patients with active ileal disease but there was a low recovery in healthy controls. Recently Darfeuille-Michaud and his colleagues found that adherent-invasive *E.coli* adhesion occurs via specific binding sites on the bacterial surface and is secondary to increased expression of a carcinoembryonic antigen-related cell adhesion molecule 6 propagated by the action of IFN-gamma or TNF-a stimulation, prior adherence by adherent-invasive *E. coli* (AIEC) bacteria (224).

Although an infection from a potentially unknown pathogen cannot be excluded completely, currently there is not enough evidence to implicate one in the pathogenesis of IBD. In addition, the strongest argument against a persistent infection in IBD aetiology is the beneficial effect of host immunosuppression and steroids in disease management that indirectly precludes a pathogen as the sole causative mediator in IBD.

1.7.2.3.2. Defective intestinal barrier function

Preservation of intestinal homeostasis and inactivation of GALT depends on the maintenance of an impermeable mucosal barrier, and rapid repair of epithelial defects when the latter is breached. Abnormalities in the mucus-epithelial layer composition may facilitate

direct contact between colonic bacteria and the intestinal mucosa. This may exert an antigenic stimulus that activates the immune system, initiates inflammation, and further promotes intestinal permeability and tissue injury.

There are several lines of evidence suggesting enhanced mucosal permeability in IBD (489;490) but it is still unknown whether this is a primary defect in IBD patients or a consequential event following disease onset. The importance of effective barrier exclusion in the pathogenesis of IBD is better illustrated by experimentally altered tight epithelial junctions in mouse models tend to predispose to severe gut inflammation (491). Genetically determined changes in the mucus, particularly glycosylation of the mucous glycoproteins may result in weakening of the mucous barrier in patients with IBD. Endogenous luminal bacteria or their components could degrade mucus, penetrate into the mucus layer, activate the local immune system, enhance recruitment of inflammatory cells and provoke inflammation (492;493).

1.7.2.3.3. Defective host immune response to bacteria

A defect in the innate immune response of the GALT may be implicated in the inflammatory process seen in IBD. Recognition of commensal bacteria and prevention of an acute immune response is regulated through bacterial-host crosstalk at the level of the mucosal epithelium. This cross-talk occurs through microbial associated molecular patterns that are recognized by pattern recognition receptors, such as toll-like receptors. Toll like receptor expression appears to be carefully regulated to mute a proinflammatory response towards mutualistic organisms in healthy individuals but to initiate inflammatory response in the case of pathogens (30).

Polymorphisms in toll like receptors have been linked to CD pathogenesis. In 2001 two independent groups identified the CARD15 gene on chromosome 16q as a susceptibility gene in CD (27;28). Three common polymorphisms were found to be associated with development of CD. The CARD15 protein functions as an intracellular receptor for a structural motif (muramyl dipeptide) of peptidoglycan a common component of bacterial cell walls. Upon recognition of muramyl dipeptide, CARD15 activates the NF-κB pathway a key step in the inflammatory response. Because of this CARD15 is considered a proinflammatory molecule. In CD the associated mutations impair the proinflammatory function of CARD15 that has potentially serious consequences involving reduced antibacterial action from impaired production of defensins and other antimicrobial peptides from Paneth cells in both ileal and colonic CD (26;494;495). Other CD related genetic polymorphisms in the pattern recognition receptors such as toll like receptor 4, the autophagy gene ATC 16L1 which regulates intracellular microbial processing and killing further support the hypothesis that a subset of CD patients may have defective innate immune response to microbial antigens

(496;497). Defective mechanisms of bacteria killing could lead to defective clearance of bacterial antigens with parallel activation of the T cells mediated immune response.

1.7.2.3.4 Dysbiosis of commensal microbiota

Failure to identify a pathogen in IBD along with supportive clinical and experimental evidence suggesting involvement of normal gut microbiota in IBD, directed the focus of the subsequent research on the dominant intestinal microbiota. It is now generally accepted that commensal enteric bacteria provide the constant antigenic stimulus that continuously activates pathogenic T cells, causing chronic intestinal injury. The exact mechanism still remains unidentified but a disruption of colonic bacterial balance is incriminated.

It has been postulated that disruption of commensal microbiota homeostasis may spark an aberrant immune response. A decreased ratio of protective to unprotective bacterial species, might reduce the production of epithelial energy substrates, increase exposure of bacterial proinflammatory antigens to mucosal T cells, activate the local immune response and overall disrupt the host tolerance to its endogenous bacteria. This concept of a breakdown of balance between putative species of “protective” versus “harmful” intestinal bacteria has been termed “dysbiosis” (498) and has been the objective and subsequent outcome of several investigations that tried to explain the association of gut bacteria with IBD pathogenesis.

Previous studies that used conventional microbiology techniques and cultures on selective growth media, reported substantial differences in the microbiota composition between IBD patients and healthy controls, with increasing prevalence of streptococci, reduced numbers of bifidobacteria (499;500) and increased presence of Bacteroides fragilis (501;502). However due to the inability of traditional microbiology methods to culture the majority of luminal bacteria these results had to be revisited in the era of the new molecular bacteriology techniques.

Review of studies on the composition of commensal microbiota in IBD

In the following section the recent evidence suggesting dysbiosis as the causative mechanism of the initiation and perpetuation of inflammatory response is summarised. Due to the plethora of relevant studies and as it is out of the primary scope of this thesis, the literature review is limited to studies published in the last decade which coincides with the flowering of applied molecular bacteriology in clinical gastroenterology.

Research strategy and description of studies

A comprehensive literature search was carried out to retrieve all studies published on the topic from 1998 to May 2008. Identification of additional articles was achieved via scrutiny of reference lists of major reviews articles. In total 27 original studies were identified with a boom in publications after 2004. At the beginning of this PhD there were six studies in total on the diversity and composition of intestinal microbiota in IBD. Twenty-one studies have been published since (Table 1.7.4) with the majority focusing on the mucosal associated microbiota.

Most studies used a healthy control group for comparison whereas others looked into differences with other GI disorders or differences between the two types of IBD (CD & UC). A few studies looked into the spatial organization of the mucosal associated microbiota and some attempted to link the bacterial diversity and phylogeny with disease activity and dissimilarities in the bacterial composition profiles between inflamed and healthy mucosa. Two studies assessed temporal changes of the intestinal microbiota using colonic contents.

Molecular bacteriology with analysis of bacterial community profiles using molecular fingerprinting techniques (TGGE, TTGE, DGGE) and/or characterization of the microbiota with species-specific bacterial probes (FISH, PCR) were most common methods used. Some authors used PCR amplification, with subsequent cloning and sequencing and recently two studies used a broad metagenomic approach to characterize the whole indigenous bacterial intestinal diversity in IBD patients. It is noteworthy that all but one study (503) assessed the microbiota of adult patients with IBD. The only paediatric study explored the mucosal microbiota of children with IBD. Thus far no study has been published on the bacterial composition of faecal microbiota of paediatric CD.

Table 1.7.4: Evidence table of studies on the diversity and composition of faecal and mucosal microbiota of IBD patients

Study	Aim	Disease	Methods	Results-Comments
Martinez et al 2008 (504)	Temporal changes in faecal microbiota on longstanding remission	UC on remission for 1 year; H	DGGE	Similarity index declined in UC but was stable in H; Biodiversity was significantly lower in UC than H
Swidsinski et al 2008 (505)	Composition & spatial organization of faecal microbiota; Disease activity effect	CD, UC, IC, coeliac, self limiting colitis, other GI disorders	Microscopy & FISH	Bacterial profile opposite for 6 out of 11 FISH probes between UC & CD; Disease diagnosis based on analysis of the spatial organization & composition of bacteria & leucocytes in stool samples; Spatial differences between bacteria of IBD & H or other G disorders; Common habitual bacteria lower in IBD than H; *C Coccoides* group reduced in CD & UC; *Bacteroidaceae* higher in UC than CD; *Faecalibacterium prausnitzii* reduced in CD only but not UC; *Bifidobacteriaceae* absent in 56% of CD; *Bifidobacteriaceae* mean concentration higher in UC than H; *Enterobacteriaceae* increased in CD with increasing activity but not in UC; Concentration of habitual decreased with increasing inflammation in UC & CD; Occurrence & concentrations of *Bifidobacteriaceae* in CD lower in active disease than in remission & significantly lower than UC
Baumgart et al 2007 (487)	Composition & spatial organization of ileal mucosal microbiota	CD colitis & ileitis, H	Culture, FISH, quantitative PCR, RAPD-PCR, cloning sequencing	In ileitis *E. coli* higher & *Clostridiales* depleted compared with CD colitis, or H; The number of *E. coli* correlated with severity of ileal disease; Invasive *E coli* was restricted to inflamed mucosa; *E coli* novel in phylogeny with pathogen like behaviour; No evidence of *Mycobacterium avium* subspecies paratuberculosis, Shigella & Listeria
Frank et al 2007 (483)	Composition of mucosal microbiota	CD, UC non-IBD patients	Metagenomics, quantitative PCR	Depletion of *Firmicutes-Lachnospiraceae* subgroup & *Bacteroidetes* phyla; Increase in *Proteobacteria & Bacillus* subgroup; Abnormal microbiota was associated with occurrence of abscesses
Sokol et al 2007 (506)	Comparison of mucosal microbiota in inflamed & healthy tissue	UC	TTGE	No difference was found.
Vasquez et al 2007 (507)	Composition of mucosal microbiota; comparison between healthy & inflamed ileal tissue	CD surgical samples	TTGE, FISH	High similarity between inflamed & non inflamed, No intraepithelial bacteria were detected or bacteria in direct contact
Martinez et al 2006 (508)	Composition of mucosal microbiota	CD, UC, ischemic colitis, H	DGGE, sequencing	Patient-to-patient bacterial variability higher in CD than in H & UC; In CD prevalence of Clostridia, Ruminococcus torques, E. coli higher whereas for *Faecalibacterium prausnitzii* lower than H; Some opportunistic pathogens (*Enterobacter, Proteus, Haemophilus, Klebsiella* occasionally found in CD but not in H

Conte et al 2006 (503)	Composition of mucosal microbiota	Newly diagnosed CD, UC, IC, lymphonodular hyperplasia, H, **children**	Culture, real time RT-PCR	Higher number of aerobic & facultative anaerobic bacteria in IBD than in H; Increased concentration of mucosa associated bacteria; Occurrence of *B. vulgatus* lower in IBD whereas *E. coli* higher than H; *Klebsiella* occurred in IC; Pathogens were not isolated; No differences for bifidobacteria, *C coccoides* group, *Faecalibacterium prausnitzii*; Higher colonization in rectum & ileum
Bibiloni et al 2006 (509)	Composition of mucosal microbiota; comparison between healthy & inflamed tissue	Active, newly diagnosed, untreated CD & UC, H;	DGGE, cloning, PCR & RT-PCR	UC patients higher number of mucosa associated bacteria than CD or H; No difference in bacteria between inflamed & healthy tissue; High number of unclassified bacteria belonging to Bacteroidetes in CD; Different bacteria composition between CD & UC & with healthy; No differences for *M. avium, paratuberculosis, H. Pylori, C. difficile*, SRB, lactic acid bacteria & bifidobacteria
Gophna et al 2006 (510)	Composition of mucosal microbiota; comparison between healthy & inflamed tissue	CD, UC, H	PCR, cloning, sequencing	*Proteobacteria (Acinetobacter junni, Klebsiella pneumoniae, SRB), Bacteroidetes (B. fragilis)* higher in CD than H or UC but Firmicutes (clostridia class) lower; No difference between UC & H; No differences between inflamed & healthy tissue
Ott et al 2006 (511)	Composition of mucosal fungal microbiota	IBD, H	FISH, DGGE, cloning, sequencing	Diversity has increased in IBD; different composition with H
Sokol et al 2006 (512)	Composition of faecal microbiota	CD, UC, infectious colitis, H	FISH	IBD microbiota comprises of unusual bacteria; C. coccoides group was reduced in UC & C. leptum group in CD
Manichanh et al 2006 (513)	Composition of faecal microbiota	CD in remission, H	Metagenomics & FISH	Reduced diversity of Firmicutes phyla in CD; C leptum group was less abundant in CD than H; Presence of unknown species was more common in CD than in H
Scanlan et al 2006 (514)	Temporal changes in faecal dominant & selective subdominant microbiota; Disease activity effect	CD, H	DGGE	Dominant microbiota stability changed in CD; Reduced diversity in CD compared with H; In remission diversity & stability more stable compared with active disease; Clostridium & Bacteroides were reduced in a proportion of CD but in none of the H; Inter-individual similarity was higher in H than CD; No temporal changes for bacterial stability or diversity for Bifidobacteria for CD or H; Lactic acid bacteria were complex & host specific & changed with time in both CD & H. Diversity higher in H than CD
Seksik et al 2005 (515)	Composition of mucosal microbiota; comparison between healthy & inflamed tissue	CD	TTGE	Bacterial diversity remained high in ulcerated & non ulcerated mucosa

Mylonaki et al 2005 (516)	Composition of rectal mucosa microbiota	CD, UC, H	FISH	In UC less bifidobacteria but more *E coli; E coli* & Clostridia higher in active than inactive UC; *E coli* higher in CD; Individual bacteria & clusters were detected in the lamina propria of CD & UC. None was detected in H
Swidsinski et al 2005 (517)	Composition & spatial organization of mucosal microbiota	CD, UC, self limiting colitis, IBS, H	FISH	Higher concentrations of mucosal bacteria in IBD; *Bacteroides fragilis* prevalent in IBD; *Eubacterium rectale-clostridium coccoides* less prevalent in IBD; No patient with bacterial infiltration of the mucosa
Lepage et al 2005 (518)	Comparison of mucosal microbiota composition along the GI tract; Comparison with faecal microbiota	CD, UC, H	TTGE	Mucosal microbiota diversity stable along the GI tract; No differences with disease activity but differences with faecal microbiota
Bullock et al 2004 (519)	Composition of faecal microbiota; Disease activity effect	UC	FISH & DGGE	Lactobacilli number lower in active disease
Ott et al 2004 (520)	Composition of mucosa microbiota	CD, UC, non-IBD GI inflammatory conditions, H	SSCP, cloning, sequencing, real time PCR	Diversity was reduced to 50% in CD & to 30% in UC; Anaerobic bacteria (*Bacteroides, Eubacterium, Lactobacillus* species were reduced)
Macfarlane et al 2004 (340)	Composition of mucosal rectal microbiota	UC, H	Culture, FISH	UC lower ratio of anaerobes to facultative anaerobes than H; Bifidobacteria counts 30 times higher in H than UC: Peptostreptococci were only detected in UC; Bacteria diffused in the mucus of UC but not in H
Prindiville et al 2004 (521)	Composition of mucosal microbiota	CD (some surgical specimens too), H;	PCR, cloning, sequencing	In CD colon more facultative aerobes than H; In small bowel *Ruminococcus gravus (C Coccoides)* was increased whereas *C leptum & Prevotella nigrescens* decreased in CD; No difference between healthy & inflamed mucosa
Mangin et al 2004 (522)	Composition of faecal microbiota	CD, H	PCR, cloning, sequencing	All CD shared *B. Vulgatus* but none in H; E coli isolated in 50% of CD but not in H; Numerous other clones belonged to uncommon bacteria in CD
Seksik et al 2003 (523)	Composition of faecal microbiota; Disease activity effect	CD colitis, H	TTGE, quantitative dot-blot hybridisation	Enterobacteria higher in CD; Diversity remained high but varied in active & quiescent disease 30% of microbiota belonged to yet undefined phylogenetic groups
Kleessen et al 2002 (524)	Composition & spatial organization of mucosal microbiota	CD, UC, H	FISH	Mucosa adherent & invading bacteria in IBD; Bacterial invasion of the mucosa was evident in 83.3% of colonic specimens from the UC patients, in 55.6% of the ileal and in 25% of the colonic specimens from the CD patients; No bacteria were detected in the tissues of the H.

Swidsinski et al 2002 (525)	Composition & spatial organization of mucosal microbiota	CD, UC, IC, self limiting colitis, H	Culture, FISH, quantitative PCR, cloning, sequencing, electron microscope	Mucosal bacteria concentration high in IBD; Higher in CD; Concentration increasing with severity; no phylogenetic differences; No evidence of mycobacteria or listeria; Bacteroides & E coli higher in IBD; Intracellular bacteria observed in mucosa with the high bacterial load; No bacteria in lamina propria
Schultsz et al 1999 (493)	Composition & spatial organization of mucosal microbiota of the rectum	Active CD, UC, H	In situ hybridization	Bacteria more common & in higher concentration in IBD; Bacteria were localized within mucus layer, but were not adherent to epithelium; No presence in lamina propria; No correlation between the bacterial counts & the degree of inflammation or the use of anti-inflammatory medication

H: healthy

Characterization of intestinal microbiota in IBD

Microbial diversity and abundance is reported to be decreased in IBD compared with healthy controls (504;513;520) with a decrease up to 50% in CD and to 30% in UC. Only one study investigated the diversity of yeasts which was found to be increased in IBD patients (526). Inter-individual similarity of bacterial community profiles is reportedly high in healthy or UC but not CD subjects indicating that the former two groups share more common bacteria among individuals than CD patients (504;514).

With regard to the composition of the intestinal microbiota, higher numbers of aerobic and facultative anaerobic bacteria have been reported in IBD (503;521;527) signalling a different intestinal ecosystem compared with healthy people. Frequently characterization of the bacterial composition revealed increased numbers of *enterobacteriaceae* (523), mainly *E. coli* (516;522) and decrease in *Firmicutes* (483;513) with selectively decreased *Clostridium* species (487;505;510;512;514;517;521;525). Recently in a comprehensive analysis of 190 IBD tissue samples Frank et al, using a metagenomic approach, (483) confirmed these previous observations and found decreased numbers of the phylum *Firmicutes* and *Bacteroidetes* and a parallel increase in *Proteobacteria* and *Actonibacteria* (483).

An “unhealthy” colonic microbiota mucosa with reduced putative beneficial species like bifidobacteria or lactobacilli and occasionally an increase in commensal opportunistic pathogens like *E. coli* has been reported by few (505;516;519;520) but not all investigators (509). It is noteworthy that bifidobacteria was found at 30-fold higher levels in healthy than UC mucosal cultures and the presence of peptostreptococci was only identified in the latter specimens (527).

Undefined new bacterial species that do not belong to common dominant microbiota were typically seen in IBD mucosal and faecal specimens and the possible contribution of these strains to IBD remains elusive (509;513;520;522;523;528).

Comparisons of the faecal microbiota of patients with CD, UC and infectious colitis showed significant differences suggesting that observed modifications of the flora are not only because of the ecological changes induced by colitis (512).

Evidence of bacterial pathogens in IBD

Identification of pathogens was only a secondary objective in the studies reviewed, and the majority did not find clear evidence for pathogens such as *Mycobacteria, Listeria*, (487;525) *H. pylori, C. difficile*, and SRB (509). Occasionally some authors detected opportunistic pathogens in mucosa samples of patients with CD only but not in healthy or UC subjects (503;504;510). *Bacteroides vulgatus,* a mucus degrading bacterium which initiates colitis in

transgenic HLA-B27 rats (477), was detected, in most of the faecal samples of patients with CD but was rarely retrieved from healthy participants in a French study and this is in agreement with previous studies using traditional microbiology on growth media.

IBD microbiota and disease activity

The notion that intestinal microbiota composition might influence disease activity and explain the remitting and relapsing disease course has been challenged by a few researchers, although it is difficult to distinguish whether any difference seen has a primary causal effect or is a secondary to the disease process. Some authors found that the diversity of microbiota or individual bacterial species (505) were different between active and inactive disease (523) (Table 1.7.4).

An association between disease activity and specific bacterial species was recently reported by Baumgart et al (487) who found that in CD with ileal involvement *E. coli* numbers in situ correlated with the severity of ileal disease. Likewise in a previous study Swidsinski et al found increased concentration of mucosa adherent bacteria with increasing severity and Bullock et al (519) found lower concentration for lactobacilli in active compared to quiescent UC disease. Swidsinski et al (505) recently found that with increasing intestinal inflammation, loss of the habitual microbiota occurred in both CD and UC. In the same study, species-specific changes were observed between active and quiescent disease in CD with *Enterobacteriaceae* increased and bifidobacteria decreased in active compared with inactive CD.

A localized bacterial dysbiosis, and alterations in the mucosal microbiota between inflamed and healthy tissue has not been found in the majority of studies (506;507;509;510;521). Only Baumgart et al (487) observed a high presence of adherent and invasive *E. coli* in the inflamed but not in healthy mucosa of CD patients with ileum involvement. Failure to identify any difference between the microbiota of healthy and inflamed areas is contrary to the hypothesis of a localized “dysbiosis” which could explain the intestinal lesions and the disease distribution mainly in CD.

Spatial organization of bacterial microbiota

The well documented differences between faecal and mucosal associated microbiota (347) and the direct contact of the latter to the intestinal epithelium prompted a considerable amount of research to be directed towards the spatial organization of the mucosal associated microbiota.

Several studies have repeatedly shown that the epithelial surface or mucus layer of IBD patients contains a higher number of bacteria compared with controls

(487;493;509;517;524;525). Bacterial infiltration and detection of intracellular bacteria in the intestinal epithelium and lamina propria of patients with IBD were reported by some investigators particularly, in specimens with increased number of mucosal associated bacteria, but not by all (493;507;517). An interesting observation is that intracellular *E. coli* were observed in the inflamed tissue of ileal but not CD colitis, despite the higher concentration of bacteria lining the villi of the latter.

Differences in the gut microbiota between CD and UC

Disease specific differences in the commensal microbiota profiles were described in several studies (505;509;510;524;525). Swidsinski et al (525) found a higher concentration of mucosal adherent bacteria in CD than in UC, but no phylogenic differences were described. In a following study, the same group (505) showed a different bacterial profile between UC and CD for six out of 11 FISH probes used. In particular *Faecalbacterium prausnitzii* was dramatically reduced in CD only but was unaffected in UC. In 56% of the CD patients bifidobacteria were undetectable, whereas the mean concentration of the latter was higher in UC than in healthy faecal specimens. Based on these overt disease specific bacterial differences and in conjunction with differences in the faecal leucocytes concentrations, diagnostic cutoffs were proposed by the authors to discriminate between active CD and UC. Similarly Gophna et al (510) found significant phylogenetic differences between CD and UC and an overall more unbalanced microbiota in CD than in UC.

Temporal changes of microbiota in IBD

Stability of the diversity and composition of the dominant intestinal microbiota is characteristic of healthy adults. Disturbance occurs in some GI diseases as well as with dietary or pharmaceutical intervention. Only two studies assessed temporal changes of the intestinal microbiota in IBD. Martinez observed a decline in the bacterial similarity index of UC patients on longstanding remission of one year. No changes were seen for the healthy subjects who preserved both their bacterial diversity and similarity (504). Likewise Scanlan et al (514) found unstable microbiota profiles and decreasing diversity in CD patients who had achieved clinical remission with steroid treatment. However no changes in the bacterial profile stability or diversity of bifidobacteria species were observed. A disease activity effect was observed with a more stable diversity in quiescent than in active disease (514).

Microbiota diversity along the GI tract in IBD

No major differences were observed in the diversity of the dominant mucosal-associated microbiota along the digestive tract in IBD patients (518) although Baumgart et al (487) found that invasive-adherent E. coli occurred more often in CD with ileal involvement than in inflammation confined to the colon.

Paediatric studies of intestinal microbiota in IBD

To the best of my knowledge only one study has investigated the intestinal microbiota of paediatric patients with IBD. Conte and his colleagues (503) investigated and characterized the predominant composition of the mucosa associated intestinal microbiota in colonoscopic specimens of paediatric patients with newly diagnosed IBD, children with lymphonodular hyperplasia and healthy controls. Using traditional culturing procedures linked with novel molecular biology techniques, the authors found more mucosa associated bacteria and measured higher numbers of aerobic and facultative aerobic bacteria in IBD than in healthy controls. Pathogens were not isolated (*Yersinia, Campylobacter*, etc), nor were differences for bifidobacteria, *C. coccoides* group, *Faecalibacterium prausnitzii*, observed, as opposed to some adult studies (Table 1.7.4). Occurrence of *B. vulgatus* was lower and *E. coli* was increased in IBD compared with healthy children.

1.7.2.3.5. Conclusion

Bacterial dysbiosis in the luminal microenvironment rather than a single pathogen has been proposed as a putative mechanism in IBD aetiopathogenesis (498), and overall this comprehensive review supports this hypothesis. However a pathogen as causative factor in

IBD cannot be entirely excluded. One should bear in mind that these studies have mostly assessed the dominant intestinal microbiota qualitatively and therefore cannot fully exclude quantitative differences, qualitative differences in the subdominant bacteria or a pathogen that which is unable to be isolated and be studied with contemporary techniques.

In summary commensal intestinal microbiota is distinguishable in IBD from that of healthy subjects:

a. With increased numbers of bacteria in the mucus layer or in contact with epithelial cells
b. Reduced bacterial diversity
c. Unstable bacterial diversity over time
d. Colonisation by uncommon, unidentified bacteria
e. Reduction in several “beneficial” species and increase of opportunistic pathogens
f. Disease activity associated changes

Changes in the microbiota of patients with IBD are difficult to interpret clinically although many of the depleted bacteria are major butyrate producers and their reduction might therefore compromise production of epithelial energy substrates (513).

In considering dysbiosis as a key factor in the pathogenesis of IBD, perhaps the most difficult question is to distinguish between cause and effect: “Is dysbiosis just a secondary phenomenon of IBD or is it actually the cause of it?” All the former studies were unable to answer this as they recruited patients with established disease. Only a large cohort longitudinal study with follow up measurements prior to disease onset could identify differences in gut microbiota diversity and metabolism between healthy people and patients who developed IBD at follow up. It was surprising that evidence concerning gut microbiota in paediatric IBD comes from a single study in patients with active disease. Extrapolation of the results from adult studies may not be appropriate. Although both adults and children share common pathology and clinical characteristics, the phenotypic characteristics and response to treatment are different. Paediatric IBD is more extensive and aggressive than in adults and perhaps differences in adult microbiota might explain that. More studies are needed to explore the composition of the intestinal microbiota in paediatric IBD and how this fluctuates with disease activity.

1.7.2.4. Enteral nutrition, gut flora and metabolism in patients with IBD

Although the commensal gut microbiota is stable over time (341), its composition can fluctuate under circumstances such as in health and disease. Nutrition is a strong determinant of the microbiota composition and metabolic activity, and although daily changes in a normal diet are coupled with minor modifications of the faecal flora, radical changes of a

person's diet such as artificial nutrition can affect the composition and metabolic activity of the commensal bacteria (529).

On the other hand commensal intestinal microbiota is implicated in the disease pathogenesis in IBD, and "dysbiosis" is considered as one of the putative mechanisms that may be implicated in intestinal inflammation and mucosal injury (Section 1.7.2.3.4). Thus modification of the commensal microbiota to a more beneficial/balanced microbiota could be a possible mechanism of action for EEN and could also explain the better efficacy of EEN in sites of the GI tract (264) where abnormal microbiota is usually encountered. Although modification of the intestinal microbiota has always been suggested as one of the potential mechanisms of action in relevant topic reviews (276;530) none of those cited an appropriate study. Thus far the only peer-reviewed evidence comes from an Italian study by Lionetti et al (531) and a recent Australian by Leach et at (532).

The first by Lionetti et al (531) in a clinical study, collected serial stool samples from nine patients on EEN treated with a polymeric feed for eight weeks. Using molecular methods of bacterial community profile analysis, they found evident changes in the dominant gut microbiota as a consequence of EEN treatment. Ongoing changes were also seen beyond eight weeks whilst children maintained supplementary formula. In contrast, samples from healthy children showed stable bacterial profiles with no variation over time. In the second study Leach et al (532) collected serial stools samples from six CD children during EEN, and at four weeks and at four months after treatment, and compared their bacterial composition patterns and diversity with seven healthy children. The diversity of bacteria was assessed with DGGE and changes in the global bacterial, and group specific diversity and similarity patterns were measured. Although bacterial diversity did not differ significantly between healthy and CD children at treatment initiation a lower diversity was found at the end of treatment in the CD group compared with the healthy controls. *Bacteroides-prevotella* and *Clostridium coccoides* bacterial species were significantly decreased in the CD group compared with the healthy controls at the end of EEN treatment. Moreover the similarity index of bacterial diversity was low during treatment for all bacterial groups studied, and was significantly lower than healthy subjects. However no pairwise comparison of changes in bacterial diversity and similarity patterns were reported during EEN to investigate changes during treatment.

Disease improvement and amelioration of intestinal inflammation might be a result of changes in the production of bacterial metabolites which have been implicated in the pathogenesis of IBD, like SCFA and sulphide. Such a putative mode of action has been proposed by Day et al (533) in a recent review whereas possible prebiotic properties have also been attributed to the feeds (531). However to the best of our knowledge most of the commercial feeds commonly used for the management of CD, and particularly those tested

by the studies above lack non-digestible carbohydrate which could impart a prebiotic effect to the supplement.

Beyond the limited evidence of relevant studies in the area of IBD, studies that assessed the effect of EEN on the gut microbiota of healthy people or patients with other morbidities may be of relevance. The evidence on this topic has been recently reviewed by Whelan et al. (529) In a series of studies, it was concluded that the evidence of an effect of EN on the intestinal bacterial metabolism is inconclusive. Results from individual studies were contradictory with some showing large reductions in the total number and type of bacteria, and others suggesting no changes or increase in particular bacterial species. Apart from differences in study design, methodological flaws, and different types of nutritional formulae, the additional limitations of the use of conventional bacteriological techniques might explain these discrepancies (529). In keeping with the ability of nutritional therapy to affect colonic fermentation, most of the studies observed reduction in the production of SCFA, particularly butyrate, in healthy subjects on low residue feeds (529).

In a recent double blind RCT with cross over design, Whelan et al (324) administered a standard low residue diet or one high in fructoligosaccharides and fibre in 10 healthy adults exclusively for 14 days with a six week wash out period. In both treatments reductions in total faecal bacteria were observed with FISH analysis although these were higher in the low residue regime. Although the low residue diet did not alter the diversity of microbiota, the high residue diet increased bifidobacteria and reduced clostridia concentration and relative proportion compared with baseline. Similarly the low residue feed reduced the concentration of total and major SCFA compared with baseline values whereas in the fortified feeds group, reductions were observed only for butyrate. Marked changes were observed in the relative proportion of each SCFA, following consumption of the feeds. Consumption of the low residue feed resulted in an increase in the relative proportion of acetate, isobutyrate, and isovalerate and a reduction in the percentage of butyrate compared with baseline values. For the high residue formulae similar changes with the alternative feeds were observed only for acetate and butyrate. Regarding changes in faecal pH, administration of both low and high residue diet, increased pH significantly although the increase was lower in the latter.

In other GI disorders Schneider et al (534) in a cross sectional study of eight patients on nutritional therapy with feeds low in residue, demonstrated concentrations which were lower for faecal anaerobes and higher for aerobes, despite no difference compared with healthy controls for bacteroides, clostridia, and bifidobacteria. In a third group on total parenteral nutrition, both aerobes and anaerobes decreased dramatically. Despite differences in bacterial populations, there were no differences between patients, and healthy controls in the concentrations of total SCFA, acetate, propionate or butyrate. In the total parenteral group, total SCFA were decreased compared with the EN group.

Extrapolating the results from studies in healthy subjects or similar investigations in other digestive disorders, in the context of IBD is inappropriate. Changes in gut bacteria population and metabolic activity can be attributed to both the effect of nutritional therapy and disease-mediated effects as commensal microbiota are strongly implicated in IBD pathogenesis. In the studies reviewed by Whelan (529) in healthy subjects any changes could be ascribed mainly to the effect of the nutritional therapy. In IBD this could be the additional outcome of disease improvement. The effect of EEN on the gut microbial flora deserves further exploration. Alterations in the bacterial populations and metabolic activity may alter interactions with the intestinal epithelium, thereby leading to modulation of inflammation. With the advent of new methods of analysis of bacterial flora, more evidence is anticipated.

1.8. Overall conclusions and proposed areas of research

From the pathogenesis of the disease to its management, nutrition plays an important role in paediatric IBD. The extensive literature review presented above was a compilation of the evidence on nutritional aspects in IBD and aimed to identify areas of interest for further research. Although good evidence is emerging from research and clinical experience to advocate the role of EEN in disease management there is a substantial lack of studies investigating its mechanisms of action. This combined with the evidence that implicates the commensal microbiota in disease pathogenesis led the researcher to postulate a mechanism of EEN action mediated through modulation of intestinal microbiota diversity and/or metabolic activity. In 2004 when this PhD started there was no published evidence, which explored this although leading reviews on the topic often cited this as a mechanism of action. More evidence was therefore needed to address these anecdotal speculations. The two studies that followed were of small number of patients and were restricted only to changes in bacterial diversity. None of these explored changed in the metabolic activity of the commensal microbiota. A study that assessed changes in metabolic activity and bacterial diversity is presented in Chapter 7.

As was described in Section 1.5, nutritional therapy has a double role in paediatric CD. This is mainly to induce clinical remission, and in parallel, to improve nutritional status in active paediatric CD. Although changes in basic anthropometry have been described in almost all of the studies, changes in body composition and micronutrient status have not been reported extensively. New methods of body composition are now available which allow measurements at bedside. These are explored in Chapter 4.

Micronutrients play an important role in health, and in IBD, there is evidence to suggest an additional role in disease pathogenesis, and intestinal injury. Consensus

guidelines from North America suggest that all patients should be screened regularly for micronutrient deficiencies. However any speculation on micronutrient deficiencies in IBD is based on studies from adult studies, case reports and studies of individual micronutrients. Moreover low levels observed in many of the and their association with disease activity has to be revisited as recent evidence suggests that disease activity and acute phase response can spuriously change the serum levels of micronutrients and any association with activity markers may actually be artefactual. This is investigated in Chapter 5.

On the other hand the role of other unconventional nutritional therapies, such as probiotics, and special diets are increasing in the general population but the evidence from paediatric IBD studies is limited. As many of these therapies, have a good record of efficacy, increase in their use, by IBD patients should be anticipated. Moreover avoidance of specific food groups, as part of adherence on exclusion diets, can impact on the micronutrient status of the patients and affect their health negatively. The use of nutritional therapies, special diets and alternative medicine is explored in Chapter 2.

The aim of this doctorate therefore was to study the use of unconventional nutritional therapies by paediatric IBD patients and the effect of EEN on the intestinal microbiota and nutritional status, by measuring body composition, micronutrient status and disease activity.

CHAPTER TWO

Dietary modifications, nutritional supplements and alternative medicine in paediatric patients with IBD.

OUTLINE

This chapter describes a questionnaire survey to assess the use of special diets, nutritional supplements and alternative medicine in a paediatric population of Scottish children with IBD. Predictors and reasons of use are discussed. This study has already been published. *Alimentary Pharmacology and Therapeutics. 2008 15;27(2):155-65*

2.1. Introduction

The aetiology of IBD remains unknown and the most effective medications commonly have side effects that limit patients' acceptance and long-term compliance. In children, the additional issues of growth, maturity and body image make disease management more complicated and troublesome for health professionals, the patients themselves and their guardians (34). Although most patients rely on conventional medicine, some may combine or replace this with unconventional-alternative remedies for the management of their condition (60;68).

Complementary an alternative medicine (CAM) use by the general population is widespread around the world (48) and increasing over time (Section 1.2.2). CAM use is reported as high in chronic illness and gastrointestinal patients (47;50;51;56;60-62;66;69;70;535). In particular, the prevalence of CAM use in IBD patients has been reported to be higher than in healthy people (61) and although early studies estimated that 4% of patients use CAM (62), recent surveys reported a sharp increase to 72% (69). However no definite figures can be drawn. The use of CAM varies widely between studies and this could reflect differences in study design, responding populations (68;536), cultural specificities or preferences (46), but most of all the different definitions of CAM (48). There is no clear definition of which specific methods and remedies comprise CAM and which do not. Some studies included therapies like prayer (46;537) and exercise in the definition and others excluded the use of multivitamins purchased over the counter (63). Therapies with good evidence of clinical efficacy, but which are not prescribed as conventional medicine, like probiotics, were included in only a few studies (64;66). In fact with more scientific evidence accumulating around the efficacy of such products (75;538) and their commercialization, it is anticipated that more patients will use them in the future. New branches of established CAM disciplines are continually being developed, taught in medical schools, used in hospital or in primary healthcare (54;56;539), reimbursed by insurance companies, and integrated into conventional medicine (540).

Although there have been some studies on the use of CAM in adult IBD patients (46;63;68;539) the prevalence of CAM in paediatric populations is not well studied.

Differences in disease management regimes between adult and paediatric patients (34), as well as the issues of growth, maturity and image make the extrapolation of the results from adult studies difficult. Indeed the use of CAM by paediatric patients reflects the choice of their guardians and not the patients', and rather how the former perceive and cope with their child's disease.

Most paediatric surveys have been conducted in the US (64) and Australia (66) with only one multinational study which included only a small sample of UK patients from a single hospital in London, UK (65). Disease management protocols differ between countries (256) and as the use of CAM in adults was associated with the type and use of specific medications (68;541), different patterns of CAM use should be expected worldwide.

The main purpose of the present study was the evaluation of current and past CAM use by a paediatric IBD population in the West of Scotland, including types of CAM with good records of clinical efficacy, the reasons of their choice, and parental attitudes on CAM use. A secondary outcome was to report any factors associated with CAM use including new predictors like growth and anthropometry and serial post diagnostic laboratory markers of disease activity.

2.2. Materials and methods

All paediatric (<18 y old) IBD patients, along with their guardians, who attended their follow up clinical appointments at Yorkhill Royal Hospital of Sick Children, Glasgow were eligible to participate. This is the biggest tertiary paediatric IBD referral centre in Scotland, serving approximately half of the Scottish paediatric population and is predominately in charge of the management of almost all children with IBD in the greater region of the West of Scotland. At the time of the study, there was no private paediatric clinic. This allowed inclusion of nearly all paediatric patients diagnosed with IBD and residing in the West of Scotland.

Given that each patient visits the IBD outpatients clinic at least once a year for medical review (independent of disease activity), it was anticipated that all IBD patients, and their guardians, could be approached within a year. All recently diagnosed patients (< 3 months) were excluded, as they would not have had much time to consider CAM treatment. Patients with other severe concomitant chronic illness were also excluded.

The study protocol was approved by the Local Research Ethics Committee and the Research and Development Office at Yorkhill Hospitals.

2.2.1. Recruitment

An independent clinical researcher explained the research to the patients and their guardians. An information leaflet was given to the guardians and an age adapted version to

the patients themselves. If both agreed to participate, a blank questionnaire was provided with a post-paid, pre-addressed envelope and the method of completion was demonstrated on a template questionnaire by the researcher. Participants could ask questions on clarity. The guardian filled in the questionnaire at home and returned it by post. A second questionnaire was posted to all participants that had not responded within 15 days.

2.2.2. Questionnaire

The instrument consisted of 40 questions and took approximately 15 min to complete (Appendix). A preliminary questionnaire was compiled using two existing questionnaires (64;66). The questionnaires were then modified with the inclusion and exclusion of questions to reflect the use, and types of CAM commonly used in the UK. CAM was defined as those unconventional remedies and treatments that are not normally taught in UK medical schools, as established approaches to IBD management, are not reimbursed by the NHS nor recommended by our medical staff. Exercise and prayers were not included.

The face validity of the questionnaire was checked by several health professionals and the first 10 participants were asked anonymously to comment on questionnaire clarity and ease of use. Appropriate amendments were made to the questions and the structure of the instrument.

The first section of the questionnaire described the patient's socio-demographic and disease characteristics. The following section focused on the use of CAM and listed four groups of therapies namely Dietary modifications, Herbals, Nutritional Supplements, and Alternative Therapists. The guardians answered whether the child used, or had recently used any of these especially for the management of IBD. The time from symptoms onset to diagnosis is quite variable and patients may have had the opportunity to use CAM before diagnosis, however to ensure all patients had significant time to try CAM after diagnosis this survey asked carers to document any type of CAM used three months post-diagnosis.

In total, 35 different types of CAM were described and there was an open question at the end of each group for any CAM not on the list. Although multivitamins and dietary modifications were included, it was made clear to participants to report only those that were purchased over the counter or not recommended by their hospital medical staff.

The next section, applied only to those patients that had used CAM. Participants were asked to report sources of information on CAM, reasons they had chosen these, their effectiveness, cost and doctor's awareness of their CAM use. The last section, directed to all the respondents consisted of five questions addressing parental attitudes about CAM, and the use of CAM by other members of their family or the patients themselves for other health reason.

To ensure anonymity, confidentiality and undistorted responses the questionnaires were not numbered and parents asked not to add any identifiers. An invisible number was located on each questionnaire to identify non-respondents for subsequent mailing.

On a supplementary form, detached from the main questionnaire, but with the same invisible number, the researchers recorded information on disease characteristics, post diagnostic laboratory data, conventional treatments, growth and anthropometry (Appendix). This form was reattached to the main questionnaire when the latter was returned to the researchers by the participants.

2.2.3. Statistical analysis

The prevalence of CAM use was estimated as the total number of patients reporting CAM use of any type divided by the total number of respondents. Categorical variables were presented as frequencies and means with standard deviations and medians with inter-quartile range for parametric and non parametric variables respectively.

Binary and nominal logistic regression analyses (Minitab 13) were undertaken to determine potential predictors of CAM use. Candidate predictors were suggested *a priori*. These included age, sex, disease type, phenotype and duration, total number and types of medication since diagnosis, serial post diagnostic laboratory data on CRP (C-Reactive Protein), haemoglobin, albumin, ESR (Erythrocyte Sedimentation Rate), IBD operation, BMI (Body Mass Index), height and height velocity z-score at recruitment, six and 12 months before, parental use of CAM, CAM use for other reason, social deprivation score (Scottish Index of Multiple Deprivation; http://www.scotland.gov.uk/Topics/Statistics/SIMD/Overview), parental education and age. For purposes of analysis, patients were grouped into “Self-prescribed CAM Users” (including dietary modifications, nutritional and herbal supplement users) and “CAM Therapist Users” (those who consulted an alternative therapist) as proposed by Harris and Rees (48) to permit comparison with other studies (537). The strength of association between use of CAM and associated factors was measured by odds ratio and 95% CI.

The process of selection and inclusion of variables in the multivariate model was based on univariate logistic regression analysis. Associated predictors with p-values < 0.1 were entered in a multivariate model and their independent association tested using backward stepwise regression. The final model included only those predictors with p-value less than 0.05 as independent predictors of CAM use for the binary analysis, whereas for the nominal regression analysis the final model considered only predictors with p-value less than 0.05 for at least one of the comparisons.

2.3. Results

2.3.1. Recruitment

Approximately 480,000 children are serviced by the Yorkhill hospitals, and 159 patients visited the IBD outpatient clinic between June 2005 and July 2006 and this covers the IBD population serviced by the IBD clinic. Twenty four patients were not eligible to participate. From 135 eligible patients 104 were approached (77% of the eligible population) along with their guardians and all consented to participate. Due to logistic constraints it was not possible to approach all patients and 31 who visited the clinic in the time of the study were not recruited. Eighty six including 40 female patients completed and returned the survey (83 % response) and all were used in the analysis. All but one patient were of Caucasian origin.

2.3.2. Respondents demographic characteristics

The median age of patients at recruitment was 12.7 (4.8 – 17.5) y. There was almost equal distribution of the family social deprivation score (Scottish Index of Multiple Deprivation) (Table 2.1). The majority of the responding parents were more than 31 y old and 56% had higher education (college and university combined) (Table 2.1).

Table 2.1: Demographic characteristics of participants in a Scottish survey on the use of CAM by children with IBD

Demographics		**Parental age†**	**N (%) patients**
Gender (M/F)	46/40	18-30	5 (6)
Age (y mean ± SD)	12.7 ± 2.8	31-45	58 (68)
		46-60+	22 (26)

Social deprivation* (quintiles)	**N (%) patients**	**Parental education†**	**N (%) patients**
1	16 (20)	Standard Grade (year 11)	25 (33)
2	15 (18)	Highers (year 12)	8 (11)
3	13 (16)	Further Education/College	18 (24)
4	22 (27)	University	24 (32)
5	15 (19)		

*Scottish Index of Multiple Deprivation 2004; based on six individual domains of current income, employment, housing, health, education, skills and training and geographic access to services and telecommunications † responding parent

2.3.3. Patients disease characteristics

Crohn's disease patients accounted for two thirds of participants, whereas 26% had UC, and 9% were classified as IC according to standard endoscopy, histology and radiology criteria. The median age at diagnosis was 10.3 y (range: 1.9-14.5) with median disease duration of 2.4 y (0.4-7) at time of recruitment. The majority of patients had been treated with 5-aminosalicylates, azathioprine, steroids and EEN (Table 2.2). The majority of them had a repeat course of EEN or oral steroids. 12% of patients had had a major operation for IBD that included resection of part of their gut.

2.3.4. Patients anthropometry and growth data

The mean BMI z-score of the respondents was 0.2 SD with 2.4% classified underweight (BMI z-score < 2 SD) and 3.6% obese (BMI z-score > 2 SD).

Table 2.2: Disease characteristics and post diagnostic medication of participants in a Scottish survey on the use of CAM by children with IBD

Disease type (n=86)	N (%) patients	Disease location	N (%) patients
Crohn's disease	56 (65)	Lower GI	33 (42)
Ulcerative colitis	22 (26)	Upper & Lower	42 (53)
Indeterminate colitis	8 (9)	Upper	4 (5)

Total post-diagnostic medication No	N (%) of patients	Total post-diagnostic medication type	N (%) of patients
≤2	6 (7)	5-ASAS	16 (81)
3	8 (10)	Azathioprine	58 (68)
4	19 (23)	Steroids	55 (65)
5	10 (12)	Vitamins / Minerals	55 (65)
6	12 (14)	EEN	51 (60)
7	8 (10)	Caloric Supplements	37 (43)
8	10 (12)	Antibiotics	36 (42)
≥9	10 (12)	Omeprazole	20 (24)
Gut Resection	10 (12)	Methotrexate	15 (18)
		Infliximab	8 (9)

No of oral steroid courses	N (%) of patients	No of EEN courses	N (%) of patients
0	36 (42)	0	36 (42)
1	17 (20)	1	24 (28)
2	15 (18)	2	15 (18)
3	12 (14)	3	4 (5)
>3	5 (6)	>3	6 (7)

Biochemistry*	Median; (Range)	Hospitalization (last 2 y)	N (%) of patients
Albumin (g/l)	39 (24-47)	0	34 (40)
CRP (mg/l)	7 (7-100)	1	20 (24)
Haemoglobin (g/dl)	11.8 (8.4-14.7)	2	14 (17)
ESR (mm/hr)	20.7 (3-66)	>2	16 (19)

* Median of all available post-diagnosis values

The mean height z-score was –0.4 SD with 7.2% presenting values less than -2 SD suggesting possible growth retardation. Their median height velocity at six and 12 months was 0.9 and 0.8 SD respectively.

2.3.5. Use of CAM

Fifty two of the eighty six respondents (61%) reported prior use of CAM for the management of IBD and 37% were using some form of CAM on recruitment. A quarter of the respondents (21/86) had consulted at least one alternative therapist whereas only three patients were using that kind of CAM on recruitment (Table 2.3).

Table 2.3 Past and recent users of CAM in a Scottish survey of children with IBD grouped into "self-prescribed CAM users" and "CAM therapist users"

Type of CAM User	N (%) Ever used (n=86)	N (%) Recently used (n=86)
"Self-prescribed CAM users"	31 (37)	29 (34)
"CAM therapist users"	21 (24)	3 (3)
Overall CAM use	52 (61)	32 (37)

Probiotics, dairy free diet, omega-3/fish oils and aloe were the most common therapies, followed by gluten free diet, homeopathy, massage and over the counter multi and megavitamins (Table 2.4). The median and interquartile range of total number of CAM therapies ever used was three (range: 2-6) CAM and two (range: 1-2) in the recent users.

Table 2.4: Use of dietary modifications, nutritional supplements, herbals and alternative therapists in a Scottish survey on the use of CAM by children with IBD

	N (%) Ever used (n=86)	N (%) Recently used (n=86)
Dietary modifications		
Dairy free diet	24 (28)	12 (14)
Gluten free diet	13 (15)	2 (2)
Low residue diet	8 (9)	3 (3)
Low sugar diet	4 (5)	0 (0)
Vegeterian diet	4 (5)	0 (0)
Other diets	5 (6)	1 (1)
Herbals		
Aloe	16 (19)	2 (2)
Garlic	8 (9)	0 (0)
Echinacea	8 (9)	1 (1)
Evening primrose	7 (8)	0 (0)
Other herbals	19 (23)	1 (1)
Nutritional supplements		
Probiotics	38 (44)	17 (20)
Fish / Omega-3 Oils	25 (27)	7 (8)
Vitamins / Minerals	12 (14)	3 (3)
Prebiotics	8 (9)	1 (1)
'Active' dairy products	8 (9)	2 (2)
Other nutritional supplements	11 (13)	3 (3)
Alternative therapists		
Homeopathy	12 (14)	1 (1)
Massage	11 (13)	1 (1)
Aromatherapy	8 (9)	0 (0)
Yoga	5 (6)	0 (0)
Relaxation	5 (6)	1 (1)
Chiropractic	4 (5)	0 (0)
Reflexology	4 (5)	0 (0)
Other alternative therapists	14 (16)	0 (0)

Seventy three percent (38/52) of CAM users answered the relevant section on CAM use. Of 14 respondents that skipped the section, only one was a "CAM Therapist User". Of these 22 revealed the use of CAM to their doctor. 50% reported that their doctor reacted positively, 37% neutrally and 12% negatively to the use of CAM. Most participants that did not report

the use of CAM to their doctor, thought that it was not important for their doctor to know or they forgot to do so. Parents reported that they received information about CAM from personal recommendations, magazines, newspapers and the Internet (Table 2.5).

Table 2.5: Reasons of use and sources of information on CAM in a Scottish survey on the use of CAM by children with IBD

Reasons of CAM use	N (%)	Sources of information	N (%)
Complement conventional treatment	21 (45)	Read about it	18 (47)
Personal experience	16 (42)	Recommended by friends	18 (47)
CAM is natural and harmless	12 (32)	Internet search	10 (26)
Frustration with medication side effects	11 (29)	Recommended by health professional	7 (18)
Dissatisfaction with medication	5 (13)	Other reason	7 (18)
Other reason	7 (10)		
CAM is more effective	1 (3)		
Lack of confidence in medication	1 (3)		

Reasons given for using CAM included an attempt to complement conventional treatment, personal experience with CAM use, frustration with conventional medication side effects and the belief that CAM is natural and harmless (Table 2.5).

Approximately equal numbers (16 and 17 respectively) of CAM users judged CAM use effective and not effective. One patient reported deterioration of the disease condition and one mentioned side effects. More “CAM Therapist Users” (12/16) labelled CAM effective compared to “Self-prescribed CAM Users” (p=0.022). Fifteen of 38 users found CAM expensive although most of these (10/15) had consulted an alternative therapist.

Twenty seven percent of all respondents had discussed CAM with their doctors and most were “CAM Therapist Users” (p=0.003). 16% of the children had used a type of CAM for other reasons whereas 32 % of the patients’ relatives had used CAM.

Most of the parents were positive about the use of CAM, but the majority (40/53) had already used CAM for their child’s IBD (Table 2.6). 89% of the respondents would give their child CAM if they felt it to be useful although 50 out of 73 were already CAM users. In addition 52% of the respondents were not wary of using CAM, 57% of them would be happy for their child to use any type of CAM and 86% agreed with the statement that “doctors should be supportive of people using CAM”. On the other hand the majority of the respondents had no opinion about CAM safety and agreed with the statement that “not enough is known about CAM” (Table 2.6).

Table 2.6: Attitude on CAM use by parents of children with IBD in a Scottish survey of the use of CAM

	Disagree N (%)	No opinion N (%)	Agree N (%)
Not a person that would use CAM	53 (74)	11 15)	8 (11)
Doctors should be supportive of CAM	3 (4)	8 (10)	66 (86)
Wary of using CAM	39 (52)	3 (4)	33 (44)
CAM is very safe	16 (21)	36 (49)	22 (30)
Not enough is known about CAM	8 (11)	15 (20)	51 (69)
Would be happy my child to use any CAM	19 (25)	14 (18)	44 (57)
Would give CAM only if doctor says so	31 (39)	9 (12)	39 (49)
Would give CAM if I felt to be helpful	6 (7)	3 (4)	73 (89)

2.3.6. Predictors of CAM use

Parents under 46y and of high parental educational level were the strongest independent predictors that distinguished CAM users in the multivariate analysis (Table 2.7). Use of CAM for other health reason, and an increased number of oral steroid courses were additional variables that differentiated “CAM Therapist Users” from non users although a positive association between disease duration and use of “CAM Therapist Users” revealed in the univariate analysis was lost in the multivariate model.

2.4. Discussion

Knowledge of CAM use by children with IBD is imperative. Some of these treatments have severe adverse effects with reports in the literature of death, anaphylaxis, renal failure and malignancies (77;542) whereas for those with good safety records in adults, the same doses used in adults, may not be safe in children with a smaller drug distribution volume. Moreover possible unpredicted interactions with conventional medicine, could reduce or delay the efficacy of the prescribed treatment with subsequent detrimental prognostic effects (543).

Paediatric data on CAM use by IBD patients is scarce and to the best of our knowledge this study is the biggest paediatric study so far in the UK and Europe. Unlike previous surveys (65) only patients younger than 18 y were recruited to ensure that the measured CAM use was not confounded by “adult children”.

In this study the number of non respondents was small and any difference between respondents and non respondents should not change the overall findings significantly. Response is of utmost importance in a study where a goal is to generalize the results to the entire population. Poor response rate raise concerns that existing differences between responders and non-responders would significantly change the results.

Although three patients approached in this study were of Asian origin, none of them returned the questionnaire in agreement with an adult survey where significantly less Asians than Europeans responded (47). However no difference in the use of CAM was found

between Asians and Europeans in that study and therefore the children with Asian origin in this study would be unlikely to differ from the responders.

The study design approached a geographically diverse sample, representative of half of the whole Scottish paediatric IBD population in contrast to other surveys with predominantly severely ill patients from a single tertiary hospital (65). This was achieved because there was no other public or private paediatric clinics and the hospital management protocol ensured that each patient visits the hospital at least once a year independent of disease activity. Indeed the sample's disease and demographic characteristics, mirrored the results of a Scottish national study (16) and a prospective epidemiological survey in the UK (11). This study surveyed only those patients who were diagnosed in a conventional clinic.

Table 2.7: Predictors of CAM use by Scottish children with IBD based on univariate and multivariate logistic analysis with p-values, 95% CI and odds ratios.

	All Users			"CAM Therapist Users"			"Self-Prescribed Users"		
Univariate analysis	**P**	**OR**	**95%CI**	**P**	**OR**	**95%CI**	**P**	**OR**	**95%CI**
Diagnosis age	0.127	0.9	0.7 - 1.0	0.051	0.8	0.7 - 1.0	0.388	0.9	0.8 - 1.1
Disease duration	0.517	1.1	0.9 - 1.4	0.048	1.3	1.0 - 1.8	0.541	0.9	0.7 - 1.2
Parental age*	0.007	4.0	1.5 - 11.3	0.047	4.2	1.0 - 17.1	0.022	4.0	1.2 -12.9
Parental education†	0.001	5.8	2.0 - 16.2	0.001	12.7	2.9 - 55.9	0.017	3.9	1.3 -11.9
CAM for other reason	0.155;	2.7	0.7 - 10.5	0.028	5.4	1.2 - 24.2	0.659	1.4	0.3 - 7.0
CAM use by family	0.360	1.6	0.6 - 4.1	0.061	3.1	0.9 - 10.0	0.951	1.0	0.3 - 2.9
Steroid use	0.065;	2.3	0.9 - 5.8	0.144	2.5	0.7 - 8.5	0.116	2.3	0.8 - 6.3
No of steroids courses	0.588	1.1	0.8 - 1.5	0.074	1.4	1.0 - 2.0	0.519	0.9	0.6 - 1.3
Multivariate analysis									
Parental age*	0.005	6.6	1.8 - 24.3	0.012	21.4	2 - 232.6	0.021	5.5	1.3 - 23.8
Parental education†	0.001	9.1	2.4 - 33.7	0.002	18.9	2.8 - 125.3	0.008	7.0	1.7 - 29.3
CAM for other reason	0.128	4.5	0.6 - 30.8	0.009	25.3	2.3 - 283.7	0.434	2.3	0.3 - 18.8
No of steroids courses	0.436	1.2	0.7 - 2.0	0.021	2.5	1.1 - 5.4	0.908	1.0	0.5 - 1.7

* Age groups 18-30 and 31-45 unified

† Unified into two groups (Standard Grade and Highers; College and University)

However, it is unlikely in the area surveyed that any child with IBD will never visit an NHS clinic and therefore very few who distrust conventional treatment will have been missed. Demonstration of the questionnaire and its completion at home ensured confidentiality and clarity of the questions. The anonymity of the questionnaire allowed undistorted responses and encouraged reliable answers and may have contributed to the high response rate.

Consistent with previous paediatric studies (64-66;544), this study found that a large proportion of IBD children use CAM, to a similar or greater extent than adults (63;541). More than half of the children used some form of CAM and a quarter visited alternative therapists. This is much higher than the 10% use of alternative therapists reported for the general population in the UK National Omnibus Survey. The estimated use of CAM in this study is lower than the 72% of the survey population in an Australian study, but higher than the 41% found in a multinational study (65), 22% and 51% in a Canadian (544) and American paediatric survey (64) respectively. It is possible that the increased use of CAM in this study reflects an increase over time, but this needs to be verified in a longitudinal study in the same population. A direct comparison between paediatric studies is hampered by differences in the study design, responding population, and definitions of CAM (48). Although this study did not compare the results with those from a healthy cohort, the questionnaire used in this study was specific for the past and current use of CAM for IBD management and also asked participants whether they had used CAM for other medical reason than IBD management. The fact that different answers were given in the section on IBD CAM use and in the section of CAM use for other reasons or for parental use shows that the questionnaire used was clear and specific for CAM use for IBD management.

In the current survey, probiotics, dairy free diet and fish oils were the most prevalent types of CAM, followed by aloe and homeopathy. This is consistent with previous paediatric studies in the USA (64) and Australia (66) which used similar questionnaires. In contrast the multicentre survey by Heuschkel and his colleagues (65) found only 6% of their participants used probiotics and only 2% in their centre in London compared to the 44% found in this Scottish survey. The higher use of probiotics in the current study parallels the increase in the number of studies suggesting a beneficial effect in IBD management (75;76) and increased availability and advertising. The belief that dairy products can exacerbate the disease and that patients restrict their consumption has already been well documented (545;546). The lower incidence of multi or megavitamin use than in other studies (65) could be attributed to the questionnaire being specific for vitamin supplements not prescribed by the medical team. It is noteworthy that three of the most commonly used CAM in this survey (probiotics, omega oils and aloe vera) already have controlled trials for adults published in the literature (74;76;547), are suggested through support groups and websites and which could have affected the choice of these treatments. Although it would be interesting to know whether the

patients used these treatments to maintain remission or to suppress a relapse, the questionnaire was not designed to address this.

This study considered a wide range of possible predictors for CAM use. Demographically no relation to age, gender, or disease diagnosis (UC vs CD) has been found in both adult (46;74;76;536;541;547-549) and paediatric surveys and the results are in accordance. Young parental age and higher education level were independently associated with CAM usage, a finding commonly reported in the general paediatric population (550). It may be that young educated parents, are more open or seek information on innovative methods of disease management, or do not trust conventional medicine. It has been reported (12) previously that early onset CD is associated with affluence, however, in this study the spread of patients between deprivation scores based on (SIMD) was roughly equivalent and there was no higher number in the higher affluent scores. Social deprivation was not found to be a predictor of CAM use.

In contrast to other studies that assessed medication on recruitment, this study also considered the total number and types of medication that each patient was on since diagnosis as a better depiction of disease activity history. No association between these indices and use of CAM was found confirming results from other paediatric (66;298) and adult studies (60;537). Although an association between CAM use and steroid use was not found, in contrast to findings in adults by Hilsden (541), an independent association between the number of steroid courses and the use of alternative therapists was evident. This has been previously reported in adults on high intravenous doses of steroids (68;541). No such association was found between repeated courses of EEN and CAM use. Although both repeated steroids and EEN courses reflect disease relapses, these findings suggest that the side effects of steroids and patients' desire to terminate or avoid their use may be more important than disease activity. This was reported as a reason for using CAM in the current study and also by 63% of adults IBD patients in a German survey (68;541).

As EEN is the standard treatment for paediatric CD in the UK, caloric supplementation was not a predictor of CAM use in this study unlike that of Markowitz in the USA (64) where different management protocols are followed (256).

Reading about CAM, friends' recommendation and internet searches were the main sources of information about CAM similar to previous adult (549) and paediatric surveys (66). The chronic nature of the disease and the lack of a definite cure are highlighted by the fact that most of the participants chose CAM to complement conventional medicine. Frustration with conventional medicine side effects has been continually reported as the main reason adult IBD patients used CAM (60;65;66;68;541;551) and this coincides with the observation that only 4% of patients with gastroesophageal reflux disease (for which effective medication with minimal side effects is available) tried to treat their condition with CAM (552). Moreover

recently medication side effects have been documented as the most important parental concern for their child with IBD (553) and could justify the need for alternative therapies with fewer or no side effects.

Most surveys in both adults and paediatric patients have shown a self-reported benefit of CAM use (65;66;539). In the present study half of the users considered CAM use effective, although any possible improvement was subjectively assessed by the patients themselves and it is impossible to distinguish whether this was attributed to the concomitant conventional medication, to the CAM use per se or to a spontaneous improvement due to a documented placebo effect (373). Only two patients reported an adverse effect in contrast with adult surveys (549). This is not surprising as the majority of therapies used by participants in the present study included nutritional remedies and exclusion diets generally characterised with good safety records.

Fewer patients in this study told their doctor they were using CAM than in previous studies (65). This lack of disclosure is undesirable as CAM may impact on conventional medicine or have side effects. The majority of the respondents wanted their doctor to be supportive of CAM. Adult IBD patients in Germany (68) believed that a combination of methods using CAM and conventional medicine should be offered within one integrative clinical institution.

Most of the parents in the present study said “they would be happy for their child to use of CAM” but agreed that “not much is known about CAM safety”. The chronic nature of the condition and the desire to ensure the best possible outcome for their child is reflected in that almost 90% of the respondents stated they would choose CAM if they felt these were useful and some of them would do so even if medical advice was against it.

Use of CAM is high in paediatric IBD patients in Scotland and paradoxically parallels the development of new and more effective medications and modes of disease management. With increasing use of CAM more quality studies and clinical trials are needed to shed light on their efficacy and better inform parents, patients and medical staff. So far the results of clinical trials are too limited and most too flawed to suggest use of CAM in clinical practice.

Rather than actively discouraging or encouraging use, health professionals should encourage IBD patients to report CAM use and should be aware of all the alternative therapeutic choices that are available to their patients. Several hospitals worldwide have published policies and guidelines on CAM use and the American Academy of Paediatrics published a relevant article on counselling families who choose CAM for their child with chronic illness or disability (554).

CHAPTER THREE

Prevalence and predictors of anaemia in paediatric IBD

OUTLINE

This chapter describes a retrospective study of the prevalence of anaemia at diagnosis in paediatric patients with IBD and its progression over a one year follow up using case review analysis. Predictors of anaemia incidence at diagnosis and at follow up were explored.

3.1. Introduction

Anaemia is one of the extraintestinal co-morbidities in IBD. Overt or occult intestinal bleeding is among the major presenting symptoms of the new IBD patient (33) and a drop in haemoglobin levels occurs with each flare up (246). In IBD two types of anaemia prevail, iron deficient anaemia, due to an iron poor diet and/or haemoglobin losses through gastrointestinal bleeding, and the ACD with unspecified pathogenesis (236). This topic has been reviewed extensively in Section 1.4.

Since all human cells, tissues and organs depend on an adequate oxygen supply, the onset of anaemia is accompanied by a variety of clinical manifestations for both the children and adults (Section 1.4.2). Nonetheless compared to the average awareness of other extraintestinal disease complications, such as arthritis and poor nutritional status, the issue of anaemia in IBD disease usually receives little attention from the clinician. While a possible explanation would be a low prevalence of anaemia in IBD, this is not supported by current evidence (229;236).

Thus far the evidence of the prevalence of anaemia in IBD patients comes mainly from adult studies and relevant studies in children are scarce (Table 1.4.5). Moreover all but one of the paediatric studies (243) were undertaken in small (<100) patient groups (Table 1.4.5). In most of these studies, anaemia prevalence was a secondary rather than a primary outcome (228) and thus may have included selective samples that do not allow the extrapolation and generalization of the findings to the general IBD population. Moreover studies that recruited children with longstanding IBD (242) may have underestimated the true anaemia prevalence at diagnosis, due to a confounding effect of concomitant medical intervention. Although most of the authors used haemoglobin concentration thresholds to define anaemia, the definition varied remarkably among the studies (Table 1.4.5). In fact most of the authors (252;253) failed to cite an appropriate reference source for the definition of anaemia. As normal levels of haemoglobin vary considerably with age and gender (205), it is essential to use appropriate and specific cut offs.

Potential predictors of anaemia were addressed in a few studies. Mack and colleagues (243) showed that increased disease activity was a significant predictor of anaemia at diagnosis in paediatric IBD. Similarly Weinstein and colleagues (83), although they did not describe the prevalence of anaemia, found that haemoglobin was lower in CD

than UC, a finding stronger in the oldest age group of patients (≥ 11 y). Only one study (247), published in abstract form, measured prevalence of anaemia in paediatric CD and attempted to link it with the nutritional status of the patients and disease characteristics at diagnosis. In this study, anaemic patients had higher values of PCDAI and ESR than their non-anaemic peers, and the presence of upper GI tract disease was significantly associated with anaemia incidence. Gender, duration of symptoms prior to diagnosis, body composition, or growth parameters were not predictors of anaemia at diagnosis (247).

In summary the prevalence of anaemia in paediatric IBD is under-documented and not well assessed. Most of the evidence comes from small studies using variable definitions of anaemia and do not allow a direct comparison between studies or assessment of temporal changes of anaemia prevalence. Only one study has been published in the UK, the rest of Europe with a small sample of 32 UC children (252). Whether anaemia is more common in groups of patients, with specific disease characteristics has not been studied extensively in paediatric studies. In light of new consensus guidelines on the classification of disease location and behaviour (6) further studies should be conducted to address whether disease phenotype predisposes to anaemia. Undernutrition and growth parameters may also differ between anaemic and non-anaemic IBD patients and should be investigated further.

To the best of our knowledge, no study has measured the outcome and prognosis of anaemia, during the natural history course of the disease. As early and better diagnosis is now available and new treatments are available that induce mucosal healing and tackle the inflammatory cascade at the initial stages, a decrease in the prevalence of anaemia at presentation and at follow up should be expected.

The aim of this study was to measure the prevalence of anaemia at diagnosis in a large representative population of children with IBD. Predictors of the incidence of anaemia at diagnosis and its progression at follow up were explored.

3.2. Subjects and methods

All paediatric patients that attended the IBD outpatient clinic between 2002 and 2007 were considered for analysis. Although no prospective IBD database existed at the time of the study, all the eligible patients were identified via their electronic attendance records of the IBD outpatient clinic. These were accessible through the hospital electronic patient database.

For the purpose of this study two groups of patients were characterized. The first to describe the prevalence and predictors of anaemia at diagnosis, and a sub-group of this to look at the progress of anaemia over a 1y follow up.

Patients' demographics and disease characteristics were retrieved from the hospital internal electronic patient database (Table 3.1). Patients whose haemoglobin level was not

available at diagnosis and those with concomitant illness that could potentially affect haematological parameters, independently of IBD, were excluded.

For the sub-group analysis, the case notes of a random sample of patients were reviewed and information on disease, medication, and anthropometry (Table 3.1) was culled at diagnosis and wherever available at approximately six and 12 month follow up. Although patients who received blood transfusion for acute management of severe anaemia or were on supplementation with oral iron at follow up were included, their results are presented and discussed appropriately.

3.2.1. Statistical analysis

All data were entered into an electronic database and the prevalence of anaemia calculated as the ratio of the number of anaemic patients by the total number of patients. For the definition of anaemia, gender and age specific haemoglobin cut offs were used (205) (Table 1.4.1). A haemoglobin concentration less than 10 mg/dl was characterized as severe anaemia according to WHO recommendations (Table 1.4.1). Patients with haemoglobin concentration lower than their age and gender defined cut offs, but above 10 mg/dl were classified as mildly anaemic. Differences between groups were assessed with 2-sample t-test and analysis of variance (ANOVA) for parametric variables and Mann-Whitney or Kruskal-Wallis for non-normally distributed data. Difference for categorical data was assessed with chi-square test or Fisher's exact test for 2x2 tables with small counts. Potential predictors of anaemia were studied using univariate nominal and binary logistic regression analysis. All predictors with p-value less than 0.1 were entered in a multivariate model and their independent association with the response was assessed using backward stepwise regression analysis. A model with p-values less than 0.05 for all predictors was considered the final model. Odd ratios and 95% confidence intervals were calculated.

3.3. Results

3.3.1. Prevalence and predictors of anaemia at diagnosis

3.3.1.1. Patients demographics and disease characteristics

Between April 2002 and December 2007, 272 IBD patients visited the outpatient clinic at Yorkhill RHSC. As the Yorkhill RHSC is the only tertiary paediatric referral centre for care of

Table 3.1: Demographics, disease, medication, nutrition, biochemical and haematological, data collected for the study of anaemia in paediatric IBD at diagnosis (whole group) and at follow up (sub-group)

Data		Whole group	Subgroup
Demographics			
	Gender	√	√
	Age(decimal y)	√	√
	Social deprivation score*	√	
Disease			
	Age at diagnosis	√	√
	Diagnosis delay		√
	Type of disease	√	√
	Site of involvement	√	√
	Bowel motility at diagnosis		√
	Blood in stool at diagnosis		√
Medication			
	EEN; (No of courses)		√
	Oral steroids; (No of courses)		√
	IV steroids		√
	Aminosalicylates		√
	AZA		√
	Methotrexate		√
	Infliximab		√
	Antibiotics		√
	Vitamins-Minerals		√
	Oral iron		√
	Caloric supplements		√
	Gut resection		√
Anthropometry			
	Body weight		√
	Body height		√
	Weight loss at diagnosis		√
Haematology			
	Red blood cells count	√	√
	Haemoglobin concentration	√	√
	Haematocrit concentration	√	√
	MCV concentration	√	√
	MCH concentration	√	√
Inflammatory markers			
	ESR	√	√
	CRP	√	√
Biochemistry	Albumin	√	√
	Folate (serum & RBC)	√	
	B_{12}	√	
	Ferritin	√	

* Scottish Index of Multiple Deprivation

all IBD children in the West of Scotland region, the identifiable sample of this corresponded roughly to the whole population of paediatric IBD patients.

All patients had been diagnosed according to standard clinical, endoscopic, histological and radiological findings. Those patients whose haematology profile was not available close to the date of diagnosis (n=34) or were suffering from other concomitant conditions that could impact on their haematological profile (cystic fibrosis; n=2) were

excluded. Overall 236 patients (87% of the population) had measurements of haematology at diagnosis and fulfilled the inclusion criteria.

The majority of participants had CD whereas for 5.5% a diagnosis of IC was deemed appropriate (Table 3.2). There was a higher prevalence of males to females, due to more males than females being diagnosed with CD. No gender predominance was observed for UC or IC patients. The mean age at diagnosis was 10.6 y (SD; 3.1) with UC patients diagnosed at an earlier age than CD ($p<0.04$). No gender effect was observed regarding the age at diagnosis (Table 3.2).

The majority of the CD patients had involvement of the colon (Table 3.2) but involvement of the upper digestive tract and ileum were also common. None of the UC patients studied had isolated proctitis as opposed to the high prevalence of pancolitis, which was the commonest site of disease involvement.

No difference in the social deprivation score (SIMD 2004) was found among the different types of disease. Indeed the IBD population was equally distributed in the five classes of social deprivation (Table 3.2).

3.3.1.2. Basic haematology profile at diagnosis

Full blood counts were available for 233 of the patients whereas three had only haemoglobin measurements (Table 3.2). All these three patients had been transferred from a district general hospital and no local haematology report was available close to the time of diagnosis.

Approximately 50% of the patients had haemoglobin less than 10.8 g/dl (Fig 5) . As a group, girls had significantly lower RBC, haemoglobin and haematocrit values (all $p<0.003$) than boys. A disease dependent effect was found for RBC. Red blood count was higher in CD than UC or IC patients ($p<0.0001$) but no similar differences were observed for haemoglobin or haematocrit levels (Table 3.3). However more boys than girls had been diagnosed with CD which may explain these results. No difference was found between boys and girls for mean corpuscular volume and mean corpuscular haemoglobin.

Similar to the whole group analysis, girls with CD had lower RBC, haemoglobin and haematocrit concentration than boys. No gender effect was found for MCV and MCH. In contrast, none of the haematological parameters assessed (RBC, Hb, Hct, MCV, MCH) differed between boys and girls in UC and IBDU children.

Table 3.2: Demographics, social deprivation scores, and disease characteristics of paediatric IBD patients at diagnosis

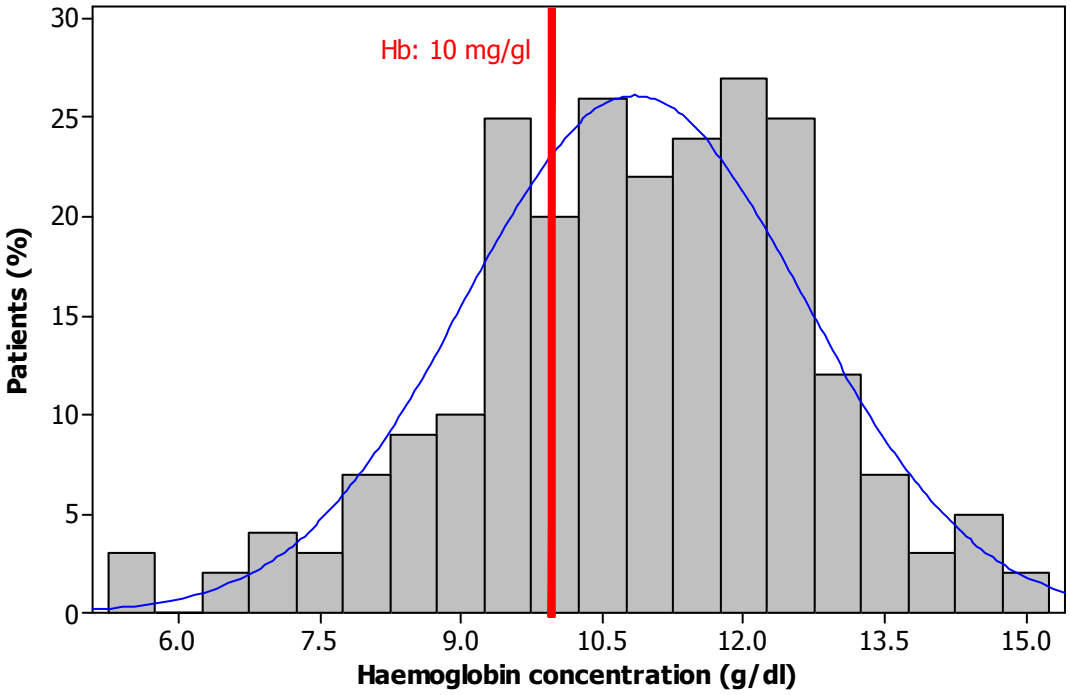
	CD		UC		IBDU		Total
	M	F	M	F	M	F	
Gender (n)	93	58	33	37	7	8	236
Age at diagnosis (mean±SD)	11.1 ± 2.9	11 ± 2.8	9.8 ± 3.5	9.9 ± 3.5	10.8 ± 1.8	8.5 ± 3.5	10.6 ± 3.1
Deprivation Score (n)							
1	17 ; (18)	11 ; (19)	9 ; (27)	5 ; (13)	1 ; (14)	2 ; (25)	45 ; (19)
2	15 ; (16)	8 ; (14)	9 ; (27)	5 ; (13)	0 ; (0)	4 ; (50)	41 ; (17)
3	15 ; (16)	11 ; (19)	6 ; (18)	11 ; (30)	2 ; (29)	0 ; (0)	45 ; (19)
4	21 ; (23)	14 ; (24)	6 ; (18)	9 ; (24)	0 ; (0)	2 ; (25)	52 ; (22)
5	25 ; (27)	13 ; (22)	3 ; (9)	7 ; (19)	4 ; (57)	0 ; (0)	52 ; (22)
Montreal classification (n)*							
L1	3 ; (3)	2 ; (3)					5 ; (2)
L2	30 ; (32)	19 ; (33)					49 ; (21)
L3	54 ; (58)	37 ; (64)					91 ; (39)
L4	61 ; (66)	32 ; (55)					93 ; (39)
B1	82 ; (88)	49 ; (84)					131 ; (55)
B2	9 ; (10)	5 ; (9)					14 ; (6)
B3	1 ; (1)	1 ; (2)					2 ; (1)
E1			0 ; (0)	0 ; (0)			0 ; (0)
E2			2 ; (7)	4 ; (12)			6 ; (2)
E3			27 ; (93)	29 ; (88)			56 ; (24)

* P denominator to characterize perianal disease was not available

Table 3.3: Haematological characteristics of paediatric IBD patients at diagnosis (mean ± SD)

	CD		UC		IBDU		Total
	M	F	M	F	M	F	
RBC (x 10^{12})	4.6 ± 0.4	4.4 ± 0.5	4.3 ± 0.4	4.2 ± 0.5	4.5 ± 0.8	3.8 ± 0.6	4.4 ± 0.5
Haemoglobin (g/dl)	11.1 ± 1.6	10.6 ± 1.5	10.9 ± 1.6	10.5 ± 2.3	12.2 ± 2.8	9.3 ± 2	10.8 ± 1.8
Haematocrit (%)	35 ± 3.8	33.4 ± 4.0	33.7 ± 3.9	32.7 ± 5.9	35.5 ± 6.7	30.7 ± 4.9	34.0 ± 4.5
MCV (fl)	75.8 ± 6.6	76.6 ± 6.5	78.3 ± 5.1	78.1 ± 8.4	70.4 ± 18.2	80.4 ± 7.5	76.7 ± 7.4
MCH (pg)	24.2 ± 3.3	24.4 ± 3.1	25.2 ± 2.3	25 ± 3.7	26.6 ± 3.4	24.3 ± 3.4	24.6 ± 3.2

Figure 5: Distribution of haemoglobin concentration (mg/dl) in paediatric patients with CD at diagnosis



3.3.1.3. Systemic markers of disease activity at diagnosis in IBD patients

ESR, CRP, albumin and platelets levels were available for 195, 205, 202 and 225 patients respectively (Table 3.4). Compared to UC children, CD patients had significantly higher CRP concentration and ESR ($p<0.0001$ & $p=0.004$ respectively) and lower serum levels of albumin at diagnosis ($p<0.0001$). There was no difference for the same markers between UC and IBDU patients (Table 3.4). ESR and CRP were higher in CD than IBDU. No difference was found between boys and girls for any of the systemic markers of disease activity. The mean platelet concentration was higher in girls than boys but did not reach statistical significance ($p=0.057$).

Overall more than 70% of patients presented at diagnosis with raised ESR, whereas 59%, 52%, and 48% had abnormal levels of CRP, albumin and platelets respectively (Table 3.4). There was no difference between genders. More CD patients (38.5%) had all the systemic markers of disease activity abnormal compared to UC (9.5%) and IBDU patients (0%). Noteworthy none of the IBDU patients had all of the systemic markers of disease activity abnormal. In fact the majority of these patients presented with normal markers of

inflammation at diagnosis, although the numbers are too small and preclude statistical analysis. Twenty nine percent of IBD children had all the systemic markers of disease activity abnormal as opposed to 14% of the cohort with respective values within the normal reference range.

3.3.1.4. Prevalence of anaemia

Defining anaemia with gender, and age specific haemoglobin concentration cut offs (205), 73% of the subjects presented with anaemia and 32% suffered from severe anaemia (haemoglobin concentration less than 10) (Fig 5). Severe anaemia was more common in girls than in boys (Fig 6). No difference in the prevalence of anaemia was found between the different types of disease (Fig 7).

Figure 6: Prevalence of anaemia and degree of severity between IBD boys and girls at diagnosis

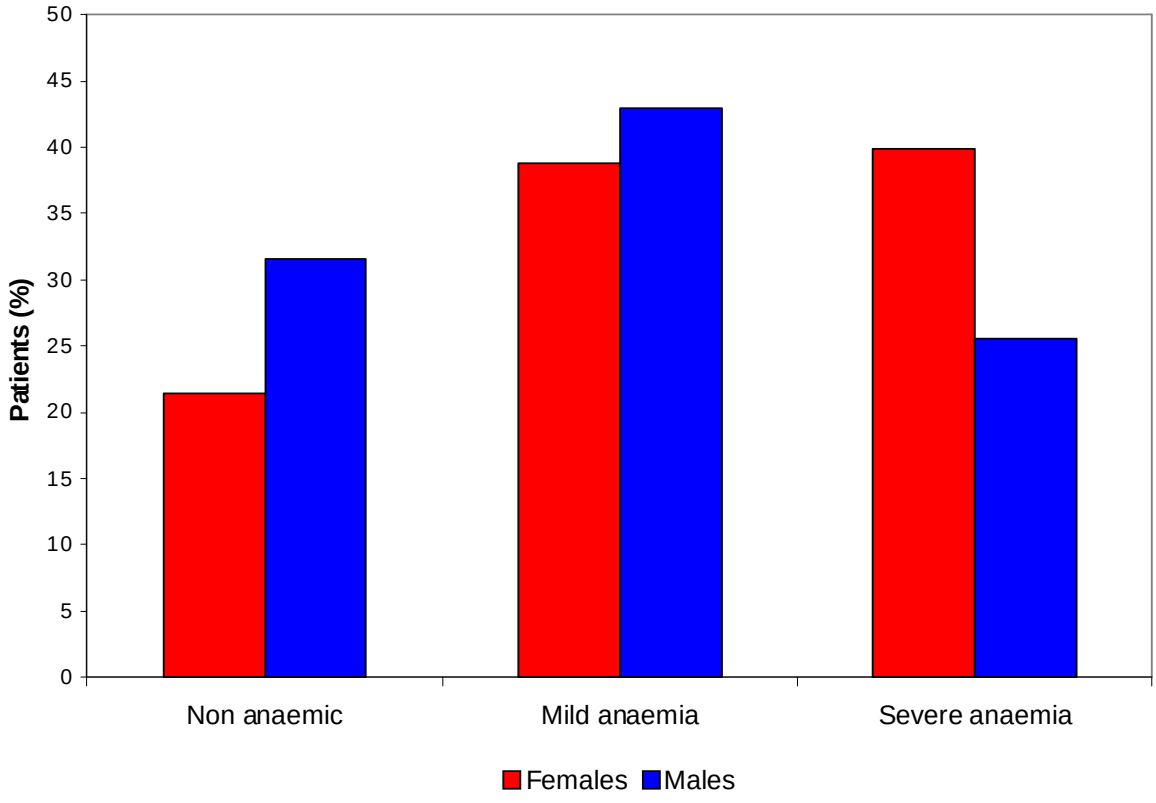
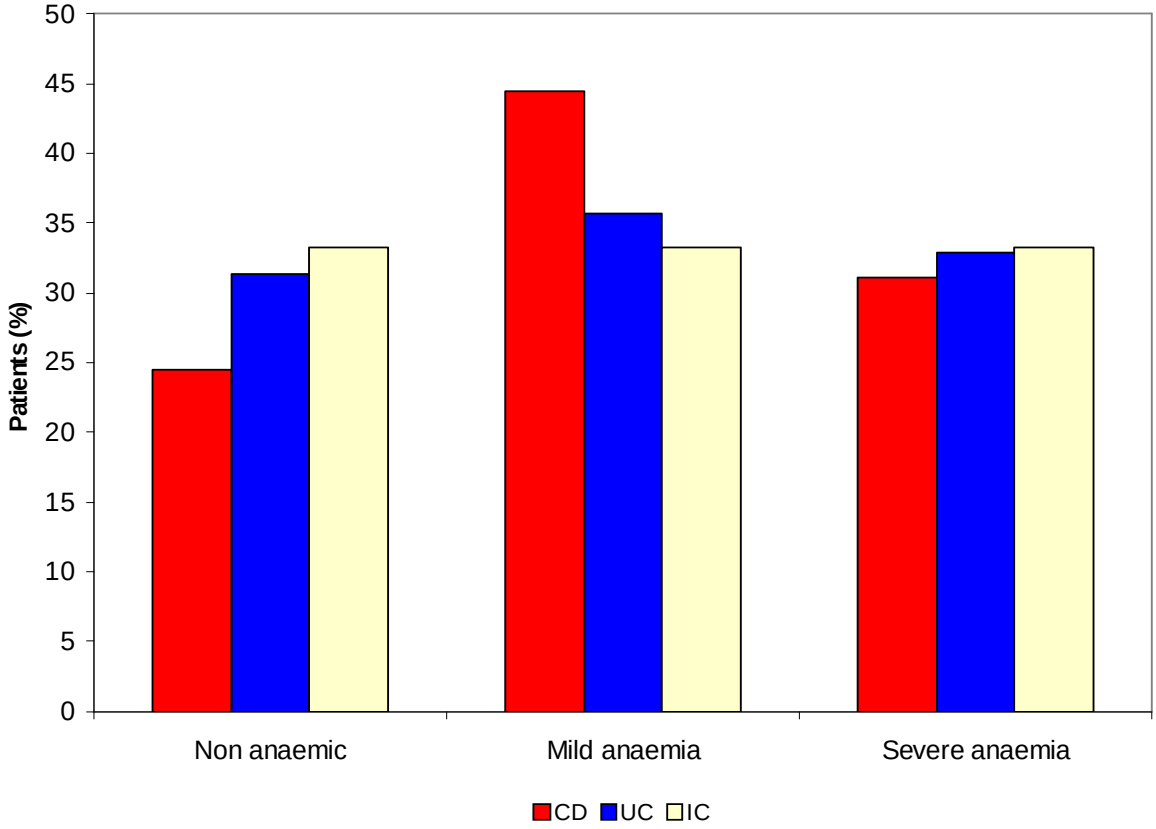


Figure 7: Prevalence of anaemia and degree of severity according to type of IBD in children



3.3.1.5. Correlation of haemoglobin concentration with systemic markers of disease activity

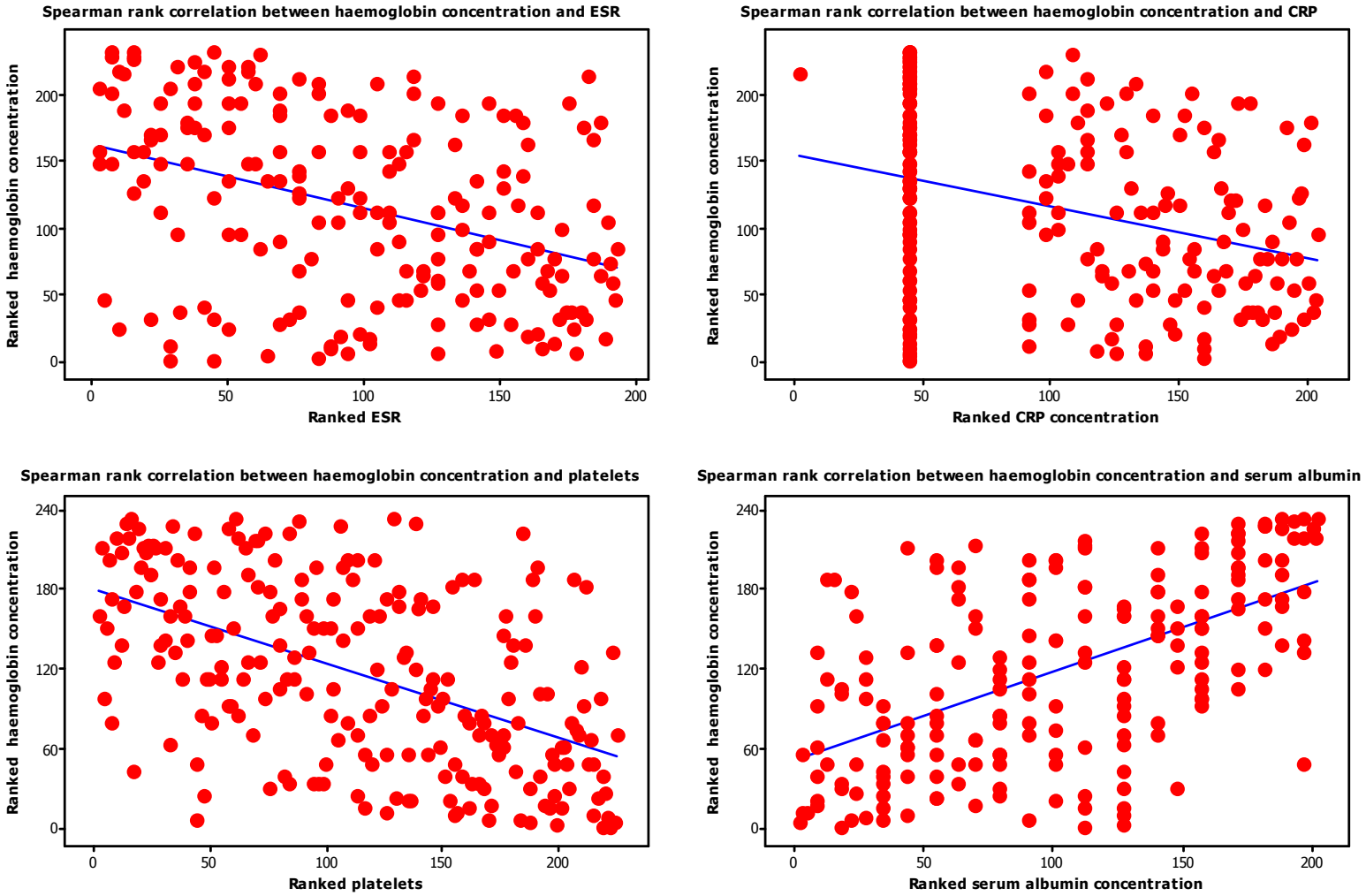
Since ESR, CRP platelets and albumin concentration did not follow a normal distribution their concentrations were ranked and Spearman correlation calculated as the most appropriate measure of their association with haemoglobin. ESR, CRP and platelets counts were negatively correlated (r=-0.38, -0.30 and –0.52 respectively) (all $p<0.0001$) with haemoglobin concentration (Fig 8). Accordingly albumin was positively and strongly associated with haemoglobin levels (r=0.56 $p<0.0001$).

Table 3.4: Systemic markers of disease activity in paediatric patients with IBD at diagnosis

	CD		UC		IBDU		Total
	M	F	M	F	M	F	
ESR (mm/hr) (mean ± SD)	40.4 ± 23.9	46.8 ± 25.7	29.9 ± 23.3	35.6 ± 24.0	25.2 ± 33.4	26.3 ± 12	39.2 ± 24.8
CRP (mg/l) (mean ± SD)	33.9 ± 39.7	45.9 ± 54.6	13.6 ± 26.2	28.7 ± 52.5	7.0 ± 0.0	20 ± 19	32.3 ± 44.6
Albumin (g/l) (mean ± SD)	32.3 ± 6.1	31.6 ± 7.4	36.2 ± 4.8	36.2 ± 7.3	34.5 ± 11.8	31.1 ± 8.5	33.3 ± 6.9
Platelets x 10^9 (mean ± SD)	474.4 ± 145.1	528.5 ± 176.6	442.1 ± 179.4	489.6 ± 171.7	394 ±158	459.1 ±164.4	482.4 ±164. 3
Abnormal ESR n;(%)	60; (77)	44; (86)	14; (58)	20; (67)	2; (40)	4; (57)	144; (74)
Adnormal CRP n;(%)	64; (74)	35; (70)	8; (29)	10; (33)	0; (0)	3; (60)	120; (59)
Abnormal albumin n;(%)	55; (65)	29; (62)	10; (38)	7; (23)	1; (17)	3; (43)	105; (52)
Abnormal platelets n;(%)	45; (51)	29; (55)	10; (34)	17; (46)	3; (43)	4; (50)	109; (48)
Disease activity score n;(%)*							
0	7; (10)	5; (13)	4; 22	4; 17	2; 40	1; 25	23; (14)
1	9; (12)	;3 (8)	5; 28	9; 37	2; 40	0; 0	28; (17)
2	9; (13)	6; (15)	1; 6	4; 17	0; 0	2; 50	22; (14)
3	20; (29)	8; (21)	6; 33	5; 21	1; 20	1; 25	41; (26)
4	25; (36)	17; (43)	2; 11	2; 8	0; 0	0; 0	46; (29)

* Sum of abnormal systemic markers of disease activity [CRP>7 mg/L), ESR>20 mm/h, Albumin <35 g/L, Platelets>450 x 10^9)]

Figure 8: Rank correlation between haemoglobin concentration and systemic markers of disease activity in cildren with IBD



3.3.1.6. Predictors of anaemia incidence at diagnosis

Potential predictors of anaemia were defined a priori. Disease type and anatomic location, age at diagnosis, gender, social deprivation, and systemic markers of disease activity were tested with nominal logistic regression to assess their independent association with anaemia prevalence and degree (moderate or severe) of severity.

In the univariate analysis females were more likely to be severely anaemic than boys (p=0.005) (Table 3.5). Increased concentrations of ESR, CRP, platelets and low albumin concentration were strongly associated with mild and severe anaemia (Table 3.5). Similarly abnormal levels of ESR (> 20 mm/h), CRP (>7 mg/l) platelets (> 450 x 10^9) and albumin (< 35 g/l) were also associated with mild and severe anaemia (Table 3.5).

In the multivariate analysis, disease activity score (sum of the number of abnormal systemic markers of disease activity) was the only independent predictor of mild and severe anaemia. Indeed the risk of being anaemic or severely anaemic was substantially higher with an increasing number of total abnormal systemic markers of disease activity. Any child with all systemic markers of disease activity abnormal at diagnosis (ESR, CRP, albumin) had 96 times more chance to be severely anaemic compared with those IBD children who presented with normal serology. The odd ratios of being anaemic augmented proportionally with the total number of abnormal systemic markers of disease activity (Table 3.5). This effect was stronger in severe than mild anaemia. Gender was also a predictor of anaemia and its degree of severity with girls being more severely anaemic than boys (Table 3.5).

Table 3.5: Predictors of anaemia incidence at diagnosis in paediatric IBD patients with univariate and multivariate regression analysis

Predictors	**Mild anaemia**			**Severe anaemia**		
	Univar p-value	OR	95%C	Univar p-value	OR	95%C
Sex (male)	NS			0.017	0.43	0.22-0.86
ESR (mm/hr)	0.0001	1.04	1.02-1.06	0.0001	1.05	1.03-1.07
CRP (mg/l)	0.007	1.02	1.01-1.04	0.003	1.03	1.01-1.04
Albumin (g/l)	0.0001	0.88	0.82-0.94	0.0001	0.78	0.72-0.84
Platelet (x 10^9)	0.0001	1.01	1.00-1.01	0.0001	1.01	1.01-1.02
Abnormal ESR*	0.001	3.78	1.74-8.21	0.0001	5.11	2.16-12.06
Abnormal CRP*	0.005	2.79	1.37-5.68	0.0001	3.78	1.79-7.97
Abnormal albumin*	0.007	2.73	1.31-5.69	0.0001	10.0	4.33-23.07
Abnormal platelet*	0.0001	4.14	1.91-8.98	0.0001	14.42	6.22-33.43
Disease activity score*						
One	NS			NS		
Two	NS			NS		
Three	0.01	5.83	1.52-22.41	0.0001	26.25	4.64-148.38
Four	0.001	13.33	2.82-63.11	0.0001	65.50	10.12-450.08

				Multivar p-value	OR	95% CI
Gender (male)	0.322	0.64	0.26-1.56	0.003	0.20	0.07-0.58
Disease activity score*						
One	0.139	2.5	0.74-8.61	0.870	1.2	0.14-10.17
Two	0.141	2.8	0.71-10.63	0.128	4.38	0.65-29.42
Three	0.007	6.5	1.65-25.65	0.0001	40.2	6.44-250.93
Four	0.001	14.63	3.03-70.61	0.0001	96.27	13.26-699.12

*Sum of abnormal systemic markers of disease activity [CRP>7 mg/L), ESR>20 mm/h, Albumin <35 g/L, Platelets>450 x 10^9)]

Age at diagnosis, social deprivation score, site of disease involvement, disease behaviour (inflammatory, stricturing, fistulising) checked independently for CD and UC did not predict anaemia.

3.3.1.7. Temporal changes of anaemia prevalence at diagnosis

No apparent trend in the prevalence or degree of anaemia severity was observed (Table 3.6) between 2001-2007.

Table 3.6: Temporal trend of anaemia prevalence and severity at diagnosis in paediatric patients with IBD diagnosed between 2001 and 2007

Patients	2001	2002	2003	2004	2005	2006	2007
All anaemic (n)	12	22	17	19	24	28	22
All non-anaemic (n)	5	8	11	8	6	10	9
Total diagnosed (n)	17	30	28	27	30	38	31
Total anaemic (%)	70.6	73.3	60.7	70.4	80	73.7	71
Mild anaemic (%)	23.5	46.7	32.1	44.4	66.7	36.8	25.8
Severely anaemic (%)	47.1	26.7	28.6	25.9	13.3	36.8	45.2

3.3.1.8. Classification of anaemia type

Due to the retrospective design of this study it was difficult to differentiate among the different types of anaemia. A rough approach was attempted evaluating haematology parameters on the size (MCV) and colour of erythrocytes (MCH). No patient had features of macrocytic anaemia (MCV > 100). Four patients had MCV values greater than 90 fl. Of these four patients, one was severely anaemic (Hb; 7.7), one was mildly anaemic (Hb; 11.9) and two had normal levels of haemoglobin. Overall 41% of the patients had low MCV according to Dallman's (205) reference range. Similarly setting 27 pg as the lower reference threshold of mean corpuscular haemoglobin, 76% of the subjects had suboptimal levels consistent with hypochromic anaemia.

3.3.1.9. Ferritin, folate and B_{12} concentration

For 122 children information on ferritin concentration was available within four months of diagnosis. Ninety one of them were anaemic but 29 patients had normal levels of ferritin (> 10 μg/l) and normal mean corpuscular volume reflecting normocytic anaemia possibly attributed to ACD. Serum ferritin concentration was positively associated with CRP (Spearman r: 0.33 $p<0.001$) and negatively with serum albumin (Spearman r: –0.21 $p<0.03$) and was significantly higher in CD than in UC patients.

Twenty five and 26 patients had measurements of folate (serum & red blood cells) and B_{12} respectively within four months of diagnosis. No patient had suboptimal levels

compared to the hospital reference range [(B_{12}:193-982 ng/l); (RBC folate: 93-641 µg/l); (serum folate: 3-17 µg/l)]. Erythrocyte folate concentration was significantly higher in CD than in UC patients (Table 3.7). Serum but not RBC concentration of folate was negatively associated with platelets concentration (Spearman r -0.50 p<0.02).

Table 3.7: Systemic concentrations of serum ferritin, folate and vitamin B_{12} levels in paediatric patients with IBD at diagnosis

Vitamin/Ferritin	CD (Median Range)	UC (Median Range)	P-value*
Ferritin (ng/l)	23.0 2.0- 343.0	12.5 2.0- 226.0	0.02
Serum folate (µg/l)	9.2 4.2- 15.8	7.5 2.7- 16.6	0.71
RBC folate (µg/l)	517.0 237.0-1195.0	251.0 236.0- 752.0	0.02
Serum B_{12} (ng/l)	621.0 246.0-1879.0	807 251.0-1611.0	0.75

* Mann Whitney Test

3.3.2 Prevalence and predictors of anaemia at follow up

3.3.2.1. Patients characteristics

The medical case notes were available for review in 179 children. Of these 12 were recently diagnosed and no follow up data were available at six months. One hundred and twenty-nine children had follow up measurements of haemoglobin at six and 12 months post-diagnosis. For the rest (38 patients) follow up measurements of haemoglobin were recorded either at six or 12 months (Fig 9). The disease and demographic characteristics of the patients are displayed in Table 3.8. There was no statistical significant difference between the whole group presented above and the subgroup of the follow up analysis.

The type of medical treatment and the total number of steroid and completed EEN courses for each patient were collected from diagnosis through one year follow up. The proportion of patients on different treatments is presented in Table 3.9. As expected the majority of CD and UC patients were treated with the two common mainstream first line treatments, EEN and steroids respectively. Aminosalicylates were the mainstream treatment to maintain disease remission in both diseases. Iron supplements had been used by 1/3 of the CD and IBDU patients and half of the UC patients. 4% of the patients had a major surgery with part of the gut resected. Blood transfusion was administered in 8% of the patients during the follow up period.

Figure 9: Flow diagram of patients included in the study on the prevalence of anaemia in paediatric IBD patients

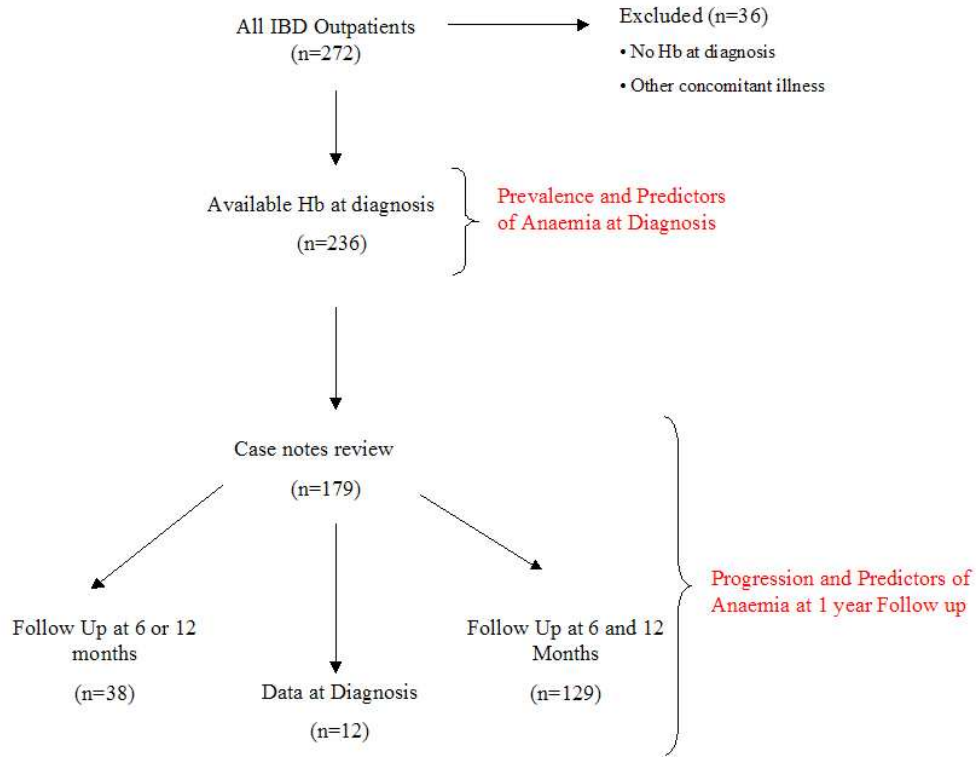


Table 3.8: Demographics, age at diagnosis and diagnosis delay of paediatric IBD patiets who participated in a study on the prevalence of anaemia at follow up

	CD		UC		IBDU		Total
	M	F	M	F	M	F	
Gender (n)	68	45	26	31	4	5	179
Diagnosis age (y) (mean ± SD)	11.0± 2.7	10.6± 2.8	9.9 ± 3.6	9.2 ± 3.3	11.3 ± 1.5	8 ± 4.6	10.3± 3.1
Diagnosis delay (mth) (mean ± SD)	11.7 ±16.8	8.4 ±10.9	10.8 ± 14.2	6.0 ± 4.9	15.2± 15.5	4.3 ± 3.4	9.6 ±13.3

Table 3.9: Total types of medical therapy used by paediatric IBD patients during one year of follow up post-diagnosis

Percent of patients n;(%)	CD	UC	IBDU	All IBD
EEN	80; (78)	2; (5)	2; (9)	84; (50)
Oral steroids	43; (42)	33; (77)	13; (59)	89; (53)
I.V. steroids	13; (13)	6; (14)	5; (23)	24; (14)
5-ASAs	74; (72)	40; (93)	15; (68)	129; (77)
Azathioprine	60; (59)	33; (33)	10; (45)	84; (50)
Methotrexate	12; (12)	0; (0)	0; (0)	12; (7)
Infliximab	6; (6)	1; (2)	1; (5)	8; (5)
Antibiotics	46; (45)	11; (26)	7; (32)	64; (38)
Vitamins	35; (34)	11; (26)	5; (23)	51; (30)
Caloric supplements	47; (46)	2; (9)	4; (9)	53; (32)
Iron supplements	36; (35)	24; (56)	7; (32)	67; (40)
Surgery	3; (3)	2; (5)	1; (5)	6; (4)

Omeprazole/Ranitidine	28; (27)	7; (16)	10; (45)	45; (27)
Rectal steroids	9; (9)	9; (21)	6; (27)	24; (14)
Laxatives	9; (9)	2; (5)	1; (5)	12; (7)
Blood transfusion	6; (6)	5; (10)	3; (13)	14; (8)

3.3.2.2. Sub group anthropometric characteristics at diagnosis and follow up

For 175 of the children basic anthropometric measurements were collected at diagnosis (Table 3.10). Standard deviation scores (z-scores) were calculated using the British normative data (94). A z-score of less than –2 SD for height and BMI were consistent with the definition of growth retardation and underweight respectively (Figs 10 & 11).

BMI z-scores did not differ between boys and girls at diagnosis and 12 months follow up. At six months girls had significantly higher BMI z-scores than boys (p=0.028) (Table 3.11). Children with CD presented with significantly lower BMI and weight z-score than patients with UC or IBDU at diagnosis, and at all time points at follow up (p-values <0.0001). For those patients with follow up anthropometric measurements recorded at six and 12 months, changes since diagnosis were assessed with ANOVA of repeated measures and Bonferroni post-hoc pairwise comparisons. BMI z-scores increased at follow with a mean increase of 0.95 and 1 SD at six and 12 months (p<0.0001). No difference in BMI z-scores was observed between six and 12 months.

At diagnosis 29% of the CD patients compared to 4% in UC and 11% in IBDU presented with BMI z-scores less than –2 SD (p<0.0001). At six and 12 months follow up fewer patients suffered from underweight. Five percent and 1% of the CD children as well as 0% and 5% of UC patients respectively had BMI z-scores keeping in line with the definition of underweight (BMI z-score <-2 SD) at six and 12 months. Although none of the patients was obese (BMI z-score >2SD) at diagnosis, at six and 12 months follow up, over 12% of the UC patients had a BMI z-score above their age and gender defined obesity threshold (BMI z-score >2). On the contrary approximately 2% of CD children were defined as obese (BMI>2 SD) at follow up. Weight loss prior to disease diagnosis was more frequently experienced in CD children than their peers with UC or IBDU (p=0.011).

Table 3.10: Anthropometric characteristics (mean ± SD), and prevalence (%) of underweight, obesity and short stature in newly diagnosed paediatric IBD patients

	CD		UC		IBDU*		Total
	M	F	M	F	M	F	
Diagnosis weight (kg)	31.3 ± 11.2	31.5 ± 11.5	32.7 ± 14.2	30.8 ± 12.4	33.1 ± 5.2	32.1 ± 20.6	31.5 ± 12.1
Weight z-score	-1.2 ± 1.4	-0.9 ± 1.1	-0.3 ± 1.1	-0.4 ± 1.0	-0.5 ± 1.0	0.2 ± 2.0	-0.8 ± 1.3
Diagnosis height (cm)	140 ± 17.6	137.5 ± 17.8	137 ± 21.9	133.6 ± 19.4	144.6 ± 7.1	127.6 ± 30.1	137.6 ± 18.8
Height z-score	-0.6 ± 1.2	-0.6 ± 0.9	-0.3 ± 1.1	-0.2 ± 1.0	0 ± 0.6	0.5 ± 2.2	-0.5 ± 1.1
BMI (kg/m^2)	15.6 ± 2.5	16.2 ± 3	17.1 ± 2.5	16.5 ± 2.5	15.7 ± 1.4	17.6 ± 3.7	16.2 ± 2.6
BMI z-score	-1.2 ± 1.6	-0.9 ± 1.4	-0.2 ± 1.2	-0.4 ± 1.0	-0.9 ± 1.0	0 ± 1.5	-0.8 ± 1.4
Height z-score<-2 n;(%)	11; (17)	2 ; (5)	1 ; (4)	0 ; (0)	0 ; (0)	1; (20)	15 ;(9)
BMI z-score<-2 n;(%)	22 ;(33)	9 ;(21)	2 ;(8)	0 ;(0)	1 ;(25)	0; (0)	34; 20
BMI z-score>2 n;(%)	0 ; (0)	0 ; (0)	0 ; (0)	0 ; (0)	0 ; (0)	0 ;(0)	0 ; (0)
Weight loss n;(%)	43 ;(74)	32 ;(82)	58 ;(11)	61 ;(11)	25 ; (1)	50 ;(2)	70 ; (103)

Table 3.11: Anthropometric characteristics (mean ± SD), and prevalence (%) of underweight, obesity and short stature of paediatric IBD patients at 6 mth post-diagnosis (mean ± SD)

	CD		UC		IBDU		Total
	M	F	M	F	M	F	
Weight (kg) at 6 mth	36.0 ± 13.0	36.8 ± 11.6	37.5 ± 14.5	38.1 ± 18.0	36.2 ± 6.2	38.6 ± 27.2	36.8 ± 14.0
Weight z-score (SD) at 6 mth	-0.6 ± 1.3	-0.4 ± 0.9	0.3 ± 1.0	0.5 ± 1.1	-0.3 ± 1.1	0.8 ± 1.7	-0.2 ± 1.2
Height (cm) at 6 mth	140.7 ± 17.6	140.1 ± 17.1	137.3 ± 21.4	134.1 ± 20.8	143.7 ± 7.8	127.6 ± 29.9	138.6 ± 18.9
Height z-score (SD) at 6 mth	-0.8 ± 1.2	-0.5 ± 0.9	-0.4 ± 0.7	-0.3 ± 1.1	-0.5 ± 0.5	-0.2 ± 1.5	-0.6 ± 1.1
BMI (kg/m^2) at 6 mth	17.6 ± 3.0	18.0 ± 2.5	19.0 ± 3.1	19.8 ± 4.3	16.3 ± 1.5	20.7 ± 5.8	18.3 ± 3.4
BMI z-score (SD) at 6 mth	-0.2 ± 1.3	0.0 ± 0.9	0.7 ± 1.0	0.8 ± 1	-0.6 ± 1.3	1.2 ± 1.1	0.2 ± 1.2
Height z-score<-2 at 6 mth n;(%)	11; (17)	3; (8)	0; (0)	1; (4)	0; (0)	1; (20)	16; (10)
BMI z-score<-2 at 6 mth n;(%)	5; (8)	0; (0)	0; (0)	0; (0)	0; (0)	0; (0)	5; (3)
BMI z-score>2 at 6 mth n;(%)	3; (5)	0; (0)	2; (11)	4; (15)	0; (0)	2; (40)	11; (7)

Table 3.12: Anthropometric characteristics (mean ± SD), prevalence (%) of underweight, obesity and short stature of paediatric IBD patients at 12 mth post-diagnosis (mean; SD)

	CD		UC		IBDU		Total
	M	F	M	F	M	F	
Weight (kg) at 12 mth	37.7 ± 12.7	37.5 ± 11.6	40 ± 18.3	40.1 ± 19.3	38.8 ± 11.1	38.4 ± 28.1	38.4 ± 14.5
Weight z-score (SD) at 12 mth	-0.6 ± 1.1	-0.4 ± 0.9	0.4 ± 1.2	0.3 ± 1.6	-0.1 ± 0.3	-0.3 ± 2.0	-0.3 ± 1.2
Height (cm) at 12 mth	144 ± 18.3	141.2 ± 16.5	140.9 ± 23.5	140 ± 18.7	147 ± 8.5	130.6 ± 27.7	142 ± 18.5
Height z-score (SD) at 12 mth	-0.9 ± 1.2	-0.7 ± 0.9	-0.2 ± 1.0	0 ± 1.2	-0.2 ± 0.6	-1.7 ± 0.7	-0.6 ± 1.1
BMI (kg/m^2) at 12 mth	17.6 ± 2.4	18.2 ± 2.6	19 ± 3.3	19.3 ± 5.6	17.8 ± 3.1	20.4 ± 7.6	18.3 ± 3.3
BMI z-score (SD) at 12 mth	-0.2 ± 1.0	0.0 ± 0.9	0.7 ± 1.3	0.3 ± 1.7	0.0 ± 1.0	0.8 ± 1.7	0.1 ± 1.2
Height z-score<-2 at 12 mth n;(%)	8; (14)	4; (11)	0; (0)	0; (0)	0; (0)	1; (50)	(9)
BMI z-score<-2 at 12 mth n;(%)	1; (2)	0; (0)	1; (5)	1; (5)	0; (0)	0; (0)	3; (2)
BMI z-score>2 at 12 mth n;(%)	2; (3)	0; (0)	1; (5)	4; (18)	0; (0)	0; (0)	7; (5)

Compared to UC children, CD patients had significantly lower height z-score at diagnosis (p=0.025) and at 12 months follow up (p=0.002) (Table 3.12). Similar to BMI z-score, no difference was found between boys and girls for height. Height z-score decreased by an average of 0.2 SD at six and 12 months follow up (p-<0.0001). No difference in the mean height z-scores change was found between six and 12 months.

Thirteen percent of the CD patients had suboptimal height consistent with the definition of short stature (height z-score <-2). No significant changes were presented at six and 12 months of follow up. Less than 2.5% of the UC patients had height z-score less than –2 SD at any time point over the follow up. Although no clear conclusions can be drawn, growth retardation tended to be more common in CD boys than girls at diagnosis (Fisher's exact test p<0.075). At follow up no difference in the prevalence of growth retardation was observed between the two genders for any type of IBD.

Figure 10: Distribution of height z-scores at diagnosis on IBD paediatric patients

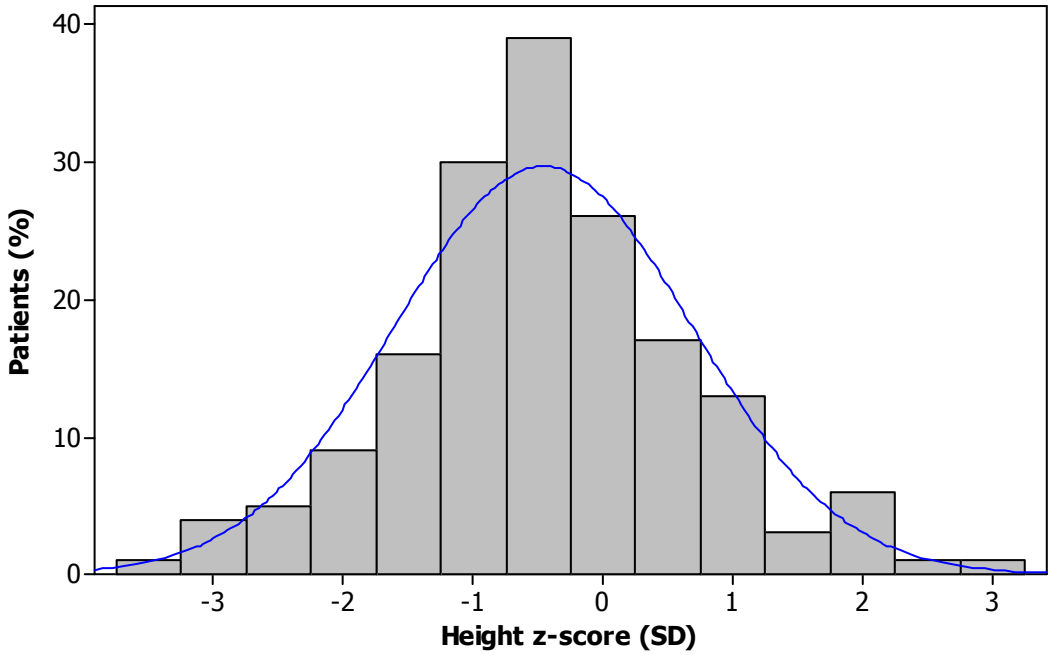
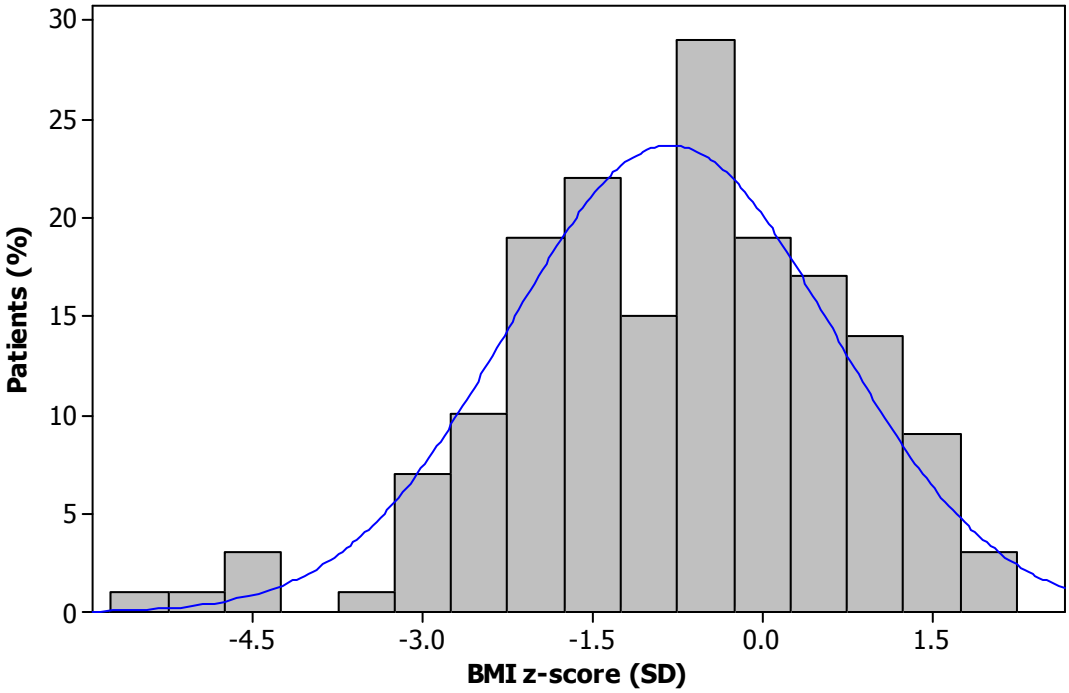


Figure 11: Distribution of BMI z-scores at diagnosis on IBD paediatric patients



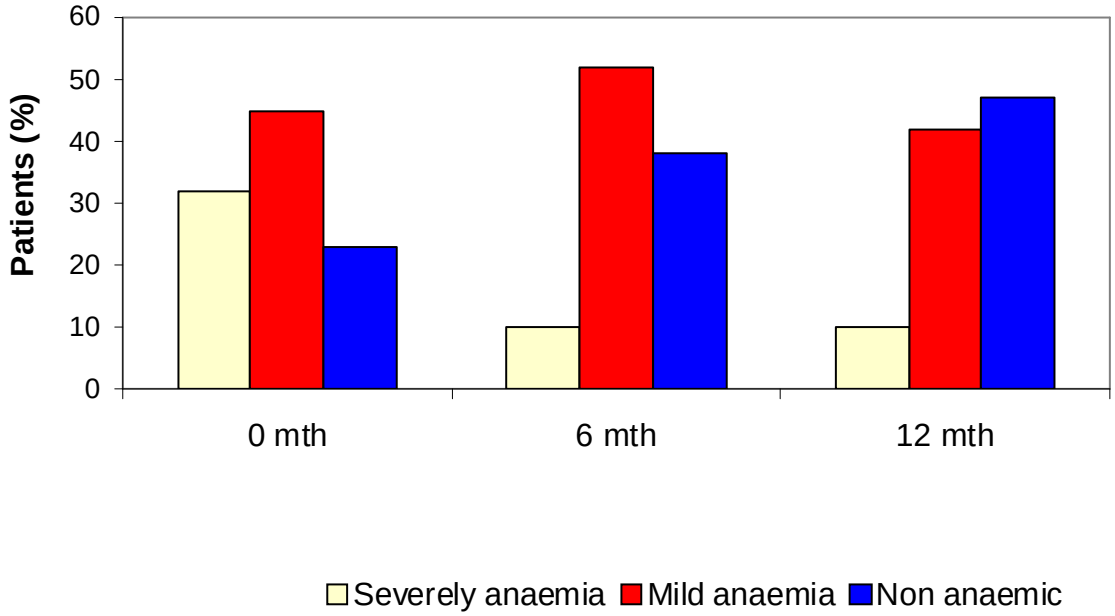
3.3.2.3. Changes in haematology at six and 12 months follow up

Haemoglobin values and full blood counts were available for 179, 153, and 143 patients at diagnosis and at six and 12 months follow up respectively (Table 3.13). The mean haemoglobin concentration was less than 11 g/dl at diagnosis but significantly improved at follow up ($p<0.0001$). Mean values were 11.8 and 11.9 g/dl at six and 12 months follow up respectively. The same trends of change were observed for the other haematological parameters of interest (Hct, MCV, MCH, RBC) (Table 3.13).

3.2.4. Changes of anaemia prevalence and severity at follow up

The progression of anaemia prevalence and degree of severity at follow up is shown in Fig 12 and displayed per disease type in Table 3.14. The proportion of patients with severe anaemia decreased from 32% at baseline to 10% at six and 12 months follow up whereas the respective number of non-anaemic patients increased at follow up (Fig 12). Similar trends were observed for CD and UC. For IBDU no changes were observed compared to baseline values although definite conclusions are difficult to be drawn due to the small numbers of patients in this group (n=9)

Figure 12: Prevalence and degree of anaemia in paediatric IBD at diagnosis and six and 12 months of follow up



3.3.2.5. Individual changes in anaemia severity classification at follow up

Patients were categorized into four groups according to the change of anaemia classification at six and 12 months follow up. Two groups included those patients who were anaemic at diagnosis and either improved to non anaemic or remained anaemic at follow up, and two other groups with those patients who were not anaemic at diagnosis and either remained so or deteriorated at follow up (Table 3.15). Approximately 30% of the anaemic patients became non anaemic at follow up and similarly around 8% who were not anaemic deteriorated at follow up (Table 3.15).

Table 3.13: Haematological characteristics at diagnosis, six and 12 mth of follow up of paediatric IBD patients (mean ± SD)

	Diagnosis			Follow up 6 mth			Follow up 12 mth		
	CD	UC	IBDU	CD	UC	IBDU	CD	UC	IBDU
RBC (x 10^{12})	4.6 ± 0.4	4.2 ± 0.5	4.3 ± 0.5	4.6 ± 0.5	4.6 ± 0.4	4.4 ± 0.5	4.5 ± 0.4	4.5 ± 0.5	4.4 ± 0.6
Haemoglobin (g/dl)	10.7 ± 1.4	10.5 ± 2.0	11.2 ± 2.1	11.9 ± 1.4	12.0 ± 1.3	11.6 ± 1.8	11.6 ± 1.4	12.3 ± 1.3	11.8 ± 1.8
Haematocrit (%)	34.1 ± 3.8	32.6 ± 4.9	34 ± 5.1	36.6 ± 3.4	37.2 ± 3.3	35.8 ± 4.2	35.8 ± 3.3	36.9 ± 3.1	36.1 ± 4.7
MCV (fl)	74.5 ± 6.3	77.8 ± 6.0	79.3 ± 7.2	79.4 ± 6.8	81.9 ± 7.9	80.3 ± 8.3	80.0 ± 6.9	82.8 ± 6.0	83.0 ± 6.6
MCH (pg)	23.4 ± 2.8	24.8 ± 2.8	26.3 ± 3.9	25.8 ± 3.0	26.5 ± 2.9	26.0 ± 3.7	26.2 ± 4.1	27.5 ± 2.4	27.1 ± 3.0

Table 3.14: Prevalence and degree of severity of anaemia at diagnosis six and 12 mth follow up in paediatric patients with IBD

	CD			UC			IBDU		
	Non anaemic	**Mild anaemia**	**Severe anaemia**	**Non anaemic**	**Mild anaemia**	**Severe anaemia**	**Non anaemic**	**Mild anaemia**	**Severe anaemia**
Baseline n;(%)	20; (19)	50; (46)	37; (35)	11; (22)	21; (43)	17; (35)	10; (43)	9; (39)	4; (18)
Follow up 6 mth n;(%)	42; (43)	46; (4)	10; (10)	25; (54)	15; (33)	6;(13)	5; (56)	4; (44)	0; (0)
Follow up 12 mth n;(%)	29; (32)	52; (57)	10; (11)	20; (51)	17; (44)	2; (5)	6; (46)	5; (39)	2; (15)

Table 3.15: Change in -individual anaemia classification patterns in paediatric IBD patients between six and 12 mth post-diagnosis

	Follow up 6 mth					Follow up 12 mth			
Anaemia pattern change*	**AA n;(%)**	**NN n;(%)**	**AN n;(%)**	**NA n;(%)**	**Anaemia pattern change***	**AA n;(%)**	**NN n;(%)**	**AN n;(%)**	**NA n;(%)**
CD (n=98)	47; (48)	16; (16)	27; (28)	8; (8)	**CD (n=96)**	58; (60)	9; (10)	22; (23)	7; (7)
UC (n=46)	17 ; (37)	7 ; (15)	18 ; (39)	4 ; (9)	**UC (n=43)**	18 ; (42)	9 ; (20)	14 ; (33)	2 ; (5)
IBDU (n=9)	4 ; (44)	3 ; (34)	2 ; (22)	0 ; (0)	**IBDU (n=4)**	3 ; (75)	1 ; (25)	0 ; (0)	0 ; (0)
Total (n=153)	68; (44)	26; (17)	47; (31)	12; (8)	**Total (n=143)**	79; (55)	19; (13)	36; (25)	6; (7)

* AA= Anaemic at diagnosis and at follow up; NN Non anaemic at diagnosis and at follow up
* AN= Anaemic at diagnosis but Non anaemic at follow up; NA= Non anaemic at diagnosis but anaemic at follow up

3.3.2.6. Changes in haemoglobin concentration at six and 12 months

Changes in the concentration of haemoglobin were calculated for each child separately as the numeric difference from diagnosis levels, at six and 12 months follow up. No difference in the mean haemoglobin change was found between the diseases (Table 3.16) ($p>0.200$). Compared to baseline, only the girls' haemoglobin levels improved at 12 but not at six months follow up.

For analysis purposes, patients were grouped into two categories based on the direction of haemoglobin concentration change at six and 12 months follow up. Two groups were assigned. Those patients whose haemoglobin concentration either increased or remained unchanged compared to diagnosis and those whose haemoglobin deteriorated at follow up. For approximately 67%-70% of the patients, haemoglobin concentration improved, compared to diagnosis, at six and 12 months follow up. No significant difference in the pattern of haemoglobin change was observed between the disease types (Table 3.16)

Table 3.16: Mean changes in haemoglobin concentration (mean ± SD) and percent of IBD patients whose haemoglobin concentration improved from diagnosis at six and 12 mth of follow up

	CD	UC	IBDU
Mean Hb change (6 mth) (mg/dl)	1 ± 1.5 (n=98)	1.3 ± 2 (n=46)	1.4 ± 2.4 (n=9)
Mean Hb change (12 mth) (mg/dl)	0.8 ± 1.5 (n=96)	1.7 ± 2.1 (n=43)	1.4 ± 3.3 (n=4)
N;(%) patients whose Hb improved (6mth)	68; (69)	31; (67)	6; (67)
N;(%) patients whose Hb improved (12mth)	68; (71)	29; (67)	3; (75)

3.3.2.7. Predictors of improvement of haemoglobin concentration at six and 12 months

Potential predictors were chosen a-priori and their association with the direction of haemoglobin change (improvement or deterioration) at follow up was examined with univariate and multivariate logistic regression analysis as described above (Section 3.2.1).

Similar to the main cohort analysis, systemic markers of disease activity, and female gender were the most significant features that characterized anaemic patients at diagnosis. Weight loss prior to diagnosis, low BMI and delayed diagnosis were the most important predictors of anaemia occurrence and degree severity at diagnosis in univariate analysis (Table 3.17). However in multivariate analysis only the level of serum albumin remained significant predictors (Table 3.17).

In subgroup analysis (follow up data), abnormal systemic markers of disease activity and low haemoglobin concentration at diagnosis were among the predictors that differentiated those patients who improved from those that deteriorated at six and 12 months follow up. Improvement of disease activity, as reflected by an increase in albumin and

decrease of ESR levels at follow up, was also associated with improvement of haemoglobin levels (Table 3.18 & 3.19).

Table 3.17: Predictors of anaemia incidence at diagnosis in paediatric IBD patients (group of patients with follow up data at six and/or 12 monts of follow up). Univariate and multivariate logistic regression analysis (p-value; OR; 95% CI)

Predictors	Mild anaemia			Severe anaemia		
	Univariate					
	p-value	OR	95% CI	p-value	OR	95% CI
Sex (male)	NS			0.038	0.42	0.19 - 0.95
ESR (mm/hr)	0.009	1.03	1.01 - 1.05	0.001	1.04	1.01 - 1.06
CRP (mg/l)	0.035	1.02	1.01 - 1.04	0.003	1.03	1.01 - 1.04
Albumin (g/l)	0.000	1.83	0.76 - 0.91	0.001	0.75	0.67 - 0.83
Diagnosis delay (mth)	0.058	0.97	0.95 - 1.00	0.022	0.96	0.93 - 0.99
BMI z-score (SD)	0.001	0.59	0.43 - 0.81	0.003	0.60	0.43 - 0.84
Weight loss (Yes)	0.042	2.47	1.03 - 5.93	0.004	4.44	1.63 - 12.10
	Multivariate					
	p-value	OR	95% CI	p-value	OR	95% CI
Albumin (g/l)	0.0001	0.83	0.76 - 0.91	0.0001	0.75	0.67 - 0.83

Although in univariate analysis iron supplementation was strongly associated with haemoglobin improvement at 12 months for both diseases in multivariate analysis this association remained significant only for CD (Table 3.20). In UC patients, improvement of serum albumin and low haemoglobin concentration at diagnosis were predictors of haemoglobin levels improvement at follow up (Table 3.20). On the other hand in CD additional characteristics differentiated patients whose haemoglobin improved from those who did not. In particular a decrease in ESR levels, and increase in BMI and growth acceleration at six and 12 months predicted improvement of haemoglobin concentration (Table 3.20).

Table 3.18: Predictors of haemoglobin concentration increase (comparison with those who deteriorated) at six mth follow up in paediatric IBD patients (univariate logistic regression; p-value; OR; 95% CI)

Predictors*		IBD			CD			UC		
		p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI
Haematology	Haemoglobin concentration at diagnosis	0.0001	1.8	1.4 - 2.3	0.004	1.6	1.2 - 2.3	0.006	2.0	1.2 - 3.2
Disease markers	ESR concentration at diagnosis	0.036	1.0	0.9 - 1.0	0.087	1.0	0.9 – 1.0	0.521	1.0	0.9 – 1.0
	ESR concentration decrease at follow up	0.008	1.0	1.0 - 1.1	0.008	1.0	1.0 - 1.1	0.700	1.0	1.0 - 1.1
	CRP concentration decrease at follow up	0.033	1.0	1.0 - 1.1	0.030	1.0	1.0 - 1.1	0.612	1.0	0.9-1.0
	Albumin concentration at diagnosis	0.0001	1.2	1.1 - 1.3	0.002	1.2	1.1 - 1.3	0.028	1.3	1.0 - 1.6
	Albumin concentration increase at follow up	0.0001	0.8	0.8 - 0.9	0.003	0.9	0.8 -1.0	0.015	0.7	0.6 - 0.9
Anthropometry	BMI z-score at diagnosis	0.015	1.4	1.1 - 1.9	0.028	1.4	1.0 -2.0	0.339	1.4	0.7 - 2.7
	BMI z-score increase at follow up	0.006	0.5	0.3 - 0.8	0.028	0.6	0.3 - 0.9	0.290	0.6	0.2 - 1.6

* Predictors studied but not significantly associated: age, type of disease, gender, CRP at diagnosis, height z-score at diagnosis, height z-score change at follow up, growth retardation (height z-score<-2SD), weight loss prior to diagnosis, underweight (BMI <-2SD)

Table 3.19: Predictors of haemoglobin concentration increase (comparison with those who deteriorated) at 12 mth follow up (univariate logistic regression; p-value; OR; 95% CI)

Predictors		IBD			CD			UC		
		p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI
Medication	Oral iron supplementation	0.001	0.2	0.1 - 0.6	0.012	0.2	0.1 - 0.7	0.009	0.1	0.0 - 0.6
Haematics	Haemoglobin concentration at diagnosis	0.0001	2.3:	1.7 - 3.3	0.001	1.9:	1.3-2.9	0.002	5.6:	2 .0- 17.9
Disease markers	ESR concentration at diagnosis	0.106	1.0	0.9 – 1.0	0.819	1.0	0.9 – 1.0	0.038	0.9	0.9 – 1.0
	ESR concentration decrease at follow up	0.010	1.0	1.0 - 1.1	0.107	1.0	0.9 – 1.0	0.055	1.1	1.0 - 1.2
	CRP concentration diagnosis	0.094	1.0	0.9 – 1.0	0.112	1.0	0.9 – 1 0	0.418	1.0	0.9 – 1.0
	CRP concentration decrease at follow up	0.068	1.0	1.0 - 1.1	0.079	1.0	1.0 - 1.1	0.508	1.0	1.0 - 1.1
	Albumin concentration at diagnosis	0.0001	1.2	1.1 - 1.3	0.001	1.2:	1.1 - 1.3	0.040	1.3	1.0 - 1.6
	Albumin concentration increase at Follow up	0.0001	0.8:	0.8 - 0.9	0.002	0.9	0.8 - 0.9	0.038	0.8	0.0 –.6-1
Anthropometry	BMI z-score at diagnosis	0.056	1.3:	1.0 - 1.7	0.157	1.3:	0.9 - 1.8	0.148	1.7	0.8 - 3.5
	BMI z-score increase at follow up	0.008	0.6:	0.4 - 0.9	0.005	0.4	0.3 - 0.8	0.818	0.9	0.5 - 1.7
	Height z-score increase at follow up	0.101	0.5:	0.3 - 1.1	0.013	0.2	0.1 - 0.7	0.825	0.9	0.4 – 2.0]
	Weight loss at diagnosis	0.002	0.3:	0.1 - 0.6	0.018	0.3	0.1 - 0.8	0.101	0.3	0.1 - 1.3

Predictors studied but not significantly associated: age, type of disease, gender, CRP at diagnosis, height z-score at diagnosis, growth retardation (height z-score<-2SD), underweight (BMI <-2SD), all types of medication used, number if oral steroid courses, number of EEN courses,

Table 3.20: Predictors of haemoglobin concentration increase (comparison with those who deteriorated) at six and 12 mth follow up in paediatric IBD patients (multivariate logistic regression ; p-value; OR; 95% CI)

Predictors	IBD			CD			UC		
6 mth Follow up	p-Value	OR	95%CI	p-Value	OR	95%CI	p-Value	OR	95%CI
Haemoglobin concentration at diagnosis	0.020	1.7	1.1 - 2.5	0.018	2.4	1.2 - 4.9			
ESR concentration at diagnosis	0.042	1.0	1.1 - 1.1	0.023	1.1	1.0 - 1.1			
Decrease in ESR concentration at 6 mth	0.025	1.0	1.0 - 1.1	0.034	1.1	1.0 - 1.1			
Increase in albumin concentration at 6 mth	0.003	0.8	0.7 - 0.9	0.006	0.7	0.5 - 0.9	0.015	0.7	0.6 - 0.9
BMI z-score at diagnosis	0.032	1.8	1.1 - 2.9	0.008	2.9	1.3 - 6.4			
Increase in BMI z-score at 6 mth	0.025	2.9	1.1 - 7.5	0.002	15.4	2.7 - 86.8			
12 mth Follow up									
Haemoglobin concentration at diagnosis	0.0001	2.6	1.6 - 4.3	0.029	1.7	1.1 - 2.8	0.002	6.0:	2.0 - 17.9
ESR concentration at diagnosis	.037	1.0	1.0 -1.1						
Decrease in ESR concentration at 12 mth	0.017	1.1	1.0 - 1.1						
Increase in albumin concentration at 12 mth	0.027	0.9	0.8 – 1.0	0.006	0.9 :	0.8 –1.0			
Oral iron supplementation				0.043	0.2:	0.1 - 0.9			
Height z-score increase at 12 mth				0.008	0.1	0.0 - 0.5			

3.4. Discussion

Anaemia is one of the extra-intestinal complications of IBD affecting body homeostasis, quality of life and, in children, may impair cognitive development (Section 1.4.2). The prevalence of anaemia in adult IBD is well reported but similar evidence in children is scarce (229).

Poor study design, with small sample size (213;242;252;253) and the use of inappropriate generic criteria to define anaemia were some of the drawbacks of previous paediatric reports. As it is well accepted that haemoglobin normal levels vary considerably with age and gender (205) the use of relevant cut offs is essential. In previous studies, both children with long-standing disease and newly diagnosed patients were recruited which may have induced selection bias to the estimation of anaemia prevalence at diagnosis (Table 1.4.5). In contrast this current study included only patients on disease presentation and before medical therapy was applied.

The Yorkhill Royal Hospital of Sick Children is the only paediatric referral hospital exclusively in charge of the care of all the children with IBD at the geographical area of the West of Scotland. This allowed to define a representative sample of the general Scottish IBD population and avoid selection bias that may have occurred in previous studies, which included only patients with active disease (242). In fact the demographic and disease characteristics of the population of the current study are in strong accordance with previous British epidemiological surveys (16;33) allowing the extrapolation of the results to the general Scottish paediatric IBD population.

In this big retrospective study, the prevalence of anaemia was high with approximately three quarters of the patients presenting with some degree of anaemia, and one third suffering from severe anaemia at diagnosis. Severe anaemia was more common in females with no difference observed between CD and UC. Blood loss from menstruation may explain this predisposition to girls despite the use of gender and age specific haemoglobin cut offs to define anaemia.

Red blood cell counts were significantly higher in UC than CD despite no significant differences in haemoglobin levels or other haematological markers. Although it is difficult to elucidate the exact reason of this difference, a possible explanation would be the different causes of anaemia in the two diseases. Overt gastrointestinal bleeding with RBC loss is more common in UC than CD (33) and may be an additional major contributor to the origin of anaemia beyond any other causes like reduced iron intake and the disease affect.

Gastrointestinal bleeding and poor dietary intake are typical clinical manifestations of the newly diagnosed IBD patient and may explain the strong association found in this study between systemic markers of disease activity and haemoglobin concentration. In fact the

total number of abnormal systemic markers of disease activity was the strongest independent predictor of anaemia occurrence and degree of its severity. A patient with abnormal CRP, ESR, serum albumin and increased number of platelet counts had an approximately 100 fold chance of severe anaemia.

Disease location with involvement of the upper digestive tract, where dietary iron is absorbed, has been associated with anaemia occurrence in previous reports (244;247) but the results of this current study are not in accordance. New guidelines were used to define the anatomic location of the disease and failed to find any difference in anaemia prevalence between patients with upper and lower gastrointestinal involvement or between patients with extensive or disease of limited extent. Indeed the absence of difference in anaemia occurrence between CD and UC advocates that iron malabsorption is not the sole determinant of anaemia onset. Other factors such as intestinal bleeding, and a diet low in iron (223) could equally contribute to anaemia aetiology. Unfortunately, due to the retrospective design of this study no dietary assessment was available to address this possibility. On the other hand no association was found between overt self-reported gastrointestinal bleeding and anaemia although faecal occult blood cannot be excluded.

Similar to disease location, disease behaviour was not significantly associated with anaemia incidence. Inflammatory, penetrating and stricture disease behaviour did not differentiate anaemic from non-anaemic patients. However perianal disease involvement was not included in the disease phenotypic characterization and consequently this study cannot answer whether there are differences in the prevalence of anaemia in patients with perianal abscesses. The lack of relevant information was due to the retrospective nature of the study and under-documentation of perianal disease in the electronic pathology reports used to assess the disease location.

A high risk of undernutrition, as reflected by prior weight loss and low at disease diagnosis, differentiated anaemic from non anaemic patients. However this association disappeared when serum albumin levels were added in the multivariate model suggesting that disease activity may be a more significant predictor of anaemia occurrence. Nevertheless albumin levels can be influenced by both inflammation and under-nutrition and a dependency with BMI may have confounded the results. Indeed when albumin substituted with ESR in the multivariate model, BMI remained a statistically significant independent predictor of anaemia.

The prevalence of anaemia varied between 2001 and 2007 but no overall temporal trends can be drawn. There was no decrease in the prevalence of anaemia over time despite our expectations. Temporal changes may have happened due to a shorter interval between symptoms onset and disease diagnosis nowadays. This would prevent the onset or allow timely correction of anaemia at early stages. However there was no difference in the

diagnosis delay within the short period assessed. Instead a significant association was found between anaemia occurrence and prompt diagnosis. This could be due to an inverse association between anaemia and diagnosis delay. It is possible that early onset of anaemia onset accelerates clinical investigations and hence prompt disease diagnosis, rather than a diagnosis delay predisposing to anaemia.

This study aimed to assess the prevalence of anaemia and its progression at follow up. Due to the retrospective design of this study it was not possible to measure additional haematological parameters to differentiate between the types of anaemia. Based on the size of RBC, no patient suffered from megalocytic anaemia and this suggests that the primary cause of anaemia in IBD is IDA or ACD. Ferritin levels were available in a few patients and a significant positive correlation was found with CRP and a negative association with serum albumin concentration. Although at first sight this seems paradoxical and may suggest that iron stores are improved with active systemic disease or undernutrition, strong evidence suggests that ferritin is a positive acute phase respondent that increases independently of the actual body iron stores in inflammatory conditions, infection and cancer (555). This study among others suggests that ferritin is an unreliable marker of body iron stores in patients with active IBD where systemic inflammation is present. The acute phase response and ACD could also explain why some anaemic patients had normal MCV and ferritin levels. Other surrogate markers should be used with better diagnostic value in order to differentiate between the different types of anaemia in IBD. Soluble serum receptor of transferrin is a potent marker but its use is limited to research only scale (228).

Although there are a few case reports of nutritional anaemias caused by folate and vitamin B_{12} deficiency, none of the 26 patients studied had suboptimal levels of vitamin B_{12} or folate. Indeed there was no association between ileal defined disease and low levels of B_{12}. This discrepancy with previous adult studies (236) may be ascribed to differences in subject characteristics and recruitment of patients with longstanding disease rather than the newly diagnosed patients this study included. It is likely that vitamin B_{12} body stores, although small, are adequate to sustain serum levels and prevent B_{12} deficient anaemia in newly presented patients. Conversely, this may be more common in patients with longstanding disease and depleted vitamin stores.

Changes in the pattern of anaemia prevalence over the natural history of the disease have not been described in adult or paediatric studies. This is the first study which assessed anaemia prevalence at follow up. Although anaemia prevalence remained high at follow up, a significant decline was observed which coincided with a significant increase in mean haemoglobin concentration by approximately 1 g/dl at follow up. Nevertheless more than half of the patients were anaemic at six months and 12 months post-diagnosis showing that a considerable number of patients remain anaemic within a year of diagnosis.

With regard to changes in the severity of anaemia, one third of the children presented at diagnosis haemoglobin levels less than 10 g/dl, but only 10% were severely anaemic at follow up. On an individual basis approximately 50% of the patients anaemic at diagnosis remained so at follow up, and 31% improved to normal haemoglobin levels. Eight percent of the patients with normal levels of haemoglobin at diagnosis deteriorated and became anaemic at follow up. Although subgroup analysis might reveal any disease or demographic differences compared to the rest of the groups, the sample sizes are too small and preclude reliable statistical analysis

Several predictors of haemoglobin concentration improvement or deterioration were evaluated at follow up. Improvement of baseline serum albumin level at follow up was among the strongest predictors in univariate and multivariate analysis which differentiated those patients whose haemoglobin improved at follow up from those who did not. On the contrary CRP and ESR changes were not strong predictors of haemoglobin improvement particularly in UC patients. This discrepancy could show that other factors beyond changes in disease activity determine haemoglobin improvement or deterioration. In particular serum albumin, a negative acute phase respondent can be further decreased by poor nutritional status and subsequently increase of haemoglobin levels in response to improvement of serum albumin may coincide not only with amelioration of disease activity but also with nutritional rehabilitation. In support of this is that a BMI increase and linear growth acceleration in CD patients were strongly associated with improvement of haemoglobin levels, an effect that was independent of any improvement in the systemic markers of disease activity.

Despite no difference in the prevalence of anaemia between CD and UC patients at any point of follow up, disease specific differences were found in the predictors of haemoglobin change. A low haemoglobin at diagnosis and improvement of albumin at follow up were the strongest predictors of haemoglobin improvement at follow up in UC children. In contrast, improvement of fundamental anthropometry and ESR were additional factors independently associated with increase of haemoglobin at follow up only in CD patients.

As might be expected oral iron supplementation was independently associated with improvement of haemoglobin levels in patients with CD. This effect was independent of changes in systemic markers of disease activity or improvement of nutritional status. Although some patients who received blood transfusion were included, this was not a significant predictor of haemoglobin anaemia improvement at follow up. It is possible that any favourable effect of blood transfusion on haemoglobin levels is either short term and counteracted by the increased disease severity and gross intestinal bleeding that is usually seen in this group of patients. In contrast to expectations, use of nutritional support did not differentiate those patients with improved haemoglobin levels at follow up from those who deteriorated. This study speculated that nutritional therapy and in particularly the number of

EEN courses would be strongly associated with haemoglobin improvement. However, frequent use of EEN nutrition also denotes increased disease activity and the number of courses corresponds to clinical relapses. As disease activity was found to be inversely associated with reduced haemoglobin levels in this study, any beneficial effect of nutritional support is counterbalanced by the negative effect of disease activity.

Although it was not among the primary aims, this study provided useful information on the nutritional status body composition and disease characteristics of children with IBD at diagnosis and over a year follow up. Disease specific differences in anthropometric characteristics were found between children with CD and UC as reported by others. CD children presented with significantly lower BMI z-scores than children with UC at diagnosis and at all time points of follow up. As a result the prevalence of underweight and growth retarded children was significantly higher in CD than UC children. Although this may be attributed to the involvement of upper digestive tract, and subsequent nutrient loss from malabsorption in CD, other factors could be implicated too. Crohn's disease and UC, although part of the same disease constellation, have different mechanisms of pathogenesis, and immunological responses that may affect nutritional status in a different way. As the findings of this study suggest, patients with CD have more abnormal systemic markers of disease activity compared to UC children, and it is perhaps due to the increased production of inflammatory markers and pro-inflammatory cytokines that CD children more often experience growth retardation and suboptimal nutritional status. This coincides with evidence that suggests that pro-inflammatory cytokines are partially responsible for growth retardation and suboptimal nutritional status (111). Future studies in this area should address this and whether different cytokines exert a different role on growth and nutritional status. It was exciting to find that the majority of the children with suboptimal weight at diagnosis caught up within six months of follow up. The proportion of patients who presented BMI lower than -2 SD at 12 months did not differ significantly from the normal distribution seen in the general healthy population. Although no IBD patient was obese at diagnosis, more than 10% percent of the UC children could be classified obese at follow up. This reverse in nutritional status could be a combination, of disease improvement, nutritional support and the side effects of the use of steroid treatment.

In the biggest study in Europe and the UK, the prevalence of anaemia is high in paediatric IBD at diagnosis. Disease activity and nutritional status at diagnosis and their change at follow up are the most important predictors of anaemia occurrence and its evolution during the course of disease. Future research should be focused on the part of patients who deteriorated at follow up. This study just explored any potential association between the use of medication and prevalence of haemoglobin at follow up and therefore it is difficult to conclude on any causative association. Further well controlled trials should

investigate the collateral therapeutic effects of new anti-inflammatory agents and nutritional therapies to correct anaemia. This is particular important in the case of ACD whose pathogenesis is predominantly attributed to the excessive production of pro-inflammatory cytokines (231). It was noteworthy to find that there is a lack of studies on the dietary assessment of paediatric patients of IBD. A diet poor in iron unable to compensate with the excessive gastrointestinal losses may equally contribute to the onset of anaemia in IBD. Iron balance studies could be designed where the equilibrium of iron balance is monitored to accurately assess intake, use, and gastrointestinal losses. In any case a diet rich in iron containing foods should be encouraged. Oral iron supplementation, although supported by the findings of the current retrospective study, should be used cautiously in the light of animal studies and in-vitro models suggesting that excessive production of free radicals and oxidative stress at the site of tissue lesion may have possible implications for increased risk of colonic carcinogenesis (236).

CHAPTER FOUR

Comparison of body composition assessment in healthy children with leg to leg bioelectrical impedance and DXA analysis

OUTLINE

This chapter describes a study that assessed the validity of a simple body composition method, based on bioimpedance, to measure accurately body composition in healthy children. The secondary aim of this study was to measure the body composition of a representative cohort of healthy Scottish children and to compare it with the body composition of CD children who participated in the studies of this thesis. The results of the second part of this study are presented in Chapter 5.

4.1. Introduction

Assessment of body composition is important in health and disease. Measurement of body fat stores is important in obesity and calorie restricting diets, as is measurement of muscle mass in chronic catabolic disorders.

Measuring body composition has been a constant challenge and at the moment no standard method is universally accepted to measure body compartments accurately and precisely (92). Chemical analysis of human cadavers is the only way body composition can be determined accurately but imaging techniques and the multi-compartment model (556) are considered the best approaches, usually cited as “gold standard” methods (557). Other techniques like stable isotope dilution, underwater weighing, total body dual x-ray absorptiometry (DEXA) known as “reference methods” rather than “gold standards”, are good alternatives with minimal error, but are mainly laboratory based methods, and hence impractical in large scale community studies or for routine clinical use.

Practical, cheap, safe and patient friendly methods of body composition assessment for clinical bedside use are needed. Fundamental anthropometry and growth charts, calculation of BMI, or use of prediction equations based on height and weight are not able to differentiate between fatness and lean mass (558). Although in healthy adults and children BMI, originally an index of morbidity risk, correlates well with fatness (93), their diagnostic value to differentiate fat from lean mass is questionable, particularly in disease. Multiple clinical conditions like oedema or dehydration, and use of steroids can affect body weight irrespective of changes in the actual muscle and fat stores. Hence the use of anthropometry can be inaccurate and misleading.

Skinfold thickness measurements have been the only method used for surveillance of body stores in routine clinical practice. However they are prone to inter-observer error, need well trained personnel and may cause discomfort in young children. Although reliable in the assessment of groups or populations, skinfold thickness measurements are not reliable in estimating the body composition of individuals. (559).

The advent of bioelectrical impedance analysis (BIA) was welcomed as a potential alternative at the beginning of the 80's. The equipment is non-invasive, portable, quick to use, provides direct results and needs less subject cooperation than any other method making it viable for field and routine use.

The fundamental principle behind BIA is the measurement of the resistance of the human body to an insensible electric current circulating through the body (560). The measured resistance/impedance or more correctly the impedance index ($Height^2$/Resistance) is proportionally related to total body water volume and consequently total fat free mass (FFM), assuming a constant hydration of FFM containing virtually all the body's conducting electrolytes (560). Nevertheless the relationship between total body water and impedance must be established empirically and there is evidence that this differs between populations, races, sexes and ages (561). The National Institute of Health published a consensus article that highlights the importance of comparing the impedance instruments with reference methods and that predictive equations are useful only for subjects who closely match the reference population from which they were derived (562;563).

The traditional method is based on the measurement of impedance between the hand and foot. A new alternative has recently been introduced that measures the impedance from an electrical circuit between the legs (564). This adapted technique resembles a household bathroom scales with the subject required to stand briefly on two metallic footplates. Unlike the conventional technique there is no need for the attachment of electrodes at precise anatomical sites and weight is simultaneously measured avoiding any transcription errors.

Many studies have compared and validated or compared the traditional hand to foot technique with other "reference methods" both in adult (565-568) and paediatric populations (569;570) but the derived equations cannot be applied to the foot to foot appliance due to difference in the measuring segments of the body (hand to foot vs foot to foot) (571). Relevant studies for the foot to foot method are scarce (572) particularly in paediatric populations (573;574) and so the validity of the new foot to foot system with its inbuilt manufacturer's equations needed to be tested.

The primary purpose of this study was to compare the foot to foot technique of body composition with respective DXA measurements in a healthy cohort of Scottish children. This would allow to consider its appropriateness for use in the assessment of body composition in paediatric patients with CD. The second aim of this study was to compare the body composition of this healthy control cohort with a cohort of children with CD which is presented in a following chapter (Chapter 5).

4.2. Subjects and methods

4.2.1. Subjects

A subgroup of children from a larger study on bone health and body composition, in mainstream and specialist sport schools, was recruited for the purpose of this study. Potential participants were approached by posted invitation letters to lists of patients kindly supplied by their general practitioners. Exclusion criteria were acute or chronic illness and concomitant medication use known to induce changes in body composition. The volunteers were from mainstream schools in the area of Glasgow. Children were selected to provide an equal distribution of ages, genders, and BMIs. Written informed consent was obtained from all children and their parents. The study protocol was approved by the local research ethics committee and the research and development office.

4.2.2. Basic anthropometry

Anthropometric measurements were conducted according to standard procedures. A trained dietitian and a paediatric endocrinologist performed all measurements. Each investigator performed a single type of measurement. Weight was measured to the nearest 100 g using a calibrated digital beam scale (SECA, Birmingham, UK). Subjects were weighed in light clothing or wearing a hospital gown. Height was measured to the nearest 1 mm using a wall-mounted stadiometer according to the Frankfurt plane position. BMI was computed as the fraction of weight to the squared height and corresponding z-scores were calculated using British national reference data (575).

4.2.3. Body composition

4.2.3.1. Foot to foot impedance measurements

Bioelectrical impedance analysis (BIA) was performed with the foot to foot technique using a TANITA body fat analyser (TBF-300, Tokyo, Japan) which provides a print out of the measured weight, impedance, and two compartment body composition analysis. Gender, age and height to the nearest centimetre were entered manually into the keypad interface. Subjects stood on the two metal sole-pad electrodes embedded on the platform scales. The electrode for each foot was subdivided into anterior and posterior electrodes. A current was applied through the anterior portion of the footpad electrodes and the voltage drop was measured in the posterior portion. The impedance measurement used a 50 kHz, 500 μA insensible current. Subjects were asked to have only a light meal at least one hour before their measurements and were asked to void their bladder before the start of the

measurements. All the measurements were done after a period of at least 10 min standing upright to minimize potential errors from acute shifts in fluid distribution. Body composition for all subjects was estimated using the standard inbuilt prediction equations of the manufacturer, as no other appropriate equation is available in the literature. Every measurement was taken in duplicate and averaged unless the difference in two impedance measurements was greater than 10 Ohms.

4.2.3.2. DXA measurements

Whole body DXA scans were performed with a narrow fan beam Lunar Prodigy densitometer (GE Healthcare) and phantoms analysed using the Encore software (Version 8.80.001). The DXA technique is based on the attenuation properties of bone, lean and fat tissues at two different x-rays energies and it measures directly the bone mineral content, lean and fat mass (FM) (576;577).

Whole body composition was analyzed for bone mass and soft tissue and the latter was subdivided into lean and FM. Bone mass content added to lean mass corresponded to FFM in accordance with the two-compartment model. Each scan took approximately five min to complete. The DXA scans were carried out randomly prior to or after the BIA measurements.

4.2.4. Statistical analysis

The difference between paired data was tested with one sample t-test and one sample Wilcoxon Sign Rank test, for normally and non-parametric distributed values. Normality of the data was checked with the Darling-Anderson test. Spearman rank correlation coefficients were calculated for BIA and DXA for all body composition measures. Linear regression analysis with best fitting line was used to predict DXA FFM measurements by the respective TANITA values. The agreement between the two methods, was evaluated with Bland-Altman plots, by plotting the individual differences of a measure, between the two methods, against their mean (578). The bias of the method is equal to the mean difference of the methods. Limits of agreement between BIA and DXA were calculated as the mean difference of the two methods ± 1.96 times the standard deviation of the mean difference. The effect of size of the measurement on the agreement of the methods was assessed by calculating the R^2 co-efficient of the linear regression model of the differences of the measures against their mean value. Statistical significance was set at $p< 0.05$. Standard deviation scores (z-scores) for % FM were calculated using British reference data (579) kindly provided by Professor Tim Cole.

4.3. Results

4.3.1. Sample characteristics and basic anthropometry

117 children aged (6.8-18.9) agreed to participate. All participants completed all measurements successfully. Most of the children were of Caucasian origin (n=107). Demographics, anthropometry and body composition data grouped by sex are presented in Table 4.1. No difference was found between boys and girls for age and fundamental anthropometry. Height z-scores did not differ significantly from zero (p-value=0.540) but BMI z-scores were slightly higher than the British reference population (575) (p <0.0001).

4.3.2. Body composition analysis

For both methods the mean FM, expressed as absolute values (kg) or as percentage of body weight, was lower in boys than girls despite no significant mean differences in BMI, body weight and height (Table 4.1). Likewise the percentage FFM but not absolute FFM was higher in boys than girls. No gender-associated difference was found for the %FM z-scores. Females had significantly higher measurements of impedance than boys as expected.

4.3.3. Comparison between DXA and TANITA

4.3.3.1. Correlation of the methods

As expected all body composition compartments (FM, FFM, %FM and %FFM) estimates by BIA were positively and highly correlated with those by DXA (Table 4.2). The Spearman rank correlations coefficient between DXA and BIA measures of FFM, FM, %FFM and %FM were high for both genders (Table 4.2 Fig 14-16). Fat free mass calculated by DXA was linearly regressed against FFM predicted by TANITA. A FFM prediction line was drawn that explained 96% of the measurements variance (Fig 13).

4.3.3.2. Biases and limits of agreement between the methods

4.3.3.2.1. Crude measurements of body composition

The differences in body composition measures between the two methods were normally distributed (Anderson-Darling normality test) and the use of Bland-Altman plot was deemed valid and no log transformation of the data was required. Mean absolute values and percentages of FM and FFM were statistically different between DXA and TANITA (all p-values <0.0001). Defining DXA as the “reference method”, TANITA overestimated %FFM by a mean of 4.5% and underestimated FM by -2.1 kg of fat (Table 4.3). Nonetheless, in all

cases, the limits of agreement between the methods were wide and therefore the methods were not interchangeable at an individual level (Table 4.3). Biases and limits of agreement for absolute measures and % FM and FFM are displayed in Figs 16-18 and summarized in Table 4.3 for the whole group and separately for boys and girls. Taking body fat as a prime example, TANITA may underestimate or overestimate %FM as much as -13.3% and 4.3% respectively. The mean bias between the two methods was higher in girls than boys but in both cases the limits of agreement for all body composition measurements were wide (Table 4.3).

A weak but statistically significant effect of the size of measurement on the agreement of the methods was observed that partially justifies the broad limits of agreement (Table 4.4). However when the data were analyzed separately for boys and girls, a size of measurement effect was observed in males but not in females (Table 4.4). With increasing body fatness the measurement bias between TANITA and DXA became progressively negative in boys. In particular TANITA consistently underestimated %FM in boys with body fat more than 20% whereas the measurement error was variable in males with lower %FM. No similar patterns were observed for females (Fig 18).

4.3.3.2.2. Z-scores of body composition measurements

Percent FM values by both methods were converted to z-scores using the British reference range (579). The age and gender adjusted bias of foot to foot BIA for %FM was -1.3 SD with wide limits of agreement (Table 4.3; Fig 19). In contrast to %FM crude values, increase of %FM z-scores influenced the bias between the two methods in a similar way for both genders (Figs 20-21). For children with normal or high %FM z-score, the bias for foot to foot BIA was small and lay close to the line of method agreement. On the other hand the limits of agreement were wide and varied considerably for children with very low %FM z-score (Table 4.3; Figs 20-21).

Table 4.1: Demographics, anthropometry and body composition characteristics (mean; SD) of healthy Scottish children (mean ± SD)

Measurement	F (n=63)	M (n=57)	Total (n=117)
Age (decimal y)	12.6 ± 3.2	12.4 ± 3.7	12.5 ± 3.4
Height (cm)	149.2 ± 14.0	152.0 ± 20.0	150.5 ± 17.0
Weight (kg)	46.4 ± 17.7	47.8 ± 19.1	47.0 ± 18.3
Height z-score	0.0 ± 1.0	0.1 ± 1.0	0.06 ± 1.0
Weight z-score	0.3 ± 1.2	0.5 ± 1.1	0.4 ± 1.1
BMI	20.2 ± 4.8	19.7 ± 3.9	20.0 ± 4.4
BMI z-score	0.4 ± 1.2	0.6 ± 1.1	0.5 ± 1.1
TANITA			
Impedance (Ohms)	601.0 ± 71.0*	564.0 ± 72.0	584.0 ± 74
FFM (%)	77.3 ± 9.5*	83.4 ± 7.4	80.1 ± 9.1
FFM (kg)	34.0 ± 8.9	39.1 ± 15.5	36.4 ± 12.6
FM (%)	22.7 ± 9.5*	16.6 ± 7.4	19.9 ± 9.1
FM (kg)	11.7 ± 10.1*	8.1 ± 5.8	10.0 ± 8.5
FM (%) z-score	-1.1 ± 4.0	-0.7 ± 2.4	-0.9 ± 3.3
Total body water	24.9 ± 6.5	28.6 ± 11.4	26.6 ± 9.2
DXA			
Total body mass (kg)	45.5 ± 17.5	47.4 ± 19.1	46.4 ± 18.2
FFM (kg)	31.6 ± 8.6	37.3 ± 14.4	34.2 ± 11.9
FFM (%)	72.0 ± 9.2*	79.9 ± 9.3	75.7 ± 10.0
FM (%)	28.0 ± 9.2*	20.1 ± 9.3	24.3 ± 10.0
FM (kg)	13.9 ± 10.1*	10.1 ± 7.6	12.2 ± 9.2
FM (%) z-score	0.6 ± 1.7	0.1 ± 1.9	0.3 ± 1.8

* Difference between genders p< 0.05 (two sample t-test or Mann-Whitney test)

Figure 13: Linear regression with best fitting line for FFM (kg) between TANITA and DXA measurements of body composition

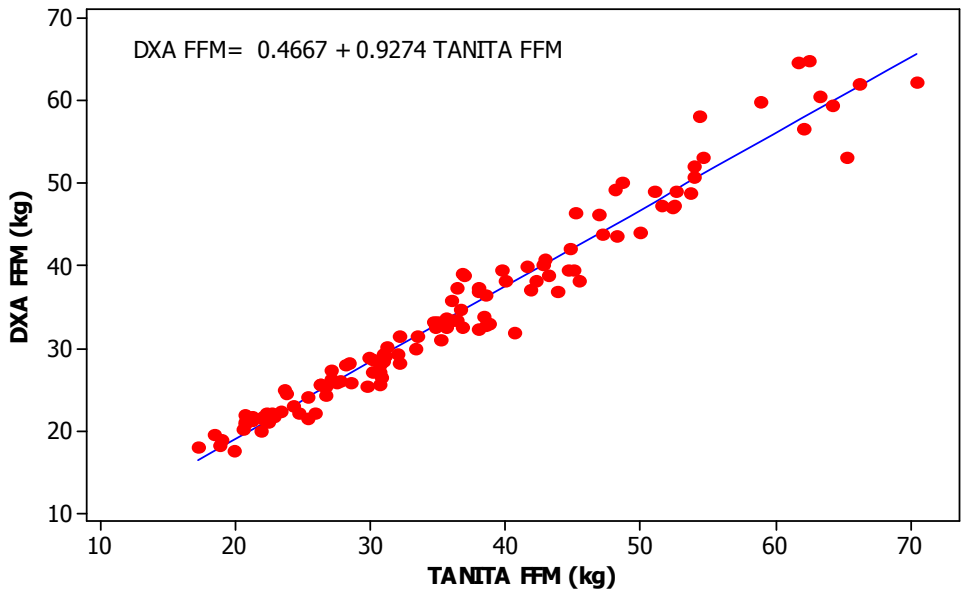


Table 4.2: Spearman rank correlation (r coefficient and p-values) for body composition variables between DXA and TANITA in healthy Scottish boys and girls

	Males		Females		Whole group	
	R coefficient	p-value	R coefficient	p-value	R coefficient	p-value
FFM (Kg)	0.99	0.001	0.96	0.001	0.98	0.001
FFM (%)	0.79	0.001	0.85	0.001	0.86	0.001
FM (Kg)	0.92	0.001	0.95	0.001	0.94	0.001
FM (%)	0.79	0.001	0.85	0.001	0.86	0.001

Table 4.3: Measurement bias with 95% limits of agreement for body composition measurement between DXA and TANITA in healthy Scottish boys and girls

	Males			Females			Whole group		
	Bias	Limits of agreement		Bias	Limits of agreement		Bias	Limits of agreement	
FFM (Kg)	1.8	-3.6:	7.3	2.4	-2.0:	6.9	2.2	-2.8:	7.1
FFM (%)	3.5	-4.8:	11.8	5.3	-3.6:	14.2	4.5	-4.3:	13.3
FM (Kg)	-2	-7.2:	3.2	-2.2	-6.4:	6.9	-2.1	-6.8:	2.5
FM (%)	-3.5	-11.8:	4.8	-5.3	-14.2:	3.6	-4.5	-13.3:	4.3
FM (%) z-score	-0.8	-4.0:	2.3	-1.2	-3.7:	1.2	-1.3	-6.0	5:4

Table 4.4: Effect of the size of the measurement on the agreement of the methods in healthy Scottish boys and girls. R coefficient, slope (β coefficient), and p-values

	Males			Females			Whole group		
	R	Slope	p-value	R	Slope	p-value	R	Slope	p-value
FFM (Kg)	15.8	0.1	0.003	2.4	0.04	0.223	6.6	0.1	0.003
FFM (%)	20.3	-0.2	0.001	0.5	0.03	0.592	4.4	-0.1	0.023
FM (Kg)	44.5	-0.3	0.001	0.0	0	0.997	7.0	-0.1	0.004
FM (%)	20.3	-0.2	0.001	0.5	0.03	0.592	4.4	-0.1	0.023
FM (%) z-score	8.3	0.26	0.019	27.3	0.35	0.001	15.5	0.29	0.001

Figure 14: Spearman rank correlation for body composition measures between TANITA and DXA in healthy children (whole group)

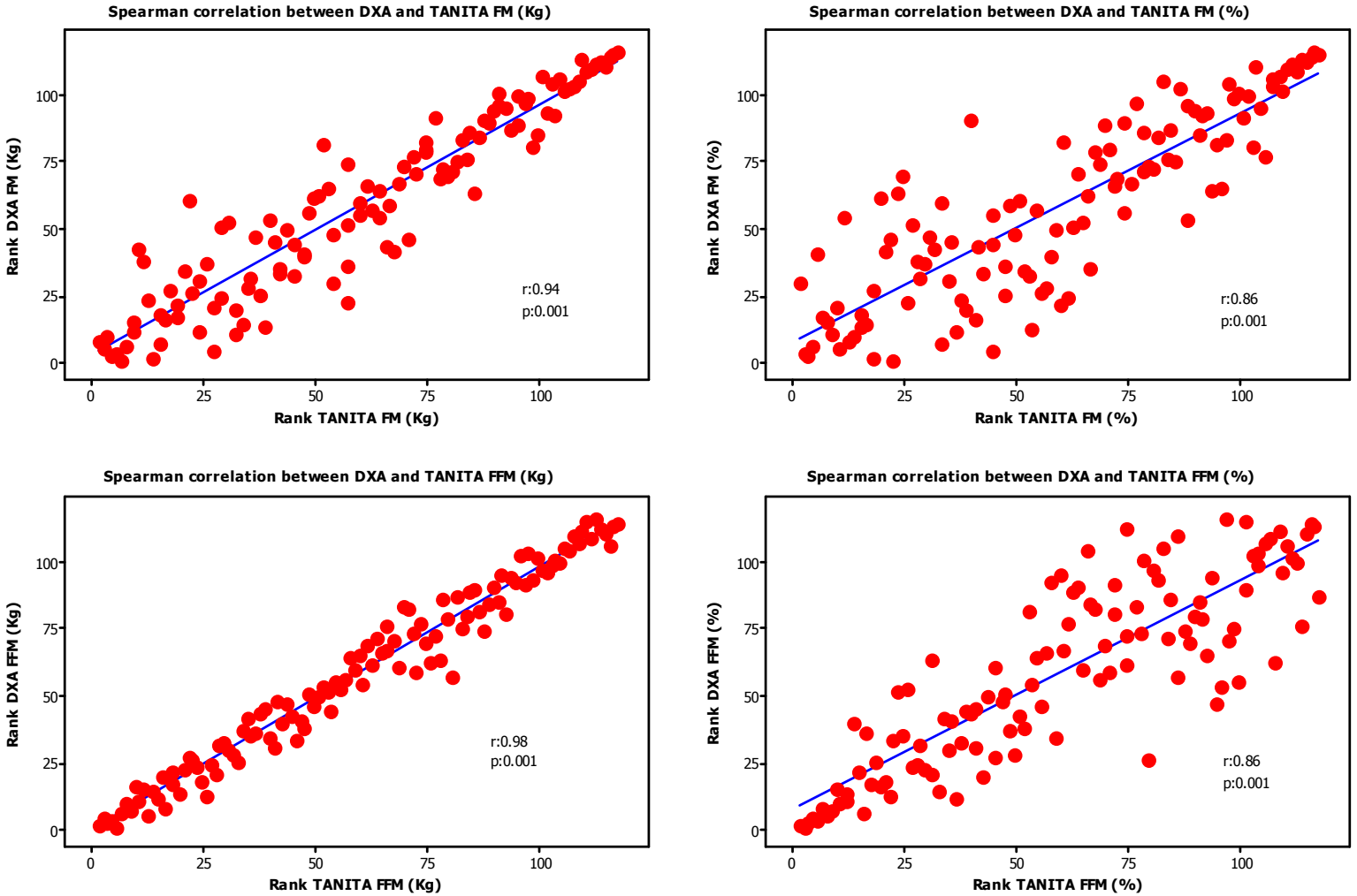


Figure 15: Spearman rank correlation for body composition measures between TANITA and DXA in healthy children (males)

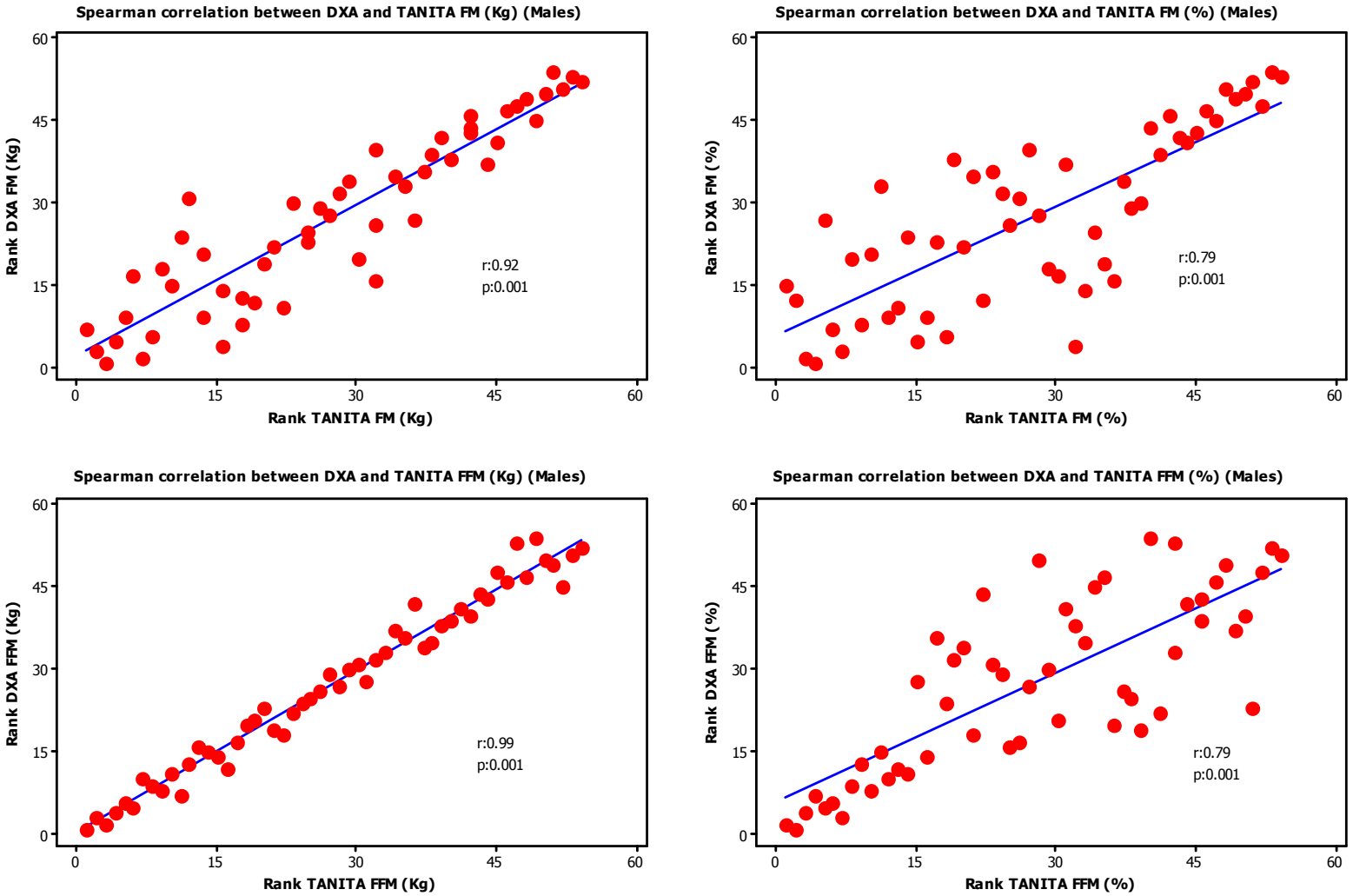


Figure 16 : Spearman rank correlation for body composition measures between TANITA and DXA in healthy children (females)

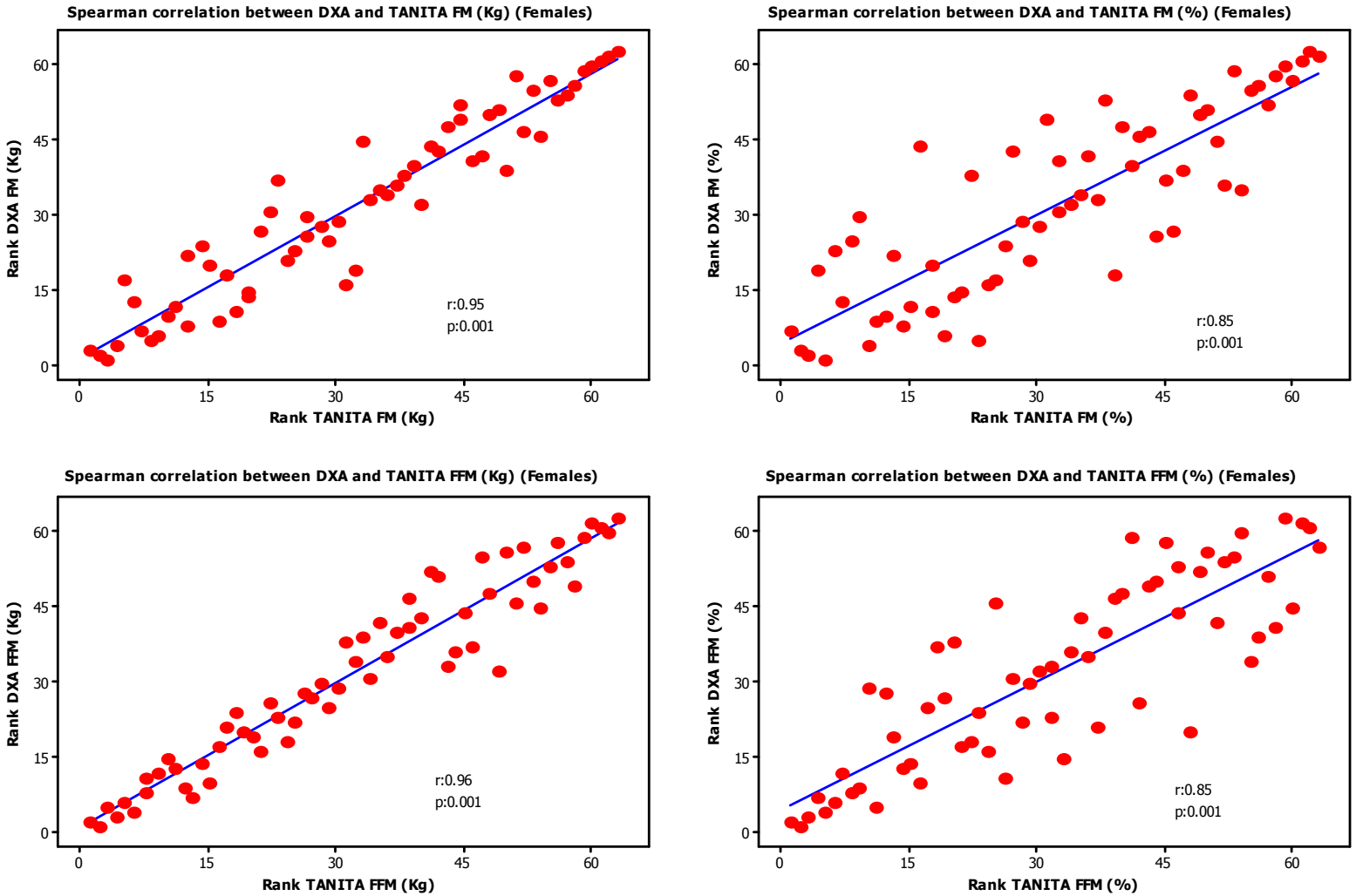


Figure 17: Bland Altman plots with 95% limits of agreement and regression line for body composition measures between TANITA and DXA in healthy children (whole group)

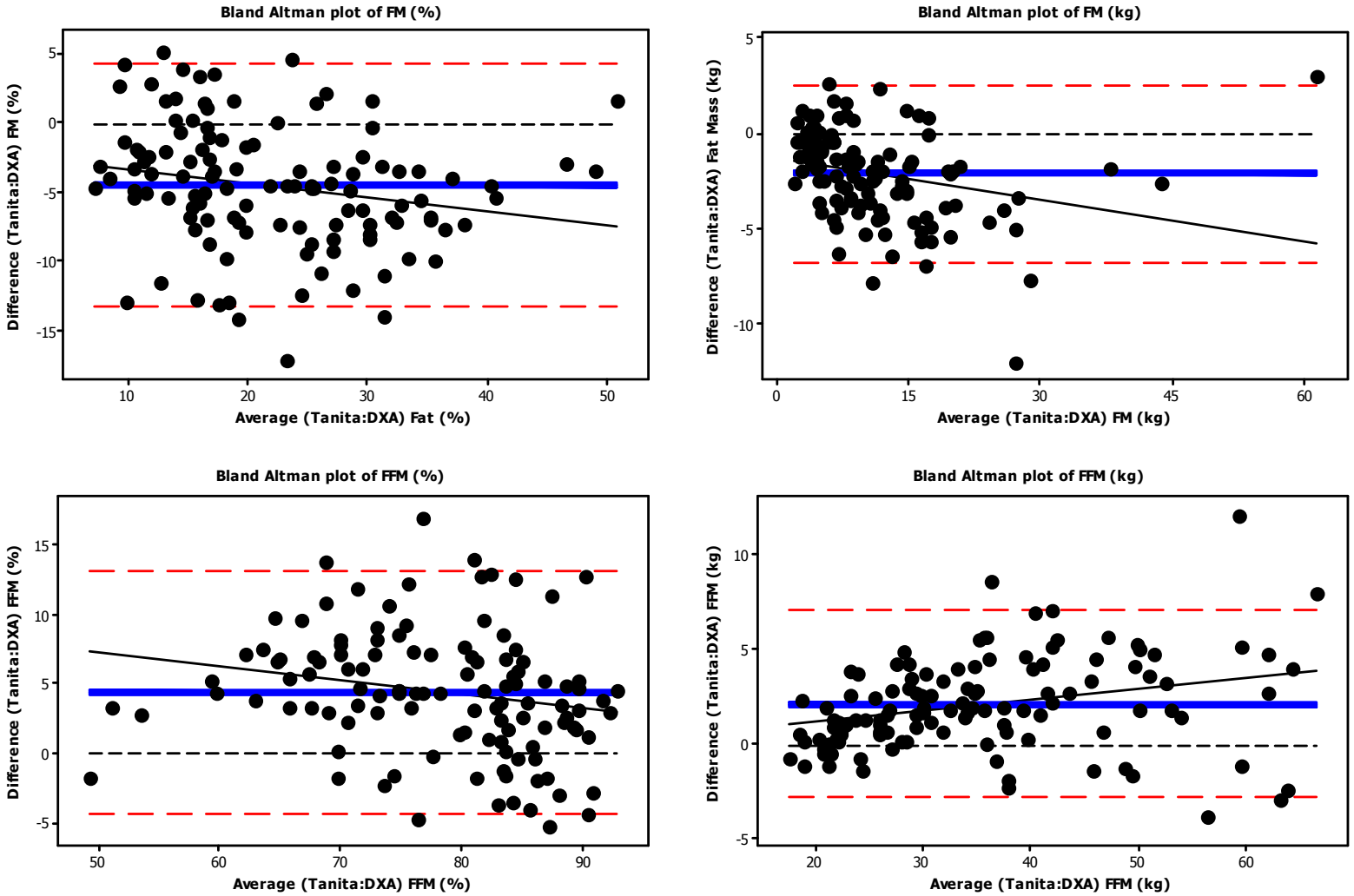


Figure 18: Bland Altman plots with 95% limits of agreement and regression line for body composition measures between TANITA and DXA in healthy children (females)

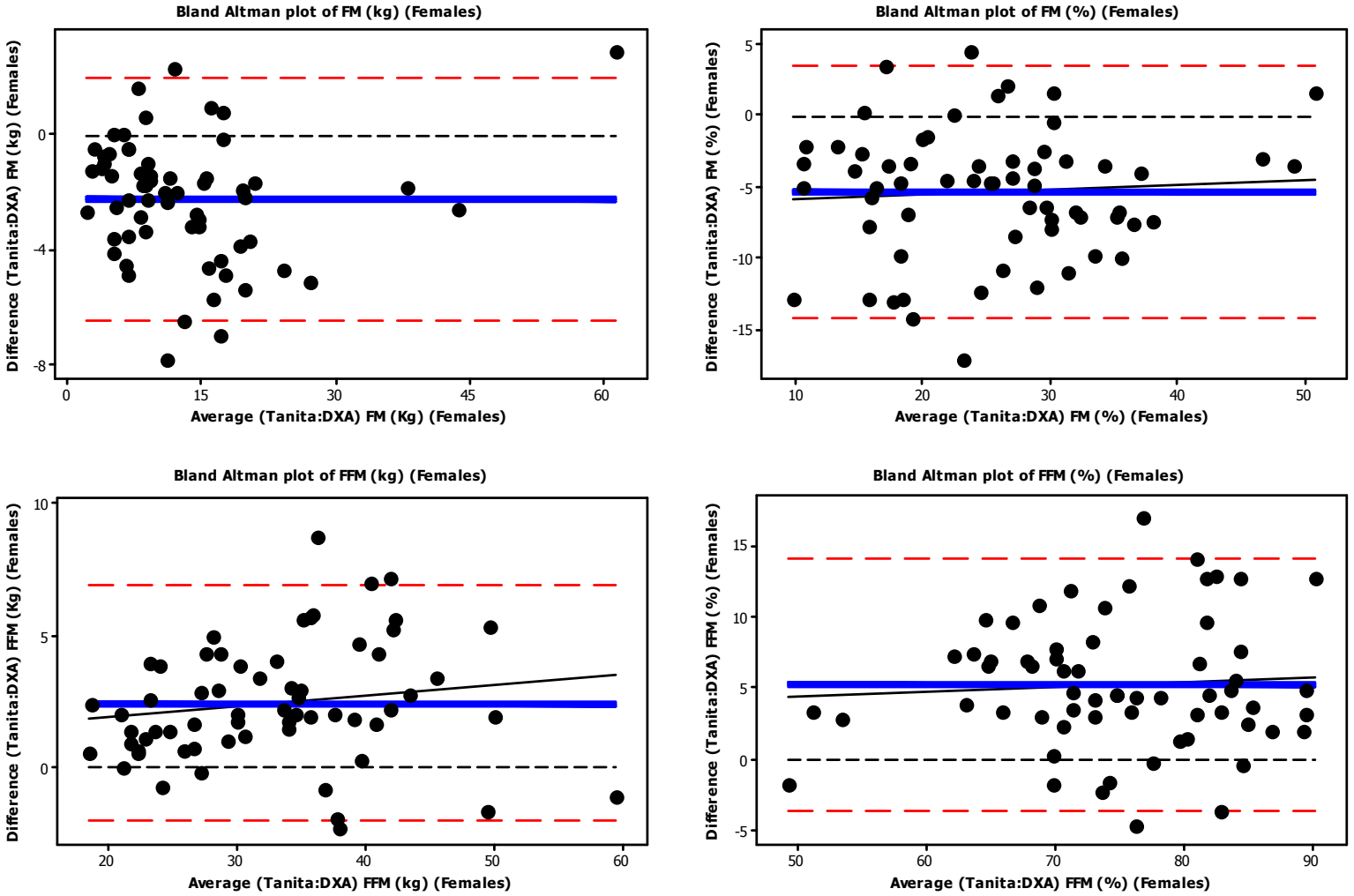


Figure 19: Bland Altman plots with 95% limits of agreement and regression line for body composition measures between TANITA and DXA in healthy children (males)

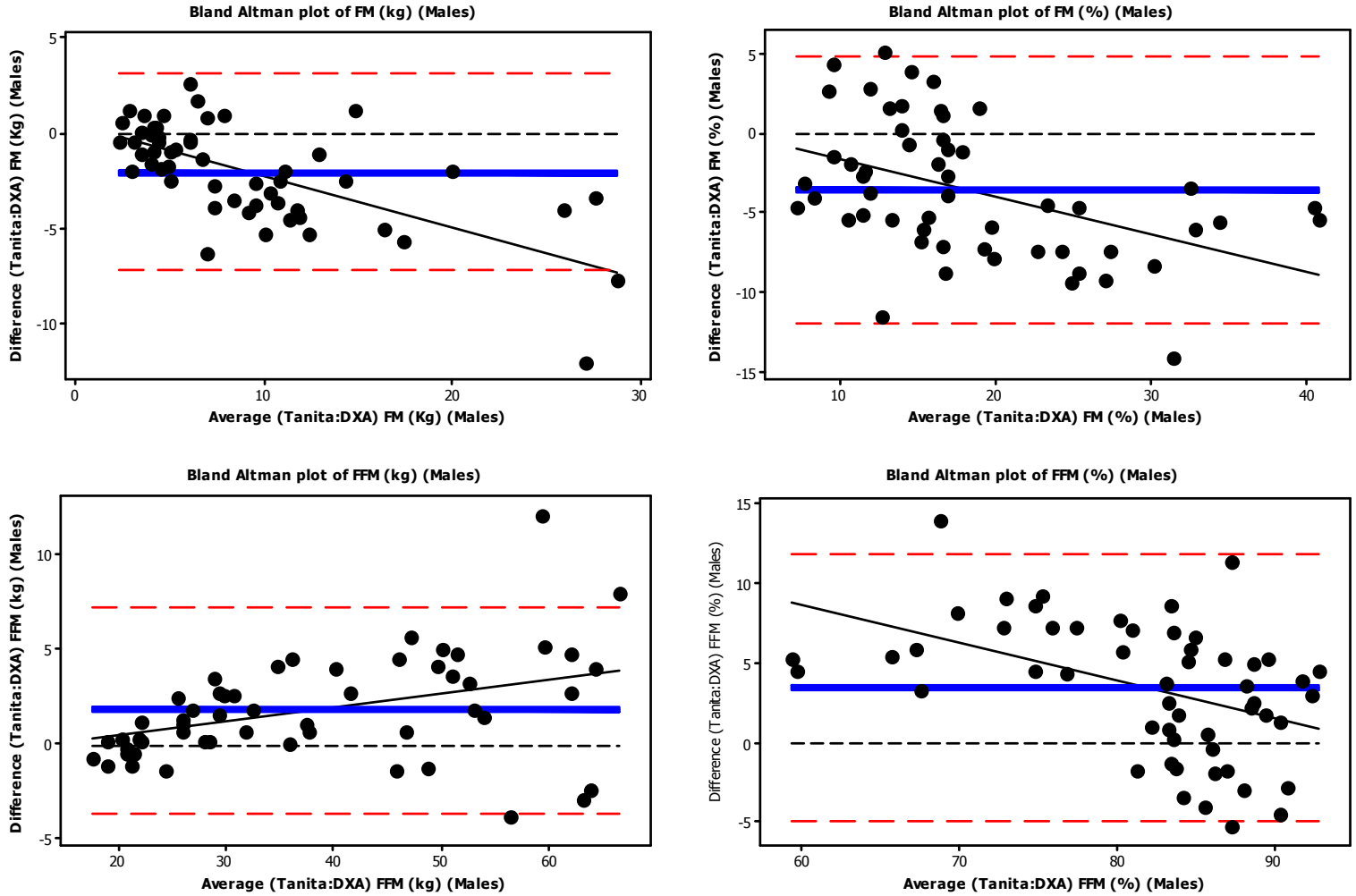


Figure 20: Bland Altman plots with 95% limits of agreement and regression line for %FM z-scores between TANITA and DXA imn healthy children (whole group)

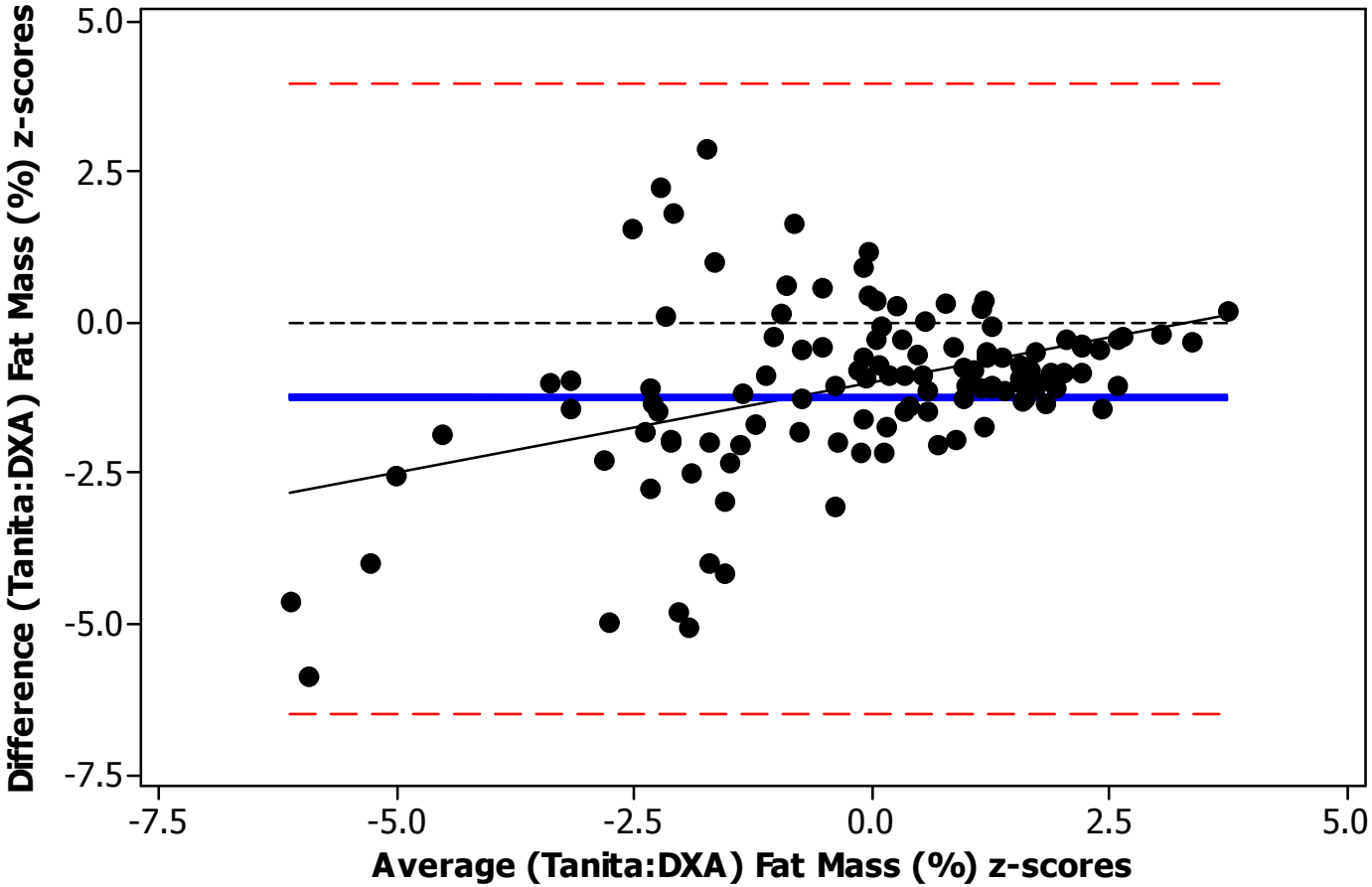
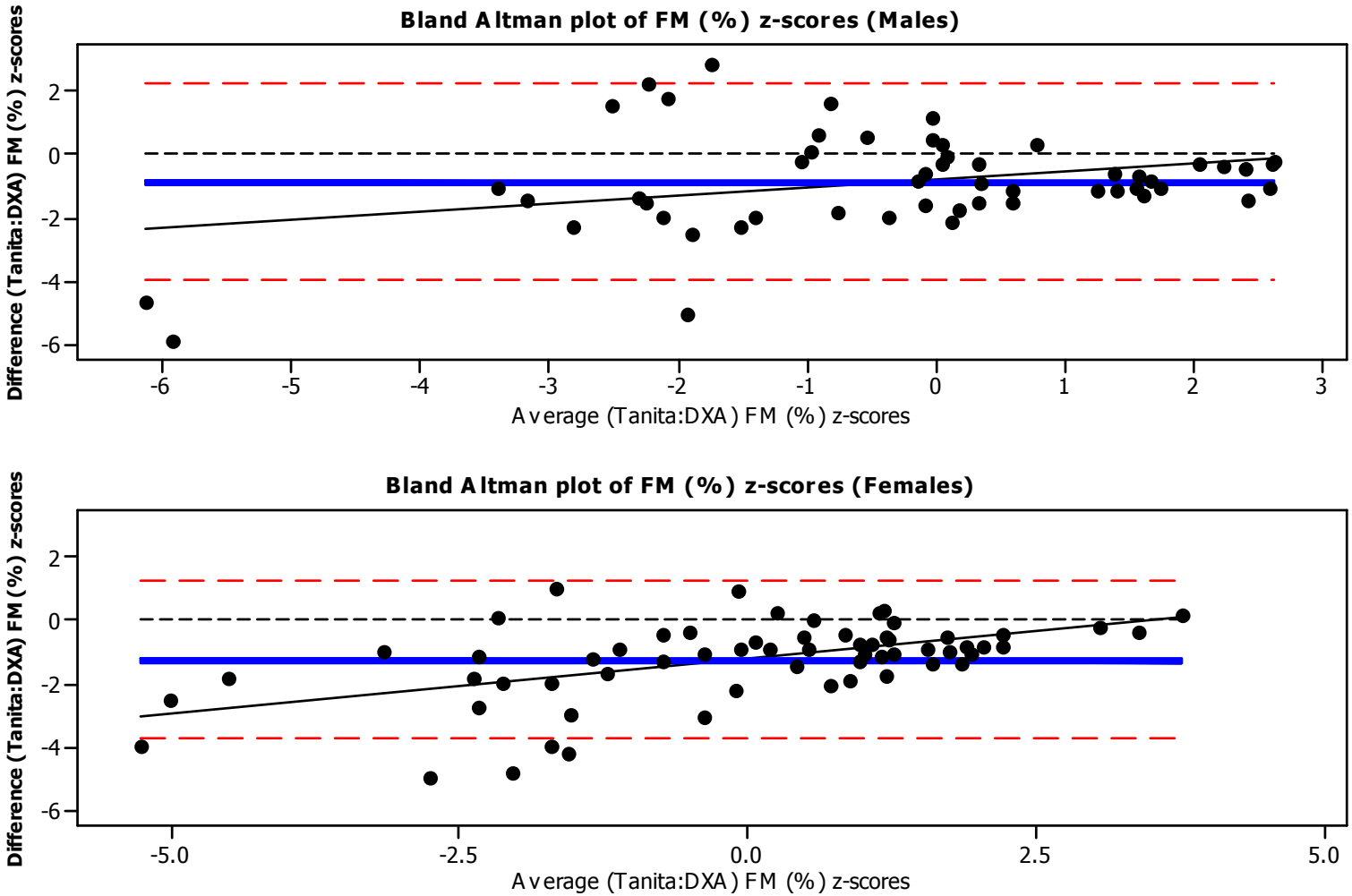


Figure 21: Bland Altman plots with 95% limits of agreement and regression line for body composition measures between TANITA and DXA in healthy children (analysis by gender)



4.4. Discussion

Body composition assessment is important in disease where changes of body weight do not always parallel alterations in body muscle and fat stores. A prime example is the chronic use of steroids, where body fat stores increase in parallel to fluid retention and muscle wasting.

There are several methods to assess body composition but at the moment none is able to assess accurately the body composition in the living human. Some of the most precise methods are impractical in clinical use or in large public health surveys whereas the accuracy of the more practical bedside methods is questionable and should always be tested against reference methods to the population intended for use. Indeed that was the primary aim of this study. To test the validity of a foot to foot BIA appliance against a body composition reference method.

In the current study, measurements of body composition carried out by the two different methods were highly correlated. This is not surprising as body composition in BIA is predicted by mathematical models, where body weight contributes substantially to the estimation of FM or muscle stores. On the other hand correlation does not parallel the agreement between the methods and whether these are inter-changeable at individual level (578). Thus the agreement of the two body composition methods was assessed using Bland-Altman plots. The overall bias of BIA was estimated to approximately +2.2 kg of FFM or -4.5% of FM and the limits of agreement were wide for all the body composition parameters. In practice this corresponds to underestimation or overestimation of real %FM by an extra 13.3% to -4.3% fat respectively. In a 35 kg child with actual body fat of 15% the BIA predicted %FM can be between 1.7% to 19.3%. Measurements of body composition with TANITA tended to underestimate FM and, accordingly overestimate FFM in a higher degree in girls than boys. However in both cases the bias was large and the limits of agreement wide.

The findings of this study are in accordance with previous work (573) where the same model of TANITA underestimated FM and overestimated FFM in a cohort of British eight y old children and, by Lazzer et al (580) in overweight/obese adolescents (Table 4.5). Opposite results and overestimation of FM by TANITA were also reported (581) and this may be due to the different devices and prediction algorithms used to calculate FM in the studies or different sample characteristics and the physiological changes that occur in body composition with age and gender (582).

In this study a gender specific effect of the size of the measurement on the agreement of the methods was found and should be considered when interpreting results with TANITA. In boys with increasing percentage fat, TANITA tended to underestimate fat more. Lazzer et colleagues (580) showed that two different foot to foot BIA appliances

underestimated low %FM but overestimated high %FM in overweight/obese children when compared with DXA body composition assessments. Both these findings are particularly important when assessing or monitoring body composition changes in overweight and obese children. On the other hand one should always bear in mind that DXA is a reference method rather than an actual “gold standard” (556;558;583) and its accuracy may be influenced by the size of the measurement (584), the location of FM (585) and the hydration of the FFM (586).

In this study it is presented for first time a new way of analyzing the agreement of body composition methods by converting %FM in z-scores using recent British normative data (579). In this way any age and gender confounding effect on the assessment of the effect of the size of the measurement on the agreement of the two methods was eliminated. Indeed similar patterns were observed for boys and girls in contrast to the crude values of %FM. A better agreement was found between the methods for children with %FM around or above the mean of the reference population. In contrast the limits of agreement were wide for negative %FM z-scores and thus TANITA consistently underestimated %FM is children with extremely low body fat. Poor validity of body composition methods is common at the extremes of body composition (573). This can be attributed to small/inadequate sample sizes recruited in order to validate body composition at the two edges of the body composition spectrum or to the invalidation of the theoretical body composition constants in obese and underweight subjects.

In this study body composition presented poor agreement with DXA to assess body composition in healthy Scottish children and therefore the two methods are not interchangeable. However the estimation error of BIA techniques is inherent to the error that the prediction equations cause when they are used in populations different from those for which they have been validated. Manipulation of body impedance values in a way that would be independent of such prediction equations, may be a better and most reliable method to use BIA. Recently Professor Wright and colleagues, University of Glasgow, developed a new method to use bioimpedance measurements and classify children as having relatively high, average or low fatness and leanness independently (587).

Although BIA may not be accurate to precisely assess body composition its use to assess longitudinal changes in follow up studies may be more useful. Its sensitivity to detect changes in body composition should be addressed with follow up studies measuring body composition at intervals with BIA and other reference methods (588;589).

This study was carried out to evaluate the validity of TANITA to assess body composition in patients with CD. However this study recruited only healthy children as this was a more convenient sample of a pre-existing ongoing study and therefore the validity of the method may be different in disease where the theoretical assumptions and constants that

each method considers in the estimation of body composition may not be valid (590;591). Separate validation studies, in patients' groups, are needed to check the validity of BIA (569;590). In paediatric IBD, Dung et al (592) tested the validity of several published hand to foot BIA prediction equations to assess FFM accurately compared to DXA measurements and proposed a new prediction equation for estimation of FFM in children with CD. In the same way validation of the foot to foot BIA appliance against reference methods of body composition, may offer a potential tool to easily, quickly and safely monitor body composition in paediatric patients with IBD.

Although it was found that the foot to foot appliance is not accurate enough to assess body composition in children, this technique was still used in the studies of this thesis. This was because there is no other alternative method of body composition to use for routine clinical and research investigations. Methods like double-labelled water, and DXA scans although more reliable than the foot to for bioimpedance are difficult to be used in clinical settings, are expensive and need much co-operation from the participants. Moreover the results of this study give more insight on how to evaluate and interpret the findings in following studies of this thesis where it was used the foot to foot bioimpedance.

Table 4.5: Paediatric studies comparing foot to foot BIA devices with DXA in the assessment of body composition in healthy Scottish children

Study	Sample	BIA equipment	Age	Bias	Size and gender effect
Hosking et 2006 (573)	203 (106 M); British	Tanita TBF-300M	8.3 ± 0.3	BIA underestimated FM & %FM and overestimated FFM & %FFM	In small boys %FM was overestimated whereas in big boys it was underestimated; No similar effect in girls
Lazzer et al 2003 (580)	53 (20 M); overweight/obese adolescents; French	Tanita TBF-625 & Tefal Bodymaster Vision	14.1 ± 1.4	Both devices underestimated %FM	Bias higher in boys than girls; Both devices underestimated low %FM but overestimated high %FM particularly in boys
Sung et al 2001 (581)	49;17 obese; Chinese	Tanita TBF-401	13 ± 3.1	BIA significantly overestimated FM but not %FM	

CHAPTER FIVE

The effect of exclusive enteral nutrition on body composition and micronutrient status in children with active CD

OUTLINE

This chapter describes a prospective observational study on the effect of EEN on body composition and micronutrient status in paediatric patients with active CD. Associations between systemic markers of disease activity, gut markers of disease activity and indices of nutritional status are explored.

5.1. Introduction

Exclusive enteral nutrition is the mainstream treatment of active paediatric CD in UK with double benefit. It induces clinical remission in more than 60% of the cases, and at the same time improves the nutritional status of the patient (297). This has been described extensively in Section 1.5.

In IBD malnutrition can occur with all its different facets. Protein-energy undernutrition, low circulating levels of micronutrients and poor bone health are all seen in IBD patients (35) (Section 1.3). Vitamins, trace elements and minerals are key nutrients of the body's harmonic function and homeostasis, and in IBD they play an additional role (Table 1.3.2). Zinc, selenium and carotenoids are important components of the body's antioxidant system and may protect biological molecules from oxidative damage caused by the excessive production of reactive oxidative species from the activated immune system in IBD.

Until now the mechanism of EEN action in inducing remission remains unexplained (Section 1.5.2). Early suggestions that nutritional replenishment is the main mechanism have been revised by recent evidence that EEN has direct anti-inflammatory effects, which precede nutritional rehabilitation (267). On the other hand a therapeutic mode of action mediated by changes in micronutrient status is possible but has not been investigated thoroughly thus far. Two previous studies were limited to the effect of EEN on the body's antioxidant systems and stores (148;336) and only one of these (148) made associations with concomitant changes in disease activity. The authors of this study (148) showed that CD children had reduced activity in enzymatic antioxidant systems in erythrocytes, which did not improve with eight weeks treatment on EEN despite parallel improvement of the clinical activity and systemic inflammatory markers (CRP and TNF-a). Although these results imply that the therapeutic action of EEN in CD is independent of changes in the antioxidant systems, changes in other micronutrient status during EEN and their association with disease activity need to be clarified further. Likewise Akobeng and colleagues (336) did not find significant changes in antioxidant mechanisms or markers of oxidative stress in children with CD after a four week course with EEN. However they did not assess disease activity or

changes in inflammatory markers levels and how these correlate to changes in the micronutrient status.

There are other valid reasons why monitoring micronutrient status during EEN is important in CD. Improvement of protein-energy status in CD children under treatment with EEN, is reflected by overt changes in body anthropometry but whether these changes coincide with replenishment or maintenance of micronutrient body stores is unknown and not easy to assess. Although most commercially available feeds provide the recommended micronutrient requirements for healthy children their corresponding adequacy in CD patients is uncertain. Malabsorption, increased consumption and increased needs in CD may require higher intakes than those established for healthy children. Abad-Lacruz et al (335), 20 years ago, failed to show improvement in the vitamin status of IBD adults on EEN despite provision of micronutrients in doses significantly higher than the recommended dietary allowance. Moreover nutritional supplements are “artificial composite food” made up of simple nutrients for which recommended allowances have been established and thus may be insufficient in non-nutrients that naturally occur in food but are rarely included in artificial feeds.

Thus far no paediatric study has assessed changes in the status of a large number of vitamins and minerals in CD under treatment with EEN. The objective of this study was to measure the body composition and micronutrient status of children with CD and to assess their change under EEN. A second aim was to explore any association between systemic markers of disease activity, gut markers of disease activity and indices of nutritional status including body composition measures and micronutrient blood status.

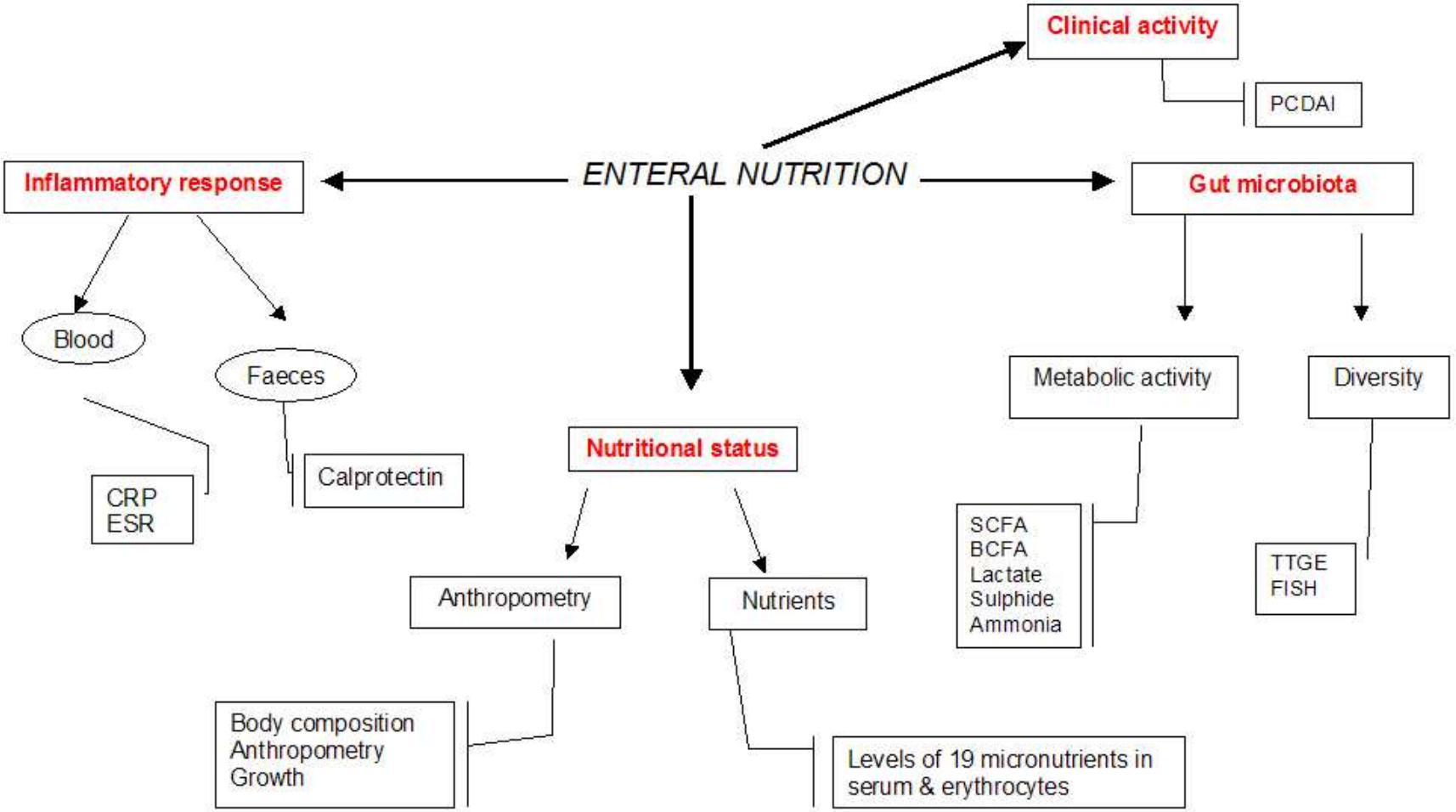
5.2. Subjects and methods

5.2.1. Subjects

Participants of this study were children CD who took part in a study on the effect of EEN on the nutritional status (Chapter 5), disease activity markers (Chapter 6), and gut bacterial metabolic activity and diversity (Chapter 7) of paediatric patients with CD (Fig 22).

All the paediatric CD patients with active disease (<16 y old) who commenced an EEN course, as part of their standard medical management were eligible to participate. These patients were either newly diagnosed or patients with longstanding disease in clinical relapse. Newly diagnosed patients were approached within two days of colonoscopy or when the biopsy histology report was announced for patients with dubious endoscopic findings. The patients with longstanding disease in relapse were recruited at the end of their outpatient clinical appointment. An introductory letter and an information leaflet (Appendix) about the study were posted to all patients with long standing disease to allow them more time to consider the study in case they were eligible for recruitment.

Figure 22: Study flowchart on the effect of EEN on inflammatory response, nutritional status and gut microbiota



5.2.2. Exclusive enteral nutrition

In all cases, the patients followed an eight weeks planned EEN course with a polymeric casein-based liquid supplement enriched with Transforming Growth Factor-β (Modulen®, Nestle). The feeds provided the recommended daily energy requirements for age and gender (COMA, 1990) favouring a higher intake of protein. A higher energy intake (200-500 kcals) was given to those children who presented underweight. The feeds were administered orally or when not tolerable via a fine bore nasogastric tube or a percutaneous endoscopic gastrostomy. No other foodstuffs were allowed during the treatment with the exception of water, tea and clear mints. At the end of treatment, a step-by-step food reintroduction program was followed before the patient returned to normal diet. Patients with suboptimal nutritional status were asked to continue supplementary feeds (500 kcals / d) in addition to their normal diet for one to two weeks after treatment completion.

5.2.3. Blood sampling and initial manipulation

For the purposes of this study, three blood samples were obtained from CD children before EEN initiation, on treatment cessation and whilst patients were on their normal diet. As it was deemed unethical to venopuncture children for research only purposes, extra blood was taken when patients were due to give clinical monitoring samples. Eight ml of venous blood were collected for research purposes in NH Trace Elements Sodium Heparin tubes (Vacuette, 456080, Austria). Six ml were centrifuged at 3500 g for 15 min at 4 ^{o}C and the plasma fraction was separated from the rest of the cellular matrices. Five hundred μl of plasma were stabilized with 6% metaphosphoric acid (Sigma Aldrich, 239275, UK) to prevent vitamin C oxidation. The samples were processed within an hour of blood sampling. All bloods and serum specimens were stored at –70^{o}C covered in aluminium foil for a maximum of six months until further analysis.

5.2.4. Micronutrient assays

In total, 19 micronutrients were measured in the serum, whole blood or erythrocytes of children with CD. Ferritin was measured as a marker of iron status and total cholesterol to adjust vitamin E concentrations for total lipid concentration.

Serum calcium and phosphate were available from the patients' biochemical reports and had been analyzed according to standard hospital protocols. The remaining nutrients were measured in the Scottish Trace Element and Micronutrient Reference Laboratory, GRI.

Trace elements (Zn, Se, Cu)

Briefly trace elements zinc, copper, and selenium were measured with an inductively coupled plasma mass spectrometer (ICP-MS) using germanium as an internal standard.

Vitamin B_1

The method measures thiamine disphosphate (B_1) in whole blood as an index of vitamin B_1 status. The thiamine disphosphate is oxidised using post column derivatisation with alkaline potassium ferriccyanide to form the fluorescent thiochrome derivative which is measured by reverse phase HPLC with fluorescent detection. The sensitivity of the method is down to 5µg/L the intra and inter CV% are 6% and 7% respectively.

Vitamin B_2

Packed red cells are precipitated with methanol, centrifuged and the supernatant is injected for HPLC analysis. Flavin adenine, dinucleotide, flavin mononucleotide and riboflavin are separated on an isocratic HPLC system with a reverse phase column and detected by fluorescence . The CV of the method is 3.6% at a mean whole blood flavin adenine dinucleotide value of 135 nmol/L and 4.8% at a red cell flavin adenine dinucleotide mean value of 2.8 nmol/gHb.

Vitamin A & E and carotenoids

Serum is deproteinised with ethanol containing internal standard. After centrifugation the vitamins, carotenoids and internal standard are extracted with hexane. The hexane is evaporated and the residue is dissolved in the developing solvent. An aliquot of this solution is injected onto a C18 reversed phase chromatographic column and absorbance measurements at 325 nm and 295 nm for vitamin A and E respectively and 450 nm for carotenoids. Peak height ratios are used to quantify vitamins and carotenoids. B-carotene is calculated by area. The intra and inter batch imprecision of the method is described in Table 5.1.

Table 5.1: Coefficient of variance for vitamin A, E and carotenoids assay

Nutrient	Intra-batch imprecision	Inter-batch imprecision
Retinol	5.4	7.8
α-tocopherol	2.0	7.0
Lutein	6.9	9.8
Lycopene	9.0	14.0
α-carotenoid	6.5	16.5
β-carotenoid	7.6	15.0

Vitamin C

Blood samples are stabilised to prevent oxidation and deproteinised with metaphosphoric acid. Following centrifugation and separation, an aliquot of the supernatant is injected on a C18 reverse chromatographic column and the ascorbic acid concentration assayed using an electrochemical detector. The within batch CV is 1.03% at a level of 31.66 µmol/L and the between batch CV is 3.69% at a level of 38.16 µmol/L.

Vitamin B_6

The method measures pyridoxal-5-phosphate concentration in red blood cells as an index of vitamin B_6 status. Pyridoxal-5-phosphate and pyridoxal are converted to their semi-carbazone fluorescence derivatives which are measured by reverse phase HPLC with fluorimetric detection. Pyridoxal is measured directly using its natural fluorescence. The intra assay CV for pyridoxal-5-phosphate is 5.2% at 367 pmol/gHb and 6.9% at 370 pmol/gHb.

Vitamin D

The 25-Hydroxy vitamin D was measured with a IDS 25-Hydroxy vitamin D EIA kit (Immunodiagnostic Systems Ltd., Boldon, UK) is an enzyme immunoassay for the quantitation of 25-OH D and other hydroxylated metabolites in serum or plasma. Calibrators, controls and samples are diluted with biotin labelled 25-OH D. The diluted samples are incubated in microtitre wells which are coated with a highly specific sheep 25-OH D antibody for two hours at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin, is added and binds selectively to complexed biotin and, following a further wash step, colour is developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures are read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 25-OH D. The intra assay %CV of the method is 5.3, 5.6, 6.7 for mean concentrations of 39, 67.1, 165 nmol/L respectively. The inter assay %CV is 4.6, 6.4, 8.7 for mean concentrations of 40.3, 72.0, 132 nmol/L respectively.

5.2.5. Markers of disease activity

Haemoglobin, erythrocyte sedimentation rate, serum albumin and CRP were assayed according to standard hospital methods and were readily available from the patients' biochemical and haematological reports. Gut inflammation was assessed by measuring the faecal levels of calprotectin in stool samples collected close to the day of blood sampling. Faecal calprotectin was measured with a commercial available ELISA kit (Phical®, CALPRO, Lysaker, Norway) and according to the manufacturer's specifications. The outline of the

assay is described in Chapter 6.2.5. Changes in the levels of disease activity markers during EEN are presented in Chapter 6.

5.2.6. Anthropometry and body composition

Changes of baseline anthropometric characteristics and body composition were assessed during treatment with EEN. Measurement of weight, height and body composition assessment by BIA, were performed before, after one month, at the end of treatment, and when patients returned to normal diet according to standard protocols as described in detail in Chapter 4. Measurements were obtained at the clinic, or when not possible, home visits were done. Triceps skinfold thickness and mid upper arm circumference were measured on the left arm midway between the acromion and olecranon according to standard procedures by a trained researcher. Measurements were performed at least twice and values averaged if the difference between the two measurements was minimal.

5.2.7. Statistical analysis

Descriptive statistics are presented as mean and standard deviation, or medians with ranges for continuous data, and with counts and proportions for categorical data. Comparisons between groups were done using 2 sample t-test and Mann-Whitney test. Changes in micronutrient concentration during EEN were assessed with Wilcoxon signed-rank test. Likewise the effect of EEN on body composition measures was evaluated using anova of repeated measures with Bonferroni post-hoc test. Anthropometry and %FM were converted to z-scores using British national data (579;593). To assess the adequacy of circulating micronutrients in house laboratory reference range was used (Appendix). Association between micronutrient levels, body composition measures and disease activity markers were assessed with Spearman's rank correlation coefficient.

5.3. Results

5.3.1. Recruitment

Between October 2005 and August 2007 approximately 60 children started on EEN as part of their standard clinical management. Thirty one children were invited to participate, six children refused participation, and two withdrew the day after recruitment. One patient's participation was terminated deliberately after a final biopsy histology report was consistent with UC diagnosis. One patient experienced a cerebral vascular accident while on treatment with EEN and stopped treatment within a week of recruitment. For two patients, disease activity further deteriorated and both had to stop EEN and initiate a regime of steroids. Two

other patients were recruited 15 days and 21 days after EEN initiation and their results are not included in the arm of the study on the effect of EEN on longitudinal changes in nutritional status.

For 25 CD children at least one blood specimen was obtained during the observational period. For 13 of these patients, three serial samples were obtained before EEN treatment, close to the time of EEN cessation and on normal diet. For the rest 12 patients the full set of bloods was not available for a number of reasons (e.g. patients withdrew, patients were not due to have routine clinical bloods).

Similarly basic anthropometry and body composition measures were obtained for 25 CD children. For 16 of these children serial measurements of body composition were available over their nutritional therapy course. For the rest no serial follow up measurements were available or obtained due to a variety of reason (disease exacerbation and EEN termination, withdrew, impractical to take measurements).

5.3.2. Body composition and micronutrient status in children with CD. (Cross-sectional data)

5.3.2.1. Comparison of body composition between CD and healthy controls

Body composition measurements were obtained for 11 children close to the date of initiation of EEN, and for 14 children at their follow up clinical appointment, and whilst patients were on normal diet (Table 5.2). The majority of participants had abnormal systemic and gut specific inflammatory markers indicative of active disease (Table 5.2). Extensive disease with involvement of the upper and lower GI tract was the predominant site of disease location (Table 5.2). There was no significant difference in disease activity markers between boys and girls or between samples collected the day before the EEN initiation and those collected at follow up and whilst patients were on normal diet.

Table 5.2: Disease location, medical treatment, concentration and percent of patients with abnormal systemic and gut disease activity markers of children with CD

Characteristics	F (n=11)	M (n=14)	All (n=25)
***Disease location (n)**			
Ileitis (L1)		1	1
Colitis (L2)	1	1	2
Upper colitis (L2L4)	3	5	8
Ileocolitis (L3)	2	0	2
Upper ileocolitis (L3L4)	5	7	12
Concomitant treatment (n)			
Steroids (tapering dose)	1	1	2
Aminosalicylates	2	2	4
Immunosuppresants	1	8	9
Antibiotics	1		1
Caloric supplements	1	1	2
None	8	5	13
Disease activity markers			
Albumin (g/l) (mean ± SD)	30.8 ± 6.4	29.4 ± 7.3	30 ± 6.8
$Abnormal albumin (n;%)	7 ; 64	8 ; 57	15 ; 83
CRP (mg/l) (mean ± SD)	17.5 ± 13.2	31.5 ± 27.4	25.7 ± 23.3
$Abnormal CRP (n;%)	6 ; 55	11 ; 79	17 ; 68
ESR (mm/h) (mean ± SD)	49.8 ± 34.7	39.5 ± 23.8	44.2 ± 29.1
$Abnormal ESR (n;%)	9 ; 82	11 ; 85	20 ; 83
Calprotectin (mg/l) (mean ± SD)	1965 ± 547	1903 ± 757	1934 ± 639
$Abnormal calprotectin (n;%)	11 ; 100	15 ; 100	25 ; 100

* Based on Montreal classification

$ Abnormal to the laboratory or manufacturer reference range

The mean body weight, BMI and % body fat z-scores of children with CD were negative and at least one SD lower than the national reference data indicating that the participants as group were substantially underweight and presented fat deficits (Table 5.3). There was no significant difference in height, weight, BMI and %FM z-scores between boys and girls. No statistics analysis was conducted for the raw data (before conversion to z-scores) as the results would be misleading due to normal biological differences that occur in body composition with gender, age and maturation.

Table 5.3: Basic anthropometry and body composition characteristics (mean ± SD) with percent (%) of underweight, short stature in paediatric patients with CD

Measurement	F	M	Total
Patients (n)	11	14	25
Age (y)	11.1 ± 2.9	12.5 ± 1.8	11.9 ± 2.4
Weight (kg)	32.8 ± 13.4	36.3 ± 9.9	34.6 ± 11.6
Weight z-score (SD)	-1.0 ± 1.4	-1.1 ± 1.3	-1.1 ± 1.3
Height (cm)	142.5 ± 19.8	149.4 ± 11.1	146.1 ± 15.8
Height z-score (SD)	-0.1 ± 1.4	-0.5 ± 0.9	-0.3 ± 1.2
Height z-score < 2 SD (n;%)	1; 10%	1; 9%	2; 9.5%
BMI (kg/m^2)	15.5 ± 2.3	16 ± 2.3	15.7 ± 2.3
BMI z-score (SD)	-1.3 ± 1.1	-1.3 ± 1.3	-1.3 ± 1.2
BMI z-score < 2 SD (n;%)	3; 30%	3; 27%	6; 29%
% Fat mass	16.9 ± 9.2	11.7 ± 3.1	14 ± 6.9
% Fat mass z-score (SD)	-4.5 ± 9.4	-2.4 ± 2.1	-3.3 ± 6.4
% Fat mass z-score < 2 SD (n;%)	5; 56%	5; 45%	10; 50%
Fat free mass (kg)	26.7 ± 8.5	32 ± 8.4	29.6 ± 8.6
Fat mass (kg)	6.6 ± 6.5	4.4 ± 2.1	5.4 ± 4.6
Impedance (Ohms)	715 ± 70	665 ± 113	687 ± 97
Total body water (kg)	19.6 ± 6.2	23.5 ± 6.6	21.8 ± 6.6
MAC (cm)	20.4 ± 3.4	20.7 ± 3.1	20.6 ± 3.2
TSF (mm)	12.3 ± 5.0	12.1 ± 4.8	12.2 ± 4.8
TSF z-score (SD)	0.1 ± 1.6	1.8 ± 2.6	1.1 ± 2.3
% TSF z-score < 2SD (n;%)	1; 11%	0; 0%	1; 5%
MAMC (cm)	16.6 ± 2.1	16.9 ± 1.8	16.8 ± 1.9

Compared to a cohort of healthy Glaswegian children (Table 5.4), CD children had significantly lower BMI and weight z-scores ($p<0.0001$). No significant difference was found between the two groups with regard to height z-score. Similar to BMI, %FM z-score was significantly lower in children with CD than in healthy controls ($p=0.002$). BMI and weight z-scores were significantly lower in CD boys and girls compared with their same gender healthy peers. However CD boys but not girls had significantly lower mean %FM z-score ($p<0.01$) compared to their healthy peers (Table 5.4). No differences between genders were found for CD patients or healthy children regarding BMI, height and weight z-score.

Table 5.4: Basic anthropometric and body composition characteristics (mean±SD) with percent of underweight, short stature in paediatric patients with CD and healthy Glaswegian children

	CD			Healthy		
Measurement	**F**	**M**	**Total**	**F**	**M**	**Total**
Patients (n)	11	14	25	63	57	117
Age (y)	11.1 ± 2.9	12.5 ± 1.8	11.9 ± 2.4	12.6 ± 3.2	12.4 ± 3.7	12.5 ± 3.4
Weight (kg)	32.8 ± 13.4	36.3 ± 9.9	34.6 ± 11.6	46.4 ± 17.7	47.8 ± 19.1	47 ± 18.3
Weight z-score (SD)	-1.0 ± 1.4*	-1.1 ± 1.3*	-1.1 ± 1.3*	0.3 ± 1.2	0.5 ± 1.1	0.4 ± 1.1
Height (cm)	142.5 ± 19.8	149.4 ± 11.1	146.1 ± 15.8	149.2 ± 14	152 ± 20	150.5 ± 17.0
Height z-score (SD)	-0.1 ± 1.4	-0.5 ± 0.9	-0.3 ± 1.2	0.0 ± 1.0	0.1 ± 1.0	0.06 ± 1.0
Height z-score < 2 SD (n;%)	1; 10%	1; 9%	2; 9%	2; 3%	1; 2%	3; 3%
BMI (kg/m^2)	15.5 ± 2.3	16.0 ± 2.3	15.7 ± 2.3	20.2 ± 4.8	19.7 ± 3.9	20.0 ± 4.4
BMI z-score (SD)	-1.3 ± 1.1*	-1.3 ± 1.3*	-1.3 ± 1.2*	0.4 ± 1.2	0.6 ± 1.1	0.5 ± 1.1
BMI z-score < 2SD (n;%)	30%	27%	29%	1; 2%	0; 0%	1; 1%
% Body fat z-score < 2 SD	16.9 ± 9.2	11.7 ± 3.1	14.0 ± 6.9	22.7 ± 9.5	16.6 ± 7.4	19.9 ± 9.1
% Body fat z-score (SD)	-4.5 ± 9.4	-2.4 ± 2.1*	-3.3 ± 6.4*	-1.1 ± 4.0	-0.7 ± 2.4	-0.9 ± 3.3
% Body fat z-score < 2 SD (n;%)	5; 56%	5; 45%	10; 50%	17; 27%	12; 22%	29; 25%
Fat free mass (kg)	26.7 ± 8.5	32.0 ± 8.4	29.6 ± 8.6	34.0 ± 8.9	39.1 ± 15.5	36.4 ± 12.6
Fat mass (kg)	6.6 ± 6.5	4.4 ± 2.1	5.4 ± 4.6	11.7 ± 10.1	8.1 ± 5.8	10.0 ± 8.5
Impedance (Ohms)	715 ± 70	665 ± 113	687 ± 97*	601 ± 71	564 ± 72	584 ± 74
Total body water (kg)	19.6 ± 6.2	23.5 ± 6.6	21.8 ± 6.6	24.9 ± 6.5	28.6 ± 11.4	26.6 ± 9.2

* Mann Whitney test, different from healthy (for same gender or total) p<0.002

5.3.2.2. Mean circulating concentration of micronutrients in CD children

Systemic levels of micronutrients are displayed in Table 5.5. Females had higher concentrations of vitamin A ($p<0.04$), vitamin E ($p<0.01$), vitamin E / cholesterol ($p<0.03$), vitamin B_6 in erythrocytes and plasma than boys ($p<0.003$) (Table 5.5).

Table 5.5: Concentration of circulating micronutrient of CD children (mean ± SD)

Micronutrient	Total	Female	Male
Vitamin A (µmol/l)	1.1 ± 0.4	1.4 ± 0.5*	1.0 ± 0.3
Lutein (µg/l)	69.7 ± 35.3	75.2 ± 35.9	65.4 ± 35.5
Lycopene (µg/l)	85.4 ± 61.0	69.5 ± 55.2	98.0 ± 64.3
A carotene (µg/l)	16.8 ± 12.8	15.6 ± 10.3	17.7 ± 14.8
b-carotene (µg/l)	87.3 ± 84.3	93 ± 94.8	82.9 ± 78.5
Vitamin E (µmol/l)	23.7 ± 5.7	27 ± 5.5*	21.1 ± 4.5
Vitamin E/Chol (µmol/mmol)	7.2 ± 2.1	8.3 ± 2.3*	6.4 ± 1.5
Vitamin D (nmol/l)	64.8 ± 38.7	54.5 ± 42.7	73.0 ± 34.6
Vitamin B_1 (wbl) (ng/g Hb)	576 ± 134	605 ± 95	552 ± 157
Vitamin B_2 (wbl) (nmol/l)	433 ± 49	448 ± 47	420 ± 49
Vitamin B_2 (rbc) (nmol/g Hb)	2.4 ± 0.5	2.6 ± 0.3	2.2 ± 0.6
Vitamin B_6 (nmol/l)	32.8 ± 39.3	53.3 ± 53.1*	16.6 ± 7.4
Vitamin B_6 (rbc) (pmol/g Hb)	560 ± 241	719 ± 220*	435 ± 178
Vitamin B_{12} (pg/ml)	762 ± 482	915 ± 488	642 ± 459
Folate (ng/ml)	4.2 ± 3.2	4.4 ± 2.9	4.1 ± 3.5
Vitamin C (µmol/l)	35.4 ± 24.5	39.6 ± 29.9	31.8 ± 19.4
Zn (µmol/l)	9.7 ± 2.5	10.4 ± 2.8	9.1 ± 2.1
Cu (µmol/l)	21.7 ± 5.6	21.3 ± 3.8	22 ± 6.8
Se (µmol/l)	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
Mg (mmol/l)	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
P (mmol/l)	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
Ca (mmol/l)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
Ferritin (ng/ml)	48.4 ± 61	56.3 ± 84.7	42.1 ± 35.8
Cholesterol (mmol/l)	3.4 ± 0.5	3.4 ± 0.7	3.4 ± 0.3

* Different from male $p< 0.05$; whl: whole blood; rbc: red blood cells

For a substantial number of children the circulating levels of micronutrients were below the lower threshold of the laboratory reference range (Appendix) (Figs 23-25). Approximately three quarters of the participants had suboptimal levels for lutein, Zn, and Se whereas two thirds had low levels for carotenoids, lycopene, α-carotene, and β-carotene (Table 5.6; Figs 23-25). Low circulating levels were found also for serum vitamin A, vitamin B_6, folate, vitamin C, copper, ferritin and calcium. Although a few patients had low serum concentration of B_6, none had suboptimal levels when the latter was measured in erythrocytes. No patient had suboptimal concentrations for vitamin D, thiamine, riboflavin, and B_{12}. Suboptimal levels of serum B_6 were seen more often in boys than girls (Table 5.6).

Table 5.6: CD children (n;%) with suboptimal concentrations of systemic micronutrients compared with the laboratory reference range

Micronutrient	F (n=11)	M (n=14)	Total (n=25)
Vitamin A	1; 9	5; 36	6; 24
Lutein	9; 82	11; 79	20; 80
Lycopene	8; 73	9; 64	17; 68
A carotene	7; 64	9; 64	16; 64
b-carotene	7; 64	9; 64	16; 64
Vitamin E	0; 0	1; 7	1; 4
Vitamin E/Cholesterol	0; 0	1; 7	1; 4
Vitamin D	0; 0	0; 0	0; 0
Vitamin B_1 (wbl)	0; 0	0; 0	0; 0
Vitamin B_2 (wbl)	0; 0	0; 0	0; 0
Vitamin B_2 (rbc)	0; 0	0; 0	0; 0
Vitamin B_6	2; 18	9; 64*	11; 44
Vitamin B_6 (rbc)	0; 0	0; 0	0; 0
Vitamin B_{12}	0; 0	0; 0	0; 0
Folate	3; 27	6; 43	9; 36
Vitamin C	3; 27	3; 23	6; 25
Zn	8; 73	13; 93	21; 84
Cu	0; 0	2; 14	2; 8
Se	8; 73	10; 71	18; 72
Mg	0; 0	0; 0	0; 0
P	0; 0	0; 0	0; 0
Ca	0; 0	4; 33	4; 21
Ferritin	5; 45	3; 21	8; 32

*Different between genders p<0.02 for chi square test; whl: whole blood; rbc: red blood cells

Figure 23: : Percent of paediatric CD patients with suboptimal systemic levels of minerals & trace elements

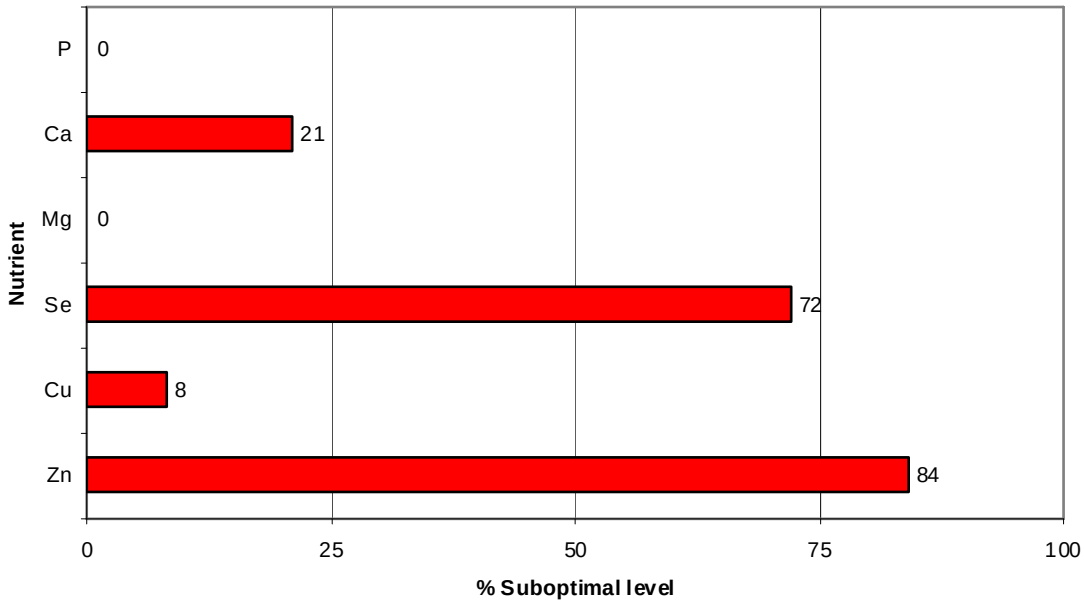


Figure 24: Percent of paediatric CD patients with suboptimal levels of water soluble vitamins & ferritin

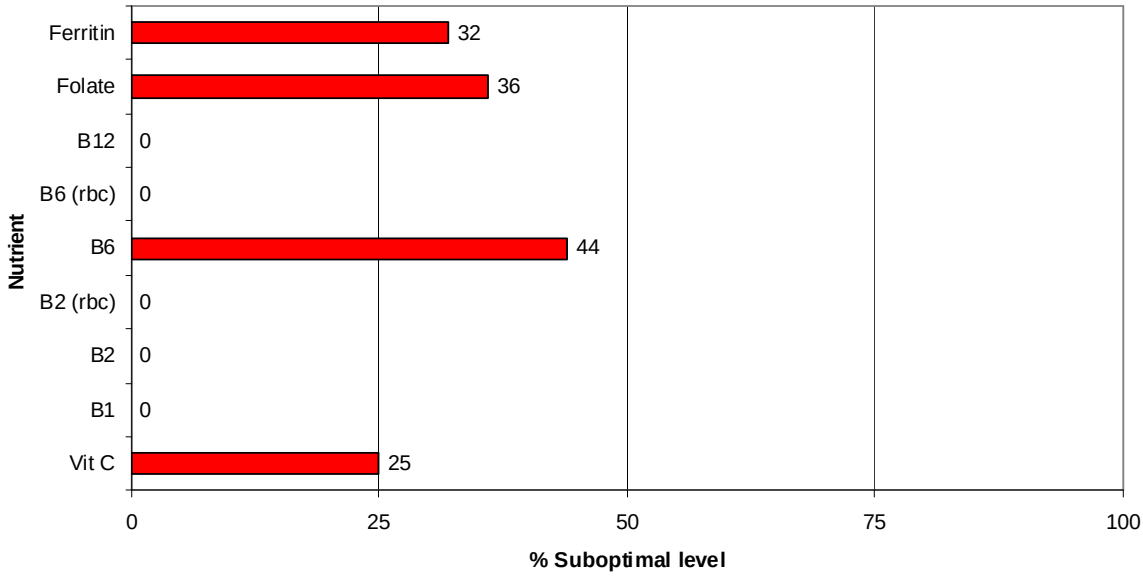
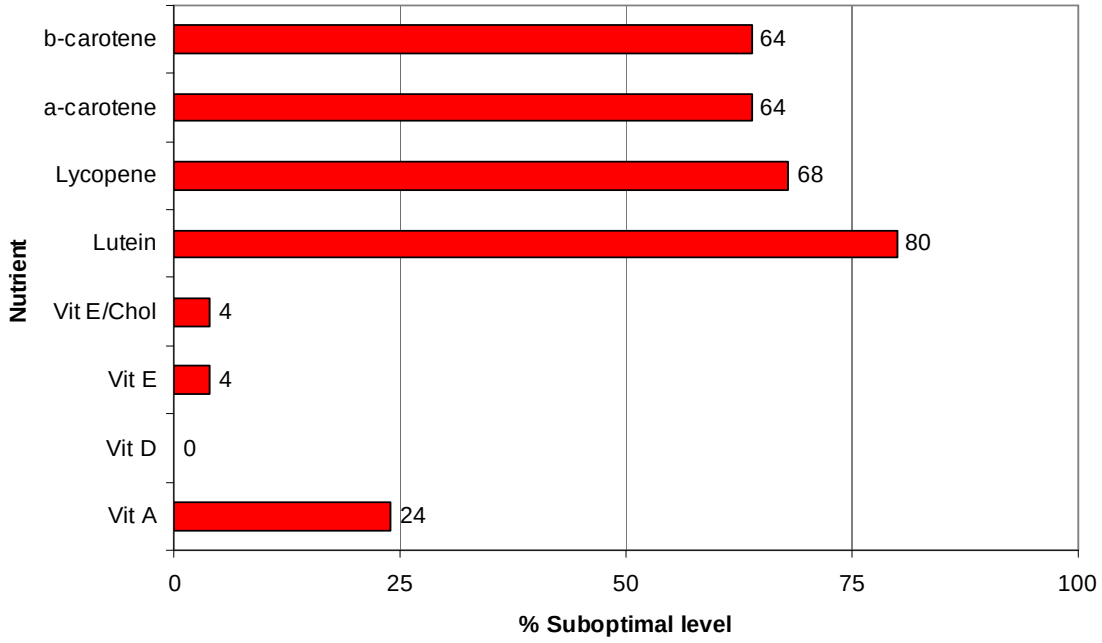


Figure 25: Percent of of paediatric CD patients with suboptimal levels of fat soluble vitamins



5.2.3. Correlation between disease activity markers, micronutrients and body composition in children with CD

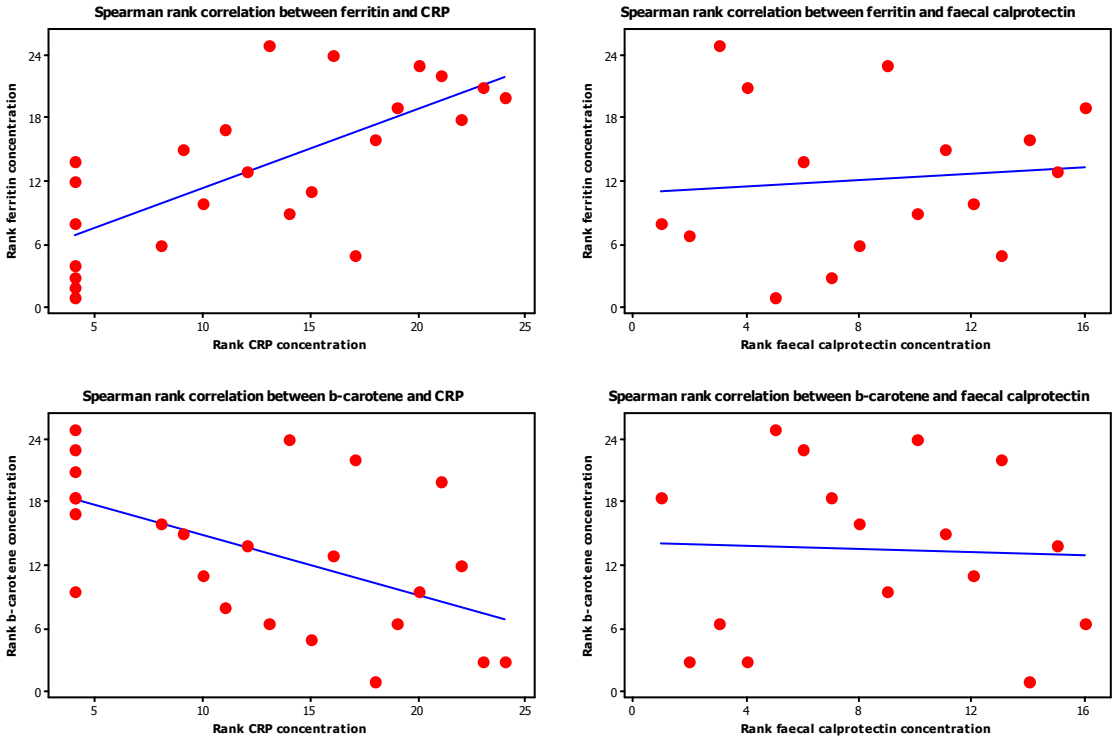
As the data were not normally distributed, values were ranked and Spearman rank correlations performed. The circulating levels of several micronutrients were strongly

associated with markers of disease activity (Table 5.7). Serum albumin and CRP were significantly associated with β-carotene (Fig 26), plasma B_6, vitamin C, zinc, ferritin (Fig 26), magnesium, calcium and folate, whereas faecal calprotectin levels were not associated with any micronutrient (Fig 26).

Table 5.7: Spearman correlation coefficients between systemic and gut disease activity markers and serum micronutrient concentrations in paediatric CD patients (r coefficient; p-value)

Micronutrient	CRP		ESR		Albumin		Calprotectin	
	R	p-value	R	p-value	R	p-value	R	p-value
Lutein	-0.50	0.01						
A carotene					0.42	0.04		
B-carotene	-0.55	0.01			0.40	0.05		
B_6 plasma	-0.39	0.06			0.50	0.01		
Vitamin C	-0.47	0.02	-0.44	0.03	0.67	0.001	All p>0.05	
Zn	-0.44	0.03			0.58	0.002		
Ferritin	0.71	0.001			-0.42	0.04		
Mg	-0.46	0.03	-0.45	0.03	0.44	0.03		
P	-0.50	0.03						
Ca	-0.55	0.02			0.92	0.001		
Folate					0.62	0.001		
Haemoglobin					0.7	0.001		

Figure 26: Spearman correlation coefficients between disease activity markers and serum micronutrient concentrations in CD children



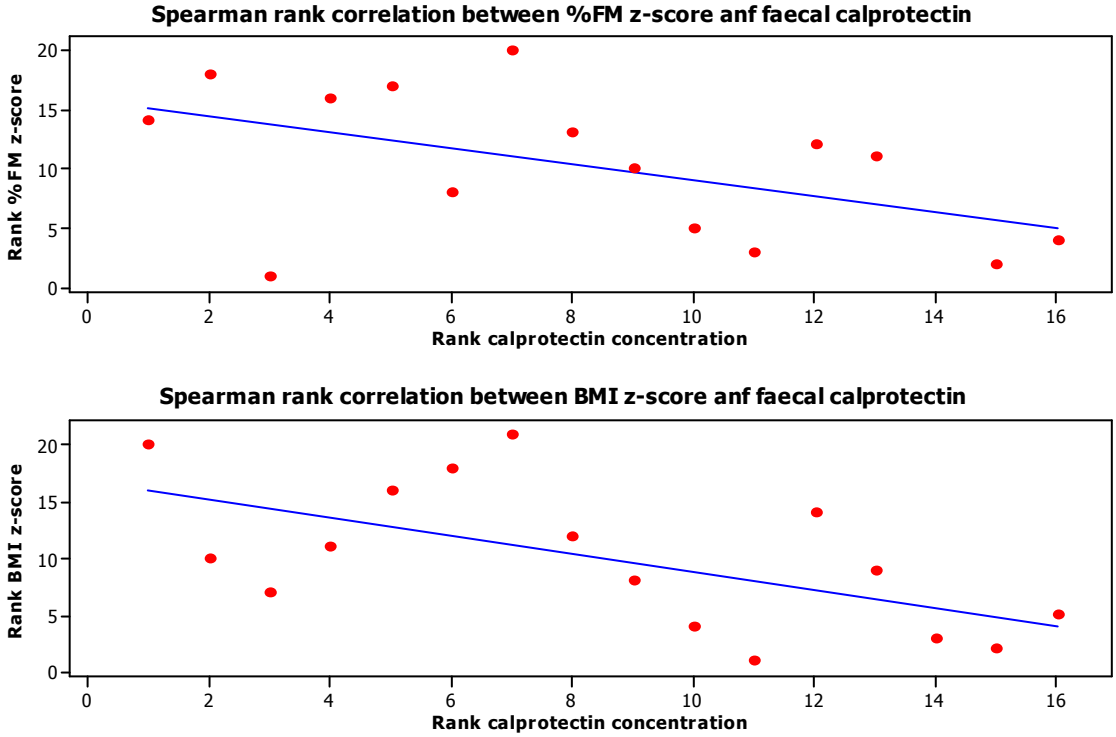
Faecal calprotectin and serum albumin concentrations were significantly associated with BMI and %FM z-scores (Table 5.8). With increasing gut inflammation, BMI and %FM were lower

suggesting that increased disease activity is strongly associated with thinness and underweight (Fig 27). A similar positive association was observed with serum albumin levels. Low albumin levels were associated with low BMI and body fat stores.

Table 5.8: Spearman correlation between systemic and gut disease activity markers and body composition measurements in paediatric CD patients (r coefficient; p-value)

Disease activity marker	BMI z-score		%FM z-score	
	R	p-value	R	p-value
Albumin	0.5	0.021	0.4	0.061
CRP	-0.4	0.097	-0.2	0.315
ESR	-0.5	0.037	-0.1	0.571
Calprotectin	-0.6	0.021	-0.5	0.073

Figure 27: Sprearman correlation between body composition measures and intestinal inflammation markers in paediatric CD patients



5.3.3. Changes in body composition and systemic micronutrient levels in CD children during treatment with EEN (prospective follow up data)

5.3.3.1. Patients characteristics

For thirteen children, follow up measurements of body composition and micronutrient levels were obtained (Table 5.9). Extensive disease with involvement of the upper GI tract and ileo-colon was the predominant site of disease (Table 5.9). Five children had other concomitant

medical treatment during EEN (Table 5.9). For all but three this was their first course on EEN. Five children entered complete clinical remission at the end of treatment (PCDAI≤10).

Table 5.9: Disease characteristics and concomitant medication of paediatric CD patients on treatment with EEN for eight weeks

	F (n=7)	M (n=6)	All (n=13)
Age	11.5 ± 3	12.9 ± 1.1	12.1 ± 2.3
Disease location* (n)			
Colitis (L2)	1	0	1
Upper & colitis (L2L4)	1	2	3
Ileocolitis (L3)	1	0	1
Upper ileocolitis (L3L4)	4	4	8
Concomitant treatment (n)			
Aminosalicylates	3		
Immunosuppresants	6		
Corticosteroids	1		
Antibiotics	1		
None	5		

* Montreal classification

5.3.3.2. Changes in systemic micronutrient status during EEN

The serum levels of several micronutrients changed significantly during EEN (Table 5.10; Figs 28 & 29). Compared to baseline, vitamin C, folate, thiamine, B_6 and selenium significantly increased at the end of treatment (Table 5.10, Fig 29). In particular a three-fold increase was observed for serum folate as opposed to a dramatic deterioration of all carotenoids levels at the end of EEN treatment (Table 5.10, Figs 28 & 29).

As mean serum concentration changes do not reflect the adequacy of body store status, the percentage of patients with suboptimal levels was calculated at the different time points of follow up. At baseline the serum levels for several micronutrients were below the laboratory reference cut offs with the most pronounced being carotenoids, trace elements, vitamin C, serum B_6 and folate (Table 10). Two thirds of the children had suboptimal levels for carotenoids whereas vitamin C, folate, and ferritin were at insufficient levels for more than 25% of the patients (Figs 30-34).

On treatment completion significantly fewer patients had suboptimal levels for most of the micronutrients apart from carotenoids, copper and ferritin which further deteriorated. Compared to 42% of the children at baseline, none of the children had suboptimal serum levels of folate at the end of treatment. Similar results were observed for selenium, which improved significantly over the treatment period, and 10 times fewer children had lower levels at the end of EEN. In contrast more than 90% of patients had carotenoid levels below the reference range on treatment cessation (Table 5.10). Indeed for many of the children, the latter were below the detection limits of the assay.

Significant changes were observed when children returned to their normal diet. Those nutrients that had improved at the end of EEN reversed to pre-treatment levels whereas

serum carotenoid concentrations increased and as a result significantly fewer patients presented biochemically insufficient levels (Table 5.10 Fig 28).

Table 5.10: Micronutrient concentrations (median) and percentage of patients with suboptimal levels before, at the end, and after treatment with EEN in paediatric CD patients

	Before EEN		After EEN		On normal diet	
Micronutrient‡	**Median**	**N; (%) with low levels§**	**Median**	**N; (%) with low levels§**	**Median**	**N; (%) with low levels§**
Vitamin A (µmol/l)	1.2	1; (7)	1.7	0; (0)	1.2†	1; (8)
Vitamin E (µmol/l)	27	0; (0)	24	0; (0)	25	0; (0)
Vitamin E:cholesterol (µmol/mmol)	7.7	0; (0)	6.6	0; (0)	5.8	0; (0)
Lutein (µg/l)	64	11; (79)	29.5*	11; (92)	108†	3; (25)
Lycopene (µg/l)	76	9; (64)	12*	12; (100)	165†	4; (33)
α-Carotenoid (µg/l)	10	9; (64)	10	12; (100)	14†	5; (42)
β-Carotenoid (µg/l)	53	9; (64)	41*	11; (92)	99†	;4 (33)
Vitamin D (nmol/l)	64	0; (0)	102	0; (0)	59†	0; (0)
Vitamin C (µmol/l)	29	3; (25)	51*	0; (0)	46	1; (8)
Thiamin (whl; ng/g Hb)	559	0; (0)	669*	0; (0)	573	0; (0)
Riboflavin (erythrocytes; nmol/g Hb)	2.5	0; (0)	2.5	0; (0)	2.1	0; (0)
Riboflavin (whl; nmol/l)	461	0; (0)	477	0; (0)	435	0; (0)
Vitamin B_6 (nmol/l)	36	4; (29)	63*	0; (0)	21	5; (42)
Vitamin B_6 (erythrocytes; pmol/g Hb)	662	0; (0)	721	0; (0)	438	0; (0)
Vitamin B_{12} (pg/ml)	662	0; (0)	843	0; (0)	505†	0; (0)
Folate (ng/ml)	2.85	5; (42)	10.5*	0; (0)	7.3	2; (17)
Zn (µmol/l)	8.5	9; (75)	11.5	7; (64)	10	11; (92)
Cu (µmol/l)	21.2	1; (8)	26	2; (18)	24.5	1; (8)
Se (µmol/l)	0.5	9; (69)	1*	1; (9)	0.7†	7; (58)
Mg (mmol/l)	0.8	0; (0)	0.9	0; (0)	0.8	0; (0)
Ferritin (ng/ml)	18.4	5; (42)	6.9*	7; (64)	15.7	2; (17)
Haemoglobin (g/dl)	10.8		11.6		11.7	

Values were significantly different from those before EEN (Wilcoxon signed rank test): *P<0.05. Values were significantly different from those after EEN (Wilcoxon signed rank test): †P<0.05.

‡Plasma, unless otherwise stated. §With reference to normal range; whole blood: wbl

Figure 28: Concentrations of carotenoids and Se before, at the end, and after treatment with EEN in paediatric patients with CD

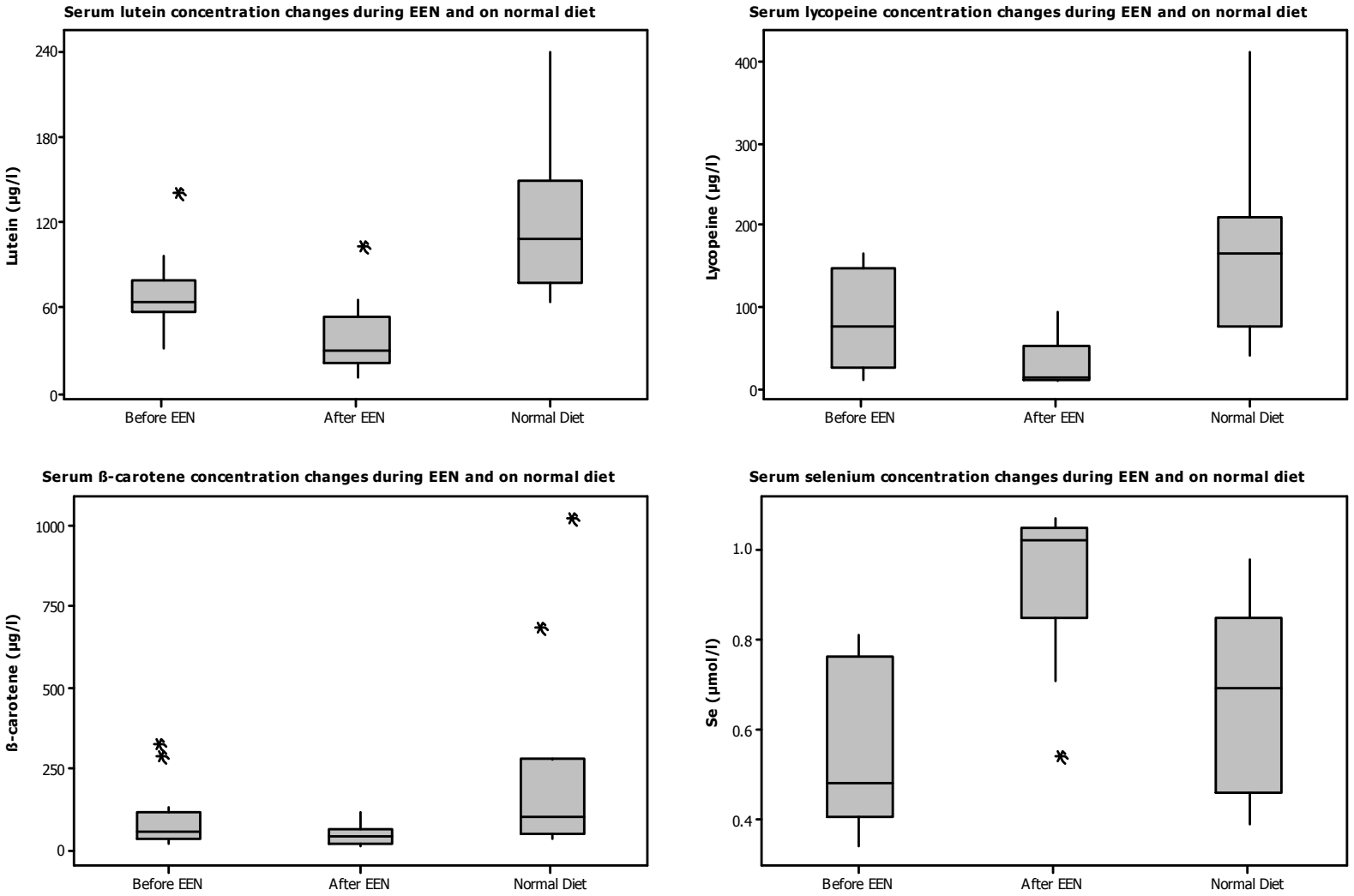


Figure 29: Concentrations of vitamin B1, B6, and folic acid before, at the end, and after treatment with EEN in paediatric patients with CD

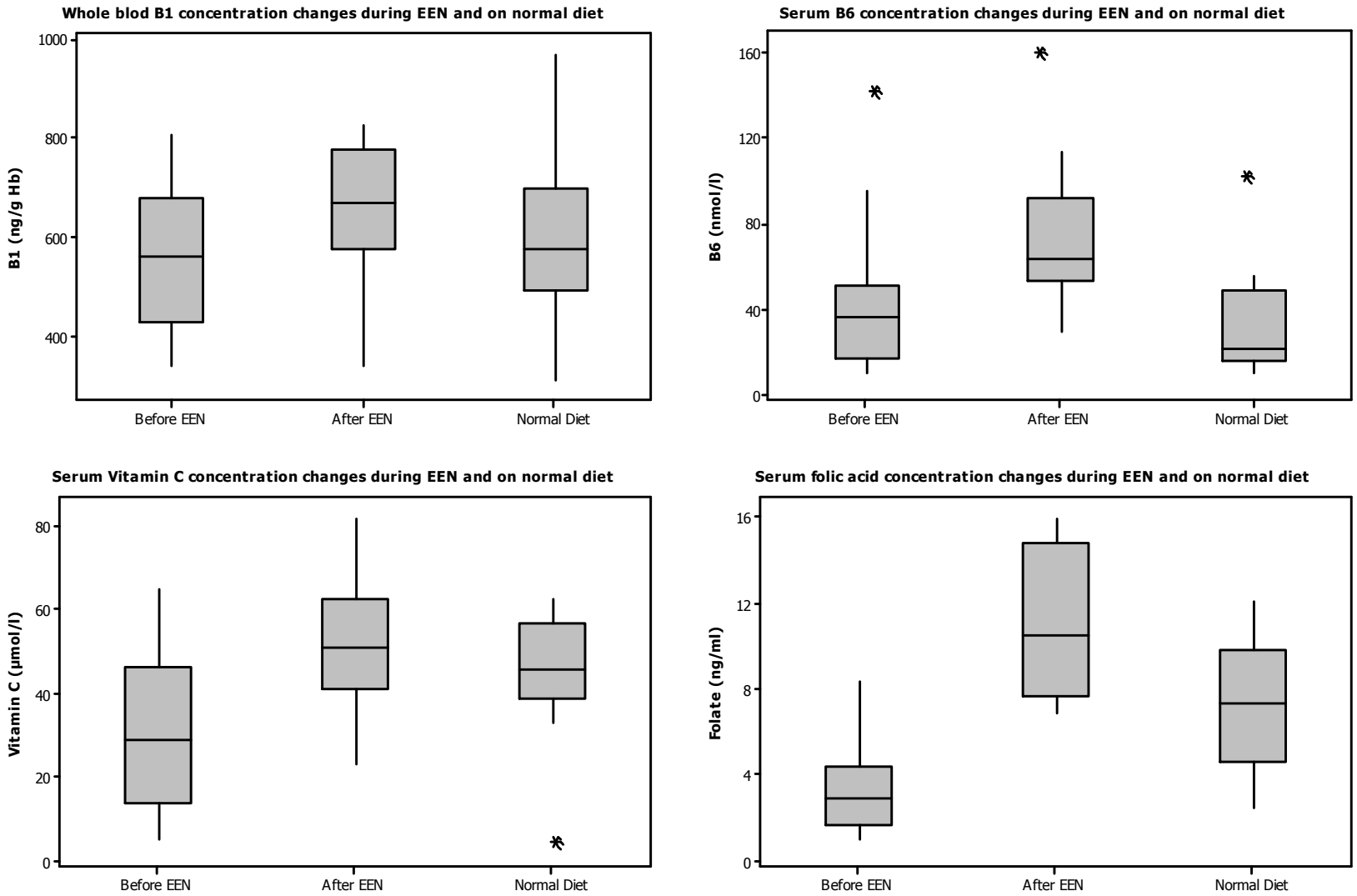


Figure 30: Percent of patients with suboptimal circulating levels of trace elements before, after EEN and on normal diet in paediatric CD

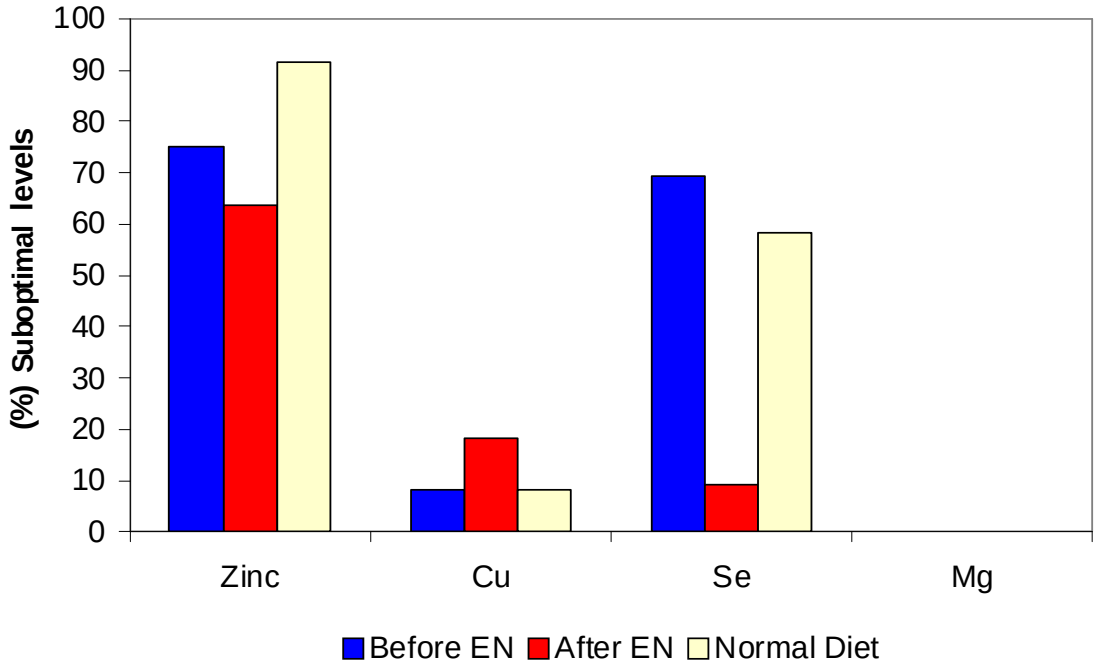


Figure 31: Percent of patients with suboptimal circulating levels of fat soluble vitamin before, after EEN and on normal diet in paediatric CD

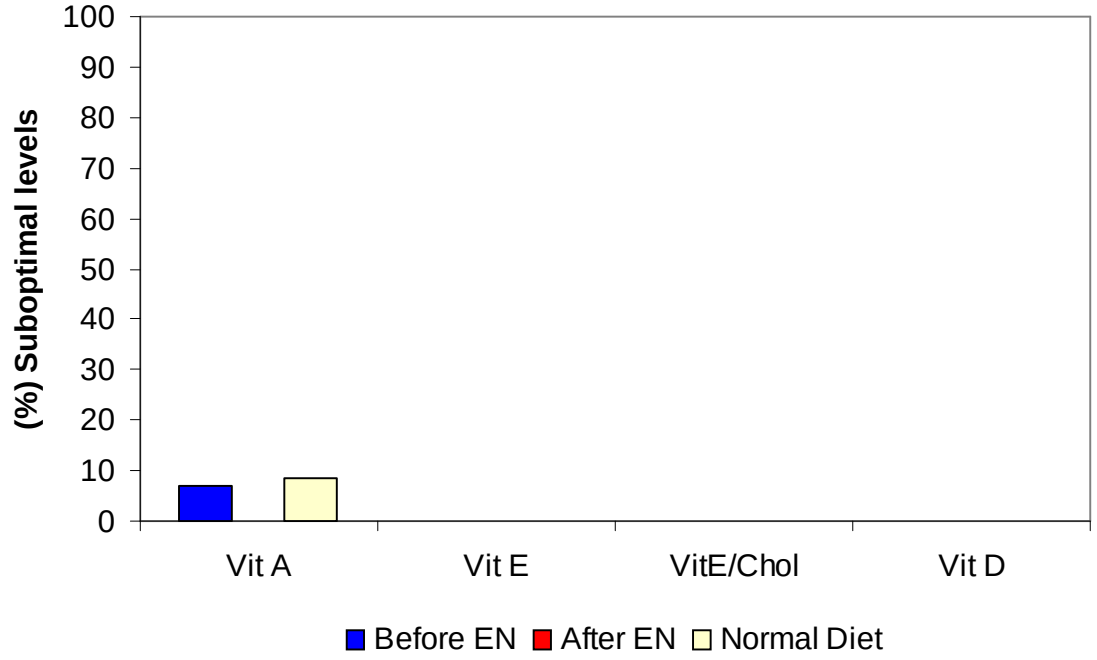


Figure 32: Percent of patients with suboptimal circulating levels of carotenoids before, after EEN and on normal diet in paediatric CD

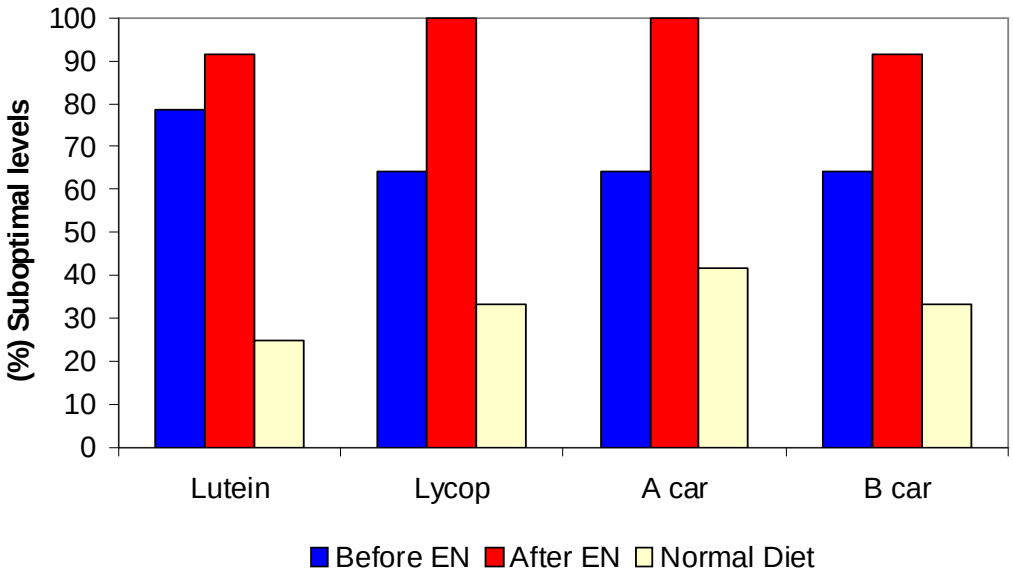


Figure 33: Percent of patients with suboptimal circulating levels of B complex vitamins before, after EEN and on normal diet in paediatric CD

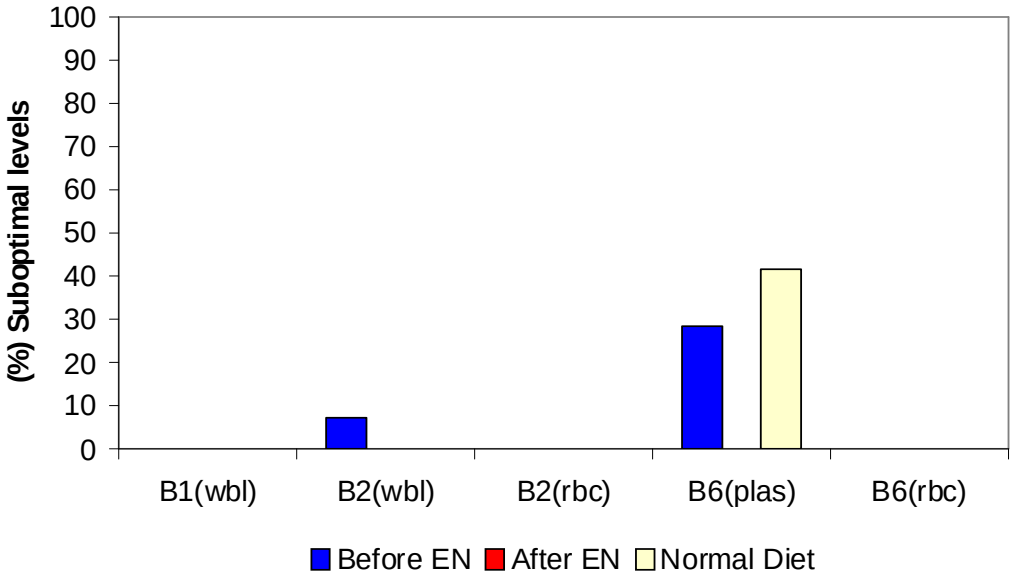
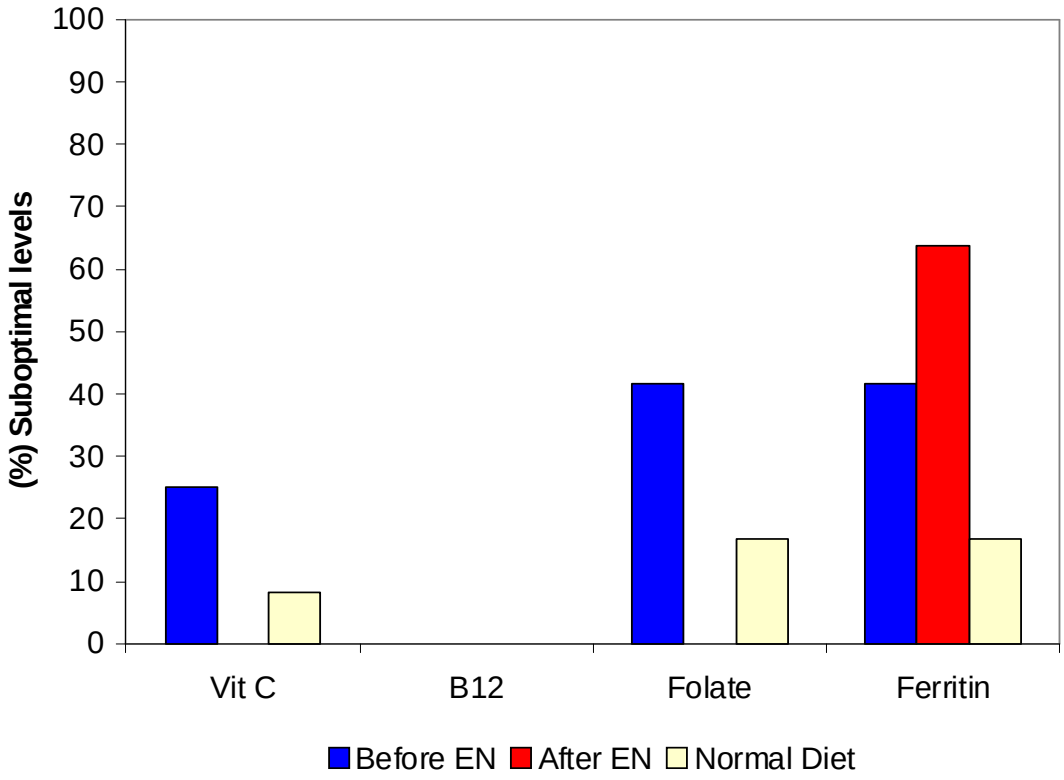


Figure 34: Percent of patients with suboptimal circulating levels of vitamins (C, B12, folate) & ferritin before, after EEN and on normal diet in paediatric CD



5.3.3.3. Changes in body composition during EEN

Body composition was assessed in 16 children whose measurements were available before treatment initiation, 30 days on treatment, at the end of EEN and on normal diet (Table 5.11). Weight (absolute mass and z-scores) and accordingly BMI and BMI z-score significantly increased within 30 days of treatment with an average increase of more than three kg. Weight increase plateaued and no further significant improvement was observed during the following month, and up till the end of treatment.

Although body fat, expressed as absolute mass (kg) or expressed as percentage of body weight increased during EEN, the difference did not reach statistical significance. In contrast, FFM in absolute mass was significantly increased at follow up compared with baseline values and achieved its highest peak at the end of treatment (all $p < 0.001$). However, when expressed proportional to total body weight, no significant difference from baseline was observed.

Similar to total body composition, all surrogate measurements of body composition (i.e. TSF, MAC, MAMC) significantly increased during EEN with a maximum increase observed at the end of eight weeks EEN. Changes in height were not assessed during EEN, due to the imprecision of the height measurements in short observation periods. Although body weight increased in both the patients who entered into remission and those who failed treatment, the magnitude of change was smaller in the latter group. However, FFM increased significantly at all time points of follow up ($p<0.006$) only in those patients who had entered clinical remission at the end of treatment. On normal diet no significant changes in body composition were observed compared to the end of treatment.

Table 5.11: Anthropometry and body composition changes during treatment with EEN and on normal diet in paediatric CD patients (mean ± SD)

Measurement	Before EEN	30 d on EEN	End of EEN	On normal diet
Weight (kg)	33.6 ± 11.9	36.9 ± 10.1*	36.3 ± 9.7*	38.2 ± 12.2
Weight z-score (SD)	-1.5 ± 1.6	-0.7 ± 1.2*	-0.9 ± 1.1*	-0.8 ± 1.3
BMI (kg/m^2)	15.1 ± 2.8	16.7 ± 2.1*	16.7 ± 1.9*	16.8 ± 2.6
BMI z-score (SD)	-2 ± 1.7	-0.7 ± 1.0*	-0.7 ± 1.0*	-0.8 ± 1.2
FM (kg)	4.8 ± 5.5	5.8 ± 5.1	5.9 ± 4.7	6.9 ± 5.9
FM (%)	12.0 ± 8.4	14.5 ± 8.4	15.1 ± 7.8	14.1 ± 7.6
FM z-score (SD)	-5.5 ± 6.7	-2.5 ± 2.6	-2.6 ± 3.5	-2.5 ± 2.6
FFM (kg)	29.1 ± 8.2	31.0 ± 7.0*	30.4 ± 6.7*	32.1 ± 8.0
Impedance (Ohms)	719 ± 100	645.0 ± 99.0*	660.0 ± 105*	626.0 ± 57
TBW (kg)	21.3 ± 6.0	21.1 ± 6.6	22.3 ± 4.9*	23.5 ± 5.8
TSF (mm)	10.5 ± 5.6	13.3 ± 5.0*	13.8 ± 4.7*	12.7 ± 4.9
TSF z-score	-0.32 ± 1.2	0.49 ± 0.9*	0.49 ± 0.25*	0.40 ± 0.95
MAC (cm)	19.8 ± 3.3	21.3 ± 3.1*	21.3 ± 2.6*	21.4 ± 3.1
MAMC (cm)	19.8 ± 3.3	21.3 ± 3.0*	21.3 ± 2.5*	21.3 ± 3.1

* Statistical significant from “Before EEN” for a-nova of repeated measures and Bonferroni post-hoc test All $p<0.02$ Mean ± SEM

5.4. Discussion

Nutritional status is a composite term that grossly describes the state of energy/protein, and other micronutrient stores in the human body. Excessive consumption causes overnutrition and the risk of toxicity whereas poor intake increases the risk of undernutrition. Protein/energy undernutrition is important as it increases the risk of disease-associated complications, mortality, and impact negatively on length of hospital stay, and in children may impair linear growth and bone health.

On the other hand micronutrients, including minerals, trace elements and vitamins are important nutrients in body homeostasis, function and metabolism. Suboptimal intake can cause nutrient-associated anaemia, osteoporosis, neurological disorders, altered taste, depression,

epithelial defects, and it is only when the body stores have been depleted that clinical manifestations occur.

Micronutrient deficiencies are uncommon in the general Westernised population, but their incidence can be high in chronic illness where an optimal balance is not achieved or maintained. With regard to IBD, poor dietary intake, malabsorption (190;199), and increased needs substantially increase the risk of micronutrient deficiency (87;173;189).

Earlier paediatric studies in IBD were restricted to assessing the energy stores and body composition (84;102) with negligible evidence existing on micronutrient status. As extrapolation of results from adult studies is inappropriate, due to differences in requirements, this study aimed to assess the body composition and systemic micronutrient levels of CD children, and secondly to assess longitudinal changes under treatment with EEN.

This study confirmed the results from previous extensive research that underweight, and increased undernutrition risk is a common finding in CD children , and at the same time offered new evidence on the micronutrient status of CD children. Compared to the national reference range CD children were significantly thinner and had more store deficits with approximately 1/3 of them having BMI values consistent with the definition of underweight and risk of undernutrition. Likewise compared to a cohort of Glaswegian children, recruited at the same time, the CD children's BMI was lower by a mean of approximately 2 SD.

Body composition is a dynamic process in childhood, which varies considerably with age and gender, and this should be considered when attempting to compare groups with different demographic characteristics. As national reference range is now available (579) this study compared differences in body composition between groups, by expressing %FM in respective z-scores. In contrast to the nutritional status classification by BMI, 50% of the children had %FM less than 2 SD of the reference population, with a mean %FM z-score of –3.3. However 25% of the healthy children had depleted fat stores too despite the fact that only 1% of them were underweight based on BMI cut-offs. Lack of concordance between these two measurements of nutritional status may denote that BMI is not a suitable surrogate of body composition, and moreover that the healthy cohort recruited was significantly slimmer than the reference population. Alternatively these results should be interpreted bearing in mind differences in socioeconomic characteristics of the reference population and the healthy cohort as well as differences in the BIA equipment and the body composition prediction algorithm that the authors of the national reference data used to assess body composition.

Weight loss, and underweight is among the major presenting symptoms of the newly diagnosed CD (33), which explains the increased prevalence of underweight in this sample,

which consisted of approximately 50% of newly diagnosed children. None of the participants were on concomitant treatment EEN, which may have biased the results.

Growth faltering is common in children with CD (109) and although no mean height difference was found with the healthy Glaswegian children, four times more children with CD were classified as growth retarded than the healthy controls. Undernutrition, the action of pro-inflammatory cytokines, delayed pubertal onset, and long-term use of steroids are all implicated in the slow linear development in CD (111).

For sixteen CD children, serial measurements of body composition were obtained during their disease management course with EEN. Although previous studies assessed changes in fundamental anthropometric characteristics during EEN, (Table 1.5.1) only a few studies measured parallel changes in body composition. As BMI is best seen as an index of nutritional status rather than a true measure of body composition (104;272), more comprehensive methods are needed to distinguish between changes in body fat and lean mass stores during therapy with EEN. Foot to foot BIA is a quick, easy to use, cost effective, patient friendly method of body composition assessment, suitable for monitoring body composition in a clinical setting. Although there is good evidence to suggest that the validity of BIA equipment is poor compared to reference methods and that was also shown in a previous chapter of this thesis (Chapter 4), these studies have assessed only the ability of the manufacturer's algorithm to predict body composition. As explained elsewhere in this thesis (Section 4.4), there are now new body composition norms which manipulate impedance measurements independently of the manufacturer's prediction equations (587), and classify children at different nutritional status scores. As these norms have been established only recently, this study relied on the use of the BIA manufacturer's equation and checked their validity against a reference method. On the other hand, use of BIA to longitudinally assess changes in body composition may be more useful than predicting accurate measurements of body composition.

Faecal calprotectin a marker of gut inflammation correlated negatively with BMI z-scores, and may show that increased intestinal inflammation and hence disease activity can cause weight loss and increase underweight and malnutrition risk. On the contrary systemic markers of disease activity like CRP did not correlate strongly with BMI which indicates that in-situ intestinal inflammation is a more important determinant of nutritional status than the acute phase response. Serum albumin was equally associated with BMI and may represent both increased disease activity and undernutrition.

In this study, serial measurements of anthropometry and body composition were assessed and showed that changes depended on the response of the patient to the treatment.

Although body weight increased in patients who achieved clinical remission and those who did not, the mean increase was higher in the former group. The mean BMI increase in the group of children who achieved complete clinical remission was 1.5 SD compared to 1.2 SD for patients with active disease at the end of EEN. It is worth mentioning that increased FFM was observed only in those patients who entered clinical remission by the end of treatment. Absolute FFM increased sharply by 2.7 kg within one month of treatment and continued so to a lower extent over the following month. The increase in FM was marginal and less than 1kg by the end of treatment. On the contrary, only TSF thickness changed significantly in the group of patients who either failed treatment or had ongoing active disease at the end of EEN. Whether clinical remission is achieved only in those patients who restore muscle body stores, or the latter is the result of disease remission should be studied further. This is supported by previous studies which observed lean mass accretion during EEN (104) and recent evidence suggesting an anabolic effect of EEN in children with CD with shift in the protein turnover (333). On the other hand these results should be considered with caution as the number of patients in each group was small and statistical results may be a random finding.

An increase in body weight and favourable changes in body composition, better represent the energy and macronutrient adequacy of the prescribed EEN regime. However improvement in energy/protein status may not parallel improvement or even conservation of body micronutrient balance.

Although clinical presentation of micronutrient deficiencies in IBD is very rare (175;180) and largely limited to case reports (176;594), suboptimal circulating levels for virtually every vitamin, mineral and trace element have been reported previously in adult IBD patients (99;157). In paediatric CD patients the few studies were limited to the assessment of a selective small number of antioxidant vitamins and minerals (Section 1.5.2.8.3) (145;177;181-183) and similar to adult studies, the serum levels of zinc, selenium, and antioxidant vitamins were significantly lower compared to healthy controls of the laboratory reference range.

In this prospective observational study, a substantial number of CD children had circulating levels of a large number of micronutrients below the laboratory reference range. Approximately 50% of the participants had suboptimal levels for serum zinc, selenium, and carotenoids. Indeed the serum concentration of carotenoids was out of the detection limit of the assay for some children. Although suboptimal circulating levels of micronutrients may reflect depleted body stores, none of the subjects presented overt deficiency symptoms. On the other hand, low levels of antioxidants may represent suboptimal antioxidant defensive systems and increased risk of oxidative stress (80;146;173). Excessive production of reactive oxygen species

by the activated immune system, in conjunction with low dietary intake (87;190) may compromise the body's antioxidant status. Unfortunately this study did not measure markers of biological molecules oxidation (eg malondialdehyde) to confirm this hypothesis.

Acute phase response in inflammatory conditions, infection and cancer can alter serum levels of many nutrients and biomolecules irrespective of actual body stores (178;595;596). Thus assessing them in serum may not represent body adequacy and results can be fallacious and misleading. There are proponents suggesting the measurement of the same nutrients in other body tissues or blood cell types (597). In this study erythrocyte levels of some B complex vitamins were measured and different results were found compared with serum levels. Approximately 50% of the participants were deemed to have insufficient serum concentrations of B_6 but none had suboptimal levels in their erythrocytes which could more correctly reflect the adequacy in the tissues. Likewise artefactual changes from the acute phase response may explain the suboptimal levels observed for some vitamins and trace elements in a significant proportion of the subjects. Among them serum carotenoids, selenium, zinc, vitamin C, and B_6 are negative acute phase respondents, whereas Cu, and ferritin are increased during inflammation (178). In this study, serum ferritin was strongly correlated with inflammatory markers as shown in Chapter 3. Measurement of ferritin levels in patients with active disease is incorrect and can be misleading. However one third of patients had ferritin levels lower than the reference range, which clearly represents depleted iron stores independently of an acute phase response artefact. Although haemoglobin levels did not change significantly during treatment with EEN, they were strongly associated with albumin levels, a marker of disease activity and malnutrition and this is in accordance with the findings of a retrospective study described in a previous chapter (Chapter 3).

Changes during the acute phase response may also explain the strong association found between the serum levels of various micronutrients and systemic inflammatory markers like CRP. Previous studies explained this as a casual association between increase disease activity and suboptimal micronutrient status (99;175;177) but we believe that these results are misleading and are actually an epiphenomenon of the acute phase response that occurs in inflammation. Reduced serum levels of micronutrients are often used to define deficiency state, but these levels may correlate better with markers of disease activity and inflammation and do not always reflect body tissue stores or functional deficits (157;177;178). A prime example is that perturbation in trace elements and vitamin concentrations in patients with cancer of the colon can be partially reversed by anti-inflammatory treatment, without supplementation (179). In this study CRP was significantly correlated with serum ($r=-0.39$; $p=0.06$) but not erythrocyte ($r=-0.21$;

p=0.32) vitamin B_6. To further support of this and challenging a causative association between micronutrients levels and disease activity, none of the micronutrients was significantly associated with faecal calprotectin concentration, a gut specific marker of disease activity that better represents intestinal inflammation. Nevertheless copper, a positive acute phase respondent was below the reference levels for 10% of the patients and this may indeed represent insufficient body stores.

For half of the participants, measurements of micronutrients were available before treatment initiation and at the end of EEN. Serum levels for many micronutrients improved but carotenoids further deteriorated. Carotenoids are negative phase respondents and someone would expect them to improve at the end of treatment as the combined effect of nutritional hyperalimentation and improvement of disease activity. However, in contrast to expectations, these further deteriorated and more than 90% of the participants had depleted stores for all carotenoids on treatment completion. Indeed their concentrations were under the detection limit of the laboratory assay. These results provide strong evidence to suggest that carotenoids are severely depleted at the end of treatment with EEN. Increased consumption during the active course of the disease and inadequate replenishment through dietary intake (190) explains these findings. The nutritional supplement the participants used, is better characterised as an “artificial food” and is nutritionally complete and balanced for the basic nutrients for which recommended amounts have been established and therefore may lack the “non-nutrient compounds” micronutrients. Indeed Modulen® Nestle composition tables (Appendix) do not outline carotenoids as part of their constituents and none of their ingredients is a carrier of them. This in conjunction with the increased utilisation of antioxidants during the active phase of the disease, and small body reserves, may explain the depleted body stores in the patients of this study.

Within a short period of time, following treatment cessation, the majority of the micronutrients reversed to pre-treatment levels. Carotenoids increased significantly whereas selenium and vitamin A decreased. Although none of the participants had suboptimal levels for vitamin D and B_{12} at any time point of follow up, their median concentration was significantly decreased compared to the end of EEN treatment reflecting a gradual deterioration of body stores. Sentogo et al (136;136) reported that 16% of CD children and young adults presented low levels of vitamin D, in line with the definition of hypovitaminosis, compared to none of the participants of this study. Different definitions of hypovitaminosis between the two studies explain this discrepancy. If like Sentogo, a threshold of 38nmol/L had been set for the definition of vitamin D hypovitaminosis in this study, 24% of participants would have been classified with suboptimal body stores.

Among the main limitations of this study are the small sample size, missing data, and the lack of a healthy control group. As this study is supplementary to the main study on the effect EEN on gut microbiota, the collection of stool samples discouraged many children to participate, and subsequently increased the recruitment period by 1,5 years (planned one year). Similarly paediatric CD is an uncommon disease with approximately 20 new cases per year in the biggest paediatric referral center in Scotland. This in conjunction to alternative treatment options (steroids, anti-TNFα agents) and the unpredictable course of the disease reduced substantially the eligible number of participants. Venupuncture for entirely research purposes was deemed unethical and this did not allow the recruitment of a healthy control group, or blood sampling when patient was not due to give monitoring clinical bloods.

This study found that the protein/energy stores and micronutrient status of children with CD are suboptimal and significant changes occurred during treatment with EEN. The findings of this study advocate the use of EEN as a nutritional complete regime to support the protein/energy stores of CD with active disease but the formula may be insufficient to sustain carotenoid body stores. Suboptimal levels for many micronutrients were found but it is difficult to differentiate between true deficiencies or an artifact of the acute phase response. If these are true deficiencies these could be attributed to suboptimal dietary intake (87;190), increased consumption by the activated immune system, malabsorption or increased enteric losses (199). On the other hand if these are an epiphenomenon of the acute phase response, measurement of the same micronutrients in other blood cells, which are unaffected by acute phase response, might be required to correctly assess body balance, and their causal association with disease activity, and intestinal injury. This needs to be studied further.

CHAPTER SIX

Changes in systemic and gut markers of inflammation during treatment with EEN in paediatric patients with CD

OUTLINE

This study assessed changes in faecal markers of intestinal inflammation in paediatric patients with CD undergoing EEN treatment. Correlation between faecal calprotectin levels, systemic inflammatory markers and clinical activity indices are explored.

6.1. Introduction

Mucosal healing rather than gastrointestinal symptom resolution could be considered the definitive target of contemporary drug therapy in IBD. However routine monitoring of intestinal inflammation and the mucosa healing process is difficult due to the inaccessibility of the digestive tract. Assessing mucosal inflammation and healing routinely with endoscopy is impractical, expensive, not acceptable to patients and needs general anaesthesia in paediatric patients. On the other hand surrogate markers of systemic inflammation are not sensitive enough or sufficiently specific to reflect mucosal inflammation (598).

The presence of active gut inflammation in patients with CD is associated with migration of leukocytes to the intestinal mucosa. This causes the production of several proteins, which can be detected in serum or stools (599). Calprotectin, a 36K Da calcium and zinc binding protein, is a neutrophil derived protein present in faeces. It represents 60% of the cytosolic proteins in granulocytes (599) and its presence in faeces is directly proportional to the migration of neutrophils and therefore gut inflammation (600).

There is good evidence that faecal calprotectin correlates with histological activity, and mucosal healing in patients with UC and colonic CD (601-604). Thus measurement of faecal calprotectin may have good potential as a non-invasive way of assessing disease activity and treatment response at the site of inflammation rather than relying on markers of systemic response.

So far, one study of paediatric UC patients showed that faecal calprotectin remained high in children with UC despite clinical improvement after treatment with steroids (312;601). Considering previous evidence, which showed that mucosal healing is achieved after treatment with EEN (Section 1.5.2.2), significant reduction in the calprotectin concentration in those patients whose disease activity improved at the end of treatment would be expected.

The aim of this study was to assess any changes of faecal calprotectin concentration in CD children on treatment with EEN and how these correlate with clinical activity indices.

6.2. Subjects and Methods

6.2.1 Subjects

As this study was supplementary to the main study of the effect of EEN on gut microbiota, eligible participants and overall recruitment is presented elsewhere (Section 5.3.1). Faecal calprotectin levels were also measured in a first-degree relative of the CD children and in healthy children with no family history of IBD.

6.2.2. Study design

Stool samples were collected from children with CD during their eight weeks course on EEN. A baseline sample was collected close to the date of EEN initiation, two during their course of treatment (15 & 30 d), and one close to the end of treatment (60 d). A final sample was collected whilst patients were on normal diet within 1-4 months after EEN treatment cessation. The samples were collected within a maximum of four hours of defecation from the patient's home, homogenized with mechanical kneading and stored at –70 ^{o}C until further analysis.

6.2.3. Systemic markers of disease activity

Systemic markers of disease activity (CRP, ESR, albumin) were measured in plasma at the beginning of EEN, at the end of treatment and whilst on normal diet. These were available from the routine clinical measurements of the patients and were assayed according to standard hospital protocols. A serum albumin concentration less than 35 g/l, and CRP and ESR more than 7 mg/l, and 20 mm/h respectively were deemed "abnormal" according to the local hospital reference range.

6.2.4. Clinical activity index

Disease activity was assessed with the Paediatric Crohn's Disease Activity Index (PCDAI) (Appendix). The PCDAI is a well established and validated clinical activity index used extensively in research to assess disease activity in paediatric CD patients (332). An overall score is calculated taking into consideration a week history recall of symptoms, laboratory markers of disease and clinical examination. The index was completed by data collected by the paediatric gastrointestinal consultants at the beginning and the end of their treatment and on the

same day as the collection of the last stool sample. An overall score more than 10 is suggestive of active disease.

6.2.5. Faecal calprotectin

Calprotectin concentration in faecal samples was measured with a commercial ELISA kit (Phical®, Norway) according to the manufacture's protocol. A sample is tested by an enzyme immunoassay specific for calprotectin. Samples and standards are incubated in separate microtitre wells coated with polyclonal antibodies against calprotectin. After incubation and washing of the wells, bound calprotectin is allowed to react with immunoaffinity purified enzyme labeled anti-calprotectin. The amount of enzyme bound is proportional to the amount of calprotectin in the sample or standard. Microtitre plates were coated with rabbit anticalprotectin for capture, and immunoaffinity-purified rabbit anti-calprotectin conjugated with alkaline phosphatase was used for development.

Approximately 100 mg of thawed faeces were suspended in manufacturer's "extract buffer" (w/v; 1:50) and homogenized for 30 sec on a vortex mixer (Fisher Scientific, FB 15024) and for 30 min on an orbital shaker (IKA VIBRAX VXR BASIC) at 1200 rpm. The homogenates (approximately 1.5 ml) were transferred into Eppendorf tubes and centrifuged for 20 min at 10 000 g at 4 ℃. The clear extract supernatant was di luted 1:50 in the manufacturer's "dilution solution" and immediately analysed by ELISA.

Fifty µl of each standard, control and diluted sample were added in duplicate in the wells of a 96 microtitre plate according to the manufacturer's protocol. The plate was covered with sealing foil and incubated at room temperature on a horizontal shaker for 45+/- 5 min. At the end of incubation, the wells were washed out six times with ample washing buffer. Fifty µl of conjugate were added to each well with a multi-channel pipette and reverse technique. The plate was covered and incubated as above. Six further washings were performed as above. One hundred µl of "substrate solution" were added to each well using a multi-channel pipette and reverse pippeting technique. The plate was incubated at room temperature for approximately 15-20 min in the dark until the O.D. value of the 1000ng/ml standard reached at 2.0 absorbance units. One-hundred µl of stop solution (NaOH 1N) was added to each well. The O.D. values were read by means of an ELISA reader at 405nm (ThermoLab Systems Multiskan Spectrum). The concentration of calprotectin in stool samples was calculated with reference to the standard curve and expressed as mg/kg of wet and dry faecal material. Values above 50mg/kg were regarded as positive calprotectin level according to the manufacturer's protocol.

Samples were measured in duplicate. All serial samples from the same patient were measured in the same kit under the same conditions and calibration curve to avoid inter-assay variance.

6.3. Results

6.3.1. Participants

Measurements of calprotectin measurements were carried out for 16 CD children with serial samples whilst on EEN (age 11.8±2.3 y, range 7.4-14.7 y) and their 14 first-degree relatives (13 healthy parents, one sibling). Calprotectin concentration was also measured in stool samples of 10 healthy children without family history of IBD. This assured the validity of the assay and also offered a group for comparison with the results of children with CD.

Nine children were on no other medication apart from EEN. The rest had other parallel medical treatments (Table 6.1). All but one child finished at least six weeks on EEN. One child stopped treatment after a month on EEN due to symptoms exacerbation. All participants provided at least three serial stool samples whilst on EEN. A final stool sample was collected on normal diet in all apart from one patient who had surgical removal of part of the large bowel. Twelve children were newly diagnosed with CD and this was their first course of EEN while for the remaining four this was a subsequent course following disease relapse. Seven children were girls. Thirteen children had extensive disease involving parts of both the small intestine and large bowel (Table 6.1).

Table 6.1: Disease location and concomitant medical treatment of CD children on EEN treatment

Disease location	N	Concomitant medication	N
Colitis (L2)	2	None	9
Ileocolitis (L3)	1	5-ASAs	4
Upper GI & Ileocolitis (L3L4)	9	Immunomodulators	5
Upper & Colitis (L2L4)	4	Steroids	2
		Antibiotics	1

6.3.2. Changes in disease activity markers

All participants had active disease as depicted by the calculation of the clinical activity index and the systemic inflammatory markers (Table 6.2). As expected the median value of the disease activity markers decreased at the end of treatment (Table 6.2). Individually, the majority of the children experienced improvement in clinical activity and improvement of the systemic inflammatory markers (Fig 35). For all apart from one patient, CRP and PCDAI were reduced compared with baseline values (Fig 35). PCDAI remained the same (PCDAI=10) for one patient.

Although most of the children had improved PCDAI in only seven out of the 16 children was complete clinical remission (PCDAI<10) achieved. There was no statistical significant difference in the baseline markers of disease activity between patients who achieved remission and those who did not.

Table 6.2: Mean changes in PCDAI and systemic markers of disease activity before, after EEN and on normal diet in paediatric patients with CD

	Baseline (mean ± SD)	End of EEN (mean ± SD)	Normal Diet (mean ± SD)
PCDAI	40 ± 17	16 ± 16[$]	18 ± 14
CRP (mg/L)	47 ± 70	14 ± 17*	23 ± 36
ESR (mm/h)	39 ± 27	22 ± 26*	28 ± 19
Albumin (g/dl)	29 ± 7	35 ± 5[$]	34 ± 6

* p< 0.05; [$] p<0.01 Wilcoxon Singed Rank Test;

6.3.3. Correlation between systemic and gut specific markers of disease activity

Correlation between systemic and gut specific markers of inflammation was assessed after pooling the results of all CD patients who participated in the EEN study. As anticipated there were significant associations with all systemic markers of disease activity and faecal calprotectin (Fig 36). Albumin was negatively and strongly associated with calprotectin whereas ESR, CRP and PCDAI were positively (Table 6.3).

6.3.4. Changes in faecal calprotectin concentrations

All participants had faecal calprotectin concentrations significantly higher than the upper normal reference range of the manufacturer's reference range (50 mg/kg) at most of the time points of the observational period (Table 6.4). Inter-individual calprotectin concentration changes varied considerably during treatment (Fig 37).

Figure 35: Individual changes of clinical activity and systemic markers of disease activity before and at the end of treatment with EEN in paediatric patients with CD

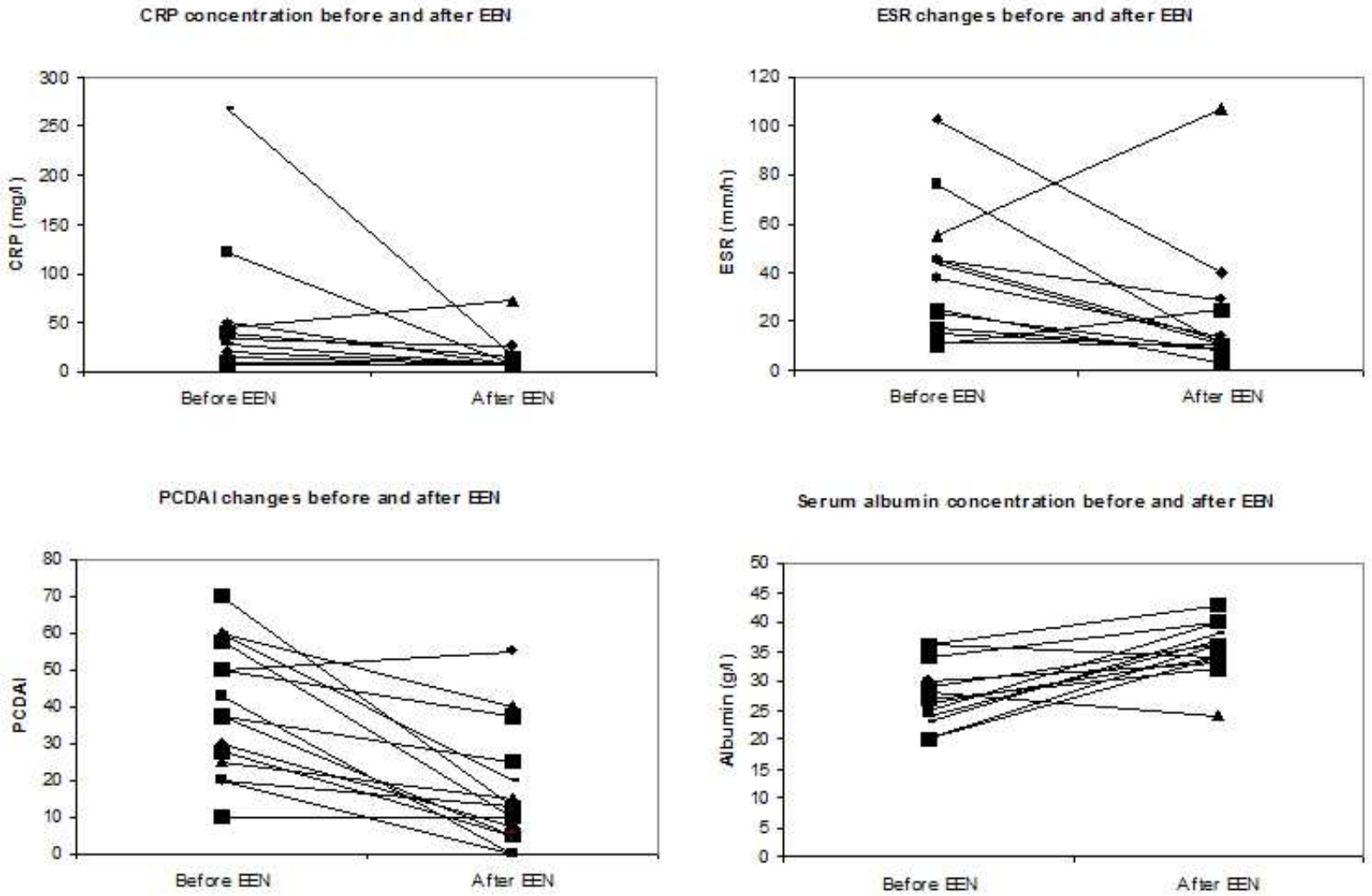


Figure 36: Spearman correlation (r coefficient and p-values) between systemic markers of disease activity, PCDAI and faecal calprotectin in paediatric patients with CD

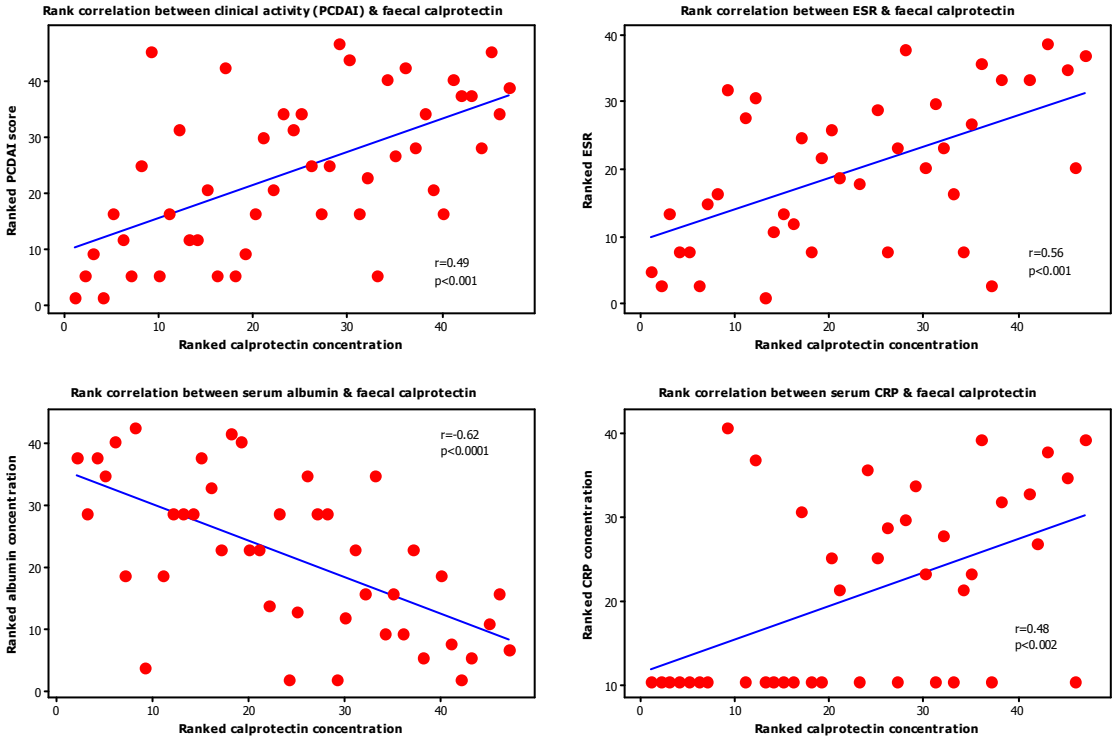


Table 6.3: Spearman correlation between systemic markers of disease activity and PCDAI with faecal calprotectin in paediatric patients with CD (R coefficient, p-value)

Disease marker	R coefficient	p-value
ESR	0.56	0.001
Albumin	-0.62	0.001
CRP	0.48	0.002
PCDAI	0.49	0.001

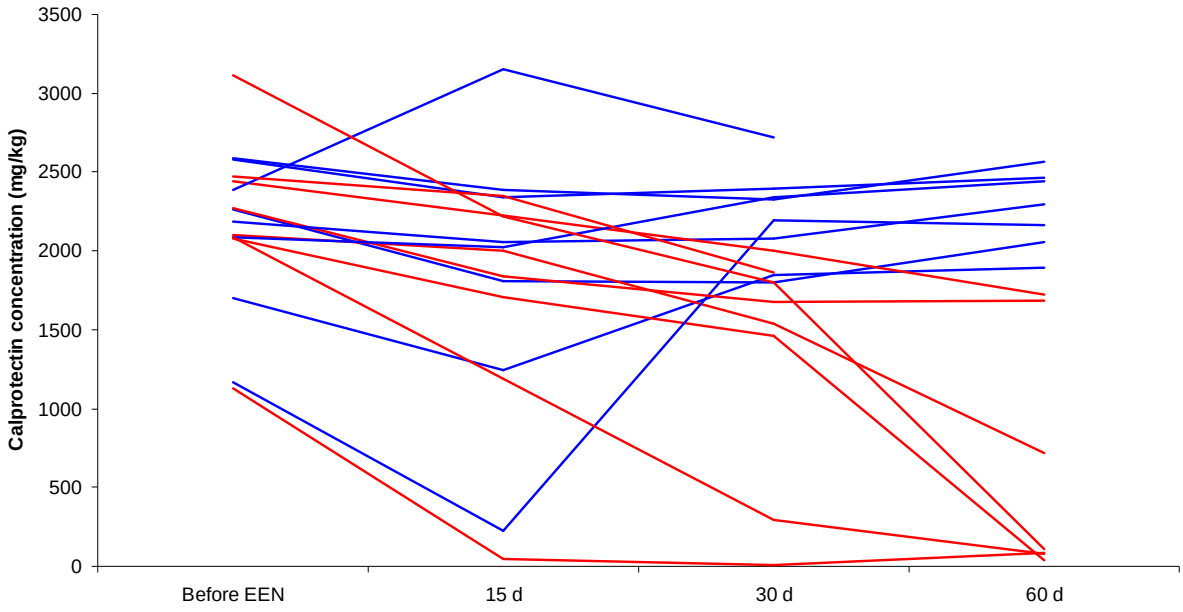
Table 6.4: Individual changes of faecal calprotectin (mg/kg) levels during EEN and on normal diet in paediatric patients with CD

Subject	Before EEN	15 d	30 d	60 d	Normal diet
A	1130.4	47.7	5.8	88.4	2054.6
B	1701.4	1241.4	1846.6	1894.8	145.6
C	2076.3	1704.8	1459.5	39.1	1712.1
D	2585.3	2390.1	2324.6	2563.7	2431.8
E	2262.3	1808	1797.6	2055.8	2470.2
F	2102.2	1999.9	1535.8	718.1	1632.2
G	1170.1	224.4	2191.6	2163.7	245.4
H	2272.3	1841.6	1673.7	1685.8	2085.3
I	2581.7	2341.1	2394.3	2460.9	2495
J	2187.9	2056.2	2076.7	2298.3	2169.5
K	2438.7	2221.6	2000.5	1723.5	2327.7
L	2389.7	3148.7	2717		1681.5
M	2086.8	2026.6	2338	2437.7	1511.9
N	2088.51		296.39	77.12	2418.17
O	2474	2347	1864.9		
P	3114.22	2216.35	1803.81	106.12	2355.18
Mean ± SD	**2166 ± 503**	**1841 ± 807**	**1770 ± 717**	**1451 ± 1 009***	**1849 ± 748**

* Significant different from “Before EEN” for ANOVA of repeated and Bonferroni post-hoc test

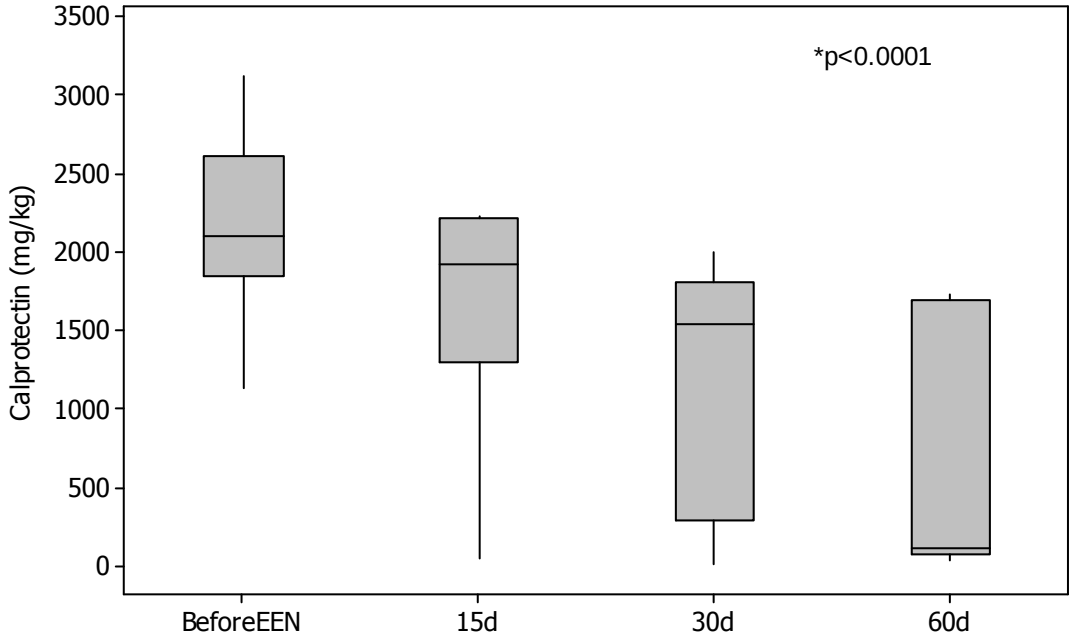
Only one of the fourteen children that completed the course of EEN had a calprotectin level within the normal range (<50mg/kg) at the end of EEN and three children had values of approximately 100mg/kg. The rest remained at much higher than normal levels and the extent of the change during treatment varied considerably (Fig 37) and (Table 6.4). Using analysis of variance of repeated measures and Bonferroni post-hoc adjustment, mean calprotectin was significantly reduced at the end of treatment compared to baseline measurements (p=0.01).

Figure 37: Individual changes of faecal calprotectin in CD children during EEN (with red line patients who achieved clinical remission and with blue line patients with active disease at the end of EEN)



The group of patients was divided into those children who entered in clinical remission (n=7) and those who did not or the treatment failed (n=9). In those patients who entered clinical remission (PCDAI <10) faecal calprotectin concentration reduced significantly (Fig 38). Faecal calprotectin was reduced during EEN treatment and was significantly lower than baseline at 30 (p<0.01) and 60 day of treatment (P<0.0001). Mean calprotectin concentration was reduced by 921 mg/kg (95%CI: -1568 to -274) and 1541 mg/kg (95%CI: -2187 to -894) after 30 and 60 days of treatment respectively. Two of these children relapsed within six months of treatment cessation, judged by the introduction of a subsequent EEN, steroid course or anti-TNFα treatment.

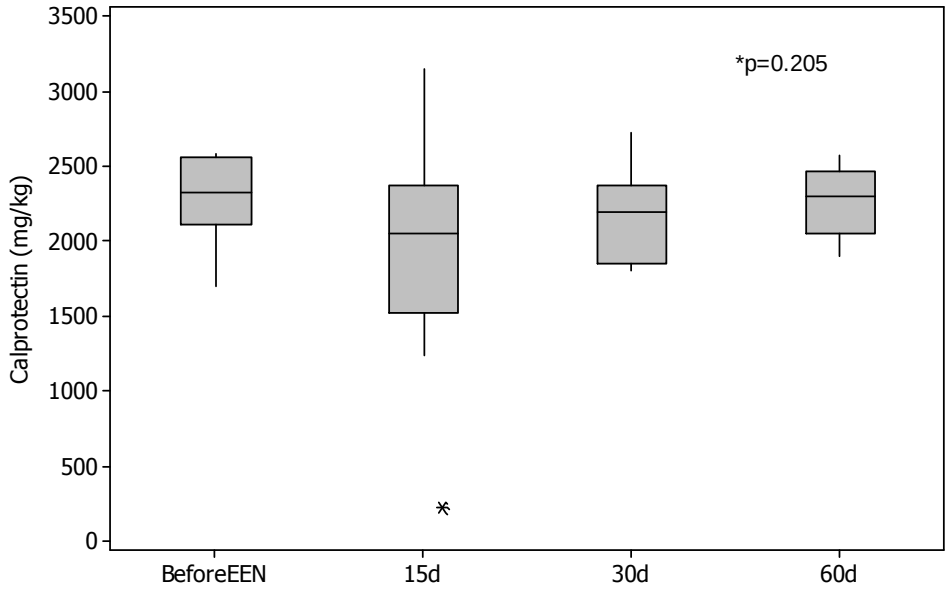
Figure 38: Calprotectin levels in paediatric patients with CD who achieved clinical remission (PCDAI≤10) on treatment with EEN



*a-nova of repeated measures and Bonferroni pot-hoc test.
All follow up points significantly different from "BeforeEEN"

In contrast, in those patients who presented clinical symptoms of disease activity disease at the end of treatment (PCDAI≥10) of discontinued treatment, calprotectin levels did not change significantly from baseline (Fig 39).

Figure 39: Calprotectin levels in paediatric patients with CD who did not achieve clinical remission (PCDAI>10) on treatment with EEN



*a-nova of repeated measures and Bonferroni pot-hoc test

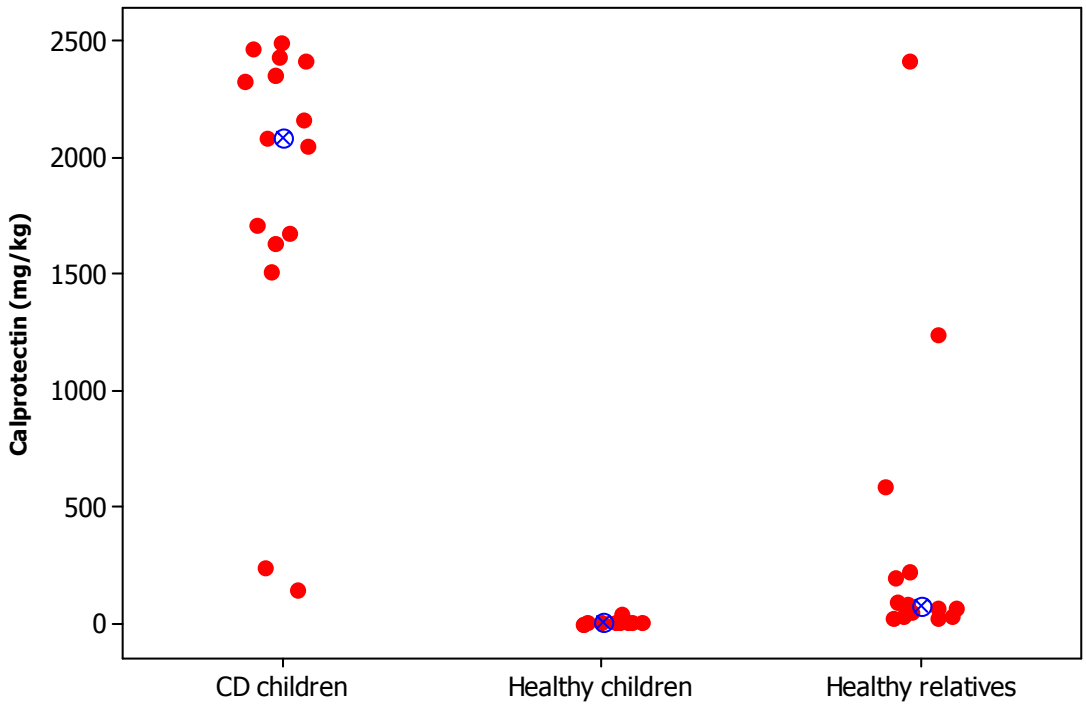
6.3.5. Changes of faecal calprotectin on normal diet

None of the patients had calprotectin levels within the normal reference range (<50 mg/kg) up to four months of EEN completion and whilst on normal diet. Calprotectin increased by approximately 10 times in those patients (n=4) who achieved calprotectin levels close to the normal range at the end of EEN (Table 6.4). Compared to the values at the end of EEN, faecal calprotectin dropped in five children and increased to nine children whilst on normal diet (Table 6.4).

6.3.6. Comparison of faecal calprotectin levels of CD patients on normal diet, their first degree relatives and healthy children

For ten healthy children, with no family history of IBD, and for 14 healthy first degree relatives of the CD children (13 parents, one sibling) faecal calprotectin was measured in a single stool sample. As expected none of the healthy children had calprotectin above the normal range (<50 mg/kg). On the contrary the majority of the healthy relatives of CD children (9/14) had abnormal calprotectin levels (Fig 40).

Figure 40: Faecal calprotectin levels in CD patients, their healthy first degree relatives and healthy children with no familial history of IBD (with blue cross the median)



6.4. Discussion

Calprotectin is a marker of intestinal inflammatory activity with increasing use in research and clinical gastroenterology. It has good diagnostic validity to discriminate between organic and functional disorders (605;606). In this study most of the patients (14/16) were newly diagnosed or had undergone recent repeat endoscopy that confirmed histological findings of active intestinal inflammation. In all of these patients faecal calprotectin concentration was significantly higher than the normal reference range, as opposed to 86%, 69%, and 73% of the same patients who presented with abnormal CRP, ESR and serum albumin respectively (data not shown). This is in accordance with substantial evidence, which suggests that calprotectin levels have excellent negative predictive value, better sensitivity and equal specificity compared to serological inflammatory markers to reflect gut inflammation (311;603;604;607;608). Moreover faecal calprotectin concentration correlates well with endoscopic and histological activity scores (311) that makes it a useful tool to monitor treatment efficacy at the site of the disease onset. So far only a few studies have assessed the effect of IBD treatments on faecal calprotectin levels (312;609), and to the best of our knowledge this is the first study in paediatric patients on treatment with EEN.

Similar to previous research (604;606;610) this study found a strong association between clinical activity indices, CRP, ESR, serum albumin and faecal calprotectin and moreover showed that calprotectin levels are decreased only in those patients who achieved clinical remission at the end of EEN treatment. Calprotectin did not change significantly in the patients where treatment failed or had ongoing clinically active disease (PCDAI>10) at the end of treatment. These results suggest that in only those patients who enter clinical remission is mucosal healing achieved. However it is noteworthy that for only one of the patients who achieved clinical remission, calprotectin levels decreased to within the normal reference range at the end of treatment. This may indicate that either there is still ongoing subclinical intestinal inflammation, despite apparent full clinical remission, or that improvement of clinical disease activity occurs more quickly than resolution of intestinal inflammation.

It was not within the scope of this research to explore whether increased calprotectin levels at the end of EEN predict an earlier clinical relapse and this must be addressed in a future prospective study. Nevertheless there is evidence to suggest that calprotectin levels can predict disease relapse in IBD (311;611;612) and if this is the case then calprotectin levels should be monitored along with clinical activity.

Apart from its growth promoting effects, the impact on the patient's nutritional status and lack of side effects, EEN is superior to steroids due to its gut mucosal healing effects

(90;261). This is in agreement with the findings of this study and a previous report in Finland, which failed to find any effect of oral steroid treatment on faecal calprotectin levels of IBD patients (312;604).

Some patients were on concomitant medical treatment that may have confounded the net effect of EEN on faecal calprotectin levels. This was unavoidable as it is unethical to withhold medical treatment for research purposes when there are clinical indications for use. Nevertheless none of the patients on parallel medical treatment experienced statistically significant reduction of calprotectin levels or entered clinical remission, which may have confounded the results of this study. Indeed all apart from one of the patients whose faecal calprotectin was reduced at follow up, were solely on treatment with EEN or on stable treatment on 5-aminosalicylates (n=1) prior to EEN introduction. Moreover there was no difference in the baseline disease activity characteristics between those patients whose faecal calprotectin improved and those did not change or increased.

Increased gut mucosal permeability and increased levels of calprotectin in CD relatives have been described before (489;490;613;614) and the findings of this study are in agreement. The majority of the healthy parents recruited had faecal calprotectin levels significantly higher than the normal reference range and this may denote that genetic and common environmental factors may predispose to intestinal inflammation. On the other hand this may be the result of lack of faecal calprotectin specificity or other unknown underlying gastrointestinal comorbidities that can affect calprotectin levels (606). Only a follow up of these subjects will address whether relatives of CD relatives with high faecal levels of calprotectin are more susceptible to develop IBD or other digestive inflammatory disorders.

For ten healthy children with no family history of IBD, faecal calprotectin was measured. These were significantly lower compared to any time point of the follow up in CD children and within the normal reference range of the kit. This ensured the validity of the assay and precluded any methodological errors. In fact the study design and the method of sample analysis are among the strongest points of this study. All of the samples were collected within four hours of defaecation, were homogenized, and all the follow up samples of the same participant were assayed in duplicate, with the same ELISA kit using the same calibration curve. Homogenization of faecal samples prior to measurement, and intra and inter assay variance are methodological issues that can affect the validity of the current assay to measure accurately calprotectin levels particularly when changes between follow up measurements are to be considered. In this study faecal calprotectin levels were also converted to per dry kg faecal material to balance for differences in the water content of the stool samples particularly between the active and inactive phase of the disease. No different results were found (data not shown) compared to the “fresh” stool” samples.

In conclusion this study found that induction of clinical remission in paediatric CD undergoing treatment with EEN is accompanied by a reduction in faecal calprotectin levels. However most patients had still high levels of calprotectin at the end of treatment and future studies should address whether these predict an earlier relapse. If this is the case then, monitoring faecal calprotectin along with changes in clinical activity will be a more appropriate indication of treatment termination or prolongation.

CHAPTER SEVEN

Nutritional therapy and gut microbiota in paediatric CD

7.1. Introduction

There is now vast clinical experience supported by a substantial number of clinical studies to advocate the use of nutritional therapy in the management of active paediatric CD. Exclusive enteral nutrition attenuates disease activity and induces clinical remission in the majority of CD children with active disease and gut mucosal healing is achieved in the majority of the cases (90;261) (Section 1.5.1.2).

As with many popular medications, the mode of action and ideal formulation of EEN remain unknown (530;615). There is still much discussion and debate about putative mechanisms of action, mainly because the gut is a difficult organ to study directly. Gut rest, direct anti-inflammatory effects, and nutritional rehabilitation have all been implicated (327;533) (Section 1.5.2) but at present there is no robust evidence to consistently support any of them in particular.

Substantial evidence over the last 10-15 years has suggested that the gut endogenous microbiota, rather than a specific microbial pathogen, plays an important role in the pathogenesis and propagation of intestinal inflammation and tissue injury, in CD (Section 1.7.2) (30). Although it remains unclear whether perturbation of the intestinal microbiota balance is a trigger of inflammation rather than the result of IBD, an unstable gut microbiota consisting of uncommon bacterial species (512;513), with mucosa invasive and adherent properties has been described in several studies (504;523) (Section 1.7.2).

Moreover studies in the early nineties described differences in the metabolic activity and production of bacterial metabolites between IBD patients and healthy controls (Section 1.7.1) which subsequently triggered the experimental use of SCFA, mainly butyrate enemas, in a number of clinical trials (439;440) and animal models of colitis (428) (Section 1.7.1.1). Recently a number of clinical and animal studies (75;538) have tested food grade bacteria (probiotics) and prebiotics to induce, or maintain remission in both CD and UC by switching a putative “dysbiotic” gut microbiota (498) towards one more “normal”.

Thus modulation of the intestinal microbiota could be an attractive potential mechanism for the action of EEN. Despite this being reported among the potential modes of EEN action in many relevant review articles (530;615) until recently no primary evidence had been cited. This was highlighted recently by an expert in paediatric CD and nutrition, Dr Anne Griffiths, who called for more research to be conducted on the effect of EEN on gut microbiota (327).

Thus far, two recent studies have described changes in the diversity of the dominant microbiota of children with CD on treatment with EEN. In the first of these studies, Lionetti et al (531) analyzed stool samples of nine CD children with TGGE and compared them with five healthy controls. A greater stability of the gut microbiota diversity was observed in the

healthy group, compared with the CD patients on EEN treatment, although no analysis of the electrophoresis gels was undertaken by the authors, who evaluated the TGGE gels only visually. The authors concluded that EEN may exert its therapeutic properties through a prebiotic effect on the large bowel microbiota of children with CD.

In the second study, Leach et al (532) collected serial stools samples from six CD children during EEN, and at four weeks and at four months after treatment, and compared their bacterial composition patterns and diversity with seven healthy children. The bacterial diversity was assessed with DGGE and changes in the global bacterial and group specific diversity and similarity patterns were measured. Although bacterial diversity (bacterial band count) did not differ significantly between healthy and CD children at treatment initiation, a lower diversity was found at the end of treatment in the CD group compared with the healthy controls. *Bacteroides-prevotella* and *Clostridium coccoides* bacteria were significantly less in the CD group compared with the healthy controls at the end of EEN treatment. Moreover the similarity index of the bacterial diversity was low during treatment for all bacterial groups studied, and significantly lower than in healthy subjects. However no comparison of changes in bacterial diversity and similarity patterns were reported during EEN to investigate changes during treatment.

Both these studies assessed changes in the bacterial diversity and composition stability of CD children on EEN. To the best of our knowledge no previous study has explored whether EEN affects the metabolism of the microbiota in paediatric CD patients.

To increase knowledge of the role of the microbiota in the action of EEN, the primary purpose of the study, described in this chapter, was to assess the effect of EEN on the gut microbiota metabolism and diversity in children with active CD. The secondary purpose was to compare the diversity of the gut microbiota and bacterial metabolism of the same children with their healthy first degree relatives and with healthy children with no family history of IBD.

7.2. Subjects and methods

7.2.1. Subjects

For the purpose of this study three groups of subjects were recruited.

i) Paediatric CD patients with active disease on EEN

ii) First degree relatives of the above (CD) children

iii) Healthy children with no family history of IBD

Crohn's disease patients on EEN

The characteristics of the eligible patients have been presented in Chapter 5. Briefly these included all the paediatric CD patients with active disease (<16 y old) who commenced an EEN course, as part of their standard medical management.

First degree relatives of patients with CD on EEN

A single stool sample was requested from one of the patients' first degree relatives for comparison purposes. This should allow a comparison between the gut microbiota diversity and metabolism of CD patients and their healthy relatives controlling partially for factors, which are known modifiers of the composition and metabolic activity of human gut microbiota (i.e. genes, diet, and environment).

Healthy children with no family history of CD

Stool samples were collected from 21 healthy children with no family history of IBD. Two stool samples were collected with at least a month gap between collections, to assess the temporal stability of bacterial diversity, composition, and metabolic activity, and to compare with the gut microbiota of CD patients under treatment with EEN.

Exclusion criteria

All participants were asked to declare recent use of antibiotics (last three months) or systematic use of probiotic products or supplements. Healthy children or family members of CD patients that had been on a recent course of antibiotics were excluded. Although CD patients with recent use of antibiotics were not excluded their results are presented and discussed with caution and, where differences were found, separately.

7.2.2. Study design

Serial stool samples were collected from CD children during their course of treatment on EEN. In total, five faecal samples were collected over a period of three to six months. A first stool sample was collected before or within five days of therapy initiation. For those patients who started treatment on the same day as endoscopy, a two day wash out period was allowed to "balance" perturbation of bacterial population and metabolism owing to the bowel preparation procedure. For these patients bowel preparation was performed with sodium picosulfate (Picolax ®) and sennosides (Senna ®) liquid preparations. Collection of stool at this time was not ideal but unavoidable. Serial stool samples were collected at 15, 30 days

on treatment and close to the last day of EEN (day 60). A final stool sample was collected at least one month after treatment cessation whilst patients were on normal diet.

7.2.3. Exclusive enteral nutrition regime

In all cases, the patients followed an eight weeks planned EEN course with a polymeric casein-based liquid supplement enriched with Transforming Growth Factor-β (Modulen®, Nestle). The details of the composition of the dietary regime is described elsewhere (Section 5.2.2).

7.2.4. Methods & materials

7.2.4.1. Samples collection and initial manipulation

Fresh stool samples were provided by all participants (CD, relatives, healthy) at home or during their routine visit to the hospital. To facilitate the collection of the stool samples a special kit was supplied to all participants. The patient passed the entire bowel movement into a plastic single use container supported on the toilet seat through a paper bedpan. Immediately after defecation the patients or their parents put the container inside a gas proof bag along with a special anaerobic kit (Anaerocult® A, Merck, Germany) to reduce the oxygen concentration in the sample and induce anaerobic conditions. A special indicator (Anaerotest®, Merck) was used to check the development of anaerobic conditions. The stool sample was kept inside an insulated bag along with frozen ice blocks to retard ongoing bacterial metabolism (Appendix).

Typically stool samples were transferred to the laboratory within a maximum of four hours of defaecation and were processed within an hour. The protocol for the initial handling of the samples ensured that the biomarkers of interest most susceptible to the effect of time would be tested first. The initial handling of the sample included mechanical blending of the whole faecal sample with a domestic hand blender (Braun, MR4050HC, Germany). Samples were blended until complete homogenization (2-3 min, depending on consistency) inside a biological flow cabinet ((MSC 12, BS 5726, Juan).

7.2.4.2. Determination of faecal pH

Measurement of faecal pH was performed on 1:3 w/v aqueous faecal slurries. Approximately 0.8-1.2 g of homogenized faecal sample was measured accurately and suspended in threefold volume of distilled water. The slurry was agitated by vortex at full power for 1 min or until the sample was completely dispersed. In the case of hard samples, a thin stick or small glass beads were used to break the lumps. The measurement of the pH was performed with an autocalibrated portable digital pH meter (Hanna HI 98140, Portugal).

7.2.4.3. Determination of SCFA and BCFA

Short chain fatty acids (C2-C8) and branched chain acids isobutyrate, isopentanoic, and isocaproic were measured with gas chromatography in ether extracts and according to the method by Laurentin & Edwards (616).

Stabilization of faecal specimens for SCFA analysis

Short chain fatty acids are particularly volatile and susceptible to oxidation. Substitution of their free carboxylic hydroxyl group, by divalent ions and conversion to their salt form in strong alkaline milieu preserves them from oxidation and decreases volatility and prevents the ongoing metabolic activity of the bacteria. Depending on the stool sample size (small samples) and its consistency (diarrhoeal samples) 0.8-1.5 g of specimens were fully dispersed in a small (7 ml) bijoux tube with an equal volume of NaOH 1M. Each tube was agitated vigorously on a vortex mixer for approximately one min or until the sample was fully dispersed. In the case of hard samples pre-weighed small beads or magnetic stirrer were used for more efficient homogenization. The procedure was repeated in triplicate. The samples were subsequently stored in –20 ^{o}C until further use.

Moisture and volatile matter determination

Moisture content was determined the day before SCFA analysis based on the freeze drying method. The pre-weighted NaOH-stabilized stool samples, were freeze dried for 24 hours in a Edwards apparatus (Freezer Dryer Micro Modulyo). The sample dry weight was measured and the moisture content was expressed as percentage of water per mass of stool sample.

Gas chromatographic determination of SCFA

Short chain fatty acids were estimated by gas liquid chromatography using a TRACE 2000 gas chromatograph (ThermoQuest Ltd, Manchester, UK) equipped with a flame ionisation detector (250 ^{o}C) and using a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 μm film thickness), made of polyethylene glycol (catalogue No. 7EK-G007, Phenomenex, Cheshire, UK). Nitrogen (30 ml/min) was used as the carrier gas.

One hundred μl of internal standard solution (86.1 mmol/l 2-Ethylbutyric acid) and 100 μls of concentrated orthophosphoric acid were added to 800 μls of distilled water containing 50 μg freeze dried faeces previously stabilized with 1:1 NaOH 1 N. The mixture was homogenized by vortex for 15 sec and was extracted immediately three consecutive times with 3 ml of diethyl ether, vortexed for 1 min each time, recovering and pooling the ether phase (supernatant) each time in a clean tube. Pooled extract (1ul) was automatically

injected (injector temp 230 ^{o}C, splitless) onto the column. The column temp was held at 80 ^{o}C for 1 min, increasing by 15 ^{o}C per min to a final oven temp of 210 ^{o}C.

The chromatograph and the peak integrals were analysed using Chrom-Card 32-bit version 2.2 (April 2003) (Thermofinnigan, Milan, Italy). For the calibration an external standard of (166.5 µmol/l acetic, 135 µmol/l propionic, 113.5 µmol/l isobutyric, 113 µmol/l butyric, 97.9 µmol/l isovaleric, 97.9 µmol/l valeric, 86.1 µmol/l hexanoic, 76.8 µmol/l heptanoic and 69.3 µmol/l octanoic, pH 8) was used. The external standard was run five times at the start of each run and the response factor for each standard averaged and the coefficient of variance calculated. The external standard was run again and the coefficient of covariance calculated after every 12 samples. All reagents used were Analytical Reagent Grade and supplied by Sigma-Aldrich Company Ltd (Dorset, UK), except acetic acid (glacial) which was supplied by Fisher Scientific (Loughborough, UK). All serial samples from each patient were analyzed at the same run to minimize inter-assay error. Each sample was extracted and analysed in duplicate (two different extractions) and in reverse order, to account for any time effect (due to evaporation of the very volatile SCFA) on the accuracy of the results. The results from the two extracts were averaged unless their variance was wide. A quality control sample (well homogenized freeze dried stock faecal material) was included at the beginning and the end of each run to assure repeatability of the assay and intra-assay comparison of the results between different runs.

7.2.4.4. Determination of faecal lactate

D- and L- Lactate were measured in freeze dried faecal samples using a modified enzymatic spectrophotometric commercial assay (D, L Lactic Acid, UV Method, Boehringer Mannheim, Roche, Cat No; 11112821035). In the presence of lactate dehydrogenase, lactic acid is oxidized to pyruvate by NAD according to the reaction:

$$Lactate + NAD \Leftrightarrow Pyruvate + NADH + H$$

The equilibrium of these reactions lies on the side of lactate. By trapping pyruvate in a subsequent one-way reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favour of pyruvate and NADH according to the reaction.

$$Pyruvate + L\text{-}glutamate \Rightarrow L\text{-}alanine + 2\text{-}oxoglutarate$$

The amount of NADH formed in the above reactions is stoichiometric to the amount of D-lactic and L-lactic acid respectively. The concentration of NADH is determined by means of its ultraviolet absorbance at 340 nm and equals to the expended lactate.

Modification and optimization of the commercial Kit

The initial protocol of the assay was based on a commercially available kit of measuring D and L lactate in foodstuffs (D, L Lactic Acid, UV Method, Boehringer Mannheim, Roche, Cat No; 11112821035). Several modification steps were applied to scale down the original method to a microtitre calibre, and additional steps were added for the purification and extraction of faecal lactate. In a two months optimization phase, different lactate extraction and enzymatic reaction conditions and protocols were tested and optimal conditions were selected. The description of the optimization steps is beyond scope of this thesis.

The final optimized protocol for the measurement of faecal lactate in stool sample had two major stages.

a. *Lactate extraction and removal of impurities*
b. *Spectrophotometric Determination of D and L lactate*

Table 7.1: Preparation method and reagents for the assay of the determination of faecal lactate

NAD working solution (0.003097 mg/µl)	Dissolve 232 mgs of NAD (Fluka 43407) in 5 ml of distilled water. The solution is stable at 4 ^{o}C for one week.
GPT solution (0.0139 Unit/µl)	Dilute 0.8 ml of well-shaken stock solution of GPT (Glutamic Pyruvic Transaminase from porcine heart 90 Unit / mg protein, 14.4 mg / ml. SAFC G9980 15UN) suspension in 4.2 ml of distilled water. Shake well before use.
D-lactase dehydrogenase (0.048 Unit/µl)	Dissolve 4.63 mg of lyophilised enzyme (SAFC 59023, D Lactate from Lactobacillus species, 1165 U / mg) in 5 ml of distilled water. The solution is stable at 4 ^{o}C for one week
L-lactase dehydrogenase (0.048 Unit/µl)	Dissolve 38 mg of lyophilised enzyme (Biochemika: L-lactate Dehydrogenase from rabbit muscles 142 U/mg 61305) in 5 ml distilled water. The solution is stable at 4 ^{o}C for one week.
Carrez I and II clarification system	Bioquant Carrez clarification 5-fold concentrated (Merck. 10537) (D, L Lactic Acid, UV Method, Boehringer Mannheim, Roche, Cat No; 11112821035)
Quality standards	Standard solutions of D and L lactate were available from the manufacturer's kit.
Buffer glycylglycine	Dissolve 1.321 g of Glycylglycine (Argos Chemicals) in 100 ml of water. Add 1.4667 g L-glutamic acid (Fisher) in 100 ml. Stir with magnetic stirrer and adjust pH with concentrated KOH (5M). Bring solution to room temperature before use.

Reagents

All reagents (Table 7.1) were kept in dark bottles or in universal tubes wrapped with aluminium foil and brought to room temperature before use.

Lactate extraction and removal of impurities

Freeze-dried stool samples (30-50 mg; same sample as for SCFA determination; fixed with 1:1 NaOH 1M) were suspended in 800 µl of distilled H_2O in a 2 ml secure lock eppendorf

tube. The suspension was agitated vigorously by vortex (1 min) and incubated in a water bath at 65 ^{o}C for 20 min. The suspension was agitated for further 45 sec and faecal impurities removed with the Carrez clarification system (Merck, UN 3082). Typically 100 µl of Carrez I were added followed by short agitation and addition of 100 µl of Carrez II. The suspension was vortexed for further 10 sec before being spun at 14000 g for 8 min. 500 µl of the supernatant were transferred into a clean secure lock eppendorf tube and 50 µl of Carrez I and Carrez II were added as above. The suspension was centrifuged at 14000g for 6 min and the clear supernatant was transferred into a clean eppendorf tube. The extract was centrifuged for a further 4 min without addition of Carrez clarification system and the final supernatant analysed immediately for the determination of the D and L lactate.

Spectrophotometric determination of D and L lactate

Each sample was analyzed twice (two separate extractions) and each extract in duplicate. The values of the two replicates and extracts from the same sample were averaged unless variance was wide. Two standard samples of D and L lactate were run each time to check completion of the reaction. A quality control faecal sample identical that used for the determination of SCFA was analysed in each run to ensure intra-assay repeatability.

In a 96 microtitre well plate (Costar 9017, US), the reactants and the under determination samples, quality controls and standards were added according to Table 7.2. The plate was covered with membrane sealer and mixed on an orbital shaker for 10 min to mix up the reagents. Absorbance was measured at 340 nm and corresponds to the background absorption. D-lactate dehydrogenase (10ul) was added, and the plate incubated on a shaker for 60 min followed by measurement of absorbance at 340 nm. Finally, 10 µl L-lactate dehydrogenase was added and the plate incubated as above for two hours and absorbance measured at 340 nm.

Table 7.2: Composition table of the faecal lactate assay

	GPT	**Buffer**	**NAD**	**Water**	**Sample**	**QC**	**D-lactase**	**L-lactase**
Blank (µl)	15	100	15	75	0	0	10	10
Control (µl)	15	100	15	65	0	10	10	10
Sample (µl)	15	100	15	45	30	0	10	10

Calculation of lactate concentration

The concentration of lactate is given by the general formula:

$$c = \left(\frac{V \times MW}{\varepsilon \times d \times v \times 1000} \right) \times DA(g/l)$$

V= Total volume of reactants in the well, v= Sample volume, MW= Molecular weight of substance to be assayed (g/mol), d= Well effective light path (cm), ε= Extinction coefficient of NADH at 340 nm = 6.3 (l x $mmol^{-1}$ x cm^{-1}), DA=the difference between absorbance: For D-lactate this was $A_1 - A_0$, and for L-lactate $A_2 - A_1$

A_0: Background Absorbance

A_1: Background Absorbance plus Absorbance due to D-lactate conversion

A_2: Background Absorbance plus Absorbance due to D-lactate and L-lactate conversion

$$\text{Concentration of } D-lactate = \left(\frac{0.215 \times 90.1}{6.3 \times 0.64 \times 0.020 \times 1000} \right) \times DA(g/l)$$

$$\text{Concentration of } L-lactate = \left(\frac{0.225 \times 90.1}{6.3 \times 0.67 \times 0.020 \times 1000} \right) \times DA(g/l)$$

Lactate concentration of initial slurry (g/l): $\frac{Concentrationoflactate}{0.9}$

Lactate concentration in dry stools (g/100g) = $\frac{Concentrationinslurry}{Weightoffreezeddriedsample} \times 100$

Lactate concentration was expressed per g of dry or wet stool sample.

7.2.4.5. Determination of free and total sulphide in faecal samples

Free and total sulphide were measured in stool samples with a spectrophotometric assay in accordance to an in-house modified method reported by Strocchi (617). This is based on the principle of the methylene blue reaction first described by Cline (618) measuring sulphide in environmental specimens. Typically sulphide reacts with a diamine reagent in an acidic environment under the oxidative effect of ferric chloride. The absorbance is measured at 670 nm and its intensity is proportional to the concentration of sulphide.

Optimization of previous assays

Several methods have been reported in the literature for determination of faecal sulphide. Most are based on the principle of the methylene blue reaction (457;617;619) and one (620)

is based on microdistillation and ion chromatography. A number of spectrophotometric assays were tested to select the most appropriate for this research and optimized them where appropriate. The protocols described by Florin (619) and Moore (457) were initially chosen as they seemed easier and more practical but our results were not encouraging particularly regarding the recovery of the assays and the sensitivity of the methods. To select the best concentrations of reactants, samples, and reaction conditions (incubation times, temperatures and reaction pH) an optimization phase of four months was undertaken. The detailed experimental description of these optimization steps and their results are out of the scope of this thesis and will not be reported in detail.

Interference of assay by formula feed ingredients and sulphate salts.

In a short experiment the influence of the ingredients of a nutritional supplement (Modulen), and particularly sulphur containing compounds (eg sulphate salts) was tested on the assay performance and mainly specificity. The free and total sulphide concentration of Modulen feeds (20%), a commercial infant milk powder (Cow & Gate), and three inorganic sulphate salts (magnesium sulphate, sodium sulphate, ferrous sulphate) were measured.

None of the inorganic salts tested presented substantial amounts of total or free sulphide and therefore interference from sulphate salts (not bacterial produced sulphide) should be negligible. Surprisingly in both Modulen and baby feed free sulphide was detected but not total sulphide. In theory this could be attributed to release of sulphide during the free sulphide stabilization step with NaOH and a possible unspecific reaction between the latter and sulphur containing ingredients in Modulen (sulphur containing amino acids).

The main assay protocol consists of two main stages:

a. *Trapping and stabilization of free and bound sulphide*
b. *Spectrophotometric determination of sulphide*

Reagents

All the reagents (Table 7.3) were of Analytical Reagents Grade and supplied from Sigma Aldrich and Fisher Scientific (Loughborough).

Trapping and stabilization of free and bound sulphide step

Sulphide occurs in stool samples in two chemical forms, typically as hydrogen sulphide or bound to divalent minerals like zinc and copper. Although bound sulphide is relatively stable, hydrogen sulphide is extremely volatile and susceptible to oxidation. Stabilization of free sulphide by converting it to the relevant salt is necessary for accurate measurements of sulphide in faecal material. A rule of thumb is to trap total (free and bound) sulphide in

insoluble zinc sulphide with zinc acetate or to increase solubility and decrease oxidation of free sulphide in an oxygen-free alkaline solution.

Table 7.3: Preparation method and reagents for assay of the determination of faecal sulphide

NaOH 1.25 M	Oxygen free NaOH 1.25 M prepared to solubilise free H^2S and to prevent oxidation. Aliquots of 1.25 N NaOH stored in 28 ml McCartney tubes (rubber sealed) and degassed by sonication (15 min) in warm water-bath (Sonomatic 375H, Jencons Scientific ltd, Bedfordshire, England).
Zn-acetate 0.11 M	Zinc acetate trapping solution prepared to preserve and stabilize both free and bound sulphide. Zinc displaces the H atoms and other divalent minerals attached to sulphide forming stable insoluble zinc sulphide salt. Zinc acetate 0.11 M (Fisher 2/0700/50) prepared by mixing zinc acetate dihydrate with distilled water and degassing by sonication as above.
Colour forming reagent (reaction reagent)	Reaction reagent (concentrated) prepared by dissolving 2g of n,n-dimethyl-p-phenylenediamine (DPD; Fluka 07750) and 3g of FeCl3 (Sigma Aldrich, 23648) in 500 ml of ice cold HCl 6M. Solution prepared fresh (weekly) and kept in dark bottle at -20 ^oC. Diluted reagent prepared by mixing equal volumes of the concentrated reagent with ice cold HCl 6N. Both dilute and concentrated reaction reagents used ice cold.
Sulphide standard stock solution (2 mM):	Sulphide standard stock solution prepared with sodium sulphide nonahydrate (Na2S • 9H2O) (Sigma Aldrich, 20804-3). A crystal of sodium sulphide nonahydrate (Na2S • 9H2O) washed out with distilled water discarding washings, wiped dry with cellulose tissue and weighed accurately. 2mmol/l solution made up with zinc-acetate 0.11 M in a gas proof glass container with rubber seal and flushed with nitrogen for 10 min to displace oxygen. Slight nitrogen over-pressure applied. Concentration of sulphide calculated from amount of sulphide dissolved (after washings) or iodometrically. Stock solution kept in 4 ^oC for 15 d and working aliquots drawn prior to assay with syringe after shaking vigorously.
Sulphide spike solution (0.5 mM)	Spikes of standard sulphide (0.5 mM) prepared fresh prior to assay to measure the recovery of faecal sulphide in the assayed samples. 0.5 mM spike was prepared by mixing 200 µl of standard (2mM) solution with 600 µl of zinc acetate 0.11 M.

Typically 1-1.5 g of weighed faecal sample was dispersed in a 50 ml pre-weighed polypropylene centrifuge tube containing 10 ml oxygen free NaOH 1,25M (free sulphide) or oxygen free zinc acetate 0,11 M (total sulphide). The sample was dispersed completely in the liquid phase by vortex. The zinc acetate faecal slurry was kept at –20 ^oC until further handling whereas the alkaline slurry was centrifuged at 15000g for 30 min to precipitate insoluble sulphide salts and recover the supernatant that contained the soluble free sulphide. The supernatant was transferred carefully in new eppendorfs and was stored at –20 ^oC.

Dilution step

Prior to analysis, frozen faecal extracts (free sulphide) and slurries (total sulphide) were thawed and brought to room temperature. The determination of sulphide was performed immediately after thawing and the time of the assay was kept minimal to avoid loss of sulphide due to oxidation. The alkaline faecal extract (free sulphide) was centrifuged for five min at 14000 g whereas the faecal slurry (total sulphide) was resuspended thoroughly before use. 1.5 ml of faecal extract (free sulphide) was diluted with 6 ml of distilled water in a dilution ratio 1:5. Similarly 0.5 ml of well-dispersed total sulphide faecal slurry were diluted to 1:20 with 9.5 ml of zinc acetate trapping solution.

Spectrophotometric determination of free and total sulphide

Samples (faecal extracts or slurries) were run in duplicate and their results averaged unless the variance between replicates was large. All serial samples from the same participant were run on the same assay using the same calibration curve to minimize intra-assay variance.

Ice cold concentrated (80 µl; blank samples) or 160 µl of diluted reaction reagent (sample and spiked sample) were added in polypropylene eppendorfs containing 900 µl of faecal slurry or extract (Table 7.4). Tubes were capped immediately to trap released sulphide and inverted gently approximately 5-10 times. Tubes were vortexed for 5 sec and incubated in the dark for 15 min at room temperature and then for 30 min at 37 ^{o}C to allow full colour development. Eppendorfs were centrifuged (6 min, 14000 g) and the supernatant transferred into disposable cuvettes. Absorbance was measured at 670 nm in a conventional spectrophotometer (Biomate® 3,Thermo, UK).

For each sample a blank replicate was prepared to correct for background absorbance of the faecal particulate and any interference due to a non specific reaction between the latter and the reaction reagent. The blank sample had identical composition to the unknown sample apart from all intrinsic sulphide being driven off, achieved by adding 80 µl of HCl 6N (Table 7.4) before development of the methylene blue color and vigorously agitating the tube on an orbital shaker (VXR Basic IKA Vibrax® SHAKER) for 1 hour with the cap on.

A spiked sample identical to the under determination sample, but spiked with 10 µl of sulphide standard (0.5 mM), was assayed to measure the recovery of the assay for each individual sample and to correct the measured absorbance of the unknown sample (Table 7.4).

Standard Calibration Curve

A standard calibration curve (Fig 41) was prepared at the beginning of each run. A working standard solution of 0.05M was prepared by mixing 200 µl of 2mM sulphide standard and 7.8 µl of zinc-acetate 0.11 M. Seven serial dilutions (0 – 37.4µM) were assayed with the same protocol for the determination of sulphide in faecal samples (Table 7.5). Absorbance of the standards was plotted against their concentrations and a linear curve was plotted.

Table 7.4: Composition of blank, sample, blank spike, spike and spiked sample in the determination of sulfide in faecal samples (all volumes in (µl)

	Blank	Sample	Spiked Sample	Spike Blank	Spike
Sample[$]	900	900	900		
Zinc acetate 0.11N	10	10		900	900
Sulphide standard 0.5mM			10	10	10
Reaction reagent	80*			80*	
HCl 6N	80	160	160	80	160
Final volume	1070	1070	1070	1070	1070

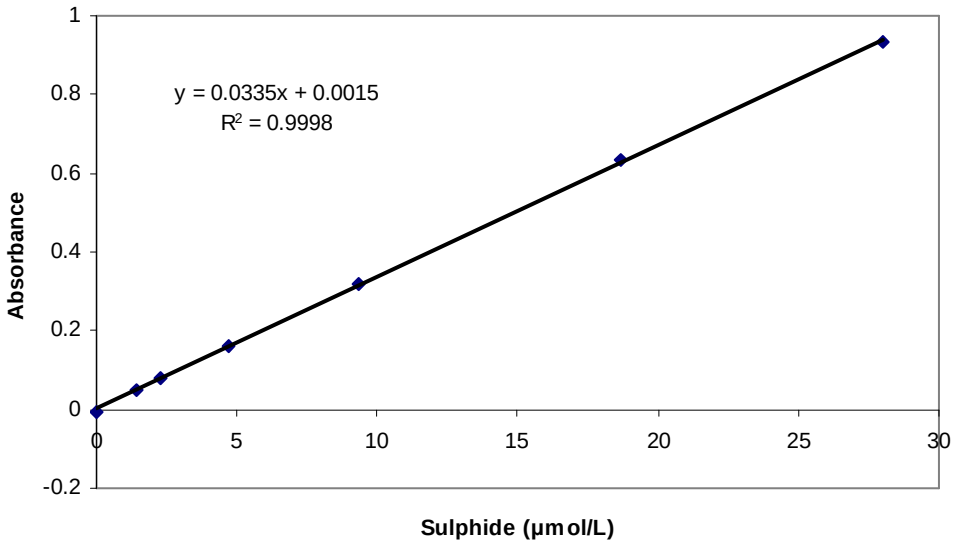
* Concentrated reaction reagent; [$] Faecal slurry or extract

Table 7.5: Composition of calibration curve for the determination of sulphide in faecal samples

Sulphide (µM)	**Blank**	**0**	**1.4**	**2.3**	**4.7**	**9.3**	**18.7**	**28**	**37.4**
Zn-Acetate	810	910	880	860	810	710	510	310	110
Standard	100	0	30	50	100	200	400	600	800
Reagent	80*	160	160	160	160	160	160	160	160
HCl	80								

*Concentrated reaction reagent

Figure 41: Calibration curve of spectrophotometric determination of sulphide



Calculation of sulphide concentration

The concentration of sulphide in samples was calculated by reference to the calibration curve equation corrected for recovery. The recovery of the assay was calculated by the formula:

$$\%\,\mathrm{Recovery}(R) = \frac{[Abs(SSp) - Abs(BS)] - [Abs(S) - Abs(BS)]}{[Abs(Sp) - Abs(BSp)]} \times 100$$

Where: Abs the absorbance of Spiked Sample (SSp), Sample Blank (BS), Sample (S), Spike (Sp), and Spike Blank (BSp).

The corrected absorbance of the sample was calculated by the formula:

$$CorrectedAbsorbance(Cabs) = \frac{Abs(S)}{R} \times 100$$

The concentrations of free and total sulphide per mass of stool sample are calculated by the formula:

$$FreeSulfide(\mu mol/g) = \frac{5.94 \times (Cabs - A)}{100 \times B \times W}$$

$$TotalSulfide(\mu mol/g) = \frac{23.78 \times (Cabs - A)}{100 \times B \times W}$$

Where: Cabs is the corrected absorbance, A and B the intercept and slope of the calibration curve, and W the weight of the stool sample.

7.2.4.6. Determination of faecal ammonia

Faecal ammonia was measured in the same faecal slurry (1:3, w/v) used for pH determination. Faecal slurry (100-500 µl) was diluted to a final volume of 40 ml with distilled water in a conical polypropylene centrifuge tube. The suspension was vortexed and filtered (0.22 µm Millipore filter). Determination of ammonia in the clear supernatant was performed with an automated ammonia analyzer (Hanna, HI 93715).

7.2.4.7. Analysis of bacterial diversity and composition with molecular bacteriology techniques

The bacterial diversity of CD children and changes during EEN treatment were assessed with molecular microbiology methods. Temporal temperature gradient gel electrophoresis (TTGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR) generated DNA amplicons of the same size (340). During TTGE, PCR products encounter increasingly higher running buffer temperature as they migrate through a polyacrylamide gel. Upon reaching a threshold buffer temperature, the weaker melting domains of the double stranded PCR product will begin to denature at which time migration slows substantially. Different sequences of DNA (from different) bacteria will denature at different temperatures resulting in a pattern of bands. Each band theoretically represents a different bacterial species present in the community. TTGE uses a polyacrylamide gel containing a constant concentration of urea.

DNA isolation from faecal samples

Approximately 200-300 mg of homogenized faecal material were stored in 1.5 ml screw cap eppendorfs on sample collection and stored at –80 ^{o}C until further analysis. Total DNA was extracted with the bead beating method according to Sokol et al (512) as originally described by Godon et al. (621) 200 mg (wet weight) of stool were suspended in 250 µl buffer 4M guanidine thiocyanate (G-9277 Sigma), 0.1M Tris (pH 7.5) and 40 µl of 10% N-lauroyl sarcosine. After addition of 500 µl 5% N-lauroyl sacrosine in 0.1 M buffer phosphate (pH 8) the eppendorf was incubated in 70 ^{o}C for 1 hour. 375 µl of 0.1 mm diameter zirconium beads (Cat. No 110791012, Biospec) previously sterilized by autoclaving were added, and the tube was shaken in a Fastprep® homogenizer (MP FastPrep 24, MP Biomedicals, California, USA) 5.5 speed twice for 5 min to lyse bacterial cells. Polyvinylpolypyrrolidone (P6755 Sigma) (15 mg) was added to the tube, which was vortexed and centrifuged for 3 min at 15,000 rpm. After recovery of the supernatant in a new eppendorf, the pellet was washed with 500 ml TENP (50 mM Tris [pH 8], 20 mM EDTA [pH 8], 100 mM NaCl, 1%

polyvinylpolypyrrolidone) and centrifuged for 3 min at 15,000 rpm. The new supernatant was added to the first supernatant. The washing step was repeated three times. The pooled supernatants (about 2 ml) were centrifuged briefly to remove particles and then split into two 2-ml tubes.

Precipitation and purification of nucleic acids

Nucleic acids were precipitated by the addition of 1 ml of isopropanol for 10 min at room temperature and centrifuged for 5 min at 15000 rpm. The supernatant was discarded and pellets resuspended and pooled in 450 ml 100 mM phosphate buffer, pH 8, and 50 µl 5 M potassium acetate (P1190, Sigma). The tube was placed on ice for 90 min or left at 4 ^{o}C overnight. Following centrifugation at 15,000 rpm for 30 min, the supernatant was transferred to a new tube and 2 µl of RNAse (RNAase ONE, M426A, Promega) (1 unit/µl) added. The mixture was incubated in a dry bath at 37℃ for 30 min. DNA was precipitated by the addition of 50 µl of 3 M sodium acetate (S2889, Sigma) and 1 ml of ice cold absolute ethanol. The tube was left for 30 min at room temperature or overnight at -20C. DNA was recovered with a sterile stick after centrifugation at 15000 rpm for 15 min. The DNA pellet was finally washed with 70% ice cold ethanol, dried, and resuspended in 30-300 µl of TE buffer.

Assessment of DNA concentration and integrity

The amount and integrity of DNA were estimated visually by electrophoresis on agarose gel. Typically 2 µl of DNA template were mixed with 3 µl of loading buffer (Bromophenol 6 X (B0126, Sigma) and loaded onto the wells of an agarose gel (0.8% containing ethidium bromide 5 µl for 50 ml of agarose). The electrophoresis run in 1 X TBE (Tris Borate EDTA) for 30 min at 100 V. A 100 bp DNA ladder (G210A, 100 bp DNA Ladder, Promega) was run in parallel to quantify the base pair size of the DNA templates.

Spectrophotometric determination of DNA concentration and purity

The concentration of total DNA was determined by measuring the ratio of the absorbance of total DNA to protein. Typically 2 µl of DNA extract were dissolved to a final volume of 50 µl with distilled water and the absorbance measured in UV at 260 (DNA) and 280 (proteins) nm corrected for the background absorbance at 320 nm. A ratio greater than 1.8 represented DNA fraction of high purity. The initial concentration of the DNA template was calculated by multiplying with the dilution factor.

Ribosomal DNA amplification

Primers with GCclamp-U968 (5’ GCclamp- GAA CGC GAA GAA CCT TAC) and L1401 (5’ GCG TGT GTA CAA GAC CC) were used to amplify the V6 to V8 regions of bacterial 16S

rDNA. Polymerase chain reaction (PCR) was performed using HotStar Taq Master Mix (Qiagen). The PCR mix (50 ml) contained 25 µl of the Hotstar mixture (HotStar Taq Master Mix Kit Cart. No 203443 QIAGEN) (1 x PCR buffer, 15 mM $MgCl_2$, 200mM each dNTP, 2.5 units of HotStar Taq DNA polymerase), 20 pmol of primers U968-GC and L1401, and approximately 20 ng of DNA. Samples were amplified in a thermocycler (PT C-200, Peltier thermal Cycler, Massachusetts, USA) using the following programme: 95℃ for 15 min, 35 cycles of 97℃ for 1 min, 58℃ for 1 min, 72℃ for I.5min, and finally 72℃ for 15 min. PCR products were identified by electrophoresis on 1% agarose gel (BPE 1356, Fisher) containing ethidium bromide to check their size (500 base pairs) and estimate their concentration.

Temporal temperature gel electrophoresis (TTGE)

The DCode Universal Mutation Detection System (Bio-Rad, Paris, France) was used for sequence specific separation of PCR products. Electrophoresis was performed through a 1mm thick, 16x16 cm polyacrylamide gel (8% wt/vol acrylamide/Bis (A7168, Sigma), 7M urea, 1.25XTAE, and, respectively, 60µl and 600µl of N,N,N',N'-Tetramethylethylenediamine (T9281, Sigma) and 10% ammonium persulfate (A3678, Sigma) using 7l of 1.25 XTAE as the electrophoresis buffer. Electrophoresis was run at a fixed voltage of 64 V for 16 hrs with an initial temperature of 66℃ and a ramp rate of 0.3° C/h. For better resolution, the voltage was fixed at 20V for 20 min at the beginning of electrophoresis. Each well was loaded with 300–400 ng of amplified DNA plus an equal volume of 2X gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70% glycerol). A DNA ladder and a mixture of PCR amplicons of known bacteria were used for each gel. Gels were stained in the dark by immersion for 30 min in a solution of SYBR Green I Nucleic Acid Gel Stain (Roche Diagnostics, GmbH, Mannheim, Germany) and read under UV light (Gel Doc EQ 170-8060, Paris, France).

Calprotectin assay

Faecal calprotectin, a marker of mucosal inflammation, was measured in the stool samples of children with CD and their relatives. The assay description is outlined in Section 6.2.5 .

7.2.4.8. Statistical analysis

The results of faecal metabolites were expressed both per g ‘dry’ and per g ‘wet’ faecal material to account for overall production (‘dry’ matter) and to demonstrate their approximate luminal concentration in the large bowel lumen that is ready available to the colonocytes (‘wet’). The relative ratio of each SCFA to the total amount of SCFA (% of total SCFA) was calculated to assess changes in the pattern of SCFA production. Likewise the ratio of D to L isomer lactate concentration was computed to assess changes in the production pattern.

Data are presented with means and SD or with medians and range for parametric and nonparametric variables respectively. To assess changes of faecal biomarkers during EEN one way analysis of variance (ANOVA) with repeated measures and Bonferroni post-hoc test was applied. For between two group comparisons the Mann-Whitney test was used and for intra-individual changes one sample Wilcoxon signed rank was applied. Fisher exact test and chi-square test were applied for differences in categorical variables between groups. Correlations between faecal calprotectin levels and faecal metabolites were assessed with Spearman rank r correlation.

TTGE gel images were captured with UV camera (Gel Doc 170-8060, Biorad, Paris, France) and analyzed with the Quantity One software version 4.5.0 (Biorad). A TTGE profile with more bands generally indicates a greater degree of bacterial species diversity compared with a TTGE profile with few bands. Therefore the number of bands that appeared on the TTGE was quantitated and used to estimate bacterial diversity in each sample. Comparison of the TTGE band patterns of individual samples was performed by Sorensen's pairwise similarity coefficient (Cs) using the similarity matrix function of the software. Cs is defined as (2j/a+b)*100, where a is the number of bands in lane one, b is the number of bands in lane two, and j is the number of common bands in both lanes. Two identical profiles result in a Cs value of 100%, whereas completely different profiles (no common band) result in a Cs value of 0%.

7.2.4.9. Power calculation

The statistical power of the study was calculated for an 80% probability of detecting a 5.5 mM increase or decrease in the faecal concentration of butyrate at a 5% significance level. For a faecal butyrate concentration of 13.4 mM with a standard deviation of 12.7 (417) and for a minimum clinical significant increase or decrease of 5.5 mM concentration the required sample size is 44 subjects.

7.3. Results

7.3.1. Recruitment

Between October 2005 and August 2007 approximately 60 children started on EEN as part of their standard clinical management. Thirty children were invited to participate in the “EEN and gut microbiota study” and three patients in clinical remission were approached as a control group. In total, six children refused participation, and two withdrew the day after recruitment. Two collected less than two stool samples but did not formally withdraw from the study. One patient’s participation was terminated deliberately after a final biopsy histology report was consistent with UC diagnosis. One patient experienced a cerebral vascular

accident while on treatment with EEN and stopped treatment within a week of recruitment. For two patients, disease activity further deteriorated and both had to stop EEN and initiate a regime of steroids. Two other patients were recruited 15 days and 21 days after EEN initiation and their results are not included in the arm of the study on the effect of EEN on gut microbiota diversity and metabolism. Fourteen children completed between six to eight weeks of EEN course. Approximately 100 stool samples were collected from children with CD. Only eight stool samples were provided at hospital despite the expectations of the single researcher. The rest were collected from patients' home by the researcher within four hours of defecation.

Fresh stool samples were collected from eighteen CD children (11 boys) at the different time points of the follow on EEN (Table 7.6). Two patients provided only two samples (one discontinued treatment and the other withdrew within 2 weeks of treatment) and therefore were excluded from analysis. Three children gave in total three serial samples during treatment (one could not provide a sample-was on holiday, two stopped at week 5 and 4 respectively due to symptoms deterioration). These patients were included in the analysis.

A final stool sample was collected whilst patients were on normal diet. For one child a final sample was not collected due to subsequent operation and resection of a significant part of the ileocolon.

From the 16 children whose data is presented (mean age: 11.8 ± 2.3 y) nine were boys, twelve were newly diagnosed and this was their first course on EEN. The remaining four patients were receiving their second course to manage a subsequent disease relapse. Nine children were on no other medication apart from EEN. The rest were on other concomitant treatments (Table 7.6). For the patients with longstanding disease, the type and dose of concurrent treatment did not change during EEN course. Thirteen children had extensive disease involving the upper and lower part of the GI tract (Table 7.6).

Table 7.6: Disease location and medical treatment of CD children on EEN treatment

Disease location (Montreal classification)	N	Concomitant medication	N
Colitis (L2)	2	None	9
Ileocolitis (L3)	1	5-ASAs	4
Upper GI & ileocolitis (L3L4)	9	Immunomodulators	5
Upper & colitis (L2L4)	4	Steroids	2
		Antibiotics	1

7.3.2. Changes of disease activity markers during EEN

Changes in disease activity markers are described in detail in Chapter 6. All participants were undergoing the active phase of their disease as evident by the increased score of the clinical activity index (PCDAI) and elevated systemic inflammatory markers (Table 6.2).

All disease activity markers improved significantly at the end of treatment as a group and individually in the majority of the children (Chapter 6, Fig 35). Regardless of any improvement in disease activity during treatment, seven out of the 16 children achieved complete clinical remission (PCDAI≤10). There was no statistically significant difference in the baseline markers of disease activity between patients who achieved remission and those who did not.

7.3.3. Changes in bacterial metabolic activity during EEN

7.3.3.1. Changes in faecal water content, pH and ammonia

No significant changes in faecal water content were observed during treatment with EEN. In contrast, faecal pH rose significantly ($p<0.0001$) (Fig 42). Compared to baseline values, a mean increase of 0.5 pH units was observed within 15 days of treatment initiation ($p<0.01$). Faecal pH remained significantly elevated at all time points of follow up ($p<0.02$) (Fig 42) but returned to pre-treatment levels when children were eating their normal diet.

Different patterns of faecal pH change were observed between those patients who achieved complete clinical remission and those who discontinued treatment or presented with still active disease at the end of treatment. In those patients who achieved clinical remission (PCDAI≤10) faecal pH was significantly increased at all follow up points compared to baseline values ($p<0.0001$; Fig 43) whereas, no significant changes were observed in those patients who did not achieve clinical remission or discontinued treatment (Fig 44).

Figure 42: Boxplots showing changes in faecal pH during EEN and on normal diet in children with CD (all patients)

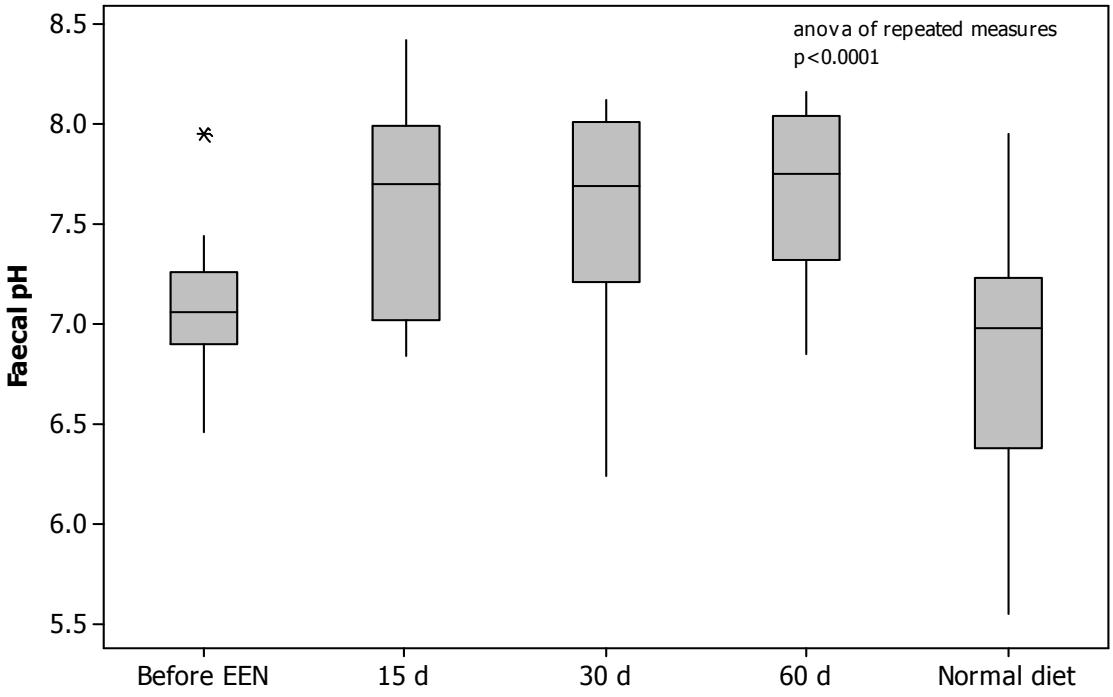


Figure 43: Boxplots showing changes in faecal pH during EEN and on normal diet in children with CD (remission)

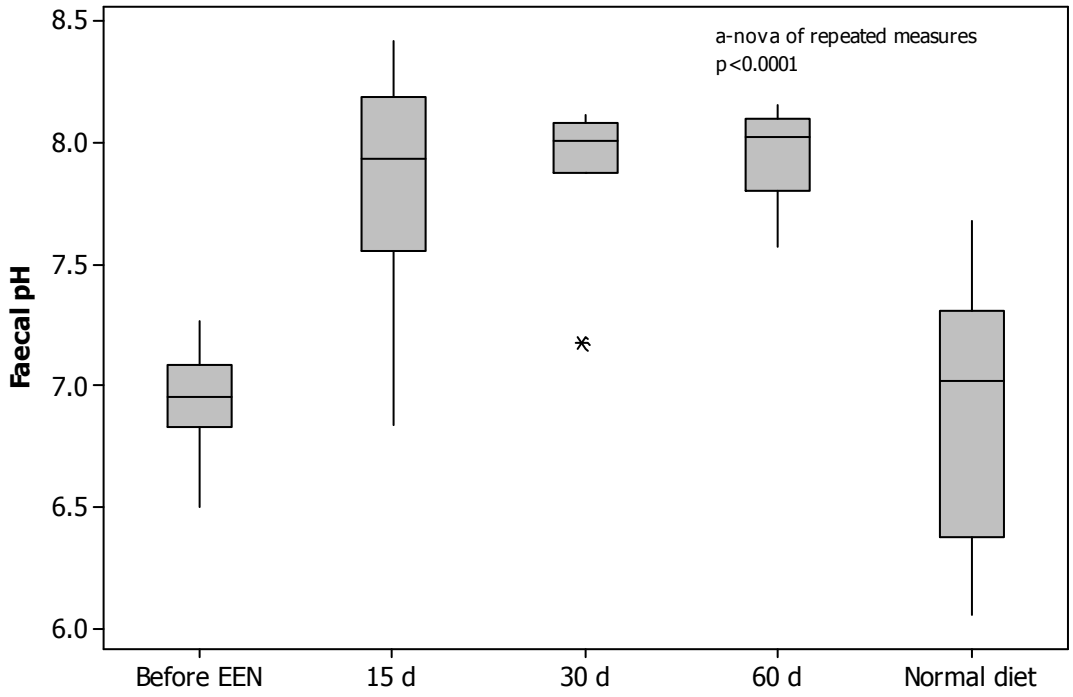
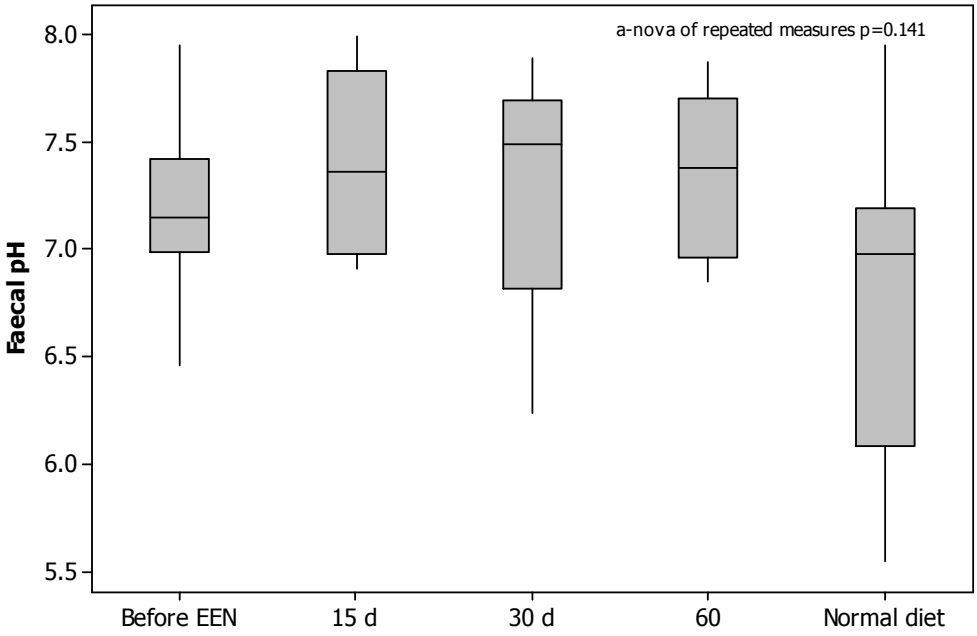
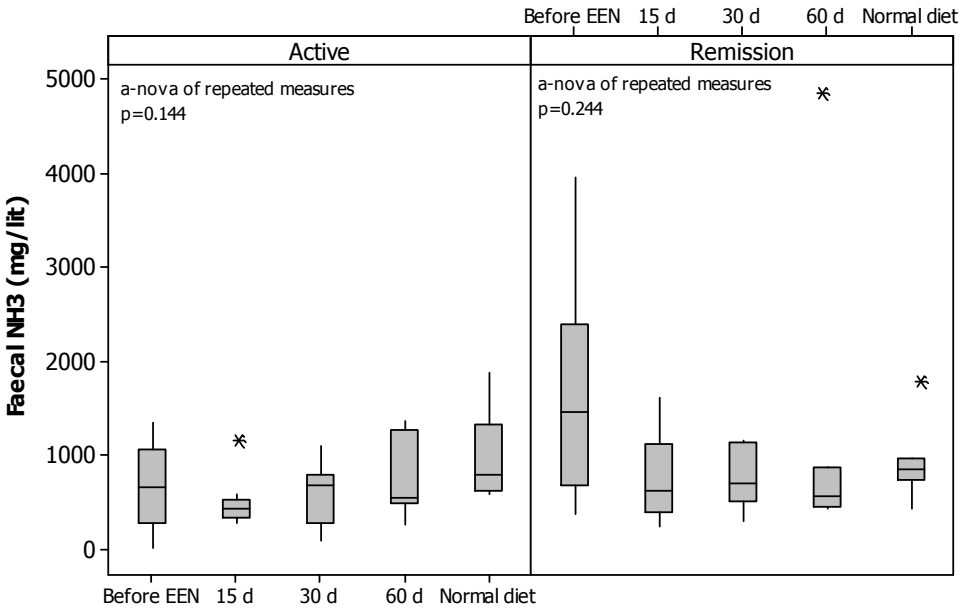


Figure 44: Boxplots showing changes in faecal pH during EEN and on normal diet in children with CD (No remission)



Treatment with EEN had no effect on faecal ammonia (expressed per litre of stool sample or per kg dry faecal material). No difference in pattern of change was found between patients who achieved clinical remission at the end of EEN treatment and those did not (Fig 45).

Figure 45: Boxplots showing changes in faecal ammonia during EEN and on normal diet in children with CD



7.3.3.2. Changes in faecal SFCA and BCFA concentration

Overall there were no major changes in the concentration of the main SCFA and BCFA during EEN treatment. Faecal butyrate concentration and its proportional fraction (% of total SCFA) were reduced during EEN treatment but this did not reach statistical significance.

However, similar to the patterns of faecal pH change, significant SCFA alterations were observed for the patients who achieved clinical remission at the end of treatment (Table 7.7). Butyrate concentration and its relative ratio (% of total SCFA) were significantly reduced within 15d of treatment initiation and continued to decrease during follow up with the maximum mean reduction observed at the end of treatment (60 d) (Table 7.7). At the end of EEN, faecal butyrate concentration was half that of baseline values, and its proportional ratio was reduced to one third of pre-treatment levels (Table 7.7). Whilst on normal diet, butyrate levels reverted to pre-treatment levels. None of the SCFA or BCFA measured changed during treatment in those patients who did not achieve clinical remission or discontinued treatment (Table 7.8).

Table 7.7: Changes in faecal concentration and % of SCFA and BCFA in paediatric CD patients who achieved clinical remission with EEN treatment

Subjects (*n*=7)	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry faecal material										
Acetate (µmol/g)	237.9	138.9	222.5	63.6	199.8	99.6	187.3	82.3	238.9	124.5
Propionate (µmol/g)	37.0	23.4	41.2	23.6	34.4	21.5	29.7	15.1	53.2	19.6
Butyrate (µmol/g)	63.0	38.9	31.7	19.0	26.3*	10.5	23.8*	11.7	59.8	32.3
Valeric (µmol/g)	5.9	6.7	4.9	6.6	4.2	5.0	4.8	4.5	5.6	5.6
Caproic (µmol/g)	0.8	0.5	2.0	1.4	1.3	0.9	0.8	0.5	1.7	0.7
Heptanoic (µmol/g)	0.2	0.6	1.1	0.7	0.4	0.7	0.0	0.0	0.7	1.3
Octanoic (µmol/g)	0.8	1.0	2.0	1.7	1.3	1.0	1.1	0.6	1.2	1.1
Isobutyrate (µmol/g)	12.2	7.0	12.2	4.5	10.0	4.3	7.9	2.1	7.0	1.3
Isovaleric (µmol/g)	17.4	8.9	17.4	7.3	14.6	6.5	11.4	2.5	11.7	3.0
Isocaproic (µmol/g)	4.2	9.4	0.7	1.0	0.4	0.7	0.1	0.4	0.0	0.0
Total SCFA (µmol/g)	379.4	160.8	335.7	91.3	292.6	119.8	266.9	98.4	379.9	174.5
Stool sample										
Acetate (µmol/g)	57.3	21.3	61.2	17.9	54.5	14.8	50.1	16.5	59.6	17.5
Propionate (µmol/g)	9.4	5.4	11.6	7.7	9.5	5.0	7.9	3.5	13.8	3.5
Butyrate (µmol/g)	15.4	7.4	9.1	6.3	7.8*	3.4	6.7**	2.9	14.8	5.6
Valeric (µmol/g)	1.6	2.1	1.2	1.6	1.3	1.5	1.4	1.3	1.5	1.5
Caproic (µmol/g)	0.2	0.1	0.5	0.3	0.4	0.3	0.2	0.1	0.5	0.2
Heptanoic (µmol/g)	0.1	0.2	0.3	0.2	0.1	0.3	0.0	0.0	0.2	0.5
Octanoic (µmol/g)	0.3	0.4	0.6	0.5	0.4	0.3	0.3	0.2	0.4	0.4
Isobutyrate (µmol/g)	3.2	1.9	3.5	1.6	2.9	1.1	2.2	0.4	1.9	0.7
Isovaleric (µmol/g)	4.9	3.7	5.0	2.6	4.3	1.8	3.1	0.6	3.2	1.2
Isocaproic (µmol/g)	0.8	1.5	0.2	0.2	0.1	0.2	0.0	0.1	0.0	0.0
Total SCFA (µmol/g)	93.1	27.6	93.2	30.5	81.3	18.3	72.0	18.3	96.0	25.9
Proportional ratio										
% Acetate	61.7	14.5	66.1	8.4	66.7	9.1	68.7	6.7	61.8	3.1
% Propionate	9.4	4.3	12.3	5.6	11.4	4.8	11.5	5.6	14.7*	2.9
% Butyrate	16.9	8.8	9.3*	3.8	9.8*	3.6	9.3**	3.5	15.2	3.1
% Valeric	1.6	1.9	1.4	1.8	1.7	2.0	1.9	1.8	1.5	1.4
% Caproic	0.3	0.2	0.6	0.4	0.4	0.4	0.3	0.2	0.5	0.3
% Heptanoic	0.1	0.2	0.3	0.2	0.1	0.3	0.0	0.0	0.2	0.4
% Octanoic	0.3	0.4	0.7	0.7	0.5	0.4	0.5	0.2	0.4	0.3
% Isobutyrate	3.5	1.8	3.7	1.2	3.7	1.1	3.2	0.9	2.1**	0.8
% Isovaleric	5.2	2.8	5.4	2.1	5.4	1.9	4.6	1.3	3.6	1.5
% Isocaproic	1.1	2.3	0.2	0.3	0.2	0.3	0.0	0.1	0.0	0.0

Values were significantly different from those “Before EEN” (repeated measures ANOVA and Bonferroni post-hoc test): *P<0.05, **P<0.02.

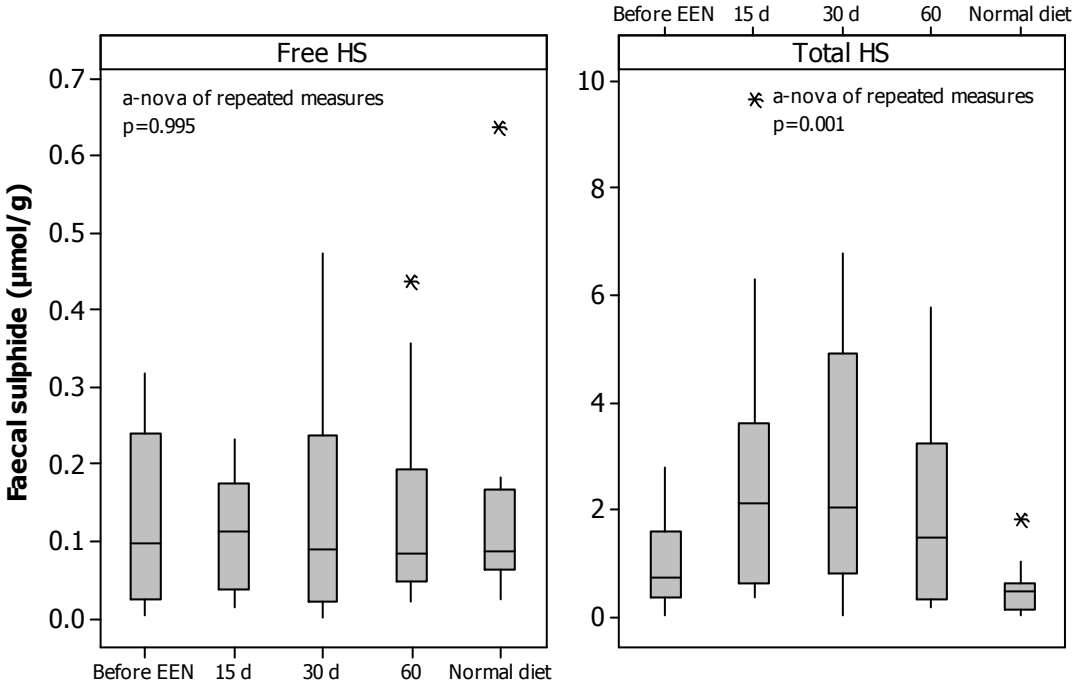
Table 7.8: Changes in faecal concentration and % total SCFA and BCFA in paeiatric CD patients who did not achieve clinical remission with EEN treatment

Subjects (*n*=9)	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry faecal material										
Acetate (µmol/g)	230.5	126.9	240.7	126.6	254.9	130.1	271.1	149.3	276.8	183.4
Propionate (µmol/g)	37.8	24.5	29.9	23.6	33.7	17.4	39.8	12.6	41.6	30.3
Butyrate (µmol/g)	26.3	25.6	20.0	16.7	20.6	15.3	25.4	15.4	39.6	42.9
Valeric (µmol/g)	5.0	7.8	3.3	4.3	4.4	5.5	4.3	4.2	3.3	4.5
Caproic (µmol/g)	1.4	1.9	1.0	1.2	1.3	0.9	1.3	0.3	1.0	0.7
Heptanoic (µmol/g)	0.5	0.6	0.4	0.7	0.7	1.1	0.1	0.4	0.3	0.5
Octanoic (µmol/g)	1.1	1.3	0.6	0.9	0.7	1.2	0.5	0.7	1.0	1.3
Isobutyrate (µmol/g)	7.8	6.9	6.1	3.9	6.0	4.3	9.3	9.0	5.7	2.1
Isovaleric (µmol/g)	10.6	7.8	6.7	3.6	7.8	6.2	10.3	6.2	9.1	4.3
Isocaproic (µmol/g)	1.1	1.4	1.8	3.0	1.3	2.1	3.9	7.0	0.0	0.1
Total SCFA (µmol/g)	322.1	130.0	310.5	135.2	331.5	123.2	366.0	159.0	378.5	203.7
Stool sample										
Acetate (µmol/g)	44.5	17.6	43.5	14.6	44.9	20.7	46.8	11.3	63.4	32.5
Propionate (µmol/g)	9.5	7.6	6.3	5.7	6.9	4.3	7.4	1.1	10.9	7.2
Butyrate (µmol/g)	7.2	7.0	4.0	2.9	4.7	5.1	5.1	2.8	10.5	9.8
Valeric (µmol/g)	1.4	2.1	0.7	0.8	1.2	1.6	1.0	1.0	0.9	1.4
Caproic (µmol/g)	0.4	0.6	0.2	0.3	0.3	0.2	0.3	0.1	0.2	0.2
Heptanoic (µmol/g)	0.1	0.2	0.1	0.2	0.2	0.2	0.0	0.1	0.1	0.1
Octanoic (µmol/g)	0.3	0.3	0.1	0.2	0.2	0.4	0.1	0.2	0.3	0.4
Isobutyrate (µmol/g)	2.1	1.9	1.3	0.7	1.4	1.2	1.8	1.4	1.6	0.9
Isovaleric (µmol/g)	2.8	2.3	1.5	1.0	1.9	2.0	2.1	1.2	2.7	1.7
Isocaproic (µmol/g)	0.3	0.3	0.3	0.4	0.2	0.3	0.6	1.1	0.0	0.0
Total SCFA (µmol/g)	68.5	34.3	58.0	18.9	62.0	28.3	65.3	9.8	90.7	39.1
Proportional ratio										
% Acetate	70.9	18.2	75.5	12.1	73.5	13.9	71.3	10.2	69.6	15.2
% Propionate	11.9	6.7	10.2	6.1	11.1	5.0	11.7	3.0	12.1	7.2
% Butyrate	8.3	6.8	6.8	4.9	7.1	4.9	7.8	3.8	10.9	7.7
% Valeric	1.6	2.3	1.3	1.6	2.0	2.8	1.6	1.6	1.2	1.8
% Caproic	0.5	0.5	0.3	0.4	0.4	0.3	0.4	0.2	0.3	0.2
% Heptanoic	0.2	0.4	0.2	0.3	0.3	0.4	0.0	0.1	0.1	0.2
% Octanoic	0.4	0.4	0.2	0.3	0.3	0.4	0.2	0.3	0.4	0.5
% Isobutyrate	2.4	1.8	2.2	1.3	2.1	1.5	2.7	1.9	2.0	1.2
% Isovaleric	3.4	2.2	2.6	1.8	2.8	2.2	3.3	1.9	3.3	2.2
% Isocaproic	0.3	0.4	0.6	0.9	0.4	0.6	0.9	1.5	0.0	0.1

7.3.3.3. Changes in faecal sulphide concentration

Total but not free faecal sulphide concentration increased significantly during EEN ($p<0.0001$) (Fig 46). A three-fold increase in total sulphide was observed within 15 d of treatment initiation ($p=0.02$). This plateaued at 30 d of treatment ($p<0.02$). At 60 d the mean total sulphide concentration was higher than at baseline but this did not reach statistical significance. Total sulphide reverted to pre-treatment levels on normal diet.

Figure 46: Boxplots showing changes in faecal free & total sulphide during EEN and on normal diet in children with CD



When sub-analysis was conducted separately for patients who achieved clinical remission at the end of treatment, and those did not or who discontinued treatment, different patterns of change were observed (Fig 47 & 48). A seven-fold increase for total sulphide was observed after 15 days of treatment introduction in the patients who entered clinical remission. From a pre-treatment concentration of 0.64 µmol/g, total sulphide increased to 4.74 µmol/g after two weeks on EEN treatment introduction ($p<0.001$). At 30 and 60 d follow up measurements, total sulphide remained at the same high levels ($p<0.03$). Concentration of total sulphide returned to pre-treatment levels when patients went back to their normal diet (Fig 48). No significant changes were seen for total sulphide concentration in patients with active disease at end of treatment or those who discontinued treatment (Fig 48).

Faecal free sulphide concentration did not change from baseline levels during EEN (Fig 47). No different patterns of change were seen between patients who achieved clinical

remission and those did not (Fig 47 & 48). Same change patterns were found when sulphide concentrations expressed per g dry sample (data not presented).

Figure 47: Boxplots showing changes in free faecal sulphide during EEN in CD children. Comparison between patients with active disease and in remission at the end of EEN

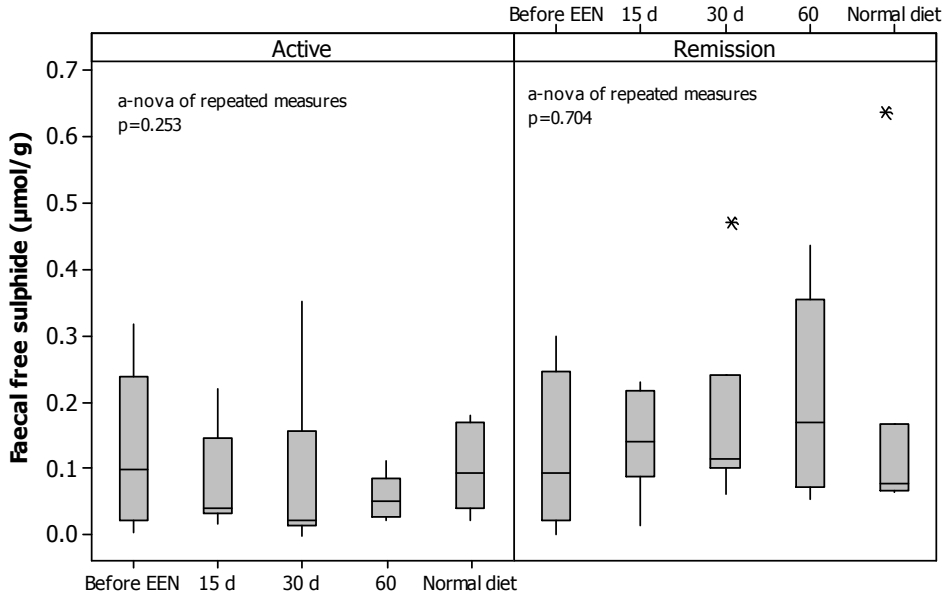
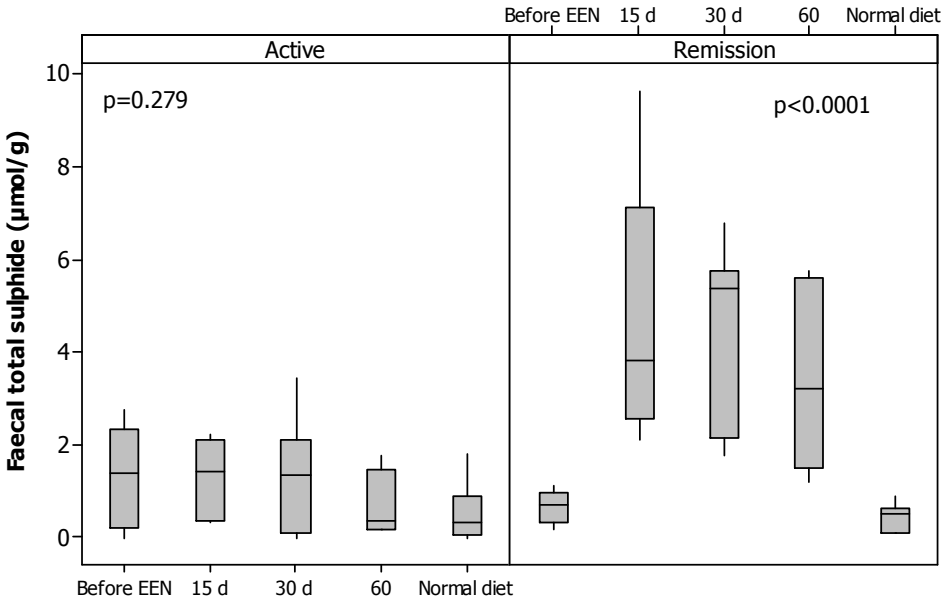


Figure 48: Boxplots showing changes in total faecal sulphide during EEN in children with CD. Comparison between patients with active disease and in remission at the end of EEN



7.3.3.4. Changes in faecal lactate concentration

Neither D nor its isomer L-Lactate changed significantly during treatment with EEN. In sub-group analysis D but not L-Lactate decreased after 15 and 30 d of treatment initiation in the group of children who achieved clinical remission ($p<0.04$; Table 7.9). At 60 d the mean decrease did not reach statistical significance ($p=0.08$). No change was found in the ratio of D and L lactate isomer concentration during the course of treatment. However children who achieved clinical remission had a higher D/L Lactate ratio on normal diet than pre-treatment.

No significant changes in concentrations of D-Lactate, L-Lactate nor their ratio were found in children who did not achieve clinical remission or discontinued treatment (Table 7.10).

7.3.4. Gut inflammation and bacterial metabolic activity

Patients were categorized into two groups based on the direction of calprotectin change at the end of treatment. The first group included those patients (n=11) whose calprotectin levels was reduced at the end of treatment, and the second group (n=5) those patients with calprotectin concentrations, at treatment cessation higher than at baseline. Four patients in the first group did not achieve complete clinical remission at the end of EEN (PCDAI>10) compared to all patients in the group in which calprotectin increased.

7.3.4.1. Changes in water content, faecal pH and ammonia

For both groups no significant changes in the faecal water content or ammonia were found during EEN (data not presented). Faecal pH increased only in the group of patients that calprotectin decreased at the end of treatment (Figs 49 & 50). In this group pH reversed to pre-treatment when patients were on normal diet.

7.3.4.2. Changes in faecal sulphide concentration

Similar to the pattern of change observed in the patients who achieved clinical remission at the end of EEN, faecal levels of total but not free sulphide increased significantly at 15 and 30 d, only in the patients with lower than pre-treatment levels of calprotectin, at the end of EEN (Fig 51). Total sulphide returned to pre-treatment levels when patients returned to normal diet. No change in total or free sulphide was found in the group of patients with higher than baseline levels of calprotectin at the end of treatment (Fig 52).

Table 7.9: Changes in faecal lactate concentration during treatment with EEN and on normal in patients who achieved clinical remission

N=7	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Dry faecal material										
D-lactate (mg/100g)	51.9	47.4	18.4*	6.5	18.9*	8.9	22.4	10.8	40.3	24.8
L-lactate (mg/100g)	160.4	262.6	29.8	10.6	29.7	11.5	32.5	15.6	49.9	31.0
Total lactate (mg/100g)	213.0	294.0	49.2	17.3	49.2	19.3	55.7	25.8	91.2	53.3
Stool sample										
D-lactate (mg/100g)	10.3	6.2	4.9$	1.1	5.2$	1.3	6.0	2.2	10.0	4.0
L-lactate (mg/100g)	28.9	41.0	8.1	2.2	8.4	2.3	8.6	3.2	12.2	4.8
Total lactate (mg/100g)	39.4	44.6	13.3	3.4	13.9	3.0	14.9	5.1	22.5	7.1
D/L-lactate	0.6	0.3	0.6	0.1	0.7	0.2	0.8	0.3	0.9*	0.7

ANOVA of repeated measures and Bonferroni post-hoc test *P<0.04 $ P<0.03 from "Before EEN"

Table 7.10: Changes in faecal lactate concentration i during treatment with EEN and on normal in patients who did not achieve clinical remission after EEN

N=9	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Dry faecal material										
D-lactate (mg/100g)	39.4	35.7	76.8	87.5	78	75.7	72.3	62.2	77.3	129.0
L-lactate (mg/100g)	76.2	77.7	187.4	214.8	173.7	168.9	151.5	192.6	106.9	170.5
Total lactate (mg/100g)	115.7	111.2	264.3	285	251.7	231.6	223.8	246.4	184.0	299.0
Stool sample										
D-lactate (mg/100g)	6.8	3.2	11.7	8.7	11.2	7.3	11.7	6.4	13.9	15.0
L-lactate (mg/100g)	12.5	8.3	29.2	28.9	23.9	17.7	22.9	22.2	19.7	19.5
Total lactate (mg/100g)	19.3	11.2	40.9	34.6	35.1	23.2	34.6	27.4	33.6	34.5
D/L-lactate	0.6	0.2	0.5	0.2	0.6	0.2	0.6	0.2	0.7	0.1

Figure 49: Boxplots showing changes in faecal pH during EEN and on normal diet in children with CD (calprotectin decreased)

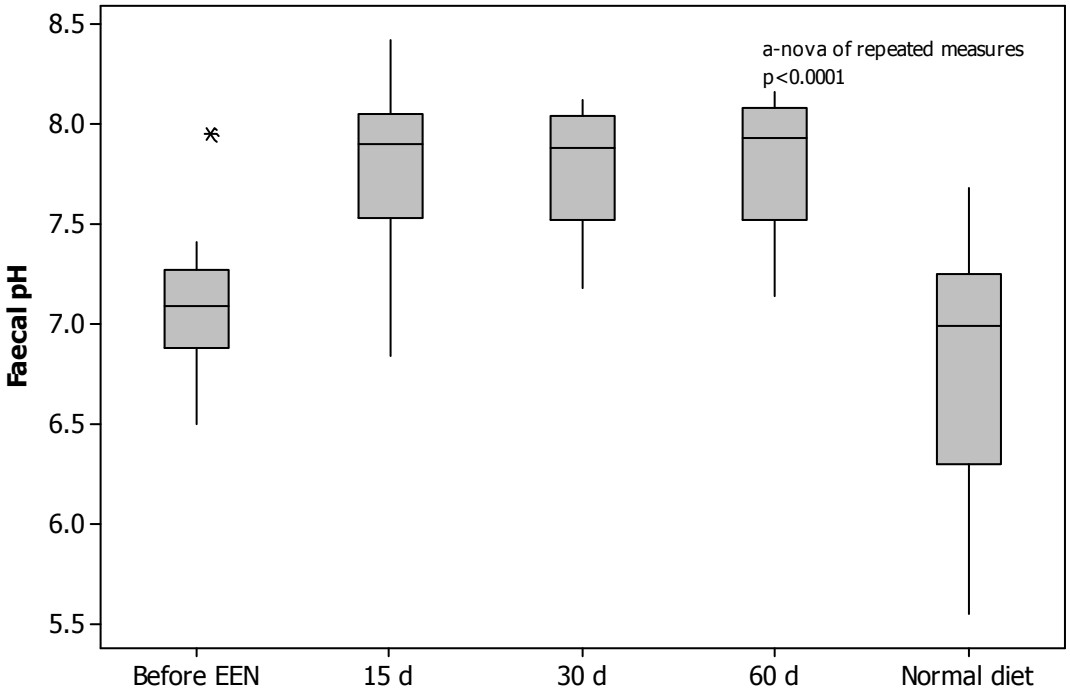


Figure 50: Boxplots showing changes in faecal pH during EEN and on normal diet in children with CD (calprotectin increased)

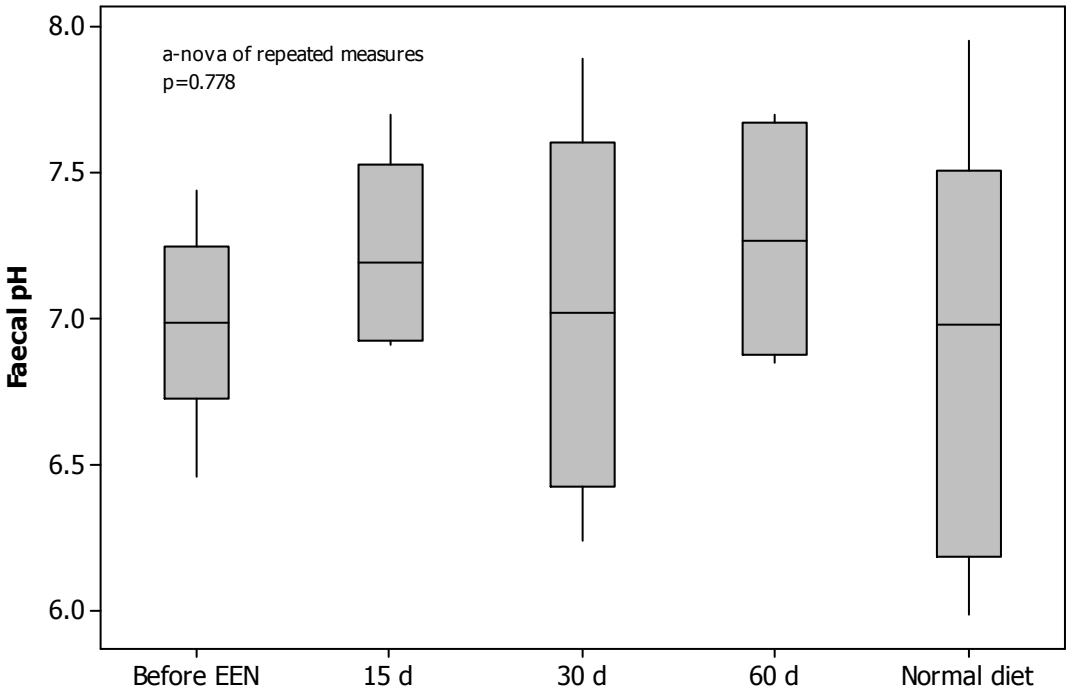


Figure 51: Boxplots showing changes in faecal free & total sulphide during EEN in children with CD (calprotectin decreased at the end of EEN)

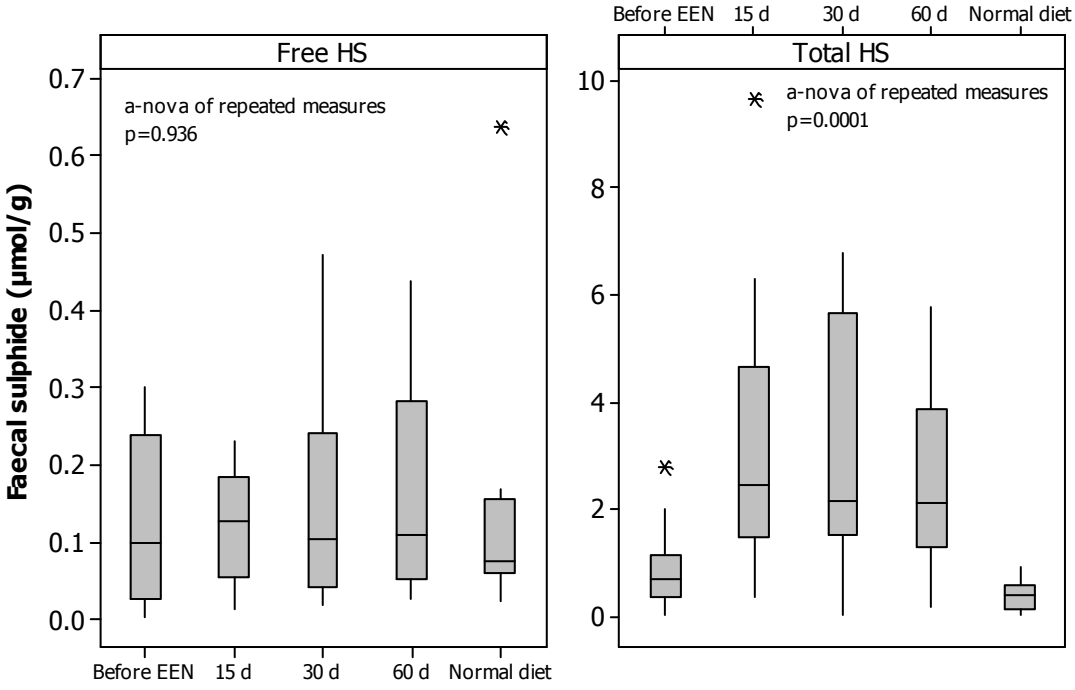
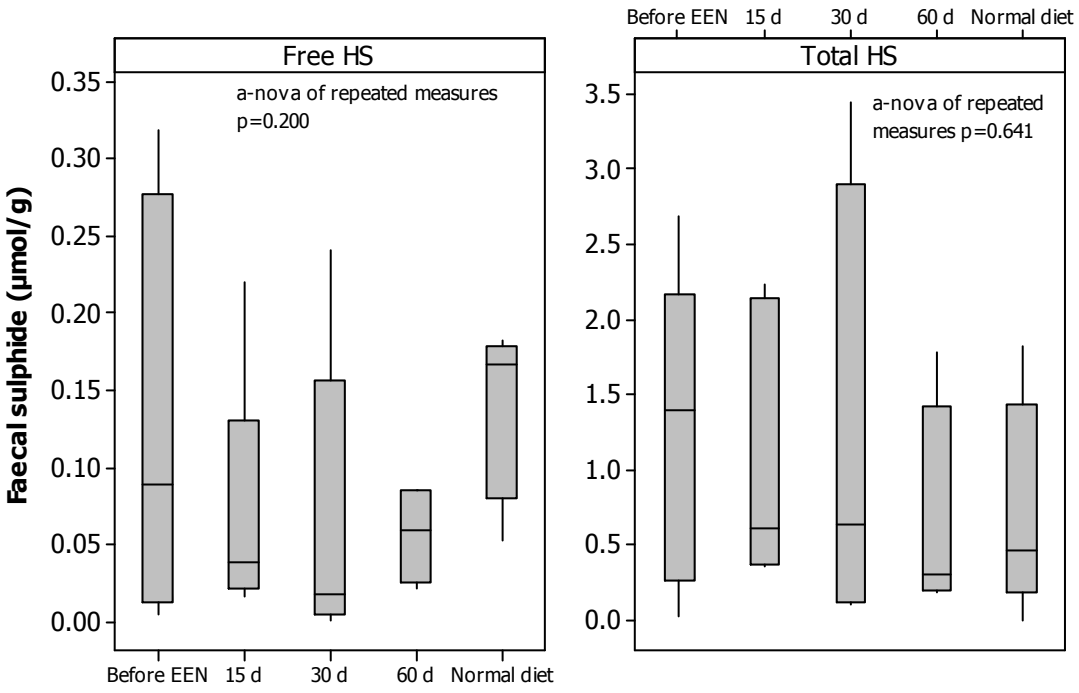


Figure 52: Boxplots showing changes in faecal free & total sulphide during EEN in children with CD (calprotectin increased at the end of treatment)



7.3.4.3. Changes in faecal SFCA and BCFA concentration

Butyrate concentration decreased by 50% within 15d on EEN only in patients whose calprotectin decreased at the end of treatment (Table 7.11). From a baseline mean concentration of 47.9 μmol/g, butyrate dropped to 24.6 μmol/g at 15 d and continued to decline further during follow up reaching the nadir mean concentration of 20.5 μmol/g at the end of treatment. No significant changes were observed for the other SCFA or BCFA (Table 7.11). None these metabolites changed in the group of patients with increased levels of calprotectin at the end of treatment (Table 7.12). On normal diet butyrate concentration returned to pre-treatment concentrations and BCFA proportional ratio were significantly lower than on treatment initiation.

7.3.4.4. Changes in lactate

Neither D or the L isomer of lactate changed significantly during EEN in any group of patients (Tables 7.13 & 7.14). On normal diet the D/L lactate concentration ratio was higher than pre-treatment in patients with a calprotectin decrease at the end of EEN treatment ($p<0.001$).

Table 7.11: Changes in faecal concentration and % of SCFA & BCFA in paediatric CD patients with decreased levels of calprotectin at end of EEN treatment

Subjects (*n*=11)	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry faecal material										
Acetate (µmol/g)	245.6	150.7	224.5	125.8	227.7	137.7	219.4	133.4	285.9	175.5
Propionate (µmol/g)	38.5	22.9	39.1	27.0	34.4	21.2	32.5	14	53.3	27.4
Butyrate (µmol/g)	47.9	38.7	24.6	17.3	21.6*	11.3	20.5*	11.0	47.8	33.7
Valeric (µmol/g)	5.8	6.7	3.9	5.4	4.5	5.1	4.0	4.2	5.0	5.5
Caproic (µmol/g)	0.8	0.5	1.5	1.2	1.4	1.0	0.9	0.5	1.7	0.6
Heptanoic (µmol/g)	0.4	0.7	0.8	0.7	0.7	1.0	0.1	0.3	0.7	1.1
Octanoic (µmol/g)	1.0	1.2	1.4	1.5	1.0	0.9	0.8	0.7	0.9	1.0
Isobutyrate (µmol/g)	10.4	6.9	9.7	5.0	8.4	4.7	7.9	1.8	6.3$	2.1
Isovaleric (µmol/g)	15.2	8.4	13.6	7.6	12	6.7	11.2	2.4	10.4	4.2
Isocaproic (µmol/g)	2.9	7.6	0.4	0.8	0.2	0.6	0.7	1.3	0.0	0.0
Total SCFA (µmol/g)	368.5	158.4	319.6	143.8	311.9	141.7	297.9	140.9	411.9	196.5
Stool sample										
Acetate (µmol/g)	53.8	20.1	55.2	18.1	53.4	19.2	49.4	15.9	64.7	28.7
Propionate (µmol/g)	10.0	6.2	10.6	7.5	9	5	7.7	2.9	13.7	5.9
Butyrate (µmol/g)	12.0	8.0	6.9	5.6	6.2**	3.7	5.5**	3.1	12.1	6.8
Valeric (µmol/g)	1.6	1.9	1.0	1.3	1.3	1.5	1.2	1.2	1.4	1.6
Caproic (µmol/g)	0.2	0.2	0.4	0.3	0.4	0.3	0.2	0.1	0.4	0.2
Heptanoic (µmol/g)	0.1	0.2	0.2	0.2	0.2	0.3	0	0.1	0.2	0.4
Octanoic (µmol/g)	0.3	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.3	0.4
Isobutyrate (µmol/g)	2.8	1.8	2.7	1.6	2.4	1.3	2	0.5	1.7	0.8
Isovaleric (µmol/g)	4.2	3.2	3.9	2.4	3.4	2.0	2.8	0.8	2.9	1.5
Isocaproic (µmol/g)	0.5	1.2	0.1	0.2	0.1	0.2	0.1	0.2	0.0	0.0
Total SCFA (µmol/g)	85.6	30.0	81.4	29.4	76.8	23	69.2	16.7	97.5	33.8
Proportional ratio										
% Acetate	64.1	15.6	68.5	10.3	69.1	12.7	70.4	8.5	65.9	13.6
% Propionate	11	5.8	12.6	6.1	11.6	5.2	11.7	5.0	14.0	5.3
% Butyrate	13.5	8.9	8*	3.9	8.2**	4.2	7.9**	4.0	12.4	6.2
% Valeric	1.8	2.1	1.4	1.8	2.1	2.7	1.7	1.8	1.5	1.8
% Caproic	0.3	0.3	0.5	0.4	0.5	0.4	0.3	0.2	0.5	0.3
% Heptanoic	0.2	0.4	0.3	0.2	0.3	0.4	0.0	0.1	0.2	0.3
% Octanoic	0.4	0.4	0.5	0.6	0.4	0.4	0.3	0.3	0.3	0.3
% Isobutyrate	3.1	1.7	3.3	1.3	3.1	1.4	3.0	1.0	1.9**	1.1
% Isovaleric	4.7	2.6	4.7	2.1	4.5	2.3	4.3	1.5	3.2**	1.9
% Isocaproic	0.7	1.8	0.1	0.2	0.1	0.2	0.2	0.3	0.0	0.0

Values were significantly different from those before EEN (repeated measures ANOVA and Bonferroni post-hoc test): $P<0.07, *P<0.05, **P<0.02.

Table 7.12: Changes in faecal concentration and % of SCFA and BCFA in paediatric CD patients with increased levels of calprotectin at end of EEN treatment

Subjects (*n*=5)	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry faecal material										
Acetate (µmol/g)	207.6	56.4	251.3	35.0	237.8	65.3	253.7	108.8	205.6	93.4
Propionate (µmol/g)	35.1	26.5	25.1	10.7	33.1	13.2	40.3	15.6	34.5	17.8
Butyrate (µmol/g)	30.1	30.0	24.7	21.4	26.4	18.2	35.0	13.4	51.5	51.0
Valeric (µmol/g)	4.4	8.8	4.1	5.3	3.9	5.6	6.0	4.3	3.1	4.1
Caproic (µmol/g)	1.9	2.6	1.1	1.5	1.0	0.7	1.5	0.1	0.7	0.8
Heptanoic (µmol/g)	0.3	0.4	0.6	0.9	0.3	0.7	0.0	0.0	0.1	0.2
Octanoic (µmol/g)	0.9	1.1	0.6	1.1	0.8	1.6	0.8	0.8	1.5	1.5
Isobutyrate (µmol/g)	8.1	7.9	6.2	4.8	6.4	4.9	10.4	12.4	6.3	1.6
Isovaleric (µmol/g)	10.1	9.3	5.7	3.5	8.0	7.9	9.9	8.5	10.3	3.6
Isocaproic (µmol/g)	1.7	1.7	3.2	3.4	2.4	2.4	5.2	9.4	0.1	0.2
Total SCFA (µmol/g)	300.2	95.6	322.6	31.9	320.2	55.8	362.9	133.3	313.6	152.8
Stool sample										
Acetate (µmol/g)	42.0	18.4	41.3	14.7	39.6	13.9	46.0	6.4	55.5	19.8
Propionate (µmol/g)	8.2	7.6	4.2	2.2	5.9	3.2	7.5	1.1	9.4	4.7
Butyrate (µmol/g)	8.2	8.5	4.4	3.8	5.7	6.8	6.8	2.0	13.5	11.2
Valeric (µmol/g)	1.2	2.5	0.6	0.7	1.1	1.6	1.4	1.0	0.7	1.0
Caproic (µmol/g)	0.5	0.7	0.2	0.3	0.2	0.1	0.3	0.1	0.2	0.2
Heptanoic (µmol/g)	0.1	0.1	0.1	0.2	0.0	0.1	0.0	0.0	0.0	0.1
Octanoic (µmol/g)	0.2	0.3	0.1	0.2	0.2	0.5	0.2	0.3	0.4	0.5
Isobutyrate (µmol/g)	2.1	2.3	1.0	0.6	1.4	1.5	2.0	1.9	1.8	0.7
Isovaleric (µmol/g)	2.6	2.7	1.0	0.6	2.0	2.6	2.1	1.5	3.0	1.5
Isocaproic (µmol/g)	0.3	0.4	0.5	0.5	0.4	0.3	0.9	1.4	0.0	0.1
Total SCFA (µmol/g)	65.4	38.5	53.4	19.6	56.5	28.2	67.2	8.4	84.6	31.6
Proportional ratio										
% Acetate	73.0	19.5	78.3	11.8	73.6	11.6	68.8	9.3	66.1	7.8
% Propionate	10.3	6.1	7.9	3.6	10.4	3.9	11.3	2.5	12.0	6.4
% Butyrate	8.9	8.1	7.3	6.0	8.5	5.4	9.9	2.1	13.9	7.0
% Valeric	1.1	2.1	1.2	1.5	1.3	1.8	2.0	1.5	1.0	1.4
% Caproic	0.6	0.6	0.3	0.4	0.3	0.2	0.5	0.2	0.2	0.2
% Heptanoic	0.1	0.1	0.2	0.3	0.1	0.2	0.0	0.0	0.0	0.1
% Octanoic	0.3	0.3	0.2	0.3	0.2	0.5	0.3	0.4	0.6	0.6
% Isobutyrate	2.3	2.0	1.9	1.4	2.1	1.7	2.9	2.6	2.4	1.0
% Isovaleric	2.9	2.4	1.8	1.1	2.6	2.5	3.0	2.1	3.8	1.7
% Isocaproic	0.5	0.4	1.0	1.1	0.8	0.7	1.2	1.9	0.0	0.1

Table 7.13: Changes in faecal lactate concentration in paediatric CD patients whose calprotectin concentration decreased at the end of EEN

N=11	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Dry faecal material										
D-lactate (mg.100g)	39.9	40.4	40.5	66.9	43.5	68.3	39.5	44.5	74.1	114.9
L-lactate (mg/100g)	117.1	212.3	73.9	129.5	70.8	119.8	91.5	168.4	95.7	154.1
Total lactate (mg/100g)	157.3	240.4	115.0	196.2	114.6	187.8	131.6	211.7	170.5	268.5
Stool sample										
D-lactate (mg/100g)	8.3	5.7	8.0	7.2	8.1	7.0	7.9	4.9	13.8	13.4
L-lactate (mg/100g)	21.7	33.4	14.3	14.1	12.9	12.3	15.7	19.9	17.5	18.1
Total lactate (mg/100g)	30.0	37.0	22.3	21.3	21.1	19.1	23.6	24.6	31.2	31.1
D/L-lactate	0.6	0.3	0.6	0.1	0.7	0.2	0.7	0.3	0.9*	0.5

* p<0.001 from “Before EEN "for ANOVA of repeated measures and Bonferroni post-hoc test

Table 7.14: Changes in faecal lactate concentration in paediatric CD patients whose calprotectin concentration increased at the end of EEN

N=5	Before EEN		15 d		30 d		60 d		Normal diet	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry faecal material										
D-lactate (mg/100g)	55.9	42.1	79.4	84.3	71.3	51.9	66.8	65.0	31.9	9.5
L-lactate (mg/100g)	104.2	98.7	225.0	241.0	198.5	166.8	93.3	78.4	49.5	10.4
Total lactate (mg/100g)	160.1	138.1	305.0	296.0	269.7	198.4	160.2	143.1	81.4	19.6
Stool sample										
D-lactate (mg/100g)	8.4	2.9	11.0	8.3	9.7	4.6	11.3	6.8	8.6	2.2
L-lactate (mg/100g)	15.3	10.4	33.7	36.2	26.3	18.5	16.0	7.1	13.6	2.5
Total lactate (mg/100g)	23.7	13.1	44.6	40.9	36.0	20.8	27.3	13.8	22.2	4.6
D/L-lactate	0.6	0.2	0.5	0.2	0.5	0.2	0.7	0.2	0.6	0.1

7.3.5. Changes in bacterial metabolic activity of healthy children without family history of IBD

The stability of the gut bacterial metabolic activity of healthy children was evaluated by comparing the concentration of faecal biomarkers between two serial stool samples of healthy children with no family history of IBD.

7.3.5.1. Recruitment

20 healthy children controls (12 boys; 9.6 y [4.5-16.9]) without known family history of IBD were recruited via advertisement and word of mouth. Eighteen children gave two serial samples at least one month apart. For two children follow up samples were not obtained (both withdrew) and these were excluded from this analysis.

7.3.5.2. Changes in faecal water content, pH and ammonia

Faecal pH did not differ significantly between the two faecal samples. The average water content was higher in the second sample but the difference did not reach statistical significance (p=0.061). Faecal ammonia concentration was not different between the two samples when this was calculated per volume of stool sample but it was marginally significant when it was expressed per kg of dry faecal material (Table 7.15).

Table 7.15: Comparison of water content, faecal pH and ammonia concentration in two serial stool samples of healthy children (n=20)

	First sample			Second sample			p-value*
	Median	Range		Median	Range		
Water content (%)	66.3	59.6	- 84.8	69.8	65.2	- 76.4	0.061
Faecal pH	6.8	6.0	- 8.0	6.8	5.7	- 7.4	0.538
Ammonia (mg/lit stool)	1151	641	-5060	1073	447	-1806	0.050
Ammonia (mg/kg dry stool)	3580	2526	-13130	3755	1797	-5569	0.276

* One sample Wilcoxon signed rank

7.3.5.3. Changes in faecal SFCA and BCFA concentration

There were no major differences in the concentration of the individual or total SCFA, between the two serial stool samples. Mean propionate concentration was slightly (p=0.05) higher in the second sample. Similarly the molar proportion of isopentanoic and octanoic acids were significantly lower in the second sample (Table 7.16).

Table 7.16: Comparison of faecal SCFA concentration and proportional ratio in two serial stool samples of healthy children

	Dry faecal material (µmol/g)			Relative ratio (%)			"Wet" stool sample (µmol/g)		
Subjects (n=18)	First sample	Second sample	p	First sample	Second sample	p	First sample	Second sample	p
	Median range	Median range		Median range	Median range		Median range	Median range	
Acetate	2312 68.4-464.3	248.8 90.3-451.7	0.163	65.2 54.2 -73.0	63.5 52.6 -76.9	0.459	73.1 26.9-103.2	73.3 28.8-110.4	0.542
Propionate	41 11.8- 86.4	58.4 8.7-116.5	0.050	11.6 9.3 -18.7	12.7 7.4 -23.0	0.055	14.8 4.6- 24.3	16.7 2.8- 36.4	0.055
Isobutyrate	5.6 0.0- 13.3	5.2 1.9- 15.4	0.695	1.9 0.0 - 2.7	1.6 0.4 - 2.6	0.089	1.9 0.0- 4.7	1.6 0.6- 5.0	0.258
Butyrate	51.3 10.9-135.5	59.6 8.6-130.8	0.223	15.0 7.4 -20.3	14.3 7.3 -27.6	0.862	16.6 3.8- 30.6	16.6 2.7- 37.6	0.360
Isopentanoic	9.2 0.0- 23.3	8.3 2.7- 26.7	0.486	3.1 0.0 - 4.8	2.6 0.4 - 4.6	0.033	3.2 0.0- 8.2	2.6 0.6- 8.6	0.139
Pentanoic	7.6 0.0- 17.0	7.7 0.0- 19.2	0.636	2.4 0.0 - 3.8	2.1 0.0 - 3.3	0.130	2.5 0.0- 5.9	2.3 0.0- 6.2	1.000
Isocaproic	0.0 0.0- 0.56	0.0 0.0- 3.4	1.000	0.0 0.0 - 0.2	0.0 0.0 - 0.7	1.000	0.0 0.0- 0.2	0.0 0.0- 0.9	1.000
Caproic	1.6 0.0- 12.9	1.5 0.5- 14.8	0.760	0.6 0.0 - 2.4	0.44 0.1 - 2.5	0.794	0.5 0.0- 3.8	0.5 0.1- 4.1	0.931
Eptanoic	0.1 0.0- 1.6	0.0 0.0- 2.4	0.563	0.03 0.0 - 0.7	0.0 0.0 - 7.0	0.351	0.04 0.0- 0.58	0.0 0.0- 0.8	0.398
Octanoic	1.8 0.0- 7.4	1.4 0.0- 3.6	0.098	0.6 0.0 - 2.0	0.39 0.0 - 1.14	0.045	0.6 0.0- 2.1	0.4 0.0- 1.2	0.050
Total SCFA	353.5 104.0-691.5	407.5 117.4-651.3	0.177	-	-		114.9 40.8-171.0	116.3 37.5-189.1	0.384

* One sample Wilcoxon signed rank

7.3.5.4. Changes in faecal sulphide and lactate concentration

Free sulphide and the dextrorotatory isomer of lactate differed significantly between the two serial samples (Table 7.17). Free sulphide was significantly higher and D-Lactate significant lower in the second sample.

Table 7.17: Comparison of total and free faecal sulphide and lactate concentration in two serial stool samples of healthy children

Subjects (*n*=18)	First sample		Second sample		p-value*
	Median	Range	Median	Range	
Dry faecal material					
Free sulphide (µmol/g)	0.38	0.11 - 1.91	0.62	0.19 - 2.3	0.005
Total sulphide (µmol/g)	1.28	0.0 - 2.91	1.13	0.34 - 2.9	0.794
D-lactate (mg/100g)	25.1	15.6 -393.6	26.2	14.9 - 54.3	0.965
L-lactate (mg/100g)	25.2	17.6 -423.8	26.9	11.7 - 44.4	0.695
Total lactate (mg/100g)	52.7	33.2 -817.4	56.0	32.5 - 80.2	0.794
D/L-lactate	0.88	[0.8 - 2.6	0.84	0.7 - 2.1	0.055
Stool sample					
D-lactate (mg/100g)	7.8	5.5 - 59.7	7.7	4.8 - 17.3	0.033
L-lactate (mg/100g)	8.3	6.2 - 64.2	8.6	3.5 - 12.6	0.794
Total lactate (mg/100g)	16.8	11.7 -123.9	16.7	10.5 - 25.5	0.207
Free sulphide (µmol/g)	0.11	0.04 - 0.62	0.17	0.06 - 0.54	0.029
Total sulphide (µmol/g)	0.46	0.0 - 0.98	0.36	0.11 - 0.92	0.408

* One sample Wilcoxon signed rank

7.3.6. Comparison of metabolic activity between healthy children and CD children on normal diet

Faecal metabolites of gut microbiota activity were compared between CD children and healthy children with no family history of IBD. Due to the substantial effect of EEN on the gut microbiota which was observed in the previous section of this thesis (Section 7.3.3), the following study chose to compare only the gut microbiota of CD children whilst on normal diet to avoid any confounding effect from the concomitant use of EEN.

7.3.6.1. Recruitment

For this arm of the study 19 children with CD (13 boys/ 6 girls) and 20 healthy controls (12 boys / 8 girls) were recruited. The healthy children (9.6; range [4.5-16.9]) were significantly younger than the patients with CD (13; range [7.6-15]). The majority of the CD children were also participated in the study of the effect of EEN on gut microbiota. Only those children who provided a stool sample whilst on normal diet were included. For healthy children the values of the faecal bacterial metabolites of two serial samples were averaged. All, apart from three

children, were on concomitant treatment (Table 7.18). No patient was on antibiotics or EEN. The majority of the children had elevated systemic and gut inflammatory markers of inflammation (Table 7.18). Nevertheless six out of 19 were in clinical remission (PCDAI ≤10).

Table 7.18: Disease activity markers and medical treatment in CD children on normal diet

Disease markers	Median	Range	Medical treatment	N (%)
PCDAI	15.0	0.0 - 55.0	Steroids	4 (21%)
ESR (mm/h)	20.5	3.0 - 55.0	Azathioprine	11 (58%)
CRP (mg/l)	7.0	7.0 - 69.0	5-ASAs	7 (37%)
Albumin (g/l)	36.0	20.0 - 41.0	Infiximab	1 (5%)
Calprotectin (mg/l)	2070	146 -2495	Nutrition supplements	2 (11%)
			Omeprazole	3 (16%)
			Methotrexate	2 (11%)
			Vitamins/Minerals	4 (21%)
			No Treatment	3 (16%)

7.3.6.2. Differences in water content, faecal pH and ammonia

No difference in faecal pH was found between CD children and their healthy peers. Faecal water and ammonia concentration were significantly lower in CD children (Table 7.19).

Table 7.19: Comparison of water content, faecal pH and ammonia concentration between CD and healthy children

	CD (n=19) Median Range	Healthy (n=20) Median; Range	p-value*
Water content (%)	73.0 64.2 - 87.2	68.2 58.1 - 77.8	0.041
Faecal pH	6.8 5.5 - 7.9	6.9 5.9 - 7.6	0.683
Ammonia (mg/lit stool)	844.9 427.0 -1882.0]	1073.0 544.0 -3200	0.044
Ammonia (mg/kg dry stool)	3169 1907 -14703	3336 2388 -8509	0.098

* Mann-Whitney test

7.3.6.3. Differences in faecal SFCA and BCFA concentration

Compared to the healthy group, total SCFA concentration was significantly lower in the stool samples of CD patients. At the individual level butyrate, valeric, caproic and octanoic acid were at significantly higher concentrations in the stool samples of healthy children (Table 7.20). Valeric and octanoic were proportionally higher in the faeces of healthy children but this difference did not receive statistical significance. In particular valeric and octanoic acid were detected in the stool samples of more healthy children than CD. Thirty seven and 47% of the CD children had undetectable amounts of octanoic and pentanoic respectively as opposed to 5% and 0% of the healthy children (p=0.003).

Table 7.20: Comparison of faecal SCFA concentration and proportional ratio between CD children and healthy children

	Dry faecal material (µmol/g)					Relative ratio (%)					"Wet" stool sample (µmol/g)				
Subjects	**CD (n=20)**		**Healthy (n=20)**		**p**	**CD (n=20)**		**Healthy (n=20)**		**p**	**CD (n=20)**		**Healthy (n=20)**		**p**
	Median	Range	Median	Range		Median	Range	Median	Range		Median	Range	Median	range	
Acetate	210.7	106.9 -602.9	227.4	99.4 -458.0	0.789	62.6	48.8 -97.1	64.3	53.4 - 74.1	0.944	56	30.2-132.5	70.4	32.6-101.6	0.106
Propionate	45.0	9.3 -103.3	49.2	11.5 - 90.8	0.423	13.4	1.9 -23.8	12.0	8.4 - 20.9	0.565	12.9	1.2 - 22.7	15.0	3.8 - 30.4	0.267
Isobutyrate	5.9	1.6 - 8.6	4.7	2.9 - 14.4	0.148	1.93	0.0 - 3.3	1.75	0.43- 2.69	0.232	1.7	0.0 - 2.95	1.62	0.6 - 4.8	0.944
Butyrate	29.8	0.0 -138.4	54.2	9.9 -125.3	0.182	11.7	0.0 -24	14.3	7.3 - 21.1	0.222	10.6	0.0 - 30.8	16.4	3.2 - 32.6	0.054
Isopentanoic	9.1	1.6 - 15.9	8.4	3.4 - 25.0	0.292	2.9	0.0 - 5.9	2.9	0.5 - 4.7	0.279	2.7	0.2 - 5.0	2.7	0.74 - 8.41	0.855
Pentanoic	0.3	0.6 - 13.9	7.7	0.0 - 18.1	0.023	0.1	0.0 - 4.8	2.41	0.0 - 3.39	0.068	0.16	0.0 - 4.0	2.5	0.0 - 6.1	0.009
Isocaproic	0.0	0.0 - 0.35	0.0	0.0 - 1.7	0.811	0.0	0.0 - 0.19	0.0	0.0 - 0.38	0.811	0.0	0.0 - 0.1	0.0	0.0 - 0.4	0.789
Caproic	1.2	0.0 - 2.6	1.6	0.36- 10.3	0.211	0.32	0.0 - 0.78	0.58	0.05- 2.18	0.089	0.32	0.0 - 0.76	0.47	0.09-3.02	0.062
Eptanoic	0.09	0.0 - 3.56	0.3	0.0 - 1.9	0.684	0.04	0.0 - 0.99	0.06	0.0 - 0.61	0.877	0.02	0.0 - 1.27	0.09	0.0 - 0.67	0.684
Octanoic	0.7	0.0 - 3.9	1.6	0.4 - 3.7	0.024	0.13	0.0 - 1.53	0.51	0.15-1.07	0.058	0.16	0.0 - 1.27	0.46	0.14 - 1.06	0.015
Total SCFA	307.0	184.5. -749.8	365.0	134.8 -671.4	0.527		-		-		83.1	50.4-164.8	109.0	44.2 - 180.0	0.054

7.3.6.4. Difference in sulphide and lactate concentration

Both D and particularly L-lactate in stool samples or dry faecal material of CD children were significantly higher than healthy children. Significant differences were found for the fractional production of the lactate isomers (Table 7.21). The D to L lactate ratio was significantly lower in stool samples of CD children denoting differences in the pattern of isomer production between the two groups. No difference was found for free or total faecal sulphide between healthy and CD children (Table 7.21).

Table 7.21: Comparison of total and free faecal sulphide and lactate concentration between CD children and healthy children

	CD (n=20) Median	Range	Healthy (n=20) Median	Range	p-value*
Stool sample					
Free sulphide (µmol/g)	0.08	0.02 - 0.64	0.14	0.03 - 0.58	0.099
Total sulphide (µmol/g)	0.46	0.0 - 1.82	0.43	0.11 - 0.95	0.922
D-lactate (mg/100g)	9.1	5.1 - 50.7	7.6	5.2 - 33.8	0.090
L-lactate (mg/100g)	12.5	6.2 - 67.6	8.4	5.5 - 37.2	0.001
Total lactate (mg/100g)	21.4	11.7 -118.3	16.2	11.1 - 98.0	0.003
Dry faecal material					
Free sulphide (µmol/g)	0.33	0.0 - 1.78	0.46	0.08 - 2.11	0.182
Total sulphide (µmol/g)	1.45	0.0 - 5.6	1.22	0.4 - 2.9	0.684
D-lactate (mg/100g)	35.1	16.5 -395.9	24.6	15.2 -210.2	0.044
L-lactate (mg/100g)	44.8	17.4 -528.3	27.5	17.6 -229.0	0.001
Total lactate (mg/100g)	77.8	46.1 -924.2	51.9	32.9 -439.3	0.001
D/L lactate	0.72	0.4 - 2.4	0.86	0.6 - 2.3	0.002

* Mann and Whitney test

7.3.6.5. Correlation of faecal bacterial metabolites with disease activity markers

Significant correlations were found between systemic markers of disease activity and the faecal metabolites checked (Table 7.22). The BCFA, isobutyrate and isovaleric acid were positively associated with CRP and ESR but no association was found with faecal calprotectin (Table 7.22). A strong negative association was found for ESR with faecal acetate and a positive with propionate (Table 7.22). D and L lactate were negatively associated with CRP levels and ESR positively with free and total sulphide.

Faecal calprotectin expressed per weight of dry faecal material was associated with acetate concentration as well as with D but not L Lactate (Table 7.22). A strong positive association was found between calprotectin in dry faecal material and faecal water content (Table 7.22).

Only the percentage butyrate and heptanoic differed significantly between the patients with active disease (PCDAI>10) and those in clinical remission (PCDAI≤10). Median butyrate in dry faecal material was significantly higher (63 vs 28.3 µmol/g; p=0.048) and

heptanoic significantly lower (0 vs 0.473 µmol/g; p=0.032) in the group of patients with quiescent disease compared with the children with clinically active disease.

Table 7.22: Spearman rank correlation between gut and systemic inflammatory markers and faecal metabolites in dry faecal material in CD children

Faecal metabolites	CRP		ESR		Calprotectin*	
	R	P value	R	P value	R	P value
Free sulphide (dry faecal material)	0.45	0.081	0.57	0.027		
Total sulphide (dry faecal material)			0.56	0.029		
Acetate (dry faecal material)					0.56	0.020
% Acetate			-0.71	0.006		
Propionate (dry faecal material)			0.63	0.014		
% Propionate			0.60	0.020		
Isobutyrate (dry faecal material)	0.52	0.042	0.54	0.037		
% Isobutyrate			0.50	0.055		
Isovaleric (dry faecal material)	0.56	0.031	0.52	0.046		
% Isovaleric			0.49	0.058		
D-Lactate (dry faecal material)	-0.49	0.059			0.51	0.037
L-Lactate (dry faecal material)	-0.57	0.028				
Faecal water					0.62	0.010

* Expressed per kg of dried faecal material

7.3.7. Comparison of faecal metabolic activity between CD children and their healthy first degree relatives

Stool samples were collected from the first-degree relatives of children with CD and were compared with samples of CD children on normal diet.

7.3.7.1. Recruitment

For 14 CD children stool samples were collected from their respective first-degree relatives. For the remaining CD children no samples were provided by their relatives and for two children their parents were also sufferers of IBD. One child with CD was excluded due to parallel use of antibiotics.

7.3.7.2. Comparison in faecal water content, Faecal pH and ammonia

Similar to the results observed in healthy control children, the water content in the relatives of CD children was significantly lower. Faecal ammonia was significantly higher in the relatives of CD children. Faecal pH was significantly higher in the relatives of CD children (Table 7.23).

Table 7.23: Comparison of water content, faecal pH and ammonia concentration between CD children and their first degree relatives

Variable	CD (n=14)			Relatives (n=14)			p-value*
	Median	range		Median	range		
Water content (%)	72.0	64.2	- 87.2	63.6	51.0	- 74.1	0.001
Faecal pH	7.0	6.0	- 7.9	7.3	6.6	- 7.7	0.046
Ammonia (mg/lit stool)	864	427	-1882	1607	1063	-3807	0.001
Ammonia (mg/kg dry stool)	2989	2179	-14703	4636	2181	-10111	0.016

* Mann and Whitney test

7.3.7.3. Difference in faecal SFCA and BCFA concentration

No substantial difference was found between CD children and their healthy relatives for butyrate concentration (Table 7.24). In contrast acetate, propionate and isobutyrate and the total amount of SCFA, were significantly higher in the dry faecal material of CD children compared to their healthy relatives (Table 7.24). Although valeric and caproic acid in “fresh” stool samples or dry faecal material did not differ significantly between the groups (CD children & healthy relatives), these were significantly higher in the healthy group when the results were expressed as the relative ratio to total quantity of SCFA (Table 7.24). Similar to the patterns observed in healthy children, valeric and octanoic acid were detected more frequently in the stool samples of the healthy relatives than CD children. Forty-three and 36% of the CD children had undetectable pentanoic and octanoic acid respectively as opposed to 7% of their healthy relatives (relatives p=0.08 for octanoic and p=0.03 for pentanoic).

Table 7.24: Comparison of faecal SCFA concentration and proportional ratio between CD children and their healthy first degree relatives

	Dry faecal material (µmol/g)							Relative ratio (%)							"Wet" stool sample (µmol/g)						
Subjects	**CD (n=14)**			**Relatives (n=14)**			*p*	**CD (n=14)**			**Relatives (n=14)**			*p*	**CD (n=14)**			**Relatives (n=14)**			*p*
	Median		Range	Median		Range		Median		Range	Median		Range		Median		Range	Median		Range	
Acetate	212.6	110.6	-491.6	124	74.4	-352.2	0.006	61.2	57.7	-97.1	66.4	53.7	-79.3	0.800	58.5	30.2	- 82.3	49.3	27	- 91.4	0.175
Propionate	43.4	9.3	- 94.8	27.7	6.3	-52.3	0.02	13.9	1.9	-23.8	12.1	6.0	-17.1	0.260	13.6	1.2	- 22.5	10.4	2.38	- 18.76	0.113
Isobutyrate	6.0	1.6	- 8.3	4.6	2.0	- 8.1	0.051	2.13	0	- 3.34	2.42	0.8	- 3.38	0.395	1.74	0.0	- 2.95	1.72	0.72	- 2.9	0.836
Butyrate	38.7	0.0	-138.4	31.1	2.6	-92.3	0.206	12.7	0.0	-24.0]	11.6	2.8	-20.2	0.629	11.6	0.0	- 30.8	10.3	0.95	- 23.96	0.323
Isopentanoic	9.7	1.6	- 14.6	8.0	4.2	-13.1	0.135	3.4	0.3	- 5.7	4.41	1.11	- 6.0	0.280	2.7	0.2	- 5.0	2.9	1.45	- 4.9	0.765
Pentanoic	0.64	0.0	- 13.9	6.3	0.0	-10.2	0.260	0.2	0.0	- 4.13	2.77	0.0	- 3.72	0.026	0.16	0.0	- 4.0	2.0	0.0	- 3.7	0.093
Isocaproic	0.0	0.0	- 0.35	0.0	0.0	-0.0	1.000	0.0	0.0	- 0.19	0.0	0.0	- 0.0	1.000	0.0	0.0	- 0.1	0.0	0.0	- 0.0	1.000
Caproic	1.18	0.0	- 2.65	1.64	0.0	- 5.2	0.206	0.31	0.0	- 0.76	1.1	0.0	- 2.77	0.032	0.30	0.0	- 0.76	0.69	0.0	- 1.98	0.103
Eptanoic	0.0	0.0	- 3.6	0.0	0.0	- 0.68	0.765	0.0	0.0	- 0.99	0.0	0.0	- 0.53	0.800	0.0	0.0	- 1.27	0.0	0.0	- 0.19	0.765
Octanoic	0.92	0.0	- 3.9	1.52	0.0	- 3.72	0.251	0.3	0.0	- 1.53	0.9	0.0	- 1.53	0.066	0.25	0.0	- 1.28	0.51	0.0	- 1.23	0.141
Total SCFA	332.6	184.5	-728.3	208.1	94.7	- 503.6	0.007		-			-		0.800	91.7	50.4	-128.3	79.4	34.0	-130.7	0.301

7.3.7.4. Difference in sulphide and lactate concentration

No difference between groups was found for free sulphide concentration expressed either per g of "fresh" stool sample or "dry" faecal material. Total sulphide in the stool samples of the relatives of CD patients was significantly higher than their offsprings. However the difference disappeared when results were expressed per g of dry faecal material (Table 7.25).

Total and L- lactate but not D-lactate was statistically higher in dry faecal material CD children compared with their healthy relatives. No difference was found when the same bacterial metabolites were expressed per g of "fresh" stool sample (Table 7.25).

Table 7.25: Comparison of total and free faecal sulphide and lactate concentration between CD children and their healthy first-degree relatives

Variable	CD (n=15)		Relatives (n=15)		p-value
	Median	Range	Median	Range	
Dry faecal material					
Free sulphide (µmol/g)	0.46	0.0 - 1.8	0.33	0.0 - 0.71	0.408
Total sulphide (µmol/g)	1.54	0.0 - 5.6	1.91	0.0 - 9.0	0.260
D-lactate (mg/100g)	34.1	16.5 -395.9	22.5	8.5 - 41.7	0.077
L-lactate (mg/100g)	45.1*	17.4 -528.3	32.8	18.0 - 61.7	0.051
Total lactate (mg/100g)	76.5*	46.1 -924.2	55.7	34.6 -103.4	0.020
D/L lactate	0.69	0.4 - 2.4	0.68	0.3 - 2.1	0.597
Stool sample					
Free sulphide (µmol/g)	0.16	0.02 - 0.64	0.11	0.02 - 0.32	0.918
Total sulphide (µmol/g)	0.49	0.0 - 1.8	0.85	0.19 - 3.35	0.049
D-lactate (mg/100g)	9.3	5.1 - 50.7	8.5	4.2 - 13.9	0.301
L-lactate (mg/100g)	13.1	6.2 - 67.6	12.1	6.5 - 20.1	0.535
Total lactate (mg/100g)	22.3	11.7 -118.3	19.7	14.3 - 33.7	0.241

*P<0.02 Mann-Whitney compared to relatives

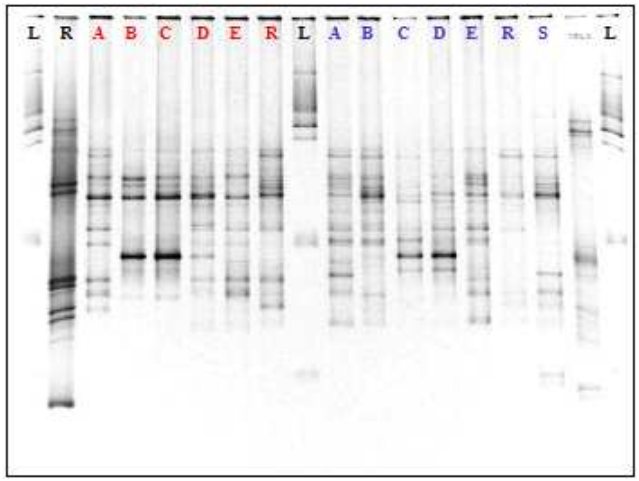
7.3.8. Bacterial diversity and composition in children with CD on EEN and healthy controls

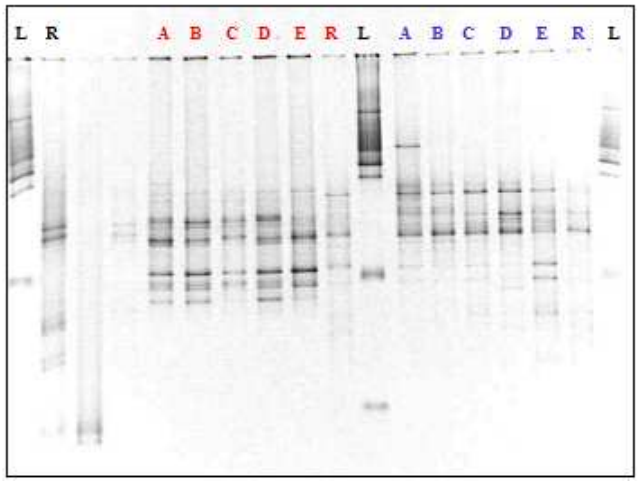
All stools collected from children with CD and control children and parents were subjected to DNA extraction and PCR-TGGE (Fig 53 & 54). Although a decreasing trend was observed there was no statistical significant difference in the total number of bacterial bands between samples collected on treatment initiation and those at any time point of the follow up (Fig 55). No different patterns were observed between those patients who achieved clinical remission and those who did not, nor when patients on antibiotic treatment were excluded. Similar to

the CD children, no statistical significant difference was found in the faecal bacterial diversity between two samples collected with at least one-month gap in healthy children. However the total number of bacterial bands in the samples of CD children was significantly lower than healthy children at all times of follow up (Fig 55). Likewise stool samples from healthy relatives of CD children had significantly less bacterial bands compared with healthy children. No such difference was found with their CD offsprings at any time point of the follow up.

A higher degree of microbiota pattern similarity was observed between the two serial stool samples in the healthy children compared with the samples of CD children on EEN. Indeed compared with any time point of the observational period, the similarity index of the bacterial pattern of the faecal microbiota in CD children was significantly lower than the healthy controls (Fig 56). The similarity index dropped by more than 40% within 15 days of start of treatment and remained low during follow up. In contrast, the similarity remained high and more than 70% in the healthy children (Fig 56).

Figure 53:TTGE bacterial profiles of serial stool samples of CD patients during EEN, on normal diet, and their healthy first degree relatives





A-D: samples during EEN at 0, 15, 30, 60 d; E: sample on normal diet; R: relative's sample; S: sibling's sample; Black R: reference bacterial scale; L: DNA ladder; Same color samples from

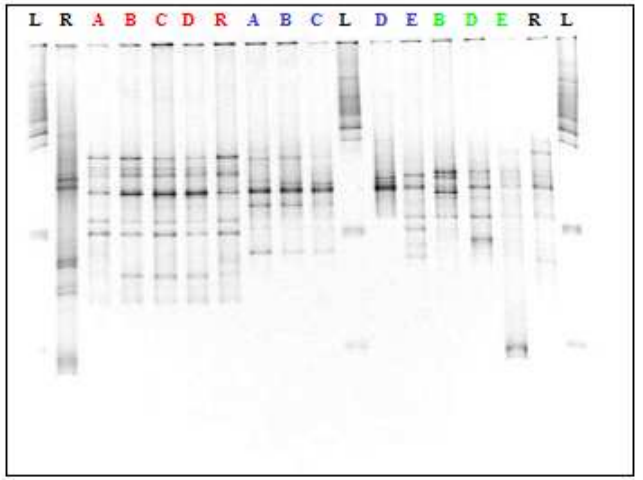
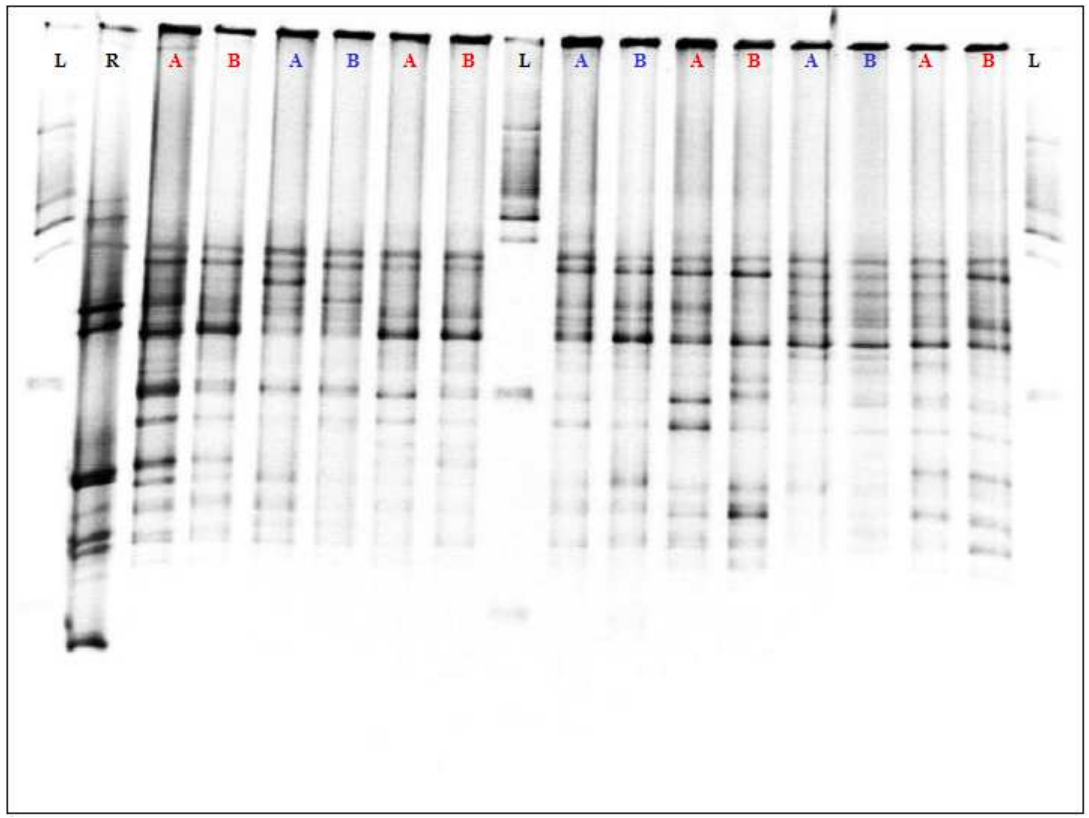


Figure 54: TTGE bacterial profiles of two serial stool samples of healthy children



A & B: serial samples from the same subject; R: reference bacterial scale; L: DNA ladder; Neighbouring samples with same color: samples from same patient

Figure 55: Number of gut bacterial bands of healthy children, healthy relatives of CD children. and CD children on EEN and normal diet (mean with 95% CI)

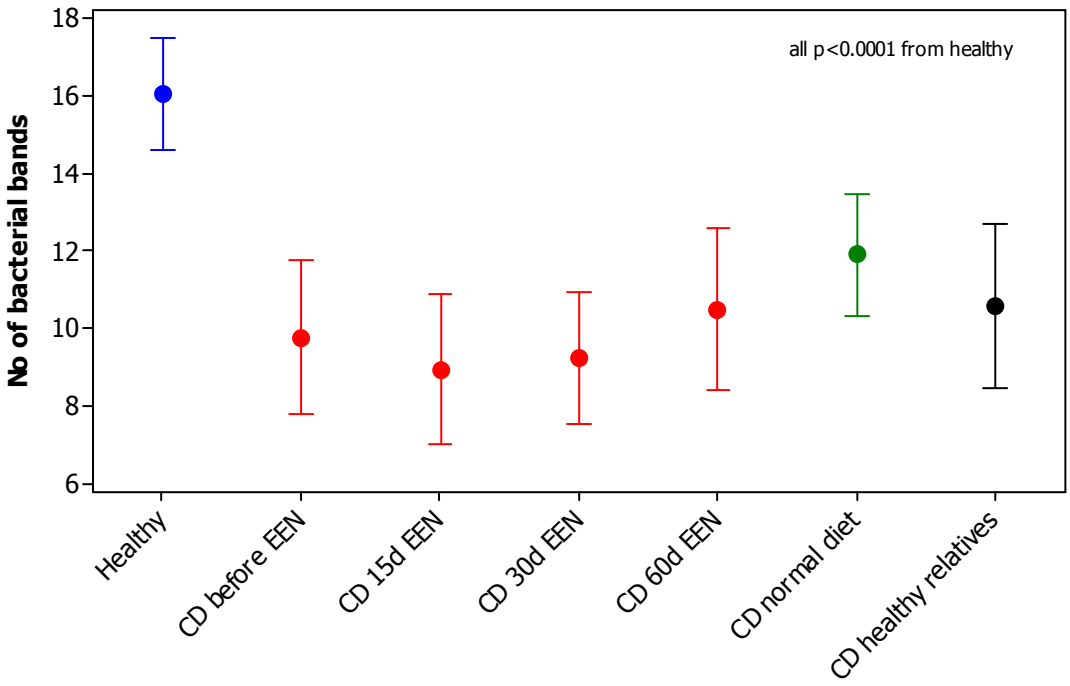
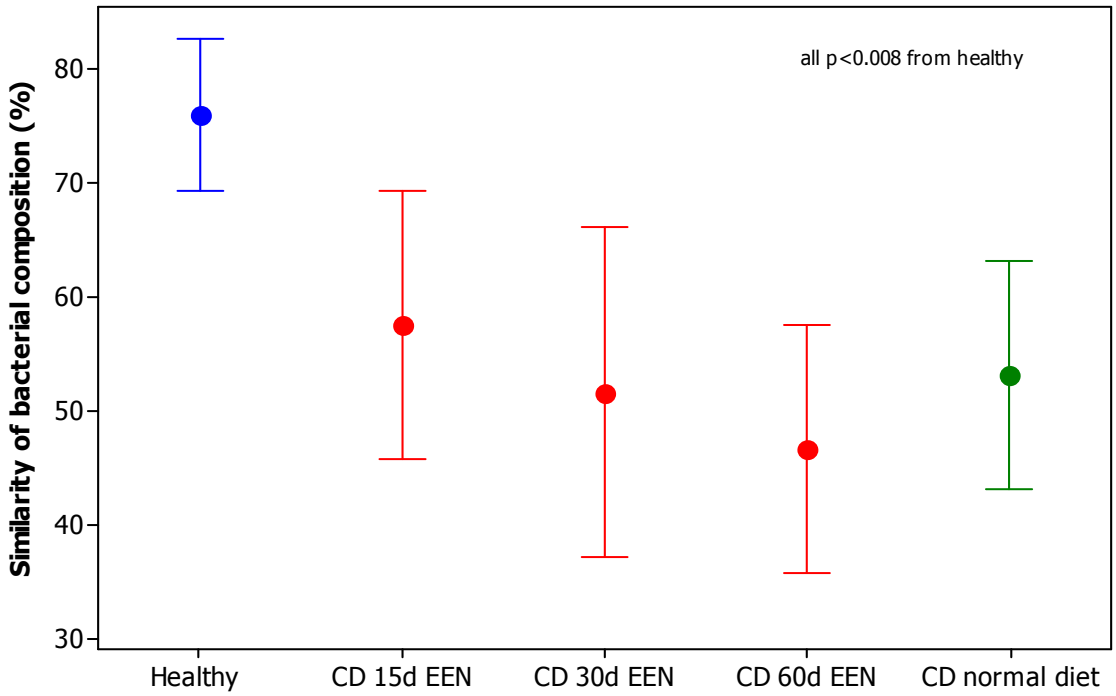


Figure 56: Percent similarity of gut bacterial composition of healthy children and CD children on EEN and on normal diet (mean with 95% CI)



7.4. Discussion

The exact mechanism of the therapeutic action of EEN in paediatric CD remains unknown. This in conjunction with robust evidence suggesting an important role of the commensal gut microbiota in IBD pathogenesis prompted the researcher to undertake this study.

Since the initiation of this work in 2004 there have been two studies which looked at changes in the bacterial microbiota diversity (531;532). The study presented here in addition to studying micorbiota diversity explored changes in the bacterial metabolic activity of CD children during treatment with EEN.

Taken as a whole the findings of this study are in contrast to the statement by Lionnetti and his colleagues (531) for a therapeutic mechanism of EEN action mediated by a "prebiotic" effect of the nutritional supplement on the gut microbiota of children with active CD. On the contrary, this study reported significant deterioration of the colonic micro-environment and a substantial increase in the faecal concentration of potentially "harmful", for the colonic health, bacterial metabolites. Indeed these effects were seen solely in those patients whose intestinal markers of gut inflammation decreased and achieved complete clinical remission at the end of the treatment course.

Changes in the metabolic dynamics of the gut microbiota, and mainly fermentation, are grossly reflected and assessed by changes in the colonic pH. In the current study faecal pH was significantly increased to the alkaline range within 15 days of EEN initiation and remained elevated during the entire course of treatment. This first observation pre-predicted the significant changes observed in the concentration of acidic, like SCFA, and alkaline, like ammonia and sulphide, bacterial metabolites.

Within 15 days of treatment initiation, butyrate, the colonocytes' preferred metabolic fuel decreased dramatically by 100% and further continued to decrease until the end of the treatment course. This was not unexpected, as the nutritional supplement that all participants received, Modulen IBD, is of low residue content and hence lacks fermentable fibre or prebiotic substrates that colonic bacteria would ferment to SCFA. This lack of fermentable substrate in the feeds may have reduced the metabolic activity of the butyrate producing bacteria and consequent production of butyric acid. This was also represented by the significant reduction in the relative molar concentration of butyrate to the total SCFA pool. On the other hand, reduced faecal concentration of butyrate may be an indication of a more efficient absorption and utilization of butyrate by the colonic epithelium following disease improvement. Although the evidence is controversial (622) some studies have proposed that butyrate metabolism (426), oxidation (422), and uptake (427) is impaired in the colonocytes of IBD patients particularly during the active phase of the disease (425). Thus a better absorption and utilization of butyrate by the colonocytes may explain the reduction in

butyrate concentration during treatment only in those patients who achieved clinical remission.

Sulphide in its free form as hydrogen sulphide has been implicated in the pathogenesis of IBD primarily due to its toxic effect to the colonocytes and inhibition of butyrate oxidation (452). Previous research in this area reported a high number of sulphate reducing bacteria, increased faecal concentration and rate of hydrogen sulphide production in UC patients (458) but overall the results are contradictory (406;457). In contrast to that and expectations for a reduction in the faecal sulphide levels during treatment with EEN, this current study reports a remarkable increase in the faecal concentration of total sulphide. Although the free fraction of faecal sulphide, hydrogen sulphide, did not change during the course of treatment, bound sulphide in the form of salts with divalent metals significantly increased within two weeks of treatment. A seven-fold increase was observed despite improvement in the disease activity and colonic inflammation. This could be attributed to the increased metabolic activity of sulphate reducing bacteria in the presence of abundant sulphur containing substrate (623) and an increase in the gut transit time (468). Modulen IBD is a nutritional supplement rich in protein and fortified with divalent metals in the form of sulphate salts (Appendix). Ferrous sulphate, manganese sulphate, zinc sulphate, copper sulphate, are added in order to increase the nutritional value of the feeds and may actually offer abundant fermentative substrate for the sulphide reducing bacteria (468). Sulphate reducing bacteria may utilize sulphate anions and subsequently increase faecal sulphide concentration.

It was a paradoxical finding that disease improvement coincided with the increase of total sulphide. This may imply that either sulphide is not implicated in the tissue injury in CD, as proposed by others (406;457) or that the biological importance of the bound compared to free sulphide is different. Moreover recent studies have now revised the role of sulphide in gut physiology (408) and current evidence suggests that it can be a potent, endogenous anti-inflammatory substance, modulating leukocyte-endothelial adhesion and leukocyte migration in the gut (466). Fiorucci et al (466) recently found that a sulphide reducing derivative of mesalamine was more effective than standard mesalamine in reducing the severity of colitis in an animal model by reducing granulocyte infiltration into the colonic tissue as well as reducing the expression of mRNA for several key proinflammatory cytokines. A new era for the role of sulphide in IBD may be emerging.

No significant changes were observed for the bacterial metabolites of protein metabolism like BCFA, ammonia and C5:C8 SCFA. We anticipated seeing an increased bacterial proteolytic activity in the absence of non-digestible carbohydrates in the feeds, and fermentation of amino acids that escaped absorption in the upper digestive tract. However no

such change was observed and the results may indicate that absorption of amino acids is efficient in the upper digestive tract and no significant residual protein reaches the colon.

Faecal lactate was relatively high on treatment initiation and significantly decreased during treatment in the group of patients who clinically improved. D and L lactate are metabolic products of bacterial fermentation that are further metabolized to butyrate and acetate (400) and they occur only in low concentration in the healthy colon. High concentration of lactate may denote malabsorption in the small intestine and increased availability of carbohydrates in the ileum, which are further fermented to lactate by the lactic producing bacteria. Increased L-lactate levels have been observed in inflamed colonic mucosal (421;434) and have been implicated in disease activity previously (421;434) but we suggest that this is not of causative association but an epiphenomenon of malabsorption, reduced gut transit time and diarrhoea (434;624). Indeed in the current study faecal water content was strongly associated with lactate concentration (data not shown) and was significantly higher in the patients with watery diarrhoea.

The use of molecular biology fingerprinting techniques in this study, allowed characterization of changes of the intestinal microbiota during treatment with EEN, which was independent of the drawbacks of the traditional microbiology culture media. No significant changes were observed in the diversity of the gut microbiota of CD children during treatment on EEN. An unstable faecal microbiota was found during treatment and the average similarity between any time points of the follow up was significantly lower than the similarity index in the healthy children. These findings are in accordance with the findings by Leach et al (532) and agree with adult IBD studies which found that commensal microbiota in IBD is unstable over time and is characterized by low bacterial diversity (504;523). Comparison of these findings with those by Leach et al (532) is difficult as the authors did not report pairwise changes in bacterial similarity and diversity changes during EEN treatment. This is surprising in a follow up study. On the other hand this study did not replicate the results by Leach et al (532) of a similar bacterial diversity with healthy controls at the beginning of treatment. In fact it was observed that bacterial diversity was consistently lower than healthy controls at all time points of the observational period. This different result may be due to differences in methodology (DGGE vs TTGE, extraction DNA with spin column vs beat beater) or the small sample size and lack of statistical power.

The molecular biology used to characterize the intestinal microbiota, although robust, can only assess changes in the composition and diversity of the intestinal microbiota without allowing for identification of changes in specific species. For this, other molecular techniques, such as cloning and sequencing, are required for group specific bacteria (532). This technique is laborious, expensive and no resources were available to conduct this during this PhD. Alternatively changes in selective bacteria can be assessed using other molecular

biology techniques, such as FISH which identify bacteria by using species specific fluorescence probes (340). Although it was among the aims of this study to assess changes using FISH analysis, and hence samples were prepared for such analysis, lack of resources, and shortage of time did not allow it.

On the other hand although significant changes in the diversity of microbiota were not observed during treatment with EEN, the method used in this study can characterize only the dominant microbiota of the digestive tract, which essentially occurs in more than 10^6-10^7 bacterial per g of faecal material. Therefore our knowledge for changes in the diversity of subdominant species remains unknown. Moreover faecal microbiota is at the best an abstract of the mucosal associated microbiota (346) and different changes may have occurred at the bacteria adherent to the gut lining. However a study with multiple serial mucosal biopsies within a short period of two months might be unfeasible and unethical.

Although it was not a primary outcome, part of this project studied differences in the gut microbiota diversity and metabolic activity between CD children, their healthy first-degree relatives, and healthy children with no family history of IBD. Prior to the current study the faecal concentration of SCFA in CD children had been reported in a single study 15 years ago (417). In contrast to this previous report, which reported increased levels of butyrate in children with CD, this current study found that total faecal SCFA and butyric acid were significantly lower in CD children compared to their healthy peers. Nevertheless this difference disappeared when results were expressed per mass of dry faecal material. This suggests that high faecal water content and diarrhoea in patients with CD may explain the different results with Treem et al (417). Alternatively, adherence to a diet low in fibre, which is commonly followed by IBD patients (545) and as in the IBD population this study recruited (625), in order to avoid symptom exacerbation, might explain the lower concentration of SCFA in CD patients.

CD children had significantly lower levels of faecal pentanoic and octanoic acids than healthy controls and in fact a substantial proportion of them had undetectable levels. Little is known about the biological significance of SCFA longer length than butyrate such as valerate (C5) and caprylic (C8) acid, although antimicrobial properties against enteropathogenic *E. coli* bacteria have been described for some of them (626). On the other hand, increased production of these metabolites may be a random finding or in conjunction with the increased levels of ammonia observed in healthy children may denote increased proteolytic fermentation due to excessive consumption of dietary protein in modern healthy children. This, in parallel with an inadequate dietary intake in children with IBD, may explain these findings. Moreover carbohydrate fermentation and SCFA production tend to occur at the ascending colon whereas protein fermentation and production of iso-carbonic acids and C5-C8 carbon SCFA occurs in the descending colon (367). Fast transit time and diarrhoea in

active CD may limit protein fermentation in the distal colon (367), and perhaps explain the significantly low levels of these bacterial metabolites compared with the healthy controls. Fast intestinal transit time and malabsorption may also explain the higher concentration of faecal lactate in CD children compared to the healthy cohort.

In accordance to the findings by Treem (417) this study did not observe any major differences in SCFA with disease activity. The higher concentration of butyrate and a lower concentration of heptanoic found in patients with quiescent, compared with those with clinically active disease, may be related to differences in water content and gut transit time between groups (627).

To the best of our knowledge this is the first study that compared the intestinal microbiota of CD patients with that of their healthy first-degree relatives. Environment, diet and genes are strong determinants of the bacterial composition and metabolic activity and this study enrolled healthy relatives of CD children to control for that. Total acetate, propionate, isobutyrate and hence total SCFA were significantly higher in the dry faecal matter of CD patients. Malabsorption of macronutrients in the small bowel may have increased the availability of fermentable substrate in the large bowel and consequently increased the production of SCFA. On the other hand, age dependent differences in the bacterial metabolic activity (628) may explain some of the differences observed between CD children and their healthy relatives although these have been reported for subjects older than 70 years old (628). To the best of our knowledge none of the healthy relatives were over 70 years of age. Similar to healthy children a higher proportion of CD children had undetectable levels of pentanoic and octanoic. These may indicate differences in the metabolic activity of the bacterial microbiota or rapid transit time in CD patients which limits the fermentation of proteins at the distal colon (367).

Another interesting finding of the current study was the low diversity of the faecal microbiota observed in the relatives of CD patients. Although no difference was found with the CD patients, the bacterial diversity was significantly lower than healthy children. Differences in age between groups may not allow a straightforward comparison with healthy children. However there is very limited data on the effect of age on bacterial metabolic activity and diversity, and mostly relates to very young infants or toddlers. A comparison with a cohort of healthy adults will show whether bacterial diversity is reduced in family members of patients with CD.

Although this study was initially powered to detect a 40% change in butyrate (417) a more than a 120% decrease was observed and therefore the study achieved significance even with a smaller number of patients. However it may lack power to detect significant differences in other parameters this study assessed, like changes in the diversity and composition of the dominant microbiota. Lack of power is common in this type of research

where long and large recruitment are required. With an intended recruitment sample size of 40 children within 1½ years 21 children on CD were enrolled within approximately two years. Although more than 60 children started on treatment in the same period only half of them were approached. Recruitment on the day of disease diagnosis, the restrictive type of treatment and in particular the type of collected sample significantly decreased out recruitment success, and increased significantly recruitment period. On the other hand due to the unpredictable course of the disease, many participants had either to stop treatment, or to introduce other treatments. This reduced the number of patients used in the final analysis.

The type of the analysis conducted and the effect of the time on the metabolites measured did not allow to use 'old' samples. According to the protocol of this study it was expected that most of the samples would be collected during the routine hospital visits of the patients. However just eight out of the approximately 187 samples were brought to or collected in the clinic. The remaining samples were collected within four hours of defaecation at any time (3 am earliest to 11:30 pm latest) and any place convenient to the participants (maximum distance 60 miles), 24 hours, seven days a week. Despite these difficulties and the discomfort this caused this study managed to recruit twice as many subjects than any similar previous report (531;532). Moreover using vigorous methods of sample collection and preservation samples were processed soon after collection ensuring rigorous and reliable findings.

As this study recruited new patients along with children in clinical relapse, many patients had received recent bowel preparation. That was inevitable and although it may have imposed an important confounding factor in study results, no differences were observed between children who had a recent colonoscopy and those who did not. The exclusive nature of the treatment on the other hand is an advantage of this study as it assessed only the effect of the treatment and results were not confounded by other dietary intake.

In conclusion the findings of this study oppose the speculation by Lionetti et al (531) for a mechanism of EEN action in paediatric CD mediated by a prebiotic effect of the nutritional supplement. Indeed results show that in those patients achieved remission, EEN induced an "unfriendly" for the colon, luminal microenvironment, with reduced availability of metabolic fuels for the colonocytes, and increased production of potent harmful substances for the colonic health. Moreover in light of the remarkable effect on EEN on gut metabolism, future studies of bacterial metabolic activity in IBD, should consider carefully the recruitment of patients on oral artificial nutritional support, in the same way as with the usage of antibiotics.

Relating the effects of changes in the concentration of faecal metabolites to events in the colon is not straightforward. It is difficult to know whether these effects have a causative association or they are simply epiphenomenon. Many factors can affect the luminal

concentrations of faecal metabolites that are difficult to distinguish. Changes in production rate (458), gut mobility (468;627), malabsorption, luminal conditions (401) or impairment in metabolism and absorption make interpretation of the results a difficult task.

On the other hand it is not possible to be sure whether the changes observed were disease specific and do not happen in healthy children too. Comparison with a parallel cohort of healthy children on treatment with the same dietary regime will better answer this. However such a study is unethical to conduct in healthy children. Previous studies summarized by Whelan et al (529) found a significant decrease in the concentration of total SCFA and butyrate in healthy human subjects.

The current study offers substantial insight on the effect of EEN on the metabolic activity and gut microbiota of CD children. The initial scope of this project was to use this baseline data to conduct a RCT on the use of a prebiotic or synbiotic supplement in parallel with EEN. The findings of this study encourage such a trial although the anticipated outcome is ambiguous. If the observed effects are the inevitable result of a lack of non-digestible carbohydrate in the EEN feeds, improvement of colonic health with synbiotic (pre and probiotic) therapy (324) may improve the clinical efficacy of the treatment. On the other hand if reduction of butyrate and a high colonic pH mediates the therapeutic effect of EEN, addition of a synbiotic supplement could potentially be harmful. This has to be addressed with a clinical trial or in-vitro studies with colonic biopsies.

CHAPTER 8

General discussion

8.1. General discussion and conclusions

Since I finished my first degree in nutrition and dietetics in 1999, I have always been interested in the importance of nutrition in diseases of the alimentary tract. Then it was the important role of the dietitian in the clinical management of the paediatric patient with IBD that motivated me to undertake this PhD. This is because nutrition is implicated in all aspects of the disease:

a) Disease pathogenesis with dietary risk factors predisposing disease onset.
b) Management of active disease with nutritional therapies; EEN being the primary treatment for paediatric CD patients.
c) Overall health, nutritional status, and growth of the patient.

Although the primary aim of this thesis was to study the effect of EEN on the commensal microbiota composition and metabolic activity, for several reasons outlined below I had the opportunity to explore other aspects of the nutrition in paediatric IBD. Thus this thesis would be better presented as a compilation of research on nutritional aspects in paediatric IBD.

Although the prevalence of juvenile onset of CD is high in Scotland (16), it still remains an uncommon GI disorder with a frequency of 20-25 new cases per year in a busy paediatric tertiary hospital like the one in which this PhD took place. This did not allow easy recruitment of a large number of patients and inevitably prolonged recruitment duration much beyond the initial expectations of this study. Moreover due to ethical constraints this study did not recruit newly presented patients before their disease diagnosis had been medically confirmed. As a result the majority of patients were approached on the same day of diagnosis, soon after the end of their endoscopy, and before the patients were discharged! This resulted in many patients being missed or not being appropriate for recruitment. In addition, the incurable and chronic nature of the disease and the restrictive dietary regime was upsetting for the majority of families and patients, and resulted in many children refusing to participate. Finally the collection of stool samples was not pleasant. For all these reasons recruitment had to be extended from one year to 2 ½ years. This in conjunction with the uncertainly of recruitment and possible patient dropout gave time for the researcher to develop parallel projects. Four of these six projects were presented in this thesis. Two other projects, which were conducted at the same time, were not included.

In summary the aims of the studies that comprise this PhD were:

1. To assess the prevalence and predictors of use of dietary modifications, nutritional supplements and alternative medicine in paediatric patients with IBD.
2. To investigate the prevalence and predictors of anaemia in paediatric IBD patients.
3. To investigate the effect of EEN on
    a. Gut specific and systemic markers of disease activity

b. Gut microbiota diversity and metabolic activity
c. Body composition, and circulating micronutrients in children with CD

4. To compare
   a. The validity of the body composition method used in some of the studies described in this thesis against a reference method
   b. The body composition of CD children with a cohort of healthy Scottish children.

Prevalence and predictors of use of dietary modifications, nutritional supplements and alternative medicine in paediatric patients with IBD

In agreement with previous studies in adult populations with IBD (63;68;70;536), this study described a high use of dietary modifications, nutritional supplements and alternative medicine in paediatric patients with IBD. Probiotic use was the most predominant form of unconventional treatment by both the current and past users.

Although the majority of the nutritional therapies that the respondents of the questionnaire used have good safety records, this study shows that patients supported and used alternative treatment options, which they did not declare to their doctors. This has important clinical applications as some of them may present adverse effects (77), may interfere with the action of conventional treatment and the avoidance of specific food groups or adherence on special diets can compromise the long-term health and nutritional status of the patients. A prime example is the use of dairy free diets, which can negatively affect the already vulnerable bone health of IBD children. Similarly exclusion of high fibre/residue foods in order to avoid exacerbation of GI symptoms can reduce the production of butyrate from the colonic microbiota and simultaneously increase the action of proteolytic bacteria. This could possibly induce an unhealthy luminal environment and lack of energy substrate to the colonocytes. This may also explain the low faecal butyrate concentrations observed in this and other studies (419).

Therefore medical and health professional staff responsible for the care of these patients should ask and record the use of unconventional therapies and this must be integrated in the standard clinical review of the patient.

Although the sample size was relatively small this study managed to recruit the majority of patients of a representative IBD population and to achieve an excellent compliance rate. The stringent recruitment process this study followed ensured anonymity and perhaps undistorted responses. Although a limitation of this study would be the lack of a cohort of healthy children to compare with the results of the IBD children this was overcome this by asking parents of IBD children for details of their use of unconventional treatment

prior to disease onset, and the questionnaire was specific to the use of remedies exclusively for the management of IBD.

Among the main reasons this study was carried out was to assess the use of probiotic supplements. It was the intention of the researcher to use this information to support the design of a clinical trial on the use of probiotic/prebiotic supplements in the same paediatric population. The findings of this study can now justify an intervention study with the systematic use of probiotic supplements in a population that is already receiving them and supports their use.

Several studies have been conducted on the composition and diversity of the colonic microbiota in IBD patients (Section 1.7.2.3.4). Although most of these considered patients' use of antibiotics, they rarely described or commented on the use of probiotic supplements. In light of these results, and the established effects of probiotic organisms on the gut microbiota and metabolism, future studies in this field should question eligible patients about probiotic use.

It is difficult to compare the results of this study with previous reports and to assess temporal changes in the use of unconventional treatments as the definition unconventional medicine and the types of therapies used varies considerably among the studies. Only a study using the same questionnaire in the same population will allow assessment of temporal changes in the use of unconventional medicine by children with IBD.

Moreover, with the increasing use of CAM reported in this and previous studies (see Section 1.2.2), and the positive attitude of parents and patients to them, more high quality and clinical trials are needed to establish their efficacy and to better inform parents, patients and medical staff.

Prevalence and predictors of anaemia in paediatric IBD patients

Anaemia is an extraintestinal manifestation in IBD. Although its prevalence is well described in adult IBD studies, in paediatrics the evidence comes from a limited number of reports with significant methodological flaws (see Section 1.4.4).

Using age and gender appropriate cut offs to define anaemia, it was found that its occurrence was high in a large population of paediatric patients with IBD and higher than previous adult reports (229). Disease severity and underweight were the strongest predictors of anaemia incidence at diagnosis and their improvement at follow up, correlated with parallel improvement in the concentration of haemoglobin. Knowledge of predictors of anaemia incidence at diagnosis and at follow up may avert early onset and accelerate its resolution at follow up.

This study failed to find a treatment that was associated with improvement or deterioration of haemoglobin levels at one year of follow up. In particular it was postulated

that nutritional replenishment on treatment with EEN would be associated with improvement of haemoglobin levels at follow up but the results of this study failed to confirm this. It is possible that the retrospective design of the study, that correlated the use of EEN at any time point over a 12-month period with changes of haemoglobin levels at 12 months, was not an appropriate method to detect any potential short-term effects of EEN on the patient's haematology profile. Nevertheless neither did haemoglobin levels improve significantly in the prospective longitudinal study in this thesis (Chapter 5), which assessed the effect of EEN on circulating micronutrient status. Unfortunately this study had a small number of participants and therefore may be underpowered. A larger prospective study is needed.

A strong positive correlation was found between CRP and serum a ferritin level, which is consistent with the evidence that suggests that serum ferritin is a positive acute phase respondent. This implies that the diagnostic value of serum ferritin to assess body iron stores can be misleading in the presence of a systemic inflammatory response and this should be considered by the medical staff responsible for the care of these patients. In particular, discrimination between the different types of anaemia, and mainly between IDA and ACD, cannot rely solely on these markers, and other serological indices with better predictive value should be used.

Despite the retrospective design of this study, it was found that iron therapy is indeed a valuable option in the treatment of anaemia in paediatric IBD, although it can exacerbate GI symptoms in patients and animal studies (236) and may increase the risk for colonic carcinogenesis. For those patients who do not improve on treatment with oral iron, the use of alternative treatment options such as intravenous iron sucrose and erythropoetin are needed. As ACD pathogenesis is attributed to the action of inflammatory cytokines (231), future studies should address whether the use of anti-inflammatory agents, which avert the activation or propagation of the pro-inflammatory cytokine cascade, can improve haematological profiles.

Although a proportion of the patients improved at follow up, some deteriorated and became anaemic. Future research should be focused on this patient group to find predictors and modifiable factors that could prevent anaemia onset with early intervention.

Anaemia in IBD can be attributed to many factors with the most important being GI bleeding. Although a diet poor in iron should be considered too, there is not good evidence to support that in paediatric IBD patients. Future studies in an IBD population should assess the dietary iron intake of these patients. An iron balance study could be designed where the equilibrium of iron will be monitored to accurately assess intake, use, and GI losses. A study which assesses the dietary intake of IBD patients and patients with other GI disorders has been designed by the researcher of this PhD and is ongoing.

The effect of EEN on gut specific and systemic markers of disease activity

Improvement in the clinical activity and systemic inflammatory markers occur within a few days on treatment initiation on EEN although they may not both be sensitive or specific enough to reflect intestinal inflammation and mucosal healing. New faecal markers of intestinal inflammation are now available with good diagnostic value to discriminate between organic and functional GI disorders. Faecal calprotectin levels was measured in the majority of patients at the time of their colonoscopy and the results showed that this correlated well with serologic markers of inflammation and presented a 100% sensitivity to identify patients with mucosal inflammation. On the contrary CRP and ESR were within the normal range for a substantial proportion of patients despite evident endoscopic and histological inflammation.

This study for first time measured changes in faecal calprotectin levels during treatment with EEN and correlated changes with clinical response to the treatment. Although the majority of the patients improved clinically and their systemic inflammatory markers decreased, only in those patients who achieved complete clinical remission, did faecal calprotectin levels decrease. This may indicate that improvement in clinical activity does not parallel changes in intestinal inflammation unless full clinical remission is achieved. This may also imply that complete clinical remission is achieved only in those patients whose intestinal inflammation attenuated. Nevertheless even in the patients who entered clinical remission, calprotectin decreased to within the manufacturer's normal reference values in only one patient. This may indicate that there is still subclinical intestinal inflammation despite induction of clinical remission or that improvement in clinical symptoms preceded improvement of intestinal inflammation. Perhaps there is a faecal calprotectin concentration cut off below which clinical symptoms of inflammation are quiescent. This should be explored in a future study.

Although recent studies (612) have shown that calprotectin levels can predict disease exacerbation this study did not explore that, and in particular whether calprotectin levels at the end of treatment on EEN can predict a subsequent flare up. This has to be addressed in a future study. If this is the case then the decision to terminate or prolong EEN treatment duration may depend also on changes in markers of GI inflammation beyond improvement in clinical activity.

A secondary outcome of this study was that a high number of healthy first-degree relatives with CD have considerably high levels of calprotectin. This has been reported previously in the literature (614) and further studies should monitor these individuals to confirm whether they are at a high risk of developing GI inflammatory conditions.

Strength of this study was the methodology followed to obtain stool samples and analyze faecal calprotectin. All samples were collected within four hours of defaecation, the whole bowel movement was homogenized, and all specimens from the same patient were analyzed

in duplicate on the same ELISA plate to avoid inter-assay variability. A limitation was the small number of participants and thus the results should be interpreted cautiously.

The effect of EEN on body composition and circulating micronutrient levels.

Reduced dietary intake, malabsorption, increased energy requirements, and the action of pro-inflammatory cytokines have all been implicated in the undernutrition and growth retardation seen in paediatric IBD patients (Table 1.3.1).

This study assessed the nutritional status of CD children, using body composition assessment and circulating micronutrient levels and explored how these changed on treatment with EEN. The results of this study agreed with previously published evidence that anthropometry is negatively affected in paediatric patients with CD (33;82). Underweight and growth failure were proportionally higher in CD patients compared with a cohort of healthy children. An interesting outcome of this study, which highlights the importance of using body composition assessment, is that FFM but not FM increased during EEN treatment only in the group of patients who achieved clinical remission. In those patients who did not enter clinical remission no significant changes in total body composition were observed. Use of BIA as a body composition assessment method may lack of validity as was found in another study of this thesis (Chapter 4) and therefore the results of this study should be confirmed by more accurate body composition reference methods such as the four compartment model (556). If these results are replicated then FFM replenishment should be further explored as a potential mechanism mediating induction of clinical remission. This is also supported by recent evidence that suggested suppressed proteolysis and increasing protein synthesis with nutritional therapy in children with inactive CD (333).

Suboptimal levels of circulating micronutrients have been described in adult patients with IBD (100;180), and this paediatric study is in accordance with that. Most of the antioxidant vitamins and trace elements measured were lower than the laboratory reference range. However none of the patients developed clinical symptoms of deficiency although this study did not specifically look at that. Some authors linked suboptimal antioxidant system with the aetiology of intestinal injury (141), but we suggest that this is an epiphenomenon of the acute phase response. Upon increase of CRP, in inflammatory conditions and infection, redistribution of many micronutrients takes place in the human body compartments, and therefore serum measurements are not reflective of the actual body stores. A lack of a causative association between inflammation and micronutrient status is further supported by the lack of association found between the serum micronutrient levels and gut specific markers of inflammation (calprotectin). We suggest that other indices of body micronutrient status should be used in patients with acute phase response in order to assess the actual body stores. Measurements in red blood cells are a potential surrogate index, as they are not

influenced by the acute phase response. Such a study, measuring micronutrients in erythrocytes, is required to reliably answer the question whether body micronutrient stores are low in children with CD and whether this is associated with intestinal inflammation.

An interesting finding of the second part of this study is that nutritional therapy with EEN improved the circulating levels of several micronutrients but the serum carotenoid concentrations deteriorated with more than 90% of the patients being biochemically depleted at the end of treatment. This is a surprising result contrary to expectations. Carotenoid concentration was predicted to increase firstly due to nutritional replenishment, and secondly because of the resolution of the inflammation and reversal of the serum levels of carotenoids (which are negative phase respondents) to higher levels. Instead carotenoids further deteriorated at the end of treatment, which may indicate increased metabolism, due to the increased oxidative stress in patients with IBD, and/or poor replenishment from the nutritional supplement. Indeed the nutritional supplements that the patients received in all cases lacks of carotenoids. Considering the importance of an effective antioxidant system in health and in mucosal injury in IBD, these results may have implications in the industrial production of artificial feeds. Depletion of antioxidant micronutrients may compromise the body's defensive mechanisms and increase oxidative stress and damage of biological macromolecules. Unfortunately this study did not measure the serum levels of oxidative stress markers like malondialdehyde, and therefore it is not possible to comment whether treatment with EEN impaired the antioxidant defensive mechanisms. If this is the case supplementation or the feeds with antioxidants may be required and perhaps increase the clinical efficacy of the treatment given the important role of antioxidants in IBD.

Similar to the cross-sectional data on the circulating micronutrient status of children with CD, the changes in the level of some of the micronutrients observed are difficult to interpret. Improvement in the levels of some of the micronutrients may be a combined effect of the nutritional replenishment, resolution of the acute phase response, and less consumption with improvement of disease activity. The use of other markers of micronutrient body stores may facilitate the interpretation of these results. The results of this study triggered the researcher to continue this study and explore changes in the levels of the same micronutrient in erythrocytes.

A limitation of this study was the small number of participants, which is frequently seen in investigations in uncommon diseases. Moreover collection of blood samples for research purposes were only allowed as an extra amount collected during routine blood samples of the patient. It was deemed unethical to venupuncture children for research purposes only and therefore, some blood samples were missed from patients who were not due to give routine clinical bloods. As this study was supplementary to the main project of the effect of

EEN on gut microbiota, recruitment was hampered by the fact that many patients did not want to participate in the arm of study, which involved the collection of stool samples.

Comparison of body composition assessment in healthy children with leg to leg bioelectrical impedance and DXA analysis

An additional experimental chapter of this work compared two methods of body composition in healthy children (Chapter 4). One of them is a common reference method to assess bone mineral content and body composition and the other one a novel bedside body composition analyzer suitable for routine measurements. The overall results of this part of the thesis do not suggest that the validity of the new bedside equipment, using the manufacturer's equation is as good as the reference method at individual level, and thus the results in the studies this used should be interpreted bearing that in mind. Manipulation of body impedance values in a way that would be independent of the manufacturer prediction equations may be better and the most reliable way to use BIA. Recently lean and fat index norms have been developed that use bioimpedance measurements and classify children as having relatively high, average or low fatness and leanness independently (587). On the other hand although BIA may not be accurate to precisely assess body composition its use to assess longitudinal changes in follow up studies, like those presented in this thesis, may be more reliable. Its sensitivity to detect changes in body composition should be addressed with follow up studies measuring body composition with BIA at different intervals and comparing it with other reference methods.

Although this chapter seems to be unlinked from the rest of the thesis, it gave important information on the accuracy of a quick method to assess body composition in healthy children. A subsequent study should be planned to check the accuracy of the same method in a paediatric cohort of IBD children. The results of a respective study will be useful in clinical practice and may help the transition from recordings of anthropometric measurements to the actual evaluation of body composition stores. Furthermore this study offered the opportunity to compare the body composition of CD children with a large sample of contemporary healthy children of the same socio-demographic background.

The effect of EEN on gut microbiota diversity and metabolic activity

A substantial number of studies have compared EEN with other mainstream treatments in the induction of clinical remission in active paediatric CD (Section 1.5.1), but there is lack of evidence to suggest the actual mechanisms of action. On the other hand the strong evidence implicating commensal microbiota in disease pathogenesis challenged the researcher to

undertake a study on the effect of EEN on the gut microbiota metabolism and diversity of these patients.

To the best of our knowledge this is the first study that explored the effect of EEN on intestinal microbiota metabolic activity in patients with CD, and one of the few that explored changes in gut bacterial diversity. A prebiotic effect of EEN proposed by the first Italian research on the topic (531) is not supported by the findings of this study which observed an increase in the faecal pH, reduced concentration of butyrate, and significant increase in the total sulphide concentration. These conditions should be better described as an “unhealthy” colonic microenvironment where colonocytes are deprived by significant energy substrates. This is a paradox finding, as the patients who presented these findings were those who clinically improved and their gut inflammation ameliorated. Similar findings were not observed in the patients who did not achieve full clinical remission. It is very difficult to interpret this discrepancy and whether this is a random finding resulting from differences in gut motility between the two patient groups or an actual phenomenon mediating the efficacy of EEN. Perhaps an impaired metabolism of butyrate, which has been described previously and in recent studies, is resolved on treatment with EEN although most of the evidence on an abnormal metabolism of butyrate in IBD, comes from studies on UC but not CD patients. Likewise the role of total sulphide should be further explored in the light of evidence that sulphide-releasing derivatives have better efficacy than standard mesalazine in animal model of colitis (466). An alternative explanation of these findings is that while on treatment with EEN, the growth, metabolic activity and microbial products of bacterial species, which may be involved in the pathogenesis or perpetuation of intestinal injury in IBD, are decreased, and the findings of this study are in fact a secondary epiphenomenon of these changes with the reduction of some as yet unidentified bacterial product which exacerbates the inflammation in the GI tract.

Although this study compared the changes of intestinal microbiota diversity and metabolic activity in CD on treatment with EEN against the changes observed in serial samples of healthy children on normal diet, a study on healthy children on EEN treatment would be more appropriate and would answer whether changes in bacterial metabolism and diversity are disease specific.

Apart from the effect of EEN on the bacterial metabolic activity this study explored parallel changes in the diversity of the commensal microbiota. Although the commensal microbiota diversity was significantly lower than in healthy controls, no significant changes in the bacterial diversity were observed during treatment. Unfortunately the molecular biology method used does not allow to comment on strain specific changes or changes in the bacterial counts. Other methods like FISH should be used to explore that. That was among the original aims of this study and samples were prepared for analysis with the FISH

technique combined with flow cytometry. However due to the lack of time, resources and essential equipment this analysis was not performed. The changes in bacterial metabolic activity observed, with significant increase in sulphide concentrations and reductions in butyrate strongly suggest the use of SRB specific probes and probes specific for major butyrate producing species for FISH analysis. Further funding and collaboration is required to allow to analyze these samples and explore the changes in bacterial diversity, composition, and metabolic activity during treatment with EEN. On the other hand both the TTGE and FISH analysis can only assess the dominant microbiota that occur in a concentration of > 10^6/g of colonic content. Thus any changes in the subdominant microbiota remain unknown and are difficult to study with current techniques. Changes in the mucosal bacterial microbiota may not coincide with changes in faecal material and this should be explored, although serial biopsies over a short period of time may be judged unethical and inappropriate.

A limitation of this study is the small number of patients and the heterogeneity in disease characteristics between patients. Lack of statistical power is a common problem in these studies due to the feasibility of large numbers given the rarity of the disease. The need to measure metabolic products in fresh stool samples, within four hours of defaecation reduced recruitment rates, imposed a significant personal burden to the researcher who had to travel in a radius of 60 miles, at any time and day to collect the samples. However the rigorous methodology followed in this study ensured reliable results. Multicentre studies are essential to achieve better recruitment and acquire adequate sample sizes. However this study is by far the biggest study carried out so far having recruited at least a double number of participants than previous report.

Conclusion

The results of this work offer new insight into several aspects of the nutrition of paediatric IBD patients and comprise the foundation for further ongoing work. Although the efficacy of EEN to induce clinical remission in active paediatric CD was well described before the initiation of this PhD, the mechanisms of action remained unknown. This PhD tried to assess potential modes of action, which involve changes in the gut microbiota and nutritional status of the patients.

Considering the results of this study, the importance of the commensal microbiota in IBD pathogenesis, and other studies showing clinical and experimental evidence on the therapeutic effect of probiotics, prebiotics and butyrate in IBD, future studies should look at the effect of dual nutritional therapies or improvement of the composition of the existing ones. Prebiotics or fibre can easily be added in nutritional feeds, whereas new vehicles of oral

butyrate derivatives which could act along the large bowel are now available. Probiotic supplements could also be used and their use is supported by a few studies.

Using a dual nutritional therapy may improve the colonic microenvironment and improve the efficacy of EEN. Such a speculation would be best addressed with well-designed clinical trials, animal models and in vitro studies using intestinal biopsies.

In this study although treatment with EEN corrected the anthropometry and nutritional status, the circulating levels of certain micronutrients deteriorated due to lack of carotenoids in the feeds. As carotenoids are important micronutrients in the human antioxidant system, and the latter have been implicated in tissue injury in IBD, future studies should compare the efficacy of standard EEN with feeds supplemented with carotenoid antioxidants. A potential superior effect of the fortified supplement may have important implications for the producers of feed companies. On the other hand, measurement of systemic micronutrients in red blood cells will better assess the actual micronutrient body stores and would better address whether there is an actual aetiological association with inflammation, intestinal injury in IBD.

"This is the END of a long journey to reach "My Ithaca". Although I avoided the rage of Poseidon and did not fight with Cyclops, and Lestrygonians, like Ulysses in Homer Odyssey, I run my journey of over four years, where I fought patience, and persistence to reach "My Ithaca". Unfortunately my Penelope was not still waiting for me…. and I had to be deceived by some Sirens!!!"

As an overall review of these last four years

- *6 studies were designed, and run of which four were presented in this thesis*
- *4 full ethical ethic applications (3 for clinical studies) and two substantial amendments were prepared*
- *Over 400 patients were actively recruited*
- *Over 180 stool samples were collected from the four points of the compass within four hours of defaecation, within a 2 ½ years of 24-hour daily recruitment.*
- *Four laboratory assays developed and established in the department*
- *Over 600 stool samples were analysed for SCFA*
- *Over 250 stool samples were analysed for calprotectin,*
- *Over 2500 stool samples were analysed for sulphide concentration*
- *Over 200 stool samples were analysed for TTGE analysis*
- *Over 200 stool were analyzed for ammonia and pH*
- *Over 400 stool samples were analysed for lactate*

- *Over 300 measurements of body composition were conducted*
- *Over 300 patient medical notes were scrutinized*
- *Hundred of hours of data analysis*
- *6 posters, 3 oral presentations and two full papers (one under review)*
- *2 prizes for best presentations won*

ALL by the same and only crazy Greek!!!!

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APPENDIX

- Paediatric Crohn’s disease activity index
- Laboratory micronutrient reference range
- Advertisement leaflet
- Instructions how to collect and store a stool sample
- Study information leaflets
- Study consent forms

Handling a Sample at Home

After sample has been produced, you should call the researcher immediately to get the sample collected. Whilst he is on his way to your house, you should follow these instructions.

1. Put on the plastic gloves provided. Cover the pot, containing the sample, with its lid. Seal it tight then place it inside the plastic bag immediately after the sample production.

2. Dip the blue area of the strip provided into the water in the tube and take it back out.

3. Then pour the water from the tube, onto the two sachets (don't tear the sachets).

4. Place the strip and the sachets at the bottom of the plastic bag as soon as possible.

5. Immediately twist neck of the bag really tight and make a firm double knot. It is very important to tie the bag firmly to prevent the loss of the gas produced by the sachets.

6. Place the bag with the sample in a cold place (perhaps outside) or if you were given a coolbag, put it inside that, along with the ice packs.

Micronutrient reference range from the Trace Element unit, Glasgow Royal Infirmary	
- copper	10-22 µmol/L (adults) 1.5 to 7.0 (0 to 3 mth) 4.0 to 17.0 (4 to 6 mth) 8.0 to 20.5 (7 to 12 mth) 12.5 to 23.5 (1 to 5 y) 13.0 to 21.5 (6 to 9 y) 12.5 to 19.0 (10 to 13 y)
- selenium	0.8 - 2.0 µmol/L (adult) 0.2 - 0.9 µmol/L (0 to 2 y) 0.5 - 1.3 µmol/L (2 to 4 y) 0.7 - 1.7 µmol/L (4 to 16 y)
- magnesium	0.7 to 1.0 mmol/L
- zinc	12-18 µmol/L
- vitamin A	1.0 to 3.0 µmol/L (adults) 0.5 to 1.5 µmol/L (< 1 y) 0.7 to 1.5 µmol/L (1 to 6 y) 0.9 to 1.7 µmol/L (7 to 12 y) 0.9 to 2.5 µmol/L (13 to 18 y)
Carotenoids	α-carotene: 14 to 60 µg/L β-carotene: 90 to 310 µg/L Lutein: 80 to 200 µg/L Lycopene: 100 to 300 µg/L
- vitamin B_1	275 to 675 ng/g Hb
- vitamin B_2	Whole blood: 220 to 410 nmol/L Red cell: 1.0 to 3.4 nmol/g Hb
- vitamin B_6	20 to 140 nmol/L 234 to 815 pmol/g Hb
- vitamin C	15 to 90 nmol/L
- vitamin E	15 to 45 nmol/L
- vitamin D	25 to 170 nmol/L
- folate	2.7 to 34 ng/mL
- ferritin	10 to 275 ng/mL
- vitamin B_{12}	180 to 883 pg/mL
- ferritin	> 10 µg/l
- Calcium	2.25 to 2.70 mmol/L
- Phosphate	0.9 to 1.8 mmol/L

Nestlé CLINICAL NUTRITION

PAEDIATRIC CROHN'S DISEASE ACTIVITY INDEX (PCDAI)[1]
Provided by Nestlé® Modulen® IBD

Surname:	**Date of birth:**
First names:	**Age:**
Sex:	**Weight (kg):**
Date:	**Height (cm):**

CALCULATION OF PCDAI
Complete the screen by ticking the appropriate points for all three sections. Add the numbers for the total screen.

1. HISTORY (recall 1 week)

ABDOMINAL PAIN	Points	STOOLS (per day)	Points
None	0	0-1 liquid stools per day, no blood	0
Mild/brief, does not interfere with activities	5	Up to 2 semi-formed with small blood, or 2-5 liquid	5
Mod/severe-daily, longer lasting, affects activities, nocturnal	10	Gross bleeding, or ≥6 liquid, or nocturnal diarrhoea	10

PATIENT FUNCTIONING, GENERAL WELL BEING (recall 1 week)

No limitation of activities, well	0
Occasional difficulty in maintaining age-appropriate activities, below par	5
Frequent limitation of activity, very poor	10

2. LABORATORY

	Age	Results	Points	Age	Result	Points
(A) HCT (%)	<10 years	>33 28-32 <28	0 2.5 5	11-14 years Male	≥35 30-34 <30	0 2.5 5
	11-19 years Female	≥34 29-33 <29	0 2.5 5	15-19 years Male	≥37 32-36 <32	0 2.5 5
(B) ESR (mm/hr)		<20 20-50 >50	0 2.5 5			
(C) Albumin (g/l)		≥35 31-34 ≤30	0 5 10			

Nestlé CLINICAL NUTRITION

3. EXAMINATION

WEIGHT	Points
Weight gain or voluntary weight stable/loss	0
Involuntary weight stable, weight loss 1-9%	5
Weight loss ≥10%	10

HEIGHT (at diagnosis)	
<1 channel decrease	0
≥1, <2 channel decrease	5
>2 channel decrease	10

HEIGHT (at follow up)	
Height velocity ≥1 SD	0
Height velocity <1 SD, >2 SD	5
Height velocity ≥2 SD	10

ABDOMEN	Points
No tenderness, no mass	0
Tenderness, or mass without tenderness	5
Tenderness involuntary guarding, definite mass	10

PERIRECTAL DISEASE	
None, asymptomatic tags	0
1-2 indolent fistula, scant drainage, no tenderness	5
Active fistula, drainage, tenderness, or abscess	10

EXTRA INTESTINAL MANIFESTATION (Fever ≥38.5 for three days over the past week, definite arthritis, uveitis, E. Nodosum, P. Gangrenosum)	
None	0
One	5
≥Two	10

Score 0-100

PCDAI >30 Moderate or severe disease 11-30 Mild disease <10 Clinical remission

Total score

If score is 11 or more, consider introducing a casein formula rich in TGF-ß$_2$ e.g. Modulen IBD.

Reference: 1. Development and validation of a pediatric Crohn's Activity Index. Journal of Pediatric Gastroenterology and Nutrition 1991; 12(4): 439-447.

Nestlé Clinutren, St George's House, Croydon, Surrey CR9 1NR. Telephone (020) 8667 5130 or fax (020) 8667 5616.

PUBLICATIONS

Full papers

- Dietary modifications, nutritional supplements and alternative medicine in paediatric patients with inflammatory bowel disease. *Aliment Pharmacol Ther. 2008 Jan 15;27(2):155-65*

Oral presentations

- Micronutrient status in paediatric CD. Association with systemic and gut inflammatory markers. European Society of Clinical Nutrition and Metabolism (E SPEN 2008), Florence, Italy

- Is Exclusive Enteral Nutrition enough for Children with Crohn's Disease? Nutrition Society summer meeting 2008, Nottingham, UK

Poster presentations

- Correlation between Crohn's disease (CD) children who achieve clinical remission on exclusive enteral nutrition (EEN) and reduction in faecal calprotectin concentration. European Society of Paediatric Gastroenterology hepatology and Nutrition meeting (ESPGHAN 2009), Budapest, Hungary

- Effect of exclusive enteral nutrition on colonic bacterial activity in paediatric Crohn's disease. Nutrition Society summer meeting 2008, Nottingham, UK

- Use of special diets, nutritional supplements and alternative therapists in a paediatric population of inflammatory bowel disease. Nutrition Society meeting 2007 (Scottish section), Glasgow UK

- Prevalence of anaemia at diagnosis in paediatric inflammatory bowel disease (IBD): A population based study in the West of Scotland. European Society of Clinical Nutrition and Metabolism (E SPEN 2008), Florence, Italy