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Short- and long-term complications in pediatric inflammatory bowel disease patients, especially Crohn's disease

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List of Abbreviations and Acronyms

ADA	Adalimumab
AZA	Azathioprine
CD	Crohn's disease
CRP	C- reactive protein
EEN	Exclusive enteral nutrition
EIM	Extraintestinal manifestation
FC	Fecal calprotectin
GI	gastrointestinal
IBD	Inflammatory bowel disease
IBD-U	Inflammatory bowel disease- unidentified
IFX	Infliximab
IM	Immunomodulator
MTX	Methotrexate
NSAIDs	Non-steroidal anti-inflammatory drugs
EGD	esophagogastroduodenoscopy
PCD	Pediatric Crohn's disease
PD	Perianal disease
PEN	Partial enteral nutrition
PIBD	Pediatric onset inflammatory bowel disease
UC	Ulcerative colitis
VEO	Very early onset

1. Introduction

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative Colitis (UC) are immune mediated chronic disorders. IBD may manifest at any age and persists in most cases lifelong. The primary aim of treating pediatric patients with inflammatory bowel disease (PIBD) is to achieve early remission, to prevent relapses, to reduce side effects of therapy to allow children and adolescents a regular physical and psychosocial development without developing any disease complications. Nevertheless, disease complications can occur in pediatric IBD (PIBD) patients at any time.

This thesis focuses on frequent and severe complications in PIBD patients, such as perianal fistulizing disease and impaired bone health and growth. At the Division of the Dr. von Hauner Children's Hospital- at the Ludwig Maximilian University (LMU) in Munich, approximately 150 to 170 children with IBD are under continuous care and followed until transferred into adult care. Most of these children are included in a registry for PIBD patients living in Germany or Austria.

CEDATA registry, established in 2004 by members of the Society of Pediatric Gastroenterology and Hepatology (GPGE) of German speaking countries collects longitudinal data of PIBD patients. This register supports quality reviews of IBD care and evaluates differences in IBD treatment and management within Germany and Austria. It also offers the opportunity to answer remarkable research questions. I was fortunate to collaborate and to analyze data from the CEDATA registry on the prevalence and incidence rate of perianal disease in Crohn's disease patients in a large PIBD cohort. During the years as PhD student under the supervision of Professor Dr. med Sibylle Koletzko I was privileged to work with my talented colleagues, Dr. med. Klara Frivolt and PD Dr. med. Tobias Schwerd on a prospective controlled intervention study investigating the effects of partial enteral nutrition on bone health, geometry and growth in children with quiescent pediatric Crohn's disease.

During the course of my PhD, I also contributed to an international multicenter study (ImageKids) with the aim to develop and validate two indices capable of measuring intestinal damage and inflammatory disease activity in Pediatric Crohn's disease by means of Magnetic Resonance Imaging (MRI) with Enterography protocol (MRE) and pelvic MRI. All participants underwent magnetic resonance enterography and ileocolonoscopy at baseline and after six months. Fecal samples were collected in this cohort to evaluate other fecal inflammation biomarkers in comparison to the established measurement of calprotectin concentrations.

Controlling disease activity is essential to reduce IBD complications. Optimizing treatment strategies with drug monitoring became an integral part of the current treatment strategies [1, 2]. In 2016, our division received funding from the European Union within the HORIZON 2020 program for a large project called PIBD Network for Safety, Efficacy, treatment and Quality improvement of care (PIBD-SETQuality). The project included a large randomized drug trial in Pediatric Crohn's Disease patients

stratified into low and high risk groups. The aim was to evaluate the efficacy of methotrexate in comparison to azathioprine (low risk) or adalimumab (high risk). Within this international consortium, we were in charge for the work package on drug monitoring and safety. Throughout my doctoral thesis, I wrote the standard operation procedures and coordinated the measurement of drug trough levels and metabolites, monitored all study subjects from the different centers and suggested adjustments where necessary. The project had been extended until June 2021. Further publications will result from this project.

The publications presented in this thesis are based on two with first authorship conducted under the supervision of Prof. Dr. Sibylle Koletzko, PD Dr. Jan de Laffolie, Dr. Katharina Werkstetter and PD Dr. Tobias Schwerd. In two further publications, not considered in this thesis, from the ImageKids project I have served as a co-author (see publication list).

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises patients with chronic relapsing bowel inflammations defined as Crohn's disease (CD), ulcerative Colitis (UC) and Colitis indeterminata (CI), later has been renamed as IBD-unclassified (IBD-U) [3]. Burrill B. Crohn firstly described Crohn's disease histological findings in 1932 [4]. The pathogenesis of IBD is multifactorial, covering genetics, environmental factors, dysregulated immune response and intestinal microbiome [5]. Sartor et al. describe the etiology of IBD as followed: "The onset and reactivation of IBD are triggered by environmental factors that transiently break the mucosal barrier, stimulate an immune response or alter the balance between beneficial and pathogenic enteric bacteria [6]."

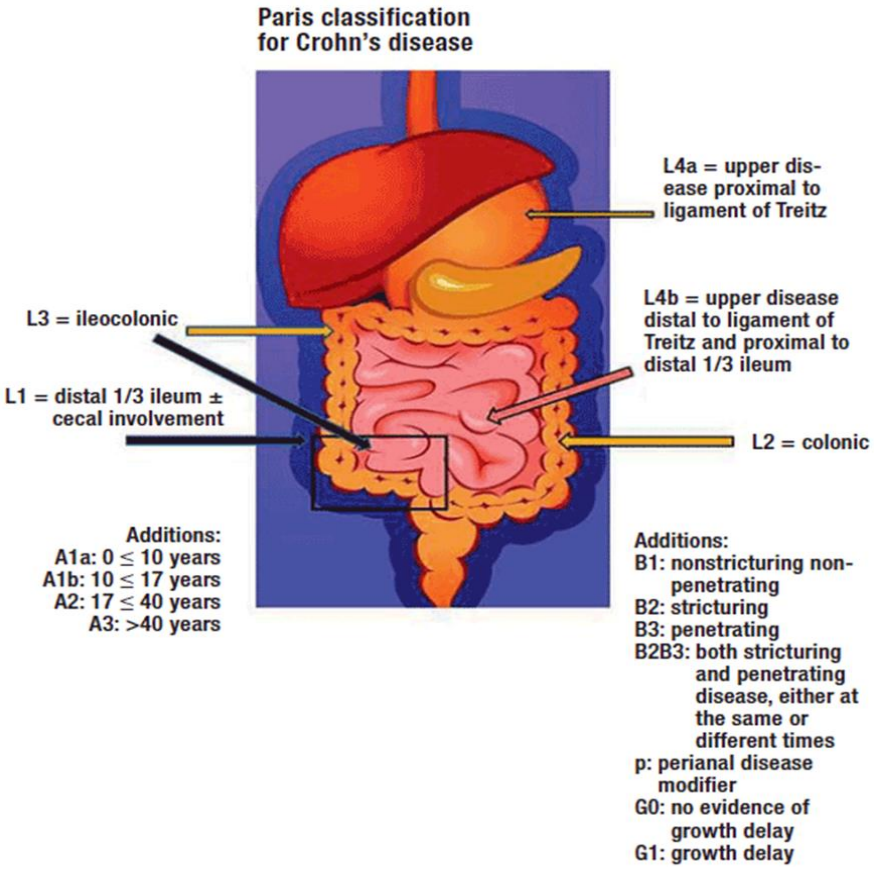
IBD may manifests at any age in up to one-fourth of affected patients it develops before reaching adulthood. Pediatric onset IBD (PIBD) differs from adult onset IBD in some aspects: (1) disease phenotype, (2) progression of disease and (3) disease complications (table 1, fig 1). This thesis will mainly focus on Crohn's disease. The dynamic features of PIBD (disease location and behavior, severe complication) have been addressed in the new Paris classification. The known Montreal disease classification was modified in Paris in 2011 and included a new sub-group for children <10 years (A1a) [7] [8] (table 1). The higher risk of monogenic disorders among the very young IBD patients has resulted in further sub-classification based on age at diagnosis: <6 years of age has been labeled as very early onset IBD (VEO-IBD), <2 years as infantile IBD [9, 10].

Table 1: IBD classification according to age of onset

IBD groups according to age	Classification	Age of onset in years
Pediatirc IBD onset	Montreal A1	<17
Pediatirc IBD onset	Paris A1b	10-17
Early onset-IBD	Paris A1a	<10
Very early onset- IBD		<6
Infantile IBD		<2

(1) *Disease phenotype*: Pediatric CD (PCD) often presents with predominant involvement of the colon and rectum, whereas adults with CD frequently have ileocecal affection [8, 11]. Figure 1 shows CD phenotypes according to the Paris classification (L1-L4) [8, 12]. **(2)** *Disease progression:* PCD indicates a more diffuse and extensive phenotype and suffers from a stronger progression of disease compared to adults [8, 13]. Long diagnostic latency is prevalent among VEO-IBD patients. These patients appear to have the most severe disease course and more complications during childhood [10, 14]. **(3)** *Disease complications:* PCD patients experience complications more often during disease progress compared to adults with IBD [3, 15]. A high proportion of CD patients are affected by delayed puberty and impaired growth (G0/G1, fig. 1) [11, 12, 15]. The Paris classification, shown in figure 1, includes stricturing, fistulizing and perianal disease (PD). Children are more affected by fistulizing and perianal disease than adults [16]. Our working group at Dr. von Haunersche Children's hospital had particular interest in bone health in PIBD. Newly diagnosed PCD patients present with reduced trabecular bone density, disturbed bone geometry, muscle mass and compensatory increased cortical density resulting in an increased risk for osteoporosis and later bone fracture [17, 18].

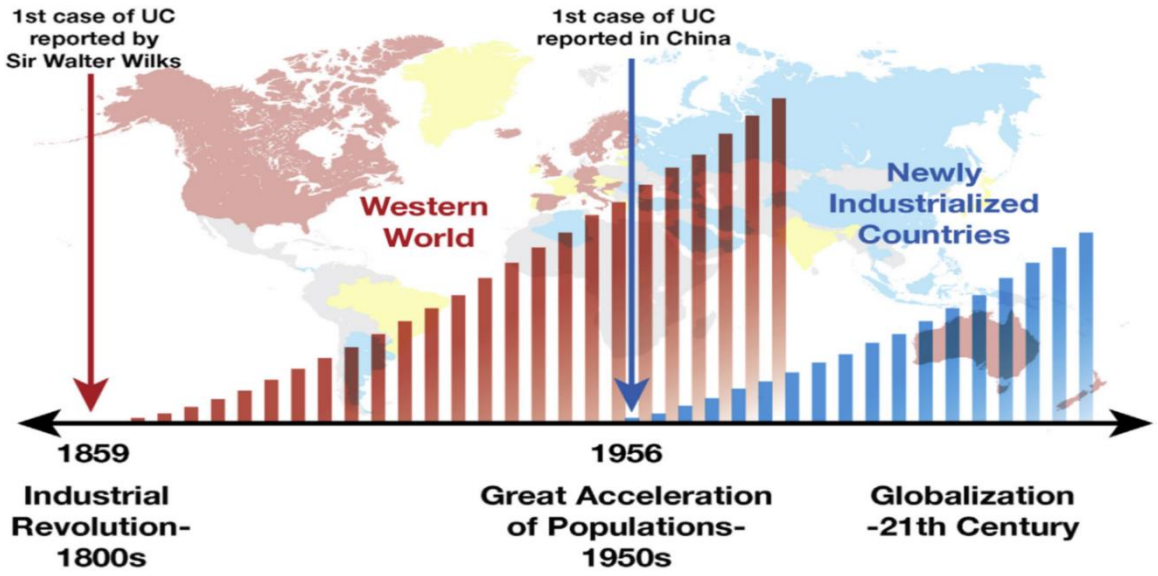
Figure 1: Buderus S. et al. (2015 Dtsch Ärzteblatt) Paris Classification of CD



1.1.2 Epidemiology of pediatric IBD

A steady increasing incidence rate of IBD has been found in the last century in industrialized and industrializing countries (fig. 2) [5]. IBD became a global disease with the highest prevalence in western countries (starting in the 19th century). However, industrializing countries or newly industrialized countries from Asia, Middle East, Africa and South America reported a highly dynamic increase of IBD incidence within the last decades (starting in the 20th century) [5, 19]. A peak of IBD incidence rate is found at the age of 33 years [20].

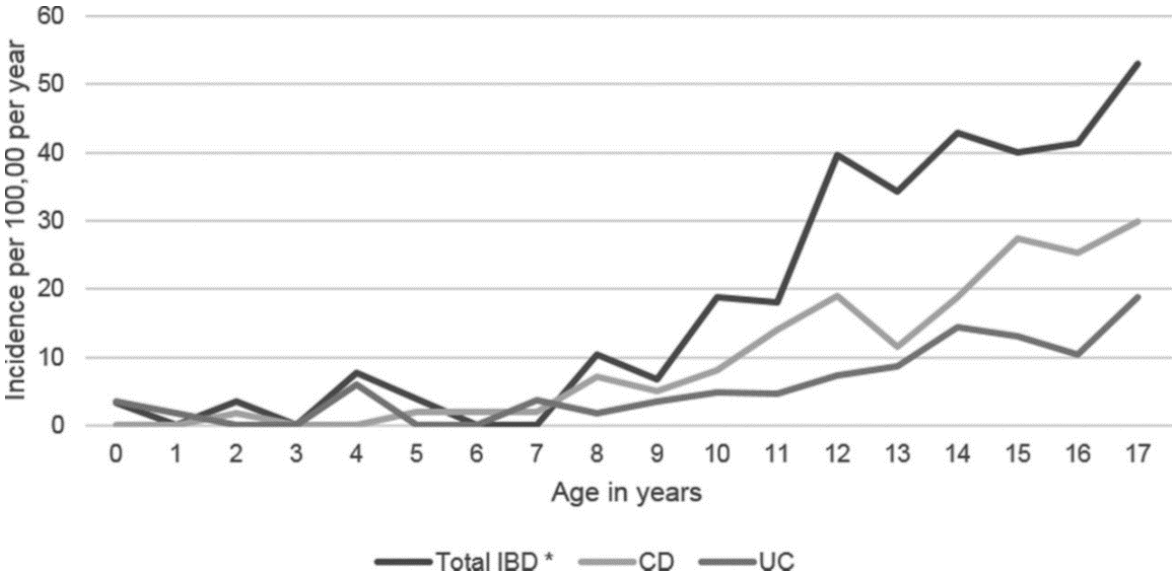
Figure 2. Kaplan G. et al (2017 Gastroenterology): Increasing trend of IBD in industrialized countries and industrializing countries



Since the beginning of the 21st century, population-based studies have shown that IBD incidence stabilizes among adults [21-23]. However, pediatric epidemiological data indicates a further steady increase in incidence and prevalence of CD in industrialized and industrializing countries, while the incidence in pediatric UC mostly remained stable in recent decades [5, 21, 24]. IBD develops before reaching the age of 18 years in up to 20% of IBD patients and out of these 25% are younger than 10 years at initial diagnosis. Age at onset less than 6 years (VEO IBD) has been reported in 15% of PIBD patients and present the fastest growing incidence rate age group [25, 26].

A systematic review from Benchimol et al. in pediatric IBD between 1950 and 2009 showed significantly increased incidence rates of CD in in 15 out of 25 studies and UC in in 4 out of 20 studies. A French study based on insurance data of children and adults reported incidence rates with 8.2/100.000 for CD [27]. Pediatric incidence rates of IBD from Stockholm (2002-2007) reported 12.8/100.000 new IBD cases (9.2/100.000 for CD) [28]. Based on German insurance data from 2007-2012, applying the same methodology and definition as Benchimol et al. in a Canadian study [21], Wittig et al. reported high IBD prevalence and incidence rates in the literature with 64.62/100.000 and 17.41/100.000, respectively [29]. Figure 3 presents the rising incidence rate of IBD with age. This German epidemiological data from 2012 are higher compared to previous data and to other European countries [12, 20]. The time trend and the most recent data collected on the incidence and prevalence of IBD could explain the higher rates compared to older studies. Country differences may also be explained by different cut offs for age (e.g. 16 years in France compared to 18 years in Germany).

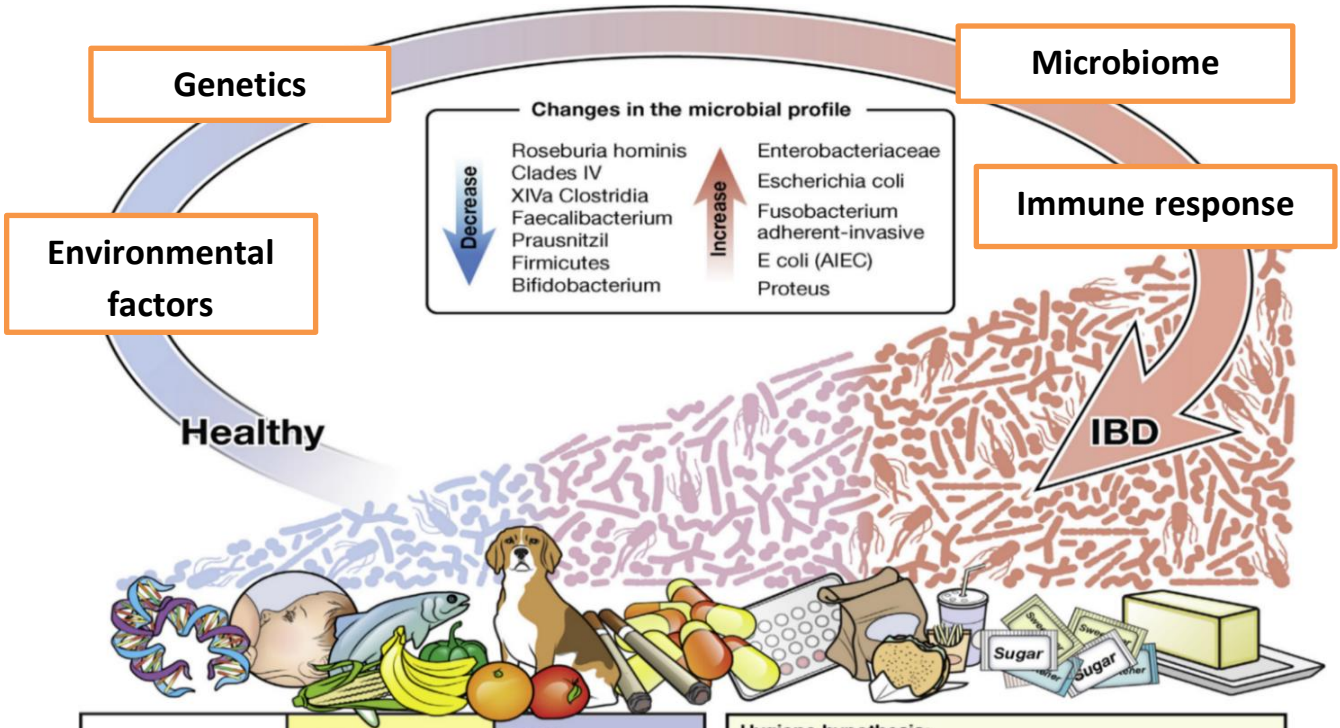
Figure 3: Wittig R. et al (2019 JPGN): German PIBD Incidence rate in 2012 per 100.000 insured patients at Barmer GEK



1.1.3 Etiopathogenesis of PIBD

The worldwide increasing incidence supports the concept of IBD being a multifactorial disease within interplay of environmental, genetic and immunological factors. Several ***environmental factors*** for PIBD development have been identified so far and include westernized diet, absence of breastfeeding, antibiotic intake during the first years of life and vitamin D levels. Diet induces shifts in the intestinal ***microbiome*** and metabolome with direct and indirect influences on the immune response and on the barrier function [30, 31]. ***Genetic risk loci***: several genetic variants/risk loci have been identified with risk increasing or reducing effect on IBD. The variants influence the innate and adaptive immune system causing ***immune dysregulations*** [32]. Although the etiology of IBD has not been fully understood yet, it involves a complex interaction among environmental factors, microbiome, genetics and immune response in individual patients. The contributing risk factors for the development of IBD are presented in figure four.

Figure 4: Kaplan G. et al (2017 Gastroenterology): Etiology and contributing risk factors for PIBD



	CD	UC
Smoking	Risk in Caucasians and Middle Eastern migrants	Protective in Caucasians and Asians
Antibiotic use in childhood	Risk in Caucasians, protective in Asians/ Middle Eastern migrants	Risk in Caucasians, protective in Asians/ Middle Eastern migrants
Breastfeeding	Protective in Asians and most studies in Caucasians	Protective in Asians and most studies in Caucasians
Oral contraceptives	Risk in Caucasians	Inconclusive
Appendectomy	Risk in Caucasians	Protective in Caucasians
Low levels of vitamin D	Risk in Caucasians	Risk in Caucasians
Tea or coffee consumption	Protective in Asians	Protective in Asians

Hygiene hypothesis:
Having pets in childhood, living on a farm, larger family size, and drinking unpasteurized milk were inversely associated with the risk of CD and UC

Changing diet:
Introduction of packaged food, fast food chains, increased use of antibiotics, increased fat (monounsaturated and polyunsaturated fatty acids) consumption and sugar intake, less dietary fibers is associated with risk of IBD

Dietary chemicals:
Food additives – saccharin, sucralose, carboxymethycellulose and polysorbate-80, common emulsifiers (including polysorbates, sorbate esters, lecithin), might increase risk of IBD (data are derived from animal models)

Over 200 IBD risk loci (37 specific for Crohn's disease and 32 for ulcerative colitis) have been discovered. However, modest fraction of predicted heritability can be explained by known genes or loci.

1.1.3.1 Genetics

Genetic IBD risk loci: Twin studies and family history have shown that Crohn's disease and Ulcerative Colitis have heritable components [33]. In monozygotic- twins, CD has a concordance rate of 20%-50% compared to 10% in dizygotic twins. Ulcerative Colitis shows lower values, 15% for monozygotic and 5% concordance for dizygotic twins indicating a weaker heritable component for UC [34]. NOD2/CARD15 is the most essential and well-known genetic risk gene in IBD that increases the risk for CD development and has been identified by linkage analyses in 2001 [35]. The gene

encodes a protein that is involved in recognizing bacteria by cells in the innate immune system [36, 37]. Genome-wide studies identified more than other 200 IBD risk loci up to date. Some of them affect the innate and adaptive immune response, endoplasmic reticulum stress, intestinal barrier function and microbial homeostasis [38-41]. The understanding of why some IBD-associated risk gene carriers remain healthy while others develop IBD remains unclear. VEO-IBD children often present severe disease progress, which may be refractory to standard treatment. Whole- exome analyses in parents and affected children revealed that among patients with VEO IBD monogenetic disorders may occur in up to 20% of cohort. About 60 different monogenetic disorders have been identified, which may manifest with IBD like clinical picture [9, 42-45].

1.1.3.2 Environmental risk factors

Migrant studies indicate the importance of environmental factors in the pathogenesis of IBD and support the hypothesis that Westernized lifestyle, diets, and especially early life events in childhood (breastfeeding, antibiotic use) influence IBD development [46, 47]. Population-based cohort- and case- studies investigated the following aspects of environmental factors.

Westernized lifestyle: Differences in nutrition and health status exist between different countries and within a country between rural and urban populations. Urban cities involve greater food abundance, less physical activity, and psychosocial stress compared to rural lifestyle [48]. The previously mentioned increasing incidence of IBD, especially in industrialized countries, indicates the Western lifestyle and the changes associated with industrialization need to be considered as risk factors for IBD [46, 49].

Westernized diet, including consumption of high animal protein, red meat, fat, processed foods, and refined sugar intake, is positively associated with the risk of IBD [50-53]. Regular consumption of fast food increases the risk for IBD, which could be related to mono- and poly-unsaturated fatty acids [54, 55]. Whereas plant fibers, particularly plantain, broccoli and Mediterranean diet, including fruits, vegetables, fish, and olive oil has been associated with reducing risk for IBD development [5, 47, 56]. Overall, diet shapes the composition of the intestinal microbiome and therefore the immune response [56, 57]. Recommendations for IBD diets include Mediterranean-diet, avoiding processed food, refined sugar, fast and highly processed food, and saturated animal fat [56]. The newly established Crohn's disease exclusion diet (CDED), is discussed in chapter 1.6.1.1 and paper II [47, 50, 58].

Breastfeeding: In 2017, a systematic review and meta-analysis of 35 studies showed breastfeeding lowers the risk for the development of CD, UC and IBD, (OR, 0.71; 95% CI,

0.59–0.85), (OR, 0.78; 95% CI, 0.67–0.9) and (OR, 0.74; 95% CI, 0.66–0.83), respectively [59, 60]. The positive effect of breastfeeding might be explained by supporting the development of the immune system and the intestinal microbiome with long-term effects on later health and disease [60, 61].

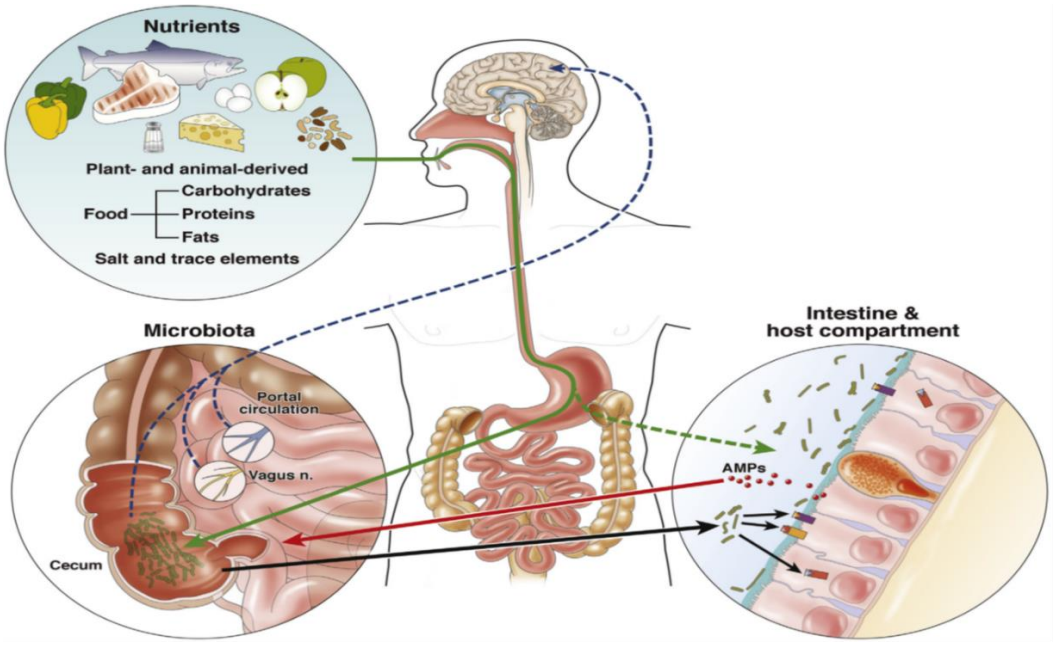
Antibiotics: The use of antibiotics during childhood increases the risk of developing IBD in Western countries. This effect increases with younger age at the first antibiotic exposure and multiple episodes of intake [62, 63]. Lewis et al. reported that early exposure to antibiotics increases microbial dysbiosis of the intestine, while reduced inflammation is associated with reduced dysbiosis [64].

Vitamin D: Low vitamin D levels are considered as a risk factor for developing initial IBD or relapses [47]. In particular, vitamin D deficiency increases hospitalization, relapses and use of escalating treatments in IBD patients. Vitamin D showed protective effects as potential immune modulator for IBD patients and should be monitored and supplemented if needed [65, 66].

1.1.3.3 Microbiome

The microbiome differs between healthy patients and patients affected by IBD [67]. However, this may be a secondary effect due to the inflammation. IBD treatment and special diets that induce remission pursue a shift in the intestinal microbiome [31, 67, 68]. Schwerd et al. from our group have shown that 6-8 weeks of treatment with exclusive enteral nutrition (EEN), a liquid nutritional complete formula, reduces alpha diversity and influences a shift in bacterial colonization [67]. The reduction in bacterial richness and lower alpha diversity induced by EEN is most likely caused by the absence of dietary fibers and fermental carbohydrates [69-71]. Data from our partial enteral nutrition (PEN) paper (Paper-II) will show that PEN is not sufficient to cause changes in the structure of the microbial community, as 6 to 8 week EEN treatment driven intestinal microbiome shifts in CD patients. Several other studies have also proven that diet affects the intestinal microbiome [72-74]. Figure 5 of Tilg et al. shows an interaction between healthy food intake, host immunity and the intestinal microbiome that starts in early childhood. Westernized diet, enriched in fat, phosphatidylcholine and L-carnitine activate intestinal inflammation by influencing the microbiome [75]. The intestinal microbiome is altered within 24 hours of dietary intervention [76, 77].

Figure 5: Tilg H. et al. (2015 Gastroenterology) Interactions of food and microbiome



1.1.3.4 Immune dysregulation

Diets and EEN effects potential interactions between immune responses and the intestinal microbiota [67, 78]. The colon epithelium and its mucus layer may get irritated by so far “unhealthy” (mainly unknown) nutrients and allow invasion and translocation of non-pathogenic bacteria or bacterial antigens that replicate within epithelial cells, dendritic cells, and macrophages and trigger the immune response and inflammation [78]. Westernized diet with nutrients including sodium caprate, gliadin, emulsifiers or carboxymethylcellulose, may affect the tight junctions in the epithelial layer and increase bacterial growth and translocation, whereas fibers reduce the risk for bacterial translocation and overgrowth [79, 80]. Alterations in susceptibility genes might lead to cell dysfunction, allowing bacteria to cause this imbalance due to loss of bacterial eradication and loss of epithelial integrity and increased penetration of bacteria leading to a vicious cycle of inflammation [81, 82].

1.4 Clinical manifestation

Classic gastrointestinal symptoms in PIBD involve abdominal pain, diarrhea (with or without blood), weight loss, decreased height-velocity, lack of energy, anorexia, anemia and/or perianal disease [15, 83, 84]. Extra-intestinal manifestations (EIM) involves arthropathy (peripheral and axial association), stomatitis, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis, pancreatitis, hepatobiliary or ophthalmologic involvement. EIM occur in 6-8.5% of PIBD patients at diagnosis and

are associated with increased baseline disease activity [85-87]. The incidence rate of EIM after diagnosis was 9% at 1 year, 19% at 5 years and 29% at 15 years [86]. EIM at childhood onset CD is more frequent compared to adult onset CD (8.5% vs 5.0%) [87] [88].

1.4.1 Complications

Children and adolescents with IBD often develop a more complicated disease course compared to adult patient. The following sub-chapters will focus on three main complications during the PIBD disease course, which are further discussed in Paper I and Paper II.

1.4.1.1 Perianal disease

Perianal disease includes perianal abscess and/or fistula. In CD patients, PD is a common complication that may lead to the initial CD diagnosis, whereas PD rarely affects UC (94% vs 6%, respectively) [89]. PD occurs more often in male PIBD patients than in females and children with PD tend to have a more severe disease progress [89, 90]. The prospective EuroKids registry initiated by the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), documents manifestation of PIBD at the time of diagnosis in more than 3000 pediatric patients and revealed a 9 % prevalence of abscess and perianal fistula at diagnosis of CD [91]. The PIBD Collaborative Research Group from the United States and Canada reports similar findings with a 10% occurrence of PD at CD diagnosis (N=247) [92]. The incidence of PD in pediatric CD patients has been reported in a large IBD cohort by Adler et al. (N=1399), with an incidence rate of 13.6%, 21.6% and 26.1% at 1, 3 and 5 years after diagnosis, respectively [90].

PD treatment and management depends on its type and severity and includes conservative management (antibiotics and/or initiating immunomodulators/ biologics), and/or surgical management (fission or drainage/leash) [93]. Moderate to severe fistulizing CD can effectively be treated with combined management (conservative (anti-TNF-alpha initiation) and surgical) [94, 95]. Higher trough levels of Infliximab (IFX) with ≥10.1 mcg/mL have been associated with favorable fistula response [94, 96]. PD treated with Adalimumab induced perianal fistula closure within 12 weeks of treatment in 44.4% of affected patients and 55% showed PD improvement [95]. Epidemiological

data, management of PD and risk factors for developing PD will be discussed in Paper I of this thesis.

1.4.1.2 Growth failure

Growth retardation and pubertal delay in PIBD patients is a well-known complication. The incidence of growth delay at diagnosis is more common in pediatric CD patients compared to UC, 15-40% versus 3-10%, respectively [24, 97]. Causative factors for growth failure are malnutrition, undernutrition, delayed puberty with decreased secretion of sexual hormones, and the inflammation itself. The prospective European study of newly diagnosed CD patients with long-term follow up (GROWTH study) published a trend for better height z-scores after 2 years in patients initially treated with EEN compared to steroids as initial induction therapy [98]. Other studies confirm that the use of exclusive enteral nutrition (EEN) compared to corticosteroids as induction therapy significantly improves the nutritional status and growth in PCD patients [99]. Besides EEN, anti-TNF biologics have been associated with improved growth [100, 101]. Controlling CD inflammation stabilizes or improves linear growth, in particular when compared to corticosteroid-treated patients [98, 102]. Further supporting factors for improving growth in PCD patients with quiescent disease will be discussed in Paper II of this thesis.

1.4.1.3 Bone health

Impaired bone health is a common complication at PIBD diagnosis attributed to decreased muscle mass, low physical activity, ongoing inflammation or nutritional deficiencies [103]. PIBD patients often present reduced trabecular density, high, cortical bone density, disturbed bone geometry and decreased muscle mass at disease onset. CD patients might have an increased risk for osteopenia and bone fractures [17, 18, 103]. Our working group at the Dr. von Hauner Children’s hospital has shown that 8 weeks of induction treatment with EEN improved bone health in CD patients with active disease, three months after starting EEN [104]. Avoiding systemic steroids and controlling inflammation by e.g. anti-TNF therapy improves bone density and structure in PCD with greatest effect in younger patients at early stage of puberty, suggesting a window of opportunity for treatment of bone deficits [105]. Bone health, bone geometry and muscle mass status in patients who have entered remission has been evaluated in Paper II.

1.5 Diagnostic work-up

According to ESPGHAN guidelines, a complete diagnostic workup for initial IBD diagnosis includes the performance of physician examination; laboratory workup, upper and lower endoscopy and small bowel imaging such as Magnetic resonance enterography (MRE) or video capsule endoscopy (VCE)) [106].

1.5.1 Diagnostic work up: laboratory

Laboratory workup for IBD patients in the blood includes the following parameters: complete blood count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as main inflammatory markers and for exclusion of thrombocytosis, leukocytosis, and anemia. Low serum albumin indicates severe ulceration with protein-losing enteropathy or lymphatic loss due to stricture. Antibodies to Saccharomyces cerevisiae (ASCA IgA and IgG) are markers for CD with a high specificity but low sensitivity. They are frequent positive in patients with early disease onset [107]. Laboratory workup for children in feces includes always the exclusion of intestinal infections including with *Clostridium difficile*. Fecal calprotectin is a marker for intestinal inflammation, but not specific for IBD. Fecal calprotectin (FC) is a neutrophil dominating and calcium- and zinc-binding protein in feces, which releases during active colorectal inflammation [108]. FC test is used for the diagnostic and monitoring of IBD [109]. It shows excellent results compared to endoscopic findings with a cut off <68 µg/g to distinguish IBD from other non-inflamed gastrointestinal conditions [110]. FC diagnostic has a high sensitivity (96%-98%) and modest specificity (69%- 72%) [111-113]. The recommended FC cut off level for children >4 years and adults for an increased value is >50 µg/g [114-116].

1.5.2 Diagnostic work up: Endoscopy

The IBD Working Group of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition developed consensus guidelines for a full diagnostic workup of pediatric IBD patients, called the Porto criteria that were published in 2014 [3]. In order to evaluate intestinal inflammation the diagnostic Porto criteria include esophagogastroduodenoscopy (EGD), ileocolonoscopy and MRE for small bowel assessment [3, 91]. ESPGHAN guidelines recommend to take at least two representative biopsies from inflamed areas, as well as five from defined colonic areas (rectum, sigmoid, descending, transverse and ascending colon) and at least two

biopsies from terminal ileum regardless the macroscopy is normal or not, during ileocolonoscopy [91, 117]. The EuroKids registry gathered data on the completeness of the initial diagnostic work up with the different participating sites between 2004 and 2009. EGD and ileocolonoscopy were performed in 64% of all cases (N=2087) and increased steadily over time (year 1 of the registry (52 %) to year 5 (71%, P <0.001)) [91].

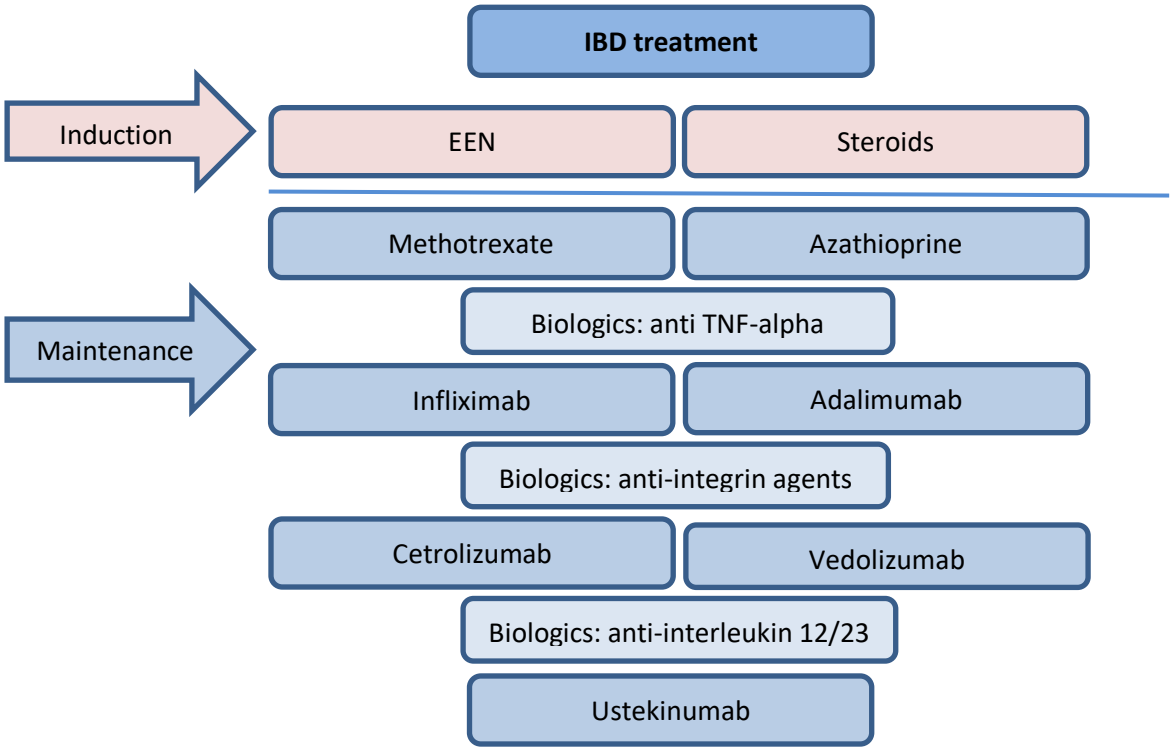
1.5.3 Diagnostic work up: Imaging Small Bowel

Magnetic resonance enterography (MRE) is preferred in PIBD over computer tomography for high diagnostic accuracy and absence of radiation. MRE requires an oral intake of a large volume of contrast agent [118]. The aliquots are ingested over one hour before the scan [119, 120]. The bowel preparation and scanning techniques are heterogenic according to a meta-analysis [121]. Contrast agents are positive, negative, or most frequently biphasic. The latest provides good contrast between the bowel lumen and wall-thickness on both T2- and enhanced T1-weighted images and improves the detection of signal changes and enhancement of an inflamed bowel [122]. A meta-analysis from Giles et al. reported a pooled sensitivity of 84% and specificity of 97% for MRE detecting active terminal ileitis in pediatric CD using ileocolonoscopy as reference [121]. Magnet resonance imaging is also very helpful for the diagnosis of perianal inflammatory bowel disease, allowing specific differentiation between perianal abscess and different types of fistula [123]. In order to evaluate small bowel parts, using MRE was superior to video capsule endoscopy but these two techniques visualize different layers and can be considered as complementary [91].

1.6 Treatment of IBD

The primary aim of pediatric IBD treatment is to induce and maintain long-lasting remission while reducing possible therapy side effects. Induction treatment for children with CD involves exclusive enteral nutrition (EEN), steroids or biologics [106]. Maintenance treatment for CD patients includes immunomodulators, biologics, and in selected cases surgery [106] (fig 5). This thesis will focus on treatment with exclusive enteral nutrition, partial enteral nutrition (PEN) and biologics.

Figure 5: IBD Treatment flow-chart



1.6.1 Exclusive enteral nutrition as induction therapy

The efficacy of exclusive enteral nutrition was discovered by chance almost 50 years ago by surgeons who used an elemental diet, created for astronauts, to stabilize malnourished IBD patients before bowel surgery. They discovered coincidentally, that severely ill CD patients, who have been treated with elemental diet, experienced weight gain and improved clinically [124]. EEN is a liquid formula containing protein (whole protein, peptides or only amino acids), lipid, carbohydrates, trace elements, and vitamins [125]. During this treatment, patients are not allowed to eat or drink any other

food than water [78, 126, 127]. According to all major societies (ECCO, ESPGHAN, NASPGHAN and ESPEN) EEN is the first- line induction therapy for pediatric CD patients with active luminal disease to induce remission. EEN induces clinical remission in up to 80%-90% of patients [99, 106, 128-131]. According to previous systematic reviews and meta-analyses, EEN is equipotent to induce clinical response in PCD compared to corticosteroids [132-137]. Patients with EEN-induced remission presented a higher rate of mucosal healing compared to patients treated with corticosteroids, evaluated by endoscopic findings [135, 138]. EEN treatment is highly demanding; therefore, induction treatment with partial enteral nutrition (PEN) has been studied. However, EEN remains superior compared to PEN in inducing remission (10/24 [42%] vs .4/26 [15%], respectively, p = 0.035) [139]. The study from Lee et al. supported the same effect on EEN vs. PEN in CD children [140].

1.6.1.1 New strategies with PEN to optimize EEN treatment

Recently, Crohn's disease exclusion diet (CDED) has been described. The diet excludes processed food, gluten, most dairy products and is based on fresh nutrients. The diet was combined with PEN of a complete liquid formula based on casein [127, 141]. CDED is divided into two phases. Phase 1 includes 50% of energy intake from the liquid formula and 50% from CDED. Followed by phase 2 with 25% formula and 75% CDED of energy intake [142]. CDED requires mandatory nutrients: chicken, eggs, banana, potatoes and optional nutrients: rice, strawberries, avocado, tomato, cucumbers ect. [57]. CD-TREAT, a study from Glasgow, developed an individualized food-based diet. This diet provides similar composition to EEN and was designed gluten-and lactose-free with macronutrients, vitamins, minerals, and fiber using ordinary food, plus substituting Maltodextrin, an artificial glucose polymer that occurs in EEN as the most common carbohydrate [143]. Five children with CD received CD-TREAT, out of these four (80%) showed clinical response and three (60%) entered remission with significant decreases in FC (mean decrease 918 ± 555 mg/kg; P=0.002). The study's overall conclusion implicates CD-TREAT diet reduced gut inflammation (evaluated by FC) and replicated EEN changes in the microbiome [143].

1.6.2 Biologics treatment

Biologics became a new potent treatment option for IBD patients within the last two decades. Three main categories of biological treatment exist. The first group involves:

anti-tumor-necrosis factor alpha agents (anti-TNF-alpha) infliximab (IFX), adalimumab (ADA), certulizumab or golimumab, followed by the second group of anti-integrin agents: natalizumab - anti integrin alpha 4, vedolizumab - anti integrin alpha 4/beta 7, and the newest development: anti interleukin 12/interleukin 23 agent: ustekinumab [144]. In children and adolescents with CD only infliximab and adalimumab have been licensed. This thesis will focus on treatment with anti-TNF-alpha in CD children. Infliximab is a chimeric protein that contains mouse-derived amino acids and it was licensed for CD in 1998 [145]. According to European consensus guidelines for pediatric CD patients, the indication of anti-TNF-alpha treatment is given in children with chronically active luminal CD despite prior optimized immunomodulatory therapy or in cases of active steroid-refractory disease to induce remission [106]. Further indications for anti-TNF-alpha treatment as first line therapy are active perianal disease in combination with surgical intervention and severe extraintestinal involvement [106]. Contraindications for treatment with anti TNF alpha drugs are patients with heart failure (NYHA III/IV), with evidence of active tuberculosis, uncontrolled HIV infection , endemic mycosis, multiple sclerosis or acute infection with Clostridium difficile or cytomegalovirus [146].

1.6.2.1 Drug monitoring and dosing of anti-TNF-alpha treatment

Therapeutic drug monitoring of biologics is essential for treatment optimization and reduces the risk of developing complications in PIBD. Dose adjustment in PCD patients with suboptimal trough levels and/or development of anti-drug antibodies lead to a significantly increased rate of clinical remission, fewer patients with loss of response and to fewer complications in PCD patients [147-151]. The use of concomitant immunomodulatory treatment with methotrexate or azathioprine reduces the formation of antibodies, therefore less immunogenic response and better drug response [152]. For pediatric CD patients IFX infusions should be given with a dose of 5 mg/kg body weight at weeks 0, 2, 6, followed by maintenance therapy with 5 mg/kg every 8 weeks [106]. There is increasing evidence that children with a body weight below 30 kg or those with low albumin serum levels need higher doses up to 10 mg/kg and/or shorter administration intervals for induction therapy. The same is true for patients losing response to IFX because of low drug level. For mucosal healing a trough level for IFX should be $\geq$ 5 µg/ml, in case of perianal fistula up even around 12 µg/ml [153]. A similar regime exists for the humanized anti-TNF-alpha treatment, Adalimumab (ADA). ADA

treatment for patients >40 kg starts with 2.4 mg/kg, followed by 1.2 mg/kg after 2 weeks, continued with 0.6 mg/kg every two weeks. Patients <40 kg treatment regime is 80mg, 40mg and 20mg every two weeks [106]. CD patients with ADA through level >8.5 µg/ml present a better response compared to earlier suggested cut-offs (>5 µg/ml) [147]. Weekly injections should be considered in patients with a trough level <8.5 µg/ml or losing response.

2. Objectives of the thesis

This present thesis is based on two studies, (1) retrospective data analyses and (2) an interventional non-randomized controlled trial. Both studies were published in peer-reviewed journals. I have co-authored in two further publications in the ImageKids trial, which are not considered for this thesis. Both have been published in the Journal for Pediatric Gastroenterology, and listed under my publications.

- **Publication I:** "Incidence and risk factors for perianal disease in pediatric Crohn's disease patients followed in CEDATA-GPGE registry" *(published in J Pediatr Gastroenterol Nutr. 2018 Jan; 66(1):73-78; PMID: 28604511)*
- **Publication II:** "Partial enteral nutrition has no benefit on bone health but improves growth in pediatric patients with quiescent or mild Crohn's disease "*(accepted for publication in Clin Nutr. J., April 2020)*

The aim of this thesis is to increase the understanding of the development of short- and long-term complications in children with IBD and to gain insights into potential risk and preventive factors in order to overcome these complications. CD patients are more likely to experience complications during the course of the disease compared to patients with ulcerative colitis (UC). We will primarily focus on CD patients and highlight the most frequent complications, which include perianal disease, low bone density and growth delay.

3. Summary

Paper I: “Incidence and risk factors for perianal disease in pediatric Crohn's disease patients followed in CEDATA-GPGE registry”

Patients are adversely affected by perianal fistulas and abscesses while suffering from secretion and pain [93, 154]. There is limited knowledge on perianal disease in pediatric CD patients with respect to basic epidemiologic data, diagnostic, and therapeutic management. The first paper aimed to evaluate the prevalence, incidence during follow up, risk factors for developing perianal disease, and PD management in a large PCD cohort. Data was obtained from the CEDATA-GPGE registry, which is a multicenter registry for pediatric IBD patients in Germany and Austria, established by the German Society for Pediatric Gastroenterology and Nutrition (GPGE). Additional questionnaires were sent to participating CEDATA sites to collect data on current PD treatment strategies in Germany. Our data analyses revealed a 5.5% prevalence of PD in newly diagnosed CD patients (N=742) and a cumulative incidence of 10% within the first three years after diagnosis. Male patients have a three times greater risk for the development of PD compared to girls and should be monitored closely. The use of steroids increased the risk of developing PD, whereas rectal disease involvement had no influence. According to our additional collected data via questionnaire only 57% of affected patients (N=46) had received a complete diagnostic workup for PD (pelvic flor MRI plus lower endoscopy with examination under anesthesia) as recommended in the international guidelines [155].

Paper II: “Partial enteral nutrition has no benefit on bone health but improves growth in pediatric patients with quiescent or mild Crohn’s disease “

Impaired bone health, decreased muscle mass, and delayed growth and pubertal development are common complications and difficult to treat in pediatric patients with Crohn’s disease (CD). Patients with active CD present a low trabecular and cortical density at initial CD diagnosis, growth delay is an ongoing complication during disease follow up [97]. Exclusive enteral nutrition (EEN) has been shown to improve nutritional status and bone health and induces growth in pediatric CD [17]. Exclusive nutrition with complete formula is highly demanding and therefore EEN is not considered as long-term treatment.

The second paper prospectively evaluated the use of partial enteral nutrition (PEN) providing 25% of daily energy intake for 12 months to improve bone and muscle development and to support growth in pediatric patients with quiescent CD. In this non-randomized controlled intervention study including 41 patients, PEN improve bone-muscle geometry over the 12 months intervention compared to the control group. However, we observed a trend for improved muscle cross sectional area and improved grip strength. This suggests that PEN may improve the nutritional status in pediatric CD patients with an increased lean body mass. The second aim of this study addressed the question whether PEN improves growth velocity in patients with quiescent disease activity. We found that in patients with well-controlled inflammation PEN over 12 months led to catch up growth those with a potential to grow at early stage of puberty (Tanner stages ≤3). Furthermore, we evaluated the effect of PEN on the intestinal microbiome and plasma metabolome and detected small effects on the microbial ecosystems but changes in the composition of the plasma metabolome. However, the proof of this affect requires further investigations in larger cohorts. Partial enteral nutrition did not have any adverse effects, was well accepted and could be implemented into clinical practice in selected patients.

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5. Publication I

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Incidence and risk factors for perianal disease in pediatric Crohn's disease patients followed in CEDATA-GPGE registry

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AB developed the study concept, performed statistical analysis, and wrote the manuscript.

KJW developed the study concept and assisted in writing the manuscript.

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Abstract

Background and Aims: Perianal disease (PD), comprising fistula and abscess, is a severe complication in Crohn's disease (CD). We examined prevalence, incidence and risk factors for PD development in a pediatric CD cohort.

Methods: CD patients from the prospective, multi-center registry CEDATA-GPGE for pediatric inflammatory bowel disease (IBD) in Germany and Austria were included if diagnosed ≤18 years, registered within three months of diagnosis and having at least two follow-up visits within the first year of registration. We examined potential risk factors for PD with Kaplan-Meier analysis and a final cox model considering sex, family history of IBD, extraintestinal manifestations, disease localization, induction therapy (corticosteroids or nutritional therapy).

Results: Of 2406 CD patients, 742 fulfilled inclusion criteria (59% male, mean age at diagnosis 12.4 ± 3.4 years). PD was present at diagnosis in 41 patients (5.5%; 80.9% male) while 32 (4.3%, 81.3% male) developed PD during follow up (mean 2.0 ±1.6 years). The cumulative incidence of PD 12 and 36 months after diagnosis was 3.5% and 7.5%, respectively. Potential risk factors for PD development during follow up were male sex (HR=3.2, [95%; CI 1.2-7.8]) and induction therapy with corticosteroids (HR=2.5 [1.1-5.5]). A guideline conform diagnostic work up for PD was performed in 60%. In half of the patient's PD resolved after one year.

Conclusion: About 10% of CD patients in our cohort suffer from PD within the first three years of their disease. Male sex and initial corticosteroid-therapy were associated with increased risk to develop this complication after diagnosis.

Key words: perianal disease, prevalence, corticosteroids

What is known/what is new:

What is known:

- Perianal disease defined as fistula and abscess formation is a serious complication and difficult to treat
- The reported prevalence of perianal disease in newly diagnosed pediatric CD patients varies from 8% to 15%, only few data on incidence after diagnosis are available.
- Corticosteroids do not induce mucosal healing in Crohn’s disease.

What is new:

- Prospective data on prevalence perianal disease, incidence during follow up and potential risk-factors in pediatric Crohn’s disease in a large cohort.
- Male sex being associated with a three times higher risk for developing perianal disease.
- Use of corticosteroids as induction therapy after diagnosis was found to be associated with the development of perianal disease during follow up.

List of abbreviations

PD- Perianal Disease

IBD- Inflammatory Bowel Disease

CD- Crohn's disease

UC- Ulcerative Colitis

IBD-U- Inflammatory Bowel Disease Unclassified

PCDAI- Pediatric Crohn's disease Activity Index

ECCO- European Crohn's and Colitis Organization

GPGE- German Society for Pediatric Gastroenterology and Nutrition

ESPGHAN- European Society for Paediatric Gastroenterology Hepatology and Nutrition

IFX- Infliximab

EEN- Exclusive Enteral Nutrition

6MMP- 6 - Methyl-Mercaptopurine

Aza- Azathioprine

MTX- Methotrexate

EM- Extraintestinal Manifestation

Introduction

Perianal disease (PD) with fistula and abscess formation is a serious clinical problem in patients with Crohn's disease (CD). Perianal fistulas are the most common manifestation of fistulising CD.[1,2] The development of PD can appear at any time during the course of disease and may be the initial manifestation leading to the diagnosis of CD.[3] Patients with PD suffer from pain and secretion. Fecal incontinence can be a long-term risk. The reported prevalence of PD in newly diagnosed pediatric CD patients varies from 8% to 15%.[4,5,6] Long term adult data on the cumulative incidence of PD in CD cohort from the US and New Zealand shows that 20-28% developed PD within 20 years.[2,3] The European Crohn's and Colitis Organization (ECCO) - guidelines recommend a full diagnostic assessment in all pediatric CD patients presenting with PD and to tailor treatment accordingly.[7] Therapeutic options especially for transsphincteric or complex fistulas are very limited. Treatment often comprises of a combination of pharmaceutical and surgical measures and depends on the inflammatory activity, localization and course of PD.[8] The identification of risk factors would allow choosing the best treatment to reduce the risk of this serious complication and provide stratification options for screening and patient education. Limited long-term data are available in pediatric CD patients on the incidence of PD after diagnosis, potential risk factors, management and outcome.

The primary objective of this project was to examine the prevalence of PD at initial CD diagnosis, the incidence during follow up and to determine potential risk factors for the development of PD in an unselected group of pediatric CD patients followed in the CEDATA-German Society for Pediatric Gastroenterology and Nutrition (GPGE) registry. A secondary aim was to investigate whether physicians in Germany and Austria follow the guidelines for the PD management in their patients.

Materials and Methods

We retrospectively analyzed data of CD patients that were entered into the German/Austrian registry CEDATA-GPGE within three months after diagnosis and followed prospectively, between April 2004-April 2014.The CEDATA-GPGE registry is a prospective, multi-center registry for pediatric IBD patients in Germany and Austria, established in 2004 by the GPGE. The 96 reporting institutions range from large Universities with >200 IBD patients to small regional hospitals.[9] Data were collected by two standardized questionnaires: one at baseline and one at follow-up visits with at least two documentations per year.

Reporting physicians were asked to complete the Baseline Case Report Form (CRF) and the first follow up CRF at the time of patient inclusion into the registry. The Baseline CRF includes general patient characteristics, onset and type of symptoms before diagnosis, date and type (CD, UC, IBD-U) of final diagnosis, extraintestinal manifestations and family history for IBD. The follow up CRF comprises data since the last documented visit on medical history including symptoms, stool behavior and extraintestinal manifestations, anthropometrics, physical examination including perianal findings, laboratory parameters, diagnostic findings (endoscopy, histology, imaging), surgical procedures, disease localization and severity, complications (fistula, abscess, stenosis and other), comorbidities, and treatment (antibiotics, IBD-specific drugs, vitamins, and supplements). Exclusive enteral nutrition as treatment option was only assessed from 2008 onwards in the registry.

Between 2004 and 2013, the institutions submitted patient’s data via hard copy CRFs and since August 2013 until censoring of the data for this analysis in April 2014 online CRFs. Due to financial constraints, enrollment of new patients was interrupted between

2010 and August 2013, while previously entered patients continued to have follow up CRFs.

Eligibility

For this study we included only CD patients fulfilling the following criteria: age at initial CD diagnosis ≤18 years, registry enrollment within three months after CD diagnosis, submission of at least two follow up CRFs in the first year with a maximum time gap of 200 days between baseline and first follow up CRF, between the consecutive follow up CRFs Eligible patients were censored if the time interval between the consecutive follow up CRFs after the first year was >200 days.

Definitions

Perianal disease (PD) was defined as perianal fistula and/or perianal abscess visualized during clinical examination or imaging. According to the perianal findings, we characterized three groups of patients:

1.) Patients with PD at initial diagnosis had a documented PD in the first CRF (prevalence at diagnosis; group 1).

2.) Patients developing PD during the course of disease had no PD documented in first CRF and a documented PD thereafter (incidence of new PD after diagnosis; group 2)

3.) Patients without PD had no documentation of PD in any CRF (no PD; group 3).

Family history of IBD included any occurrence of inflammatory bowel disease (IBD) in biological relatives of the patient. Extraintestinal manifestations comprises skin manifestations (erythema nodosum, pyoderma gangrenosum), fever (>38.5 Celsius >3 days without other focus), hepatobiliary (primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) or overlap syndrome), pancreatic, renal or

ophthalmological complications, arthralgia or arthritis of peripheral joints and the spine. Disease localization was defined according to the Paris classification with separate recording of rectal involvement.[10] Exclusive enteral nutrition therapy (EEN) was considered as exclusive feeding with a balanced elemental or whole protein formula for at least 4 weeks. Corticosteroids as initial therapy included prednisolone, methylprednisolone, or budesonide given orally, intravenously, and rectally. Immunomodulators included azathioprine, 6-mercaptopurine and methotrexate.

A complete diagnostic workup for suspected PD included pelvic MRI or trans-anorectal ultrasonography, colonoscopy or proctosigmoidoscopy, and manual examination under anesthesia by a colorectal surgeon.[7]

The diagnostic latency was defined as time between the age at onset of symptoms and the age of diagnosis**.** Physician's global assessment and a weighted pediatric Crohn's disease activity index (wPCDAI) were incompletely available at CD diagnosis and therefore not considered in our analysis.

Additional questionnaire for PD management and treatment

For all identified PD patients fulfilling the inclusion criteria, a questionnaire was sent to the respective institutions to confirm and provide additional information, particular regarding initial induction therapy, diagnostic and therapeutic management and outcome of PD during follow up.

Statistical Considerations

Descriptive analyses of the cohort and the three patients' subgroups were given as percentages with mean ± standard deviation (SD) or median (min; max) where appropriate. Chi-square and the non-parametric Kruskal-Wallis test were applied for

group comparison. Time until event was calculated by time in months from diagnosis of CD to first follow-up with documented PD, and in case of non-event censored by last follow-up. Kaplan Meier curves were performed and show the survival distribution function of the event (PD). The cumulative incidence is the difference of the curve to 1 and involved only patients with developing PD during follow up. Kaplan Meier curves were applied for the comparison of incidental PD cases and patients without PD, to identify potential risk factors for the development of PD such as sex, positive family history for IBD, extraintestinal manifestation at diagnosis, age at initial CD diagnosis, rectal disease involvement at diagnosis, disease location and corticosteroids or EEN within the first 3 months as induction therapy. For the comparison of Kaplan Meier curves the non-parametric Log Rank test was selected. For multiple analyses Cox proportional hazards models were applied and hazard rate ratios were calculated. To identify the potential risk factors, a backward selection was used starting with the previously by Kaplan Meier estimates recognized (Log Rank with p-value < 0.05) potential risk factors, which fulfilled the proportional hazard assumption. Before starting selection procedure all possible pairwise interactions were tested. The final model was found by stepwise backward elimination, decision by Wald Chi-square or by clinical importance. The statistical significance was defined as $P < 0.05$. Items with missing values of more than 30% were not considered as variable in our analysis. The statistical software SAS Enterprise for Windows, Release 9.2 (SAS Institute, Cary; NC) was used for this study.

Ethical Statement

The ethical commissions of the respective participating institutions approved the registry and all amendments. Age appropriate informed consent was signed by children

(starting at age 6) and their parents before inclusion. All data were submitted in a pseudonymized form with respective data security regulations being followed.

Results

In total, 4240 IBD patients, reported by 96 clinical institutions, were included in the CEDATA-GPGE registry until March 2014 (55.1% male, median age at diagnosis of 13.7 years (range 0.3-22.9), 2406 patients were diagnosed with CD (58.4% male; median age at diagnosis 13.9 years; range, 0.2-22.9). Of the CD patients, 742 fulfilled all inclusion criteria and were reported by 70 of 96 participating clinical institutions, (Figure 1). Basic characteristics of this final cohort are summarized in table 1, stratified for PD status.

Out of the 742 patients, 41 (5.5%) had a PD documented at initial CD diagnosis. Further 32 patients (4.3%) developed this complication after initial diagnosis. Of these 73 affected patients, 55 were identified with a perianal fistula, 33 with a perianal abscess and thereof 15 patients who were affected by both.

The cumulative incidence of new PD development during follow up within the first 12 months after CD diagnosis was 3.5% and until 36 months 7.5%. Most patients (90.0%; N=32) developed PD within the first 1.5 years of initial diagnosis. The median time of developing PD after initial CD diagnosis was 0.7 years (range, 0.2-4.6). Including patients with PD at diagnosis, the cumulative incidence of PD at 1 year after CD diagnosis was 9%.

Table 1 shows the patients characteristics in the different subgroups. Boys were more likely to be affected by PD than girls (p=0.006). Rectal inflammation at initial diagnosis was more frequent in patients with presence of PD at initial CD diagnosis (70.7%, n=41) compared to patients with PD developing during follow up (56.3%, n=32, n.s.)

and to patients without PD (49.4%, n=669, p=0.01). There were no differences among the patient groups regarding disease localization, age at diagnosis and positive family history for IBD.

Induction therapy used in the three subgroups and the total cohort is shown in table 2. More than half of patients in the cohort (55.6%) were initially treated with corticosteroids with a higher proportion in patients developing PD during follow up (75.0%; p=0.02). EEN therapy was applied in 80 out of 335 (23.9%) patients enrolled after 2008 and in 4/10 of patients developing PD during follow up. Azathioprine was induced more often in patients with developing PD than in those without PD (62.5% vs. 37.8%; p=0.0007).

Log rank tests in Kaplan Meier indicated the following two variables as potential risk factors for developing PD: male sex (log-rank=7.6; p=0.006) and the initial induction therapy with corticosteroids (log-rank=5.5; p=0.018), (Figures 2a, 2b).

No significant associations with later PD were found for positive family history of IBD, extraintestinal manifestation, rectal disease localization and age at initial CD diagnosis.

In a backward selection of the multiple Cox proportional hazards analysis, the effects of male sex and induction therapy with corticosteroids steroids were confirmed as significantly related to PD development. Testing for pairwise interactions between the given variables in the cox regression, no modification effect was found.

Hazard rate ratios of the resulting risk factors revealed that the risk of developing PD was three times higher in males than in females (HR=3.2, [95%; CI 1.2-7.8]). Induction therapy with corticosteroids within the first three months of diagnosis was associated with doubling the risk for occurrence of PD in pediatric CD patients (HR=2.5 [1.1-5.5]).

Management of PD

The additional questionnaire on PD management was returned by 46 of 73 PD patients, thereof 32 were diagnosed with PD at initial CD diagnosis. A complete diagnostic workup was performed in 57% (N=46): MRI in 67%, a sigmoidoscopy or colonoscopy in 82% and an anal sonography in 10% of patients. Information from the additional questionnaire confirmed the registry data, that two thirds of patients with PD developing after diagnosis were treated with steroids as induction therapy (74%, N=46). After diagnosis of PD, a wide variety of drugs were initiated with half of the patients receiving antibiotics and/or Aza/6MP each, another 26% biologicals, mostly infliximab, and 19 % received systemic steroids. More than half of the patients (52.2%) underwent surgical interventions. Out of them, 17 patients had an abscess incision, nine fistula incision, four drainage with seton insertion, and six patients had at least two of those interventions combined. One patient received a colostomy. After one year of PD occurrence complete healing was reported in half of the patients (51%), 25% recovered between one and two years, 12% after two years. In the remaining 12% of patients, PD did not resolve until the end of this survey. Of those, three patients received biologics in combination with rectal steroids or with Aza/6MP and one of them also had a surgical intervention with drainage. Another patient was treated with antibiotics, Aza/6MP, nutrition therapy and drainage, and the last patient had no medical treatment but abscess incision. Information on relapses after initial healing was available in 37 from 46 patients. Of these patients, 19% had relapses. Four patients received antibiotics combined with Aza/6MP or methotrexate, one infliximab combined with Aza/6MP and antibiotics, one only Aza/6MP and another one did not receive any therapy.

Discussion

In this pediatric cohort of 742 newly diagnosed CD patients, about 10% suffer from PD within the first three years after CD diagnosis. Boys had a three times higher risk for developing PD than girls. The use of corticosteroids as induction therapy after diagnosis was associated with the development of PD during follow up.

Our cohort seems representative with similar age and sex distribution compared to all CD patients in the registry. The male dominance is in concordance with figures reported from a large international prospective European registry (EuroKids) of 1221 newly diagnosed patients with IBD of which also 59% of the CD patients were boys.[11]

To avoid bias, we excluded patients with enrollment more than three months after initial CD diagnosis or less than two follow up documentations in the first year. The prevalence of PD at diagnosis (5.5%) is lower compared to the reported prevalence of 9% from the EuroKids registry.[10] Keljo et al. revealed a 10% prevalence of PD (perianal fistula and abscess) at diagnosis in 287 newly diagnosed pediatric CD patients, prospectively collected from the Pediatric IBD Collaborative Group.[12] A selection bias of more complicated cases reported from large IBD centers cannot be excluded. In contrast, our patients were diagnosed in 70 reporting sites including small hospitals and practices. A varying diagnostic latency due to different access to health care resources and specialists in the different countries may also explain differences in the cohorts. Our data revealed an incidence of 9.0% at one year, including prevalent cases at CD diagnosis. Data from Guptas et al. reported a similar incidence of penetrating disease of 8.2% at 12 months after diagnosis in pediatric CD patients from the US (N=939), also including patients with the event at diagnosis.[13] However, this cohort focused on penetrating complications and not only on perianal abscess and fistula. Data from adult cohorts in the US (N=179) and New Zealand (N=715) reported a 20%

to 28% cumulative incidence of developing PD within 20 years of initial CD diagnosis, including patients with PD at diagnosis.[2,3] Since no long-term data from pediatric onset CD on the development of PD are available it remains unresolved if age of onset has an influence on developing this complication over time. Our data did not show any association between age at initial diagnosis and PD within the pediatric range.

Disease localization did not differ between patients with PD at diagnosis, PD developing during follow up and no PD. Our data showed that a high percentage (71%) of patients with PD at diagnosis had rectal involvement which is in agreement with previous studies in adults.[1] However, no significant difference was found between children who developed PD during follow up and the remaining patients with no PD (both 47%). We also found no association with family history for IBD and the presence or absence of PD. Extraintestinal manifestations (EM) at initial diagnosis occurred in 29% of our cohort which is similar to other pediatric CD studies reports. [14,15,16] No significant association between extraintestinal manifestation and PD development was seen in our data.

In our cohort, male pediatric CD patients had a three times greater risk for developing PD than girls. We found corticosteroids as initial induction therapy to be associated with doubling the risk for developing PD in pediatric patients. Pediatric guidelines for CD recommend either corticosteroids or exclusive enteral nutrition as induction therapy in moderate to severe CD. More than half of the cohort received systemic corticosteroids as induction therapy. It is likely that corticosteroids may have been used as initial therapy in children with more severe initial manifestation, and therefore this association would be more a marker for disease severity than a causal factor. The higher frequency of azathioprine treatment in patients with PD may point in the same direction. Unfortunately, the data regarding disease activity (PCDAI and global

assessment) at diagnosis were incomplete and did not allow adjustment for corticosteroids and azathioprine in our analysis.

It has been shown that corticosteroids are not inducing mucosal healing compared to EEN and anti-TNF-alpha agents. [17,18,19] Therefore, they are a theoretical risk factor for infections including abscesses. Although it is biological plausible, only randomized intervention trials will prove a cause relationship if early treatment with corticosteroids is a risk factor for development of PD. Since EEN was assessed in our registry only after 2008 with only 23.9 % of them receiving it as induction therapy (N=80/335) we were not able to estimate its potential effects on PD development.

The additional survey on PD management showed that Infliximab (IFX) therapy was rarely used as initial therapy. The drug was licensed in September 2007 for children who had failed corticosteroids and other immunosuppressant drugs. At the time of license, almost 50% of the cohort was included. These legal limitations may explain why only 26% of children with PD received biologicals, which are now recommended as first line therapy in conjunction with surgical interventions (drainage of abscess, seton placement).[20] Antibiotics were the most commonly used medications for the treatment of PD while surprisingly 20 % received steroids.

In contrast to previous publications, the strength of this study was the prospectively collected patients' follow up data since 2004 with a high number of unselected newly diagnosed CD patients submitted by different types of clinical settings ranging from small standard care hospitals to university clinics.

The estimation of the cumulative incidence is limited due to short patient follow up data with a median of 1.6 years. Another limitation was the fact that we could include neither the Pediatric Crohn's disease Activity Index (PCDAI) nor the physician global

assessment into our final cox regression model due to an incomplete reporting of relevant data.

In spite of these limitations, our data are relevant for the management of pediatric CD. Since the majority of incident PD occurred within the first 18 months of follow up close, monitoring regarding PD development is warranted after diagnosis in pediatric CD patients, particularly boys. Further data are needed to decide whether initial induction therapy with EEN is superior to corticosteroids to reduce the risk for PD development.

Table 1: Patients' characteristics at initial diagnosis

Patients characteristics at initial diagnosis	Patients with PD at diagnosis (Group 1)	Patients with PD developing during follow up (Group 2)	Patients with no PD (Group 3)	All patients
	(N=40-41)	(N=30-32)	(N=661-669)	(N=733-742)
Age at diagnosis (mean ±SD)	12.3 ± 2.6	13.4 ±2.8	12.4 ± 3.5	12.3 ± 3.3
Male in %	78.1*	81.3*	56.6	59.0
Follow up time in the registry in years (median, range)	1.4 (0.09-6.0)	1.6 (0.2-7.0)	1.1 (0.09-7.1)	1.2 (0.1-7.3)
Positive family history for IBD in %	12.2	15.6	17.0	16.6
Extraintestinal manifestation within first 3 months of diagnosis in %	24.4	40.6	28.7	28.9
Disease localization at baseline (Paris classification) in %				
L1	2.4	9.4	6.9	6.7
L1L4	2.4	3.1	4.6	4.5
L2	4.9	12.5	13.3	12.8
L2L4	19.5	9.4	8.2	8.9
L3	17.1	9.4	22.1	21.3
L3L4	39.0	40.6	28.8	29.9
L4	2.5	0	1.2	1.2
Not available	12.2	15.6	14.9	14.7
Rectal manifestation within first 3 months of diagnosis	70.7*	56.3	49.4	50.9
Diagnostic latency in years (median, range)	0.4 (0.0-3.8)	0.3 (0.0-5.6)	0.4 (0.0-9.0)	0.4 (0.0-9.0)

*p<0.05 versus group 3

Table 2: Induction therapy in pediatric Crohn's disease patients

Induction therapy (within 3 month after diagnosis) in %	**Patients with PD at diagnosis (N=40-41) (Group 1)**	**Patients with PD developing during follow up (N=29-32) (Group 2)**	**Patients with no PD (N=611-669) (Group 3)**	**All patients (N=680-742)**
Exclusive Enteral Nutrition from Jan 2008, N= 10-335)	27.3	40.0	23.3	23.9
Corticosteroids	65.9	75.0*	54.1	55.6
Antibiotics	58.5** $^{+}$	18.8**	12.2	15.1
Methotrexate	0.0	6.9	0.0	1.5
Immunomodulator (Aza/6MP)	63.4*	62.5*	37.8	40.3%
Infliximab	2.5	3.5	1.8	1.9

*Significant <0.05 versus group 3; ** Significant <0.0001 versus group 3; $^{+}$ Significant <0.05 group 1 versus group 2

Figure 1: Patients Flow Chart

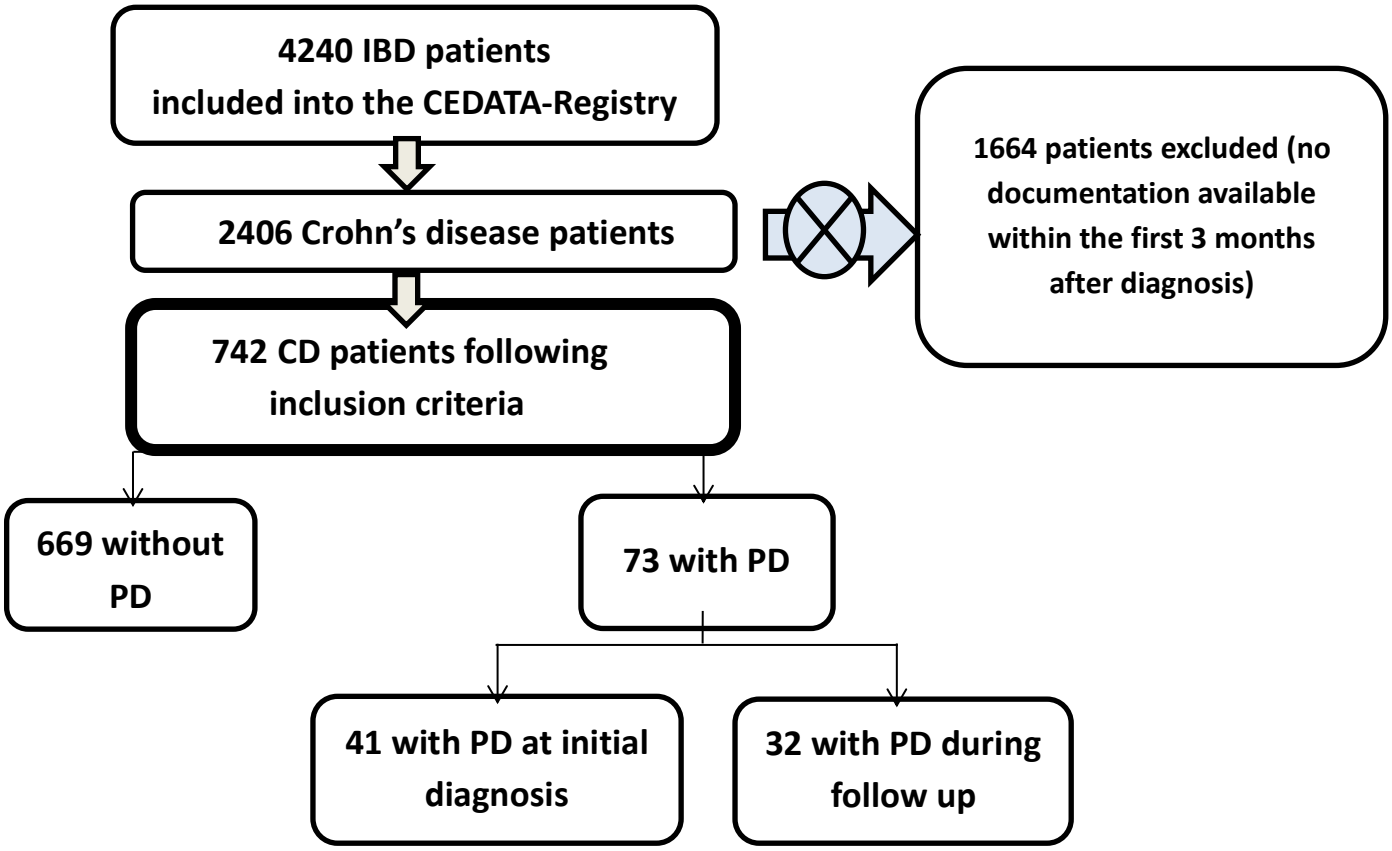


Figure 2a: Kaplan Meier of development PD stratified by sex in pediatric CD patients over time in months (N=701). Abbreviations: m= male, w=female

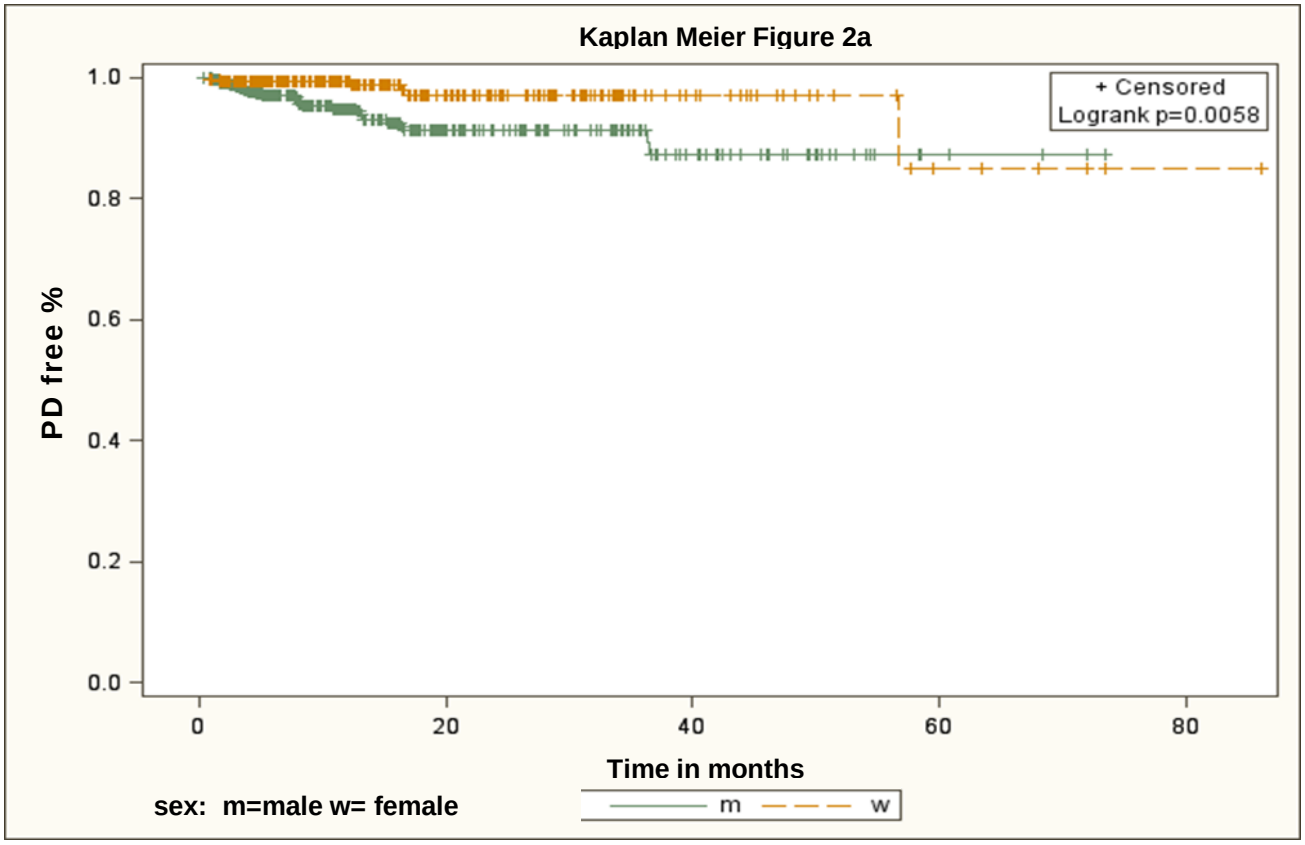
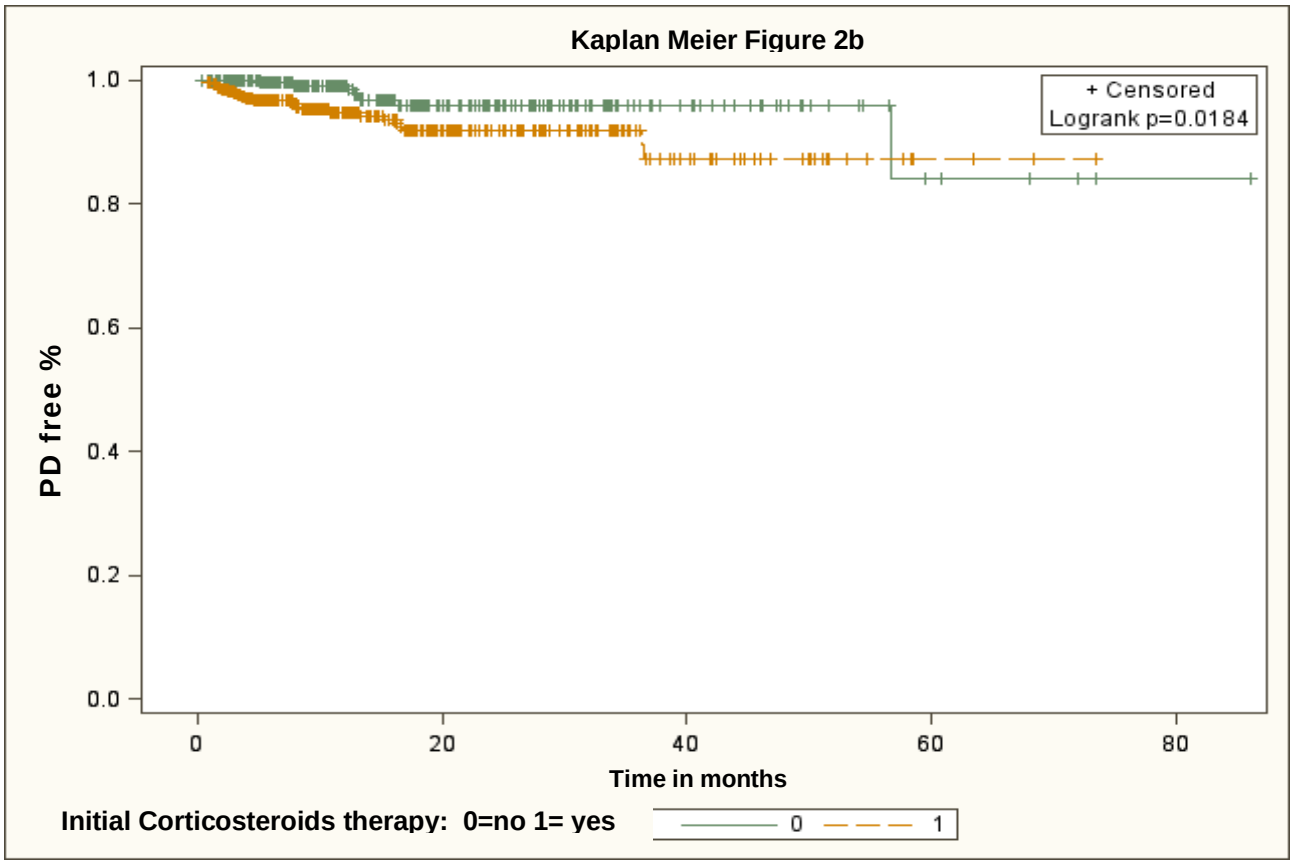


Figure 2b: Kaplan Meier of development PD stratified by induction therapy with corticosteroids within the first three months after diagnosis in pediatric CD patients over time in months (N=701).



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6. Publication II

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Partial enteral nutrition has no benefit on bone health but improves growth in paediatric patients with quiescent or mild Crohn's disease

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Abstract

Background and aims: Exclusive enteral nutrition induces remission, improves bone health and growth in paediatric Crohn's disease (CD) patients, but is highly demanding for patients. We investigated efficacy of partial enteral nutrition (PEN) on bone health, growth and course in CD patients and assessed microbial and metabolic changes induced by PEN.

Methods: We performed a two centre, non-randomized controlled intervention study in quiescent CD patients aged <18 years. Patients in intervention group received a liquid formula providing ~25% of daily energy for one year. At baseline, after 3, 6, 9 and 12 months, we collected data on bone, muscle (peripheral quantitative computertomography), anthropometry, disease activity (weighted paediatric CD activity index), metabolomic profile (liquid chromatography mass spectrometry), and faecal microbiome (16S rRNA gene sequencing).

Results: Of 41 CD patients, 22 received the intervention (PEN) (mean age 15.0 ± 1.9 years, 55% male), 19 served as controls (non-PEN) (12.8 ± 3.1 years, 58% male). At baseline, mean bone quality was comparable to reference population with no improvement during the intervention. Relapse rate was low (8/41, PEN 4/22 and non-PEN 4/19, ns). PEN was not associated with microbiota community changes (beta diversity) but significantly reduced species diversity. Metabolome changes with upregulation of phosphatidylcholines in PEN patients are likely related to lipid and fatty acid composition of the formula. PEN significantly improved growth in a subgroup with Tanner stage 1-3.

Conclusion: In our cohort of paediatric CD patients, PEN did not affect bone health but improved growth in patients with a potential to grow.

Keywords: Paediatric inflammatory bowel disease, enteral nutrition, bone and muscle geometry, microbiome, metabolome

INTRODUCTION

Crohn's disease (CD) during childhood and adolescence puts normal growth and development of the musculoskeletal system at risk (1). Impaired bone health, sarcopenia and growth deficits are frequently observed at diagnosis. Despite improvements after therapy initiation, sustained deficits persist. Therefore, additional strategies to improve these outcomes are warranted (2-4).

In paediatric CD patients, the bone development is disturbed and characterized by low trabecular and high cortical bone density, often along with reduced muscle mass (5). Consequently, paediatric CD patients may be at increased risk of osteopenia and potentially osteoporosis later in life (6). Furthermore, growth delay affects around 15-40% of patients with paediatric-onset CD at diagnosis and negatively impacts on final adult height (7). Both, bone deficits and growth stunting have been attributed to inflammatory cytokines, malnutrition and use of corticosteroids. In addition, reduced muscle mass and low physical activity are risk factors for impaired bone health as mechanical loads are crucial for an appropriate development of bone strength (8).

Exclusive enteral nutrition (EEN) is a highly effective treatment for inducing remission in paediatric CD patients, with a favourable adverse effect profile (9, 10). EEN provides improved nutritional supply thereby contributing to correcting micronutrient and caloric deficiencies. The beneficial effect on growth has been demonstrated in retrospective as well as in prospective studies (11-13). Furthermore, a small prospective study with newly diagnosed CD patients showed improved bone and muscle parameters after 8 weeks of EEN therapy (11). The exact mechanisms of action of EEN in CD remains unclear. We and others have shown that EEN is associated with changes in microbial signatures but causality behind these changes in the microbial ecosystems on outcomes has not been established (14-18).

Due to the highly demanding adherence to exclusive formula feeding, EEN is not considered a long-term maintenance treatment. Other forms of dietary intervention have been evaluated, including partial enteral nutrition (PEN) or cyclic EEN. In a retrospective study, Wilschanski et al. reported that nocturnal supplementary enteral nutrition provided to patients after achieving remission improved linear growth and prolonged remission (19). A Canadian prospective study used intermittent elemental EEN one out of four months and demonstrated significant height and weight gain in the treated group vs. controls (20). Based on these data the recent ECCO/ESPGHAN

consensus guidelines considered intermittent courses of EEN or PEN as potentially beneficial for paediatric CD patients with impaired growth (10). More recently, two novel dietary interventions have been reported, including CD exclusion diet with PEN (21-23) and an ordinary food-based diet replicating EEN (24) but long-term efficacy so far has not been established.

We aimed to determine whether PEN without exclusion diet improves bone and muscle development as well as growth in a prospective cohort of paediatric CD patients with quiescent or mild inflammation. We also explored the effects of PEN on intestinal microbiome composition and plasma metabolomics.

MATERIAL AND METHODS

Study design

We performed a two centre, non-randomized controlled intervention study in CD patients aged 6-19 years who were in remission (wPCDAI<12.5) or had mild disease activity (12.5≤ wPCDAI ≤40) based on mathematically weighted paediatric Crohn's Disease Activity Index (wPCDAI) (25). The study was originally designed as randomized controlled trial. However, during the recruitment of the first patients it turned out that randomization to PEN or control was not feasible due to individual patients' strong preferences based on their previous experience with the formula ("I liked it" or "I hated it"). Therefore, we changed the design to a non-randomized controlled trial two months after study start to achieve inclusion of a sufficient number of patients in each arm. Patients were recruited between 02/2016 and 03/2017 in the Departments of Paediatric Gastroenterology of the two University hospitals in Munich (LMU Munich and Technical University Munich). Patients were offered PEN with a casein based complete liquid formula (Modulen® IBD, Nestlé, Frankfurt/Main, Germany) that substituted ~25% of daily energy intake for 12 months in order to improve bone and muscle parameters. Patients accepting this offer represent the intervention group. The control group received no nutritional intervention. All IBD patients received recommendations on a healthy and balanced diet (26) as part our routine clinical care at time of IBD diagnosis. Both groups continued their medical maintenance treatment. Patients were evaluated at baseline and after 3, 6, 9 and 12 months. Adherence with PEN was followed by regular prescription of formula and PEN intake was evaluated at each visit by study team. Patients were asked to provide 3-days food records for the three days prior baseline, visit month 6, and visit month 12. Food records were analysed by a nutritional scientist with the software PRODI® expert 6.5 (Nutri-Science Stuttgart, Germany).

The primary endpoints of this study were improvement of bone and muscle parameters assessed by peripheral quantitative computer tomography (pQCT) and growth (improvement of z-scores for height). The latter was restricted to patients with growth potential defined as pre- or early puberty equivalent to Tanner 1-3 at study inclusion. Secondary endpoints included changes in disease activity, inflammatory parameters, microbiota composition, plasma metabolites and number of relapses. Relapse was defined as increase in wPCDAI ≥12.5 points during follow up in combination with a

parallel rise in systemic or faecal inflammation markers (CRP, ESR and/or calprotectin) suggesting relapse and/or additional step up medication.(25)

The original and amended study protocol was reviewed by the Ethical Committees of LMU Munich (Nr. 690-15) and Technical University Munich (316/16 S), and by the Federal Office for Radiation Protection (Z 5-22462/2-2016-030). Written informed consents of patients' parents/caregivers and age-appropriate informed consents of patients have been obtained. The study was registered at the German Clinical Trials Registry (Nr. DRKS00010278).

Anthropometry

Patients' height was measured with a stadiometer. Weight was quantified in underwear on a calibrated scale. Tanner stages in females (27) and males (28) were assessed by treating physicians. Z-scores for height and BMI were calculated based on reference data from the World Health Organization (29). Malnutrition was defined by decreased z-scores for BMI (undernutrition) and height-for-age (stunting) and classified as mild (z-scores −1 to −1.9), moderate (z-scores −2 to −2.9), and severe (z-scores ≤ −3) (30).

Bone and muscle parameters, grip force

Data on bone and muscle parameters were assessed by peripheral quantitative computed tomography (pQCT, XTC-2000 scanner, Stratec, Pforzheim, Germany) at baseline, 6 and 12 months as described by Werkstetter et al. (5). In brief, measurements were performed at the non-dominant forearm with the scanner positioned at 4% and 65% of the forearm length: trabecular density (at 4% forearm length, distal radius: TrbD4), cortical density and thickness (at 65% forearm length, proximal radius: CtD65 and CTth65) and muscle-cross sectional area (at 65% forearm length: muscleCSA65). With a voxel size of 0.4 mm, 2 mm thick single tomographic slices were measured at both positions (distal radius and proximal radius). The software (V 5.50; Stratec) processed images and performed calculations. Results were expressed as age- and sex-specific z-scores based on reference data from 296 healthy German children and adolescents from Neu et al. (31). As the muscleCSA65 depends on body height and some patients showed growth retardation, muscleCSA65 was corrected for height (32).

Maximal isometric grip force of the non-dominant hand was measured by an adjustable-handle Jamar Dynamometer (Preston, Jackson, MI) and age and sex-

matched z-scores were calculated based on reference data from the German DONALD (Dortmund Nutritional and Anthropometric Longitudinally Designed) study (33).

Metabolomics

Plasma samples for metabolomics were stored at -80°C until measurement. A range of 454 metabolites comprising polar lipids (acylcarnitines (Carn), phospholipids (PL) including diacyl-phosphatidylcholines (PCaa), acyl-alkyl-phosphatidylcholines (PCae), sphingomyelins (SM), acyl-lysophosphatidylcholines (LPCa), alkyl-lysophosphatidylcholines (LPCe)), sum of hexoses (H1), amino acids (AA), non-esterified fatty acids (NEFA), keto-acids, and tricarboxylic acid (TCA) cycle metabolites were measured using an established high throughput liquid chromatography mass spectrometry (LC-MS/MS) platform, as previously reported (34-38). A formula CX.Y was assigned for polar lipids and NEFA where X: length of carbon chain, Y: number of double bonds, OH: indicates presence of hydroxyl group. Letters ‘a’ & ‘e’ indicate that the acyl chain is bound via an ester or ether bond to the backbone, respectively. Concentrations were calculated in µmol/l.

High-throughput 16S ribosomal RNA (rRNA) gene sequencing

Faecal samples were collected at home in stool collection tubes with DNA stabilizer (STRATEC Molecular, Germany) (39) after detailed patient instructions. Collected faecal material was immediately submerged in DNA stabilizer and sent at ambient air by mail to our department (average time 2 days). Upon arrival samples were immediately stored at -80C degree until analysis. Metagenomic DNA was extracted from faecal samples using a modified version of the protocol by Godon et al (40). In brief, a buffer containing salt solution was added to faecal samples, and incubated for one hour at 70°C. Sterile silica beads (500 mg, 0.1 mm glass beads, Roth) were used for mechanical lysis of bacterial cells using a FastPrep®-24 bead beater (MP Biomedicals) fitted with a 24 x 2 mL cooling adaptor (3 × 40 seconds at 6.5m/s). After heat treatment (95°C, 5 min) and centrifugation (15.000 x g, 5 min, 4°C), supernatants were treated with RNAse (0.1 µg/µL) for 30 min at 37°C. Metagenomic DNA was purified using NucleoSpin® gDNA cleanup kit (Macherey-Nagel) following the manufacturer’s instructions. Concentrations and purity were controlled using the NanoDrop® system (Thermo Scientific) and samples were stored at 4°C during library preparation and at 20°C thereafter for longer storage.

The V3/V4 region of 16S rRNA genes was amplified (25 cycles) from 24 ng of metagenomic DNA using the bacteria-specific primers 341F and 785R (41) followed by a 2-step procedure to limit amplification bias (42). Amplicons were purified using the AMPure XP system (Beckmann), pooled in an equimolar amount and sequenced in paired-end modus (PE275) using a MiSeq system (Illumina Inc.) following the manufacturer's instructions and a final DNA concentration of 10 pM and 15 % (v/v) PhiX standard library.

Sequence analysis

Raw sequence reads were processed using IMNGS (www.imngs.org) (43), a platform based on UPARSE (44). First, all reads were trimmed to the position of the first base with quality score <3 and then paired. The resulted sequences were size filtered excluding those with assembled size <300 and >600 nucleotides. Paired reads with expected error >3 were further filtered out and the remaining sequences were trimmed by 10 nucleotides on each side to avoid GC bias and non-random base composition. For each sample, sequences were de-replicated and checked for chimeras with UCHIME (45). Sequences from all samples were merged, sorted by abundance, and operational taxonomic units (OTUs) were picked at a threshold of 97% similarity. Finally, all sequences were mapped back to the representative sequences resulting in one OTU table for all samples.

Only those OTUs with a relative abundance above 0.5% total sequences in at least one sample were kept to avoid analysis of spurious OTUs. The most detailed taxonomic classification for each OTU was assigned by RDP classifier (46) and SILVA (SILVA Incremental Aligner) (47). A phylogenetic tree was constructed using the Maximum Likelihood method in MEGA6 (48). Sequence proportions per sample were normalized to the mean sequence depth. For estimation of diversity within samples (alpha-diversity), the Shannon index was calculated and transformed to the corresponding effective number of species as described by (49). Effective species counts reflect better the true diversity within samples when compared with metrics like species richness as they are less affected by the number of rare species. For quantification of distances between microbial profiles (beta-diversity), generalized Unifrac distances (50) were computed and visualized by Metric Multidimensional Scaling (MDS) projections. For downstream processing of the intermediate files

generated by IMNGS, a fully modular R-based pipeline (Rhea) was used for analysis of microbial profiles (51).

Power and Sample size calculation

Based on a previous intervention study from our group, we assumed an increase in muscleCSA65 of +0.5 z-scores as a measurable improvement by the intervention (11). To reject the null hypothesis, we needed to study 18 children in each group with a type I error probability of 5% and a power of 80%.

Statistics

Descriptive analyses of this cohort were given as percentages with mean ± standard deviation (SD) or median and range according to distribution. Basic characteristics between the two groups were analysed with unpaired t-tests, while paired t-tests were applied for comparisons between different time points within a group. Mann-Whitney U test or t-test were applied for group comparison, depending on distribution of the data. Categorial data were evaluated by Fisher's exact test. Kaplan Meier curves were performed and show the relapse-free survival distribution for both groups. Time until event (relapse) was calculated by time in days from study entry. Statistical analyses were performed in the R programming environment 3.6.1 for Windows, statistical software SAS Enterprise for Windows, Release 9.2 (SAS Institute, Cary; NC) or GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). For all tests, p-values below 0.05 were considered to be indicative of significant effects (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

As for microbiome analysis, PERMANOVA test was performed to determine statistically significant differences between groups for beta diversity analysis and Kruskal–Wallis rank sum statistical test was applied to determine differences between groups for measuring species richness. For visualization of the separation between bacterial profiles, multiple dimensional scaling plots were computed using the packages vegan and ade4.

As for metabolomics, data sets from multiple batches were initially preprocessed for quality control-based signal drift correction using the statTarget® package (52). The following parameters were applied: (a) missing value filter: 0.4; QC span: 0; degree: 2; imputation method: multiple imputation using K nearest neighbor (KNN method); scaling method: pareto; permutation times: 500. Sums of some metabolite classes

were computed including ∑PC, ∑PCaa, ∑PCae, ∑lyso.PCa, ∑lyso.PCe, and ∑SM. Additionally, ratios between some metabolite species were computed including: (a) Σlyso PCa / ΣPCaa, a lipid biomarker of inflammation.(53) (b) ΣPCaa / ΣPCae, reflecting oxidative stress(54) (c) ∑PC / ∑SM as indicator of membrane fluidity in IBD(55) (d) Carn ratios (Carn 16:0/free carnitine and Carn 2:0 / Carn 16:0) as markers of carnitine palmitoyl transferase-1 activity (CPT1) and fatty acid beta-oxidation, respectively (56). Finally, 439 metabolites were used for further statistical analysis.

Considering the complex composition of data involving repeated measurements from assays where subjects of each group are exposed to different treatments (PEN/non-PEN), a multilevel multivariate approach combined with sparse partial least squares discriminant analysis (splsda) was applied. A two-factor splsda model was built within the mixOmics package (57) to assess differences between the treatment groups at different time points. To eliminate any baseline differences, relative concentrations were used to build the model, which were calculated by dividing the metabolites concentration data for the subsequent time points (3, 6, 9 and 12 months) by the respective baseline concentrations. For model optimization, a tuning process using M-Fold cross validation (CV) was carried out for selection of parameters giving the best model performance (number of principal components (PC) and key metabolites to keep in each PC (keep X)). Accordingly, three PC were selected accounting for 80% of the explained variance with keep X of 50, 30 and 20 key metabolites for the PC 1:3, respectively. For a splsda multilevel two-factor analysis, the tuning criterion is based on the maximization of the correlation between the components on the whole data set. To visualize pairwise associations between the treatment groups and the investigated metabolites, a pairwise similarity matrix was computed for the first three spls dimensions and then displayed in clustered image maps (CIM) and relevance networks, which are graphical outputs implemented in the R package mixOmics (58).

RESULTS

Study cohort

Out of 54 screened CD patients, 42 patients were recruited to the study. Screening failures were due to patients refusing study participation, unknown disease activity or anticipated difficulties to attend appointments due to long distance travel. Among the 42 participants, 22 patients agreed to PEN, and 20 patients served as controls (non-PEN). One patient in the non-PEN group transferred care to a different hospital after baseline visit and was therefore excluded from further analysis (Fig. 1A). The remaining 41 individuals were comparable for sex, BMI, height, disease location, concomitant medication and disease activity, with 37 patients in remission and 4 with mild activity (wPCDAI 17.5 – 27.5 points) (Table 1). Time since diagnosis did not differ between groups, but patients in the PEN group were diagnosed at a later age and therefore older at study inclusion (Table 1). Seven patients in the PEN group and two in the non-PEN group had previously been treated with systemic corticosteroids.

During follow-up, disease activity measurements including wPCDAI, CRP, ESR and faecal calprotectin did not differ between PEN and non-PEN patients (Supplemental Figure S1). Overall, during 12 months follow-up, four relapses occurred in each group (Fig. 1B). PEN was well-tolerated with no adverse effects.

Bone and muscle parameters, grip strength

Of 41 followed patients, pQCT measurements of 2/19 patients in non-PEN group and of 1/22 patients in PEN were excluded due to technical reasons. At baseline, z-scores for trabecular bone density (TrbD4), cortical bone density (CtD65) and muscle cross sectional area (muscleCSA65) did not differ between groups (Table 2 and Fig. 2A-C) (N=38). Means of TrbD4 and CtD65 centred around zero but muscleCSA65 and grip strength were decreased compared to reference values in both groups (Table 2) (N=38). Over 12 months follow-up, PEN did not affect TrbD4 or CtD65 (Table 2 and Fig. 2A-B). We observed a trend for increase in z-scores for muscleCSA65 corrected for individual height and age ($muscleCSA65^{height}$) in the PEN group after 12 months intervention compared to non-PEN (delta z-score +0.39 vs +0.11 respectively, p=0.22, N=34) (Fig. 2C). The grip strength only improved in the PEN group, but not significantly to the non-PEN group (p=0.26) (Fig. 2D and Table 2). Adherence to provide 3-days food records of good quality was poor in our cohort. The final analysis included only

data from 22 patients (11 each in PEN and non-PEN) who delivered thoroughly completed diaries at baseline and at 6 months (N=19) or at 12 months, if the 6 months diary was missing (N=3). No significant differences were seen at baseline between the two groups for intake of energy, protein and calcium (all calculated as % of recommended dietary intake (RDI) for sex and age group), and for vitamin D (□g/day). After at least 6 months in the study, patients in the PEN group showed a significant increased intake of calcium and vitamin D compared to baseline and a trend for higher calorie intake, while children in the control group did not improve their intake over time (Supplementary Figure S4).

Anthropometry in children with growth potential

To assess the effect of PEN on patients with a potential to grow, we analysed anthropometry in a subgroup of children with Tanner stage 1-3 at study inclusion (20/41), 10 patients each in the PEN (PEN^{T1-3}) (Tanner stage 1, N=5; Tanner stage 3, N=5) and in the non-PEN ($non\text{-}PEN^{T1-3}$) (Tanner stage 1, N=5; Tanner stage 3, N=5) group. Delayed skeletal maturation as assessed by x-ray of the left hand was present in 8/10 in PEN^{T1-3} and 6/10 in $non\text{-}PEN^{T1-3}$. At baseline, PEN^{T1-3} patients were significantly older compared to $non\text{-}PEN^{T1-3}$ patients ($p<0.01$, N=20, Table 1) and showed a trend for lower height z-scores compared to $non\text{-}PEN^{T1-3}$ individuals ($p=0.07$, N=20, Table 1). Stunting as defined by height-for-age z-scores of less than -2.0 was present in only one patient in PEN^{T1-3} group. Over the 12 months follow up, one patient in the PEN^{T1-3} group ended study participation after 3 months, due to PEN incompliance. In the remaining cohort of 9 PEN^{T1-3} patients, z-scores for height improved significantly from baseline to months 12 compared to those 10 $non\text{-}PEN^{T1-3}$ patients (delta of z-scores height 0-12 means: PEN^{T1-3}: 0.45±0.24 vs $non\text{-}PEN^{T1-3}$ 0.15±0.16; $p=0.005$, N=19) (Fig. 3A). Z-scores for BMI were significantly different at baseline, with higher BMI values in $non\text{-}PEN^{T1-3}$ controls ($p=0.02$, N=20, Fig. 3B). More patients in the PEN^{T1-3} group had BMI z-scores between -1 to -2 indicating mild underweight (1/10 $non\text{-}PEN^{T1-3}$ vs 8/10 in PEN^{T1-3}, $p=0.006$, Supplementary Table 1). Although, those differences remained stable over time and no improvement of BMI z-scores was observed despite PEN intake (delta of z-scores BMI 0-12 means: PEN^{T1-3}: 0.35±0.29 vs $non\text{-}PEN^{T1-3}$ 0.2±0.37; $p=0.35$, N=19) (Fig. 3B), the number of patients with mild underweight after 12 months halved (Supplementary Table 1). Analysis of

bone and muscle parameters in this subgroup of patients (N=19) did not reveal significant changes over time.

Microbiome and metabolome

For16S rRNA gene profiling 164 faecal samples were available from PEN and non-PEN patients. At baseline, microbial profiles did not differ between both groups (data not shown). analysis of pooled follow-up samples (all time points, except for baseline) revealed significant but rather small changes in community structure under PEN (Fig. 4A) but not in control patients, supporting that PEN affects microbial community composition (p=0.02). We observed substantial inter-personal variation between patients and across their individual disease course irrespective of dietary intervention (Fig. 4B). Community diversity at the level of species richness showed a small but significant reduction under PEN exposure (Fig. 4C).

Metabolomics, splsda analysis showed complete separation between the eight investigated groups corresponding to the two treatments (non-PEN/PEN) at the four time points (3, 6, 9 and 12 months) (Supplementary Figure S2). The CIM plot shows two main clusters corresponding to the non-PEN and PEN treatments, each composed of four clusters corresponding to the four time points of each treatment group (Fig. 5). Remarkable differences were found in the metabolic profiles of the intervention group in comparison to the control group, which were majorly persistent throughout the whole duration of the study (3, 6, 9 and 12 months). Phospholipids (PL), particularly numerous PC species, were consistently either up- or downregulated; however, the strongest associations were mostly detected after 3 months intervention. Several PC comprising palmitic acid C16.0 namely: PC.aa.32.1, PC.aa.38.4, PC.aa.32.0, PC.aa.34.2, PC.aa.36.3 were highly upregulated with the PEN therapy, particularly at 3 months; although the strongest positive association was obtained for PC.ae.36.1, a PC comprising stearic and oleic acids. Similarly, the two investigated Carn ratios and Val (AA) were uniformly upregulated in the PEN group, in addition to three NEFA, namely: NEFA 14.2; NEFA 15.1; NEFA 18.4 and fumaric acid. On the other hand, whether in the non-PEN or PEN groups, some metabolite species behaved differently across the four time points. For instance, in the PEN group, 22 metabolites (5 LPC, 5 SM, 4 Carn; 4 PC, 2 NEFA, glutamine (Glu), and Σlyso.PCa/ΣPCaa ratio) showed an initial increase at 3 months similar to the control group before consecutively decreasing afterwards starting from 6 months. In both groups (non-PEN and PEN), other

metabolites, principally phospholipids comprising polyunsaturated fatty acids (PL-PUFA), increased abruptly at 12 months (Fig. 5). Associations with values higher than a selected cut-off (threshold) = 0.5 were also visualized in relevance networks (Supplementary Figure S3).

DISCUSSION

Current guidelines suggest PEN as a strategy to improve bone health, sarcopenia, and growth development. We prospectively studied the effect of PEN (25% of daily energy intake for 12 months) on muscle mass, bone health, and growth in paediatric-onset CD patients with quiescent disease activity. Using pQCT, which provides detailed insight into changes in trabecular and cortical density and muscle mass, we did not detect any change in bone-muscle geometry with PEN over 12 months. CD patients with Tanner stage 1-3 may benefit from PEN by improving growth velocity. Furthermore, our results revealed that PEN only marginally affects the microbial ecosystems but shapes the plasma metabolome.

Bone and muscle parameters, grip strength

Poor bone quality in CD has been attributed to several factors, including glucocorticoid therapy, ongoing inflammation or nutritional deficiencies. Avoiding systemic steroids and controlling inflammation by e.g. anti-TNF therapy improves bone density and structure in paediatric CD with greatest effect in younger and growing participants, suggesting a window of opportunity for treatment of bone deficits.(59) We have recently shown that EEN treatment for 8 weeks improves bone health by week 12 in newly diagnosed patients with active CD and impaired bone density and muscle mass.(11) In contrast, our present study included children in clinical remission or with mild disease activity. Although our patients had a long history of CD, mean z-scores for bone parameters at study baseline centred on zero indicating sufficient bone quality at study inclusion and adequate anti-inflammatory therapy. The intervention with PEN, did not further improve bone parameters, but showed a trend for improved BMI, muscle-cross sectional area and grip strength in the PEN compared to the control group. This suggests that PEN may improve nutrition in paediatric CD patients and may increase lean body mass.

Growth

Growth failure has been associated with similar risk factors as bone deficits. Controlling CD inflammation by using EEN and/or biologics has been consistently associated with stabilized or even improved linear growth, in particular when compared to corticosteroid-treated patients (12, 60). Grover et al. demonstrated in a retrospective analysis of 2 years follow-up that EEN induction is superior to corticosteroids induction

for reducing growth failure (60). In the recently published prospective GROWTH study including 147 newly diagnosed paediatric CD patients there was a trend for better height z-scores after 2 years follow up in patients receiving EEN compared to steroids as initial induction therapy (12). Besides EEN, anti-TNF biologics, both infliximab and adalimumab, have been associated with improved growth with patients entering remission benefitting most (61, 62). These studies reinforce the concept of avoiding corticosteroids and preferring corticosteroid-sparing induction strategies in patients at risk for growth failure. Our sub-analysis indicated that the patients with Tanner Stage 1-3 further benefited from PEN and improved height z-scores over the 12 months intervention although they were older compared to controls. Thus, PEN may be offered to CD patients in sustained remission but persistent growth deficit.

Relapse rate/remission maintenance

Whether PEN improves maintenance therapy in paediatric or adult CD patients and reduces relapse rates remains controversial. Yamamoto et al. performed a systematic review and concluded that PEN may be useful for maintaining remission in patients with medically or surgically induced remission (63). Patients in our study received PEN as adjunct therapy in addition to medical maintenance therapy with anti-TNF or immunomodulators. We cannot draw any conclusion regarding keeping remission due to the low rate of relapses observed over 12 months follow-up. Accordingly, blood and faecal inflammatory markers were comparable in both groups during the study period.

Microbiome

We have recently shown that EEN is associated with induction of remission and substantial shifts in intestinal microbiota (14). In this study, PEN had no consistent effect on the intestinal ecosystem. We found substantial changes in microbiome profiles of individual patients during follow-up, suggesting an individualized response to PEN. In line with previous reports on EEN, we found that PEN also reduces alpha diversity. In EEN, the reduction in bacterial richness and alpha-diversity has been explained by the absence of dietary fibres, and hence reduction of fermentable carbohydrates and luminal microbial density (15-17). Taken together, our data show that PEN is not sufficient to drive changes in microbial community structure similar to what we have previously observed in CD patients treated with 8 weeks EEN.

Metabolome

In our analysis, we were able to detect remarkable differences in the metabolome following dietary intervention using PEN. This was evident in the complete separation of the non-PEN and PEN groups throughout the different time points of the study. Our results revealed that PL, especially PC was the species most impacted by PEN, similar to previous findings by our group on EEN (64). This was evident in the large number of single PC species showing strong association with the PEN therapy, particularly at 3 months, especially those comprising C16.0. This could be related to the composition of the formula used where palmitic acid (C16.0) is the major fatty acid (FA) representing >30% of the total formula FA and supports that PEN had direct effects on the metabolome of the patients in the intervention group (64). PL may therefore serve as a biomarker to assess patient adherence with PEN. We can only speculate about the particular strong effect size at month 3 which may be due to highest patient motivation and adherence after recruitment.

An interesting finding is the behaviour of Σlyso.PCa/ΣPCaa ratio. A transient elevation at 3 months was seen in both the PEN and control groups, which was followed by a consistent decrease in the PEN group in the later time points, unlike the control group. Σlyso.PCa/ΣPCaa ratio has been identified as a biomarker of inflammation in previous studies (53, 65), and its greater decrease in the PEN than in the control group may reflect a greater degree of resolution of inflammation with PEN therapy.

The principal focus of this analysis was to investigate group differences in the metabolome, which was evident in our findings. However, we were also able to detect some metabolic changes occurring in patients in remission during the study, apart from the intervention. These changes were found in a few PL-PUFA and lactic acid, which were relatively stable in both groups throughout the study duration but increased remarkably at the end of the study (at 12 months). Our findings strongly hint that metabolomics could potentially be indicative of disease activity.

Study limitations

Our study has several limitations. First, we were unable to perform this study as randomized trial as originally planned as a considerable number of eligible patients refused randomization due to individual preferences. Even inclusion of a 2nd IBD centre in Munich did not overcome this limitation. After changing to a non-randomized study

design, we successfully included a sufficient number of patients in each group as indicated by our power calculation. Patients' preferences were neither related to gender, socioeconomic background, duration or extend of the disease, nor did we see any differences in the adherence to the protocol. However, older patients chose to be in PEN group. This resulted in a significant older age at baseline and may have introduced a selection bias. Sensitivity analysis of the subgroup with a Tanner stages 1-3 revealed that those in the PEN group were leaner with a trend for lower height z-scores. Therefore, we cannot exclude that the increase in muscle CSA in the PEN group could also be, at least in part, due to pubertal development. Secondly, only 49% (20/41 patients) of our cohort had low Tanner stages 1-3 and therefore had a potential for catch up growth. We did not assess parental height in order to correct for mid parental height. Further studies in a larger number of patients are required to confirm our observation of improved growth velocity during PEN. Thirdly, although we aimed to assess accurate food intake with 3-days food records at baseline, and months 6 and 12, only about 50% of the patients provided food diaries of sufficient quality. However, we consider the significant improved intake of certain nutrients in the PEN but not in the non-PEN group as indicator for a good adherence to the formula intake. Finally, unexpectedly and different from newly diagnosed paediatric CD patients (11), this cohort showed a normally distributed bone quality at baseline comparable with the healthy reference population. Therefore, the potential for further improving the bone parameters was limited.

In conclusion, our data do not provide evidence that PEN improves bone health in adequately treated paediatric CD patients with well-controlled disease activity. Our study suggests that PEN may induce catch up growth in CD patients with quiescent disease during early stages of puberty.

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Dr Klara Frivolt, a young paediatrician in training at the University of Bratislava, died on August 5, 2017 in Hungary being pregnant with her second child. Klara had spent two research periods of 18 months each at the Division of Gastroenterology and Hepatology at the Dr von Hauner Children’s Hospital, LMU Munich, Germany. She had received several research awards including the Nestle Nutrition Award from the European Crohn and Colitis Organization (ECCO) in 2014, which she used to conduct this study. In a very enthusiastic way she supervised this study as paediatric scientist. On July 4, 2017 she successfully defended her PhD thesis. Klara Frivolt was an extremely talented young physician, scientist, mother and a wonderful person. We are in deep grief.

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AUTHOR CONTRIBUTION

A.B., K.J.W., K.F., S.K. and T.S. designed the study, evaluated and interpreted data, and contributed to manuscript writing. K.K., M.H., S.O., P.B. and S.L. recruited patients, collected clinical data and samples. S.B.D.P. analysed pQCT data and gave conceptual input. E.S., J.G.M., O.U. and B.K. performed metabolomics, statistical analysis, data interpretation and contributed to manuscript writing. M.A., A.M. and D.H. performed microbiome analysis, statistical analysis, data interpretation and contributed to manuscript writing. A.B. and T.S. summarized the first draft of the manuscript. All authors provided input on the manuscript and approved it.

CONFLICT OF INTEREST

T.S. received speaker's fees from MSD, Nutricia and travel support from Nestlé Nutrition.

D.H. received speakers fees and travel support by Nestlé Nutrition Institute.

P.B. received honorarium as speaker from GivenImaging, Abbvie, Abbott, Roche and MSD.

S.K. received a research grant from Mead Johnson and Nestle´ Nutrition, and honorarium as speaker or advisory board member from Abbott, Danone, Hipp, MSD, Pfizer, Takeda, Thermo-Fisher, Vifor.

The remaining authors report no conflicts of interest.

FIGURE LEGENDS

Figure 1

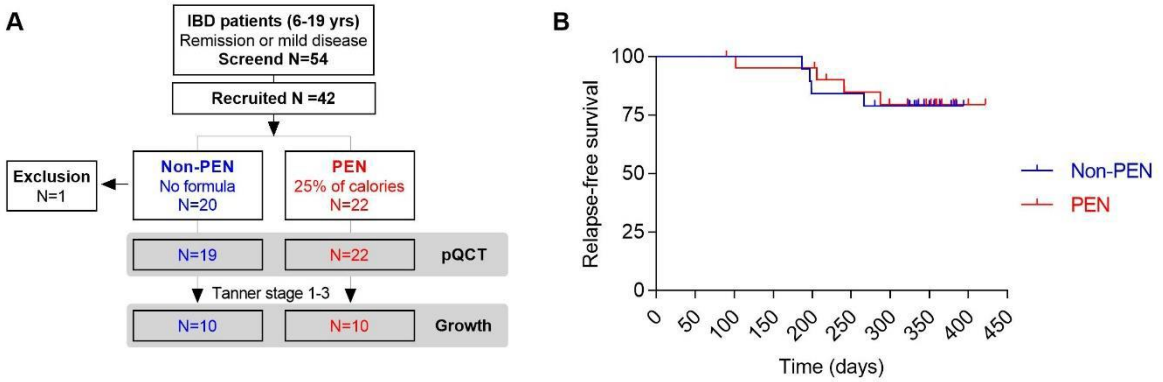

Figure 1. Study design and relapse-free survival. (A) Study design of the non-randomized controlled intervention study in CD patients aged 6-19 years who were in remission or had mild disease activity at inclusion. Patients were evaluated at baseline and after 3, 6, 9 and 12 months. **(B)** Kaplan-Meier survival analysis of relapse-free duration of remission in PEN (N=22) vs. non-PEN (N=19) groups.

Figure 2

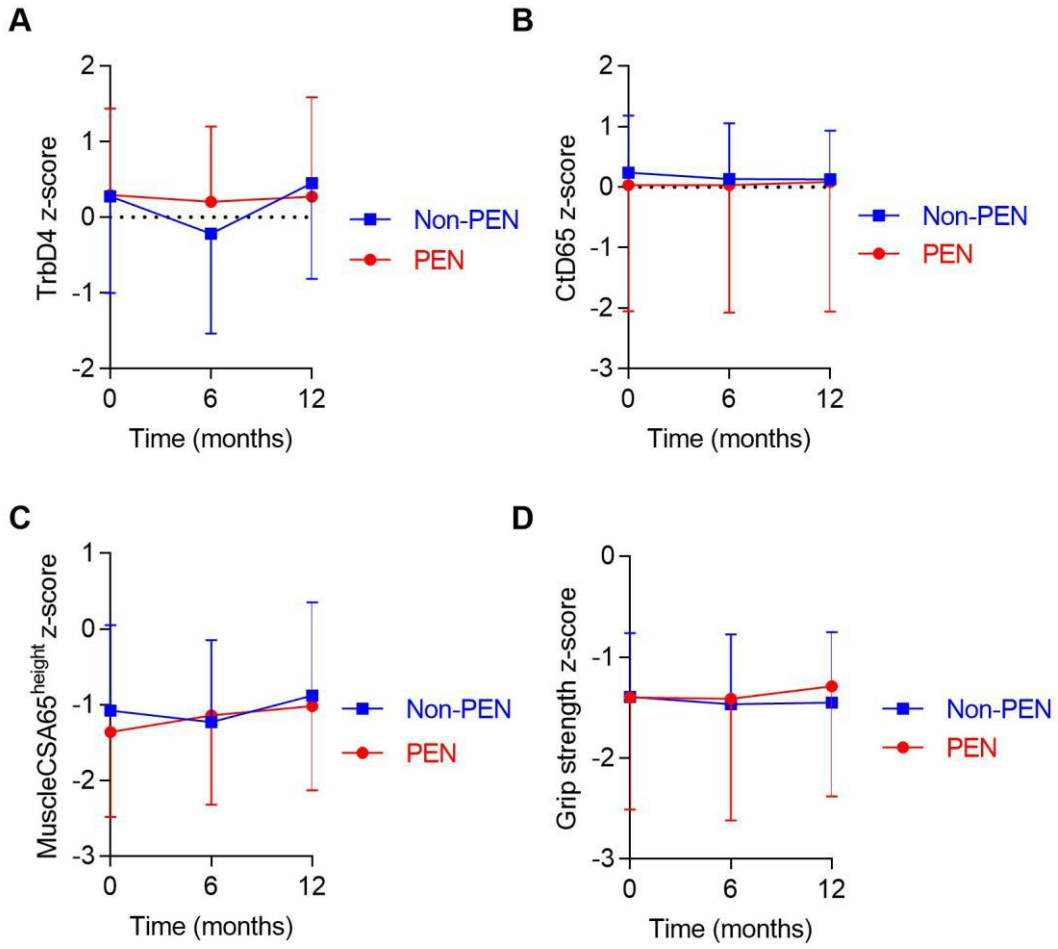

Figure 2. Development of bone and muscle parameters as well as grip strength as functional parameter of muscle over time, stratified by group. (A) Analysis of trabecular bone density (TrbD4), **(B)** cortical bone density (CtD65), and **(C)** muscle cross sectional area CSA corrected for height (muscleCSA65^{height}) by pQCT. Grip strength was determined by an adjustable-handle Jamar Dynamometer **(D)**. All available measurements are shown and parameters are expressed as z-scores.

Figure 3

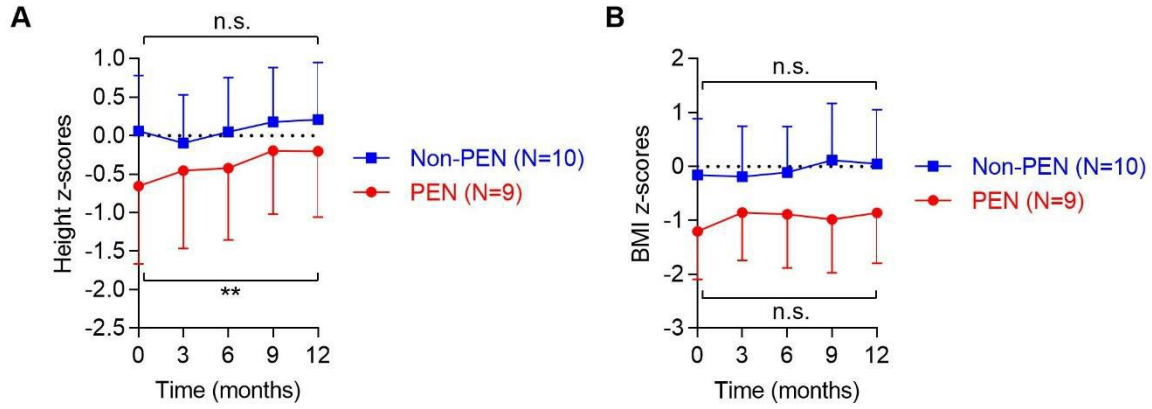

Figure 3. Development of height z-scores in children with Tanner stage 1-3. Between baseline and month 12, there was a significant increase in z-scores for height in PEN^{T1-3} patients (N=9) but not in the non-PEN^{T1-3} controls (N=10) **(A)**. BMI z-scores remained stable in both groups **(B)**. One patient without follow-up was excluded in PEN group. Differences in z-scores between baseline and 12 months were calculated for individual patients and subsequently significance between groups was assessed by comparing deltas 0-12 months between both groups by unpaired t-test (**, $p<0.01$).

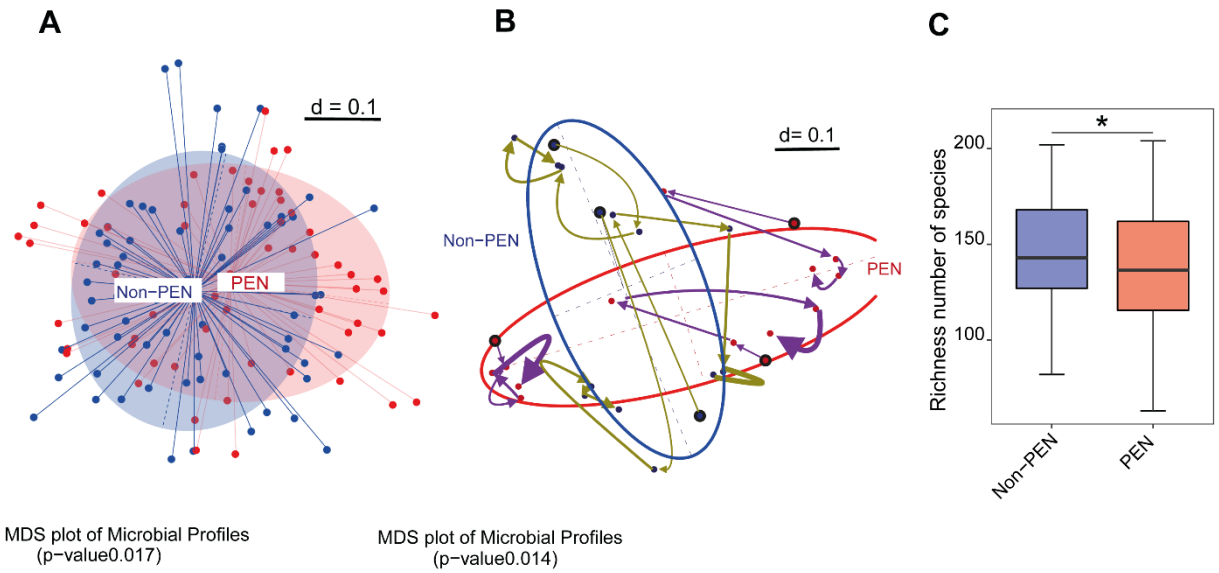

Figure 4. Small changes of microbiome signatures mediated through PEN. **(A)** Multidimensional scaling (MDS) plot shows the variance of microbial profiles in the intervention group (PEN) and control group (non-PEN). Analysis is based on pooled samples from month 3, 6, 9 and 12. **(B)** MDS plot of randomly-selected 6 patients (3 patients PEN and 3 patients non-PEN) showing changes in microbial profiles during follow-up (baseline, month 3, 6, 9 and 12). Arrows show the transition from each time-point to the next (Baseline= circles with black lining) **(C)** Bacterial species richness in both groups (PEN and non-PEN) that were analysed from month 3, 6, 9 and 12. (*, $p<0.05$, unpaired Student t-test).

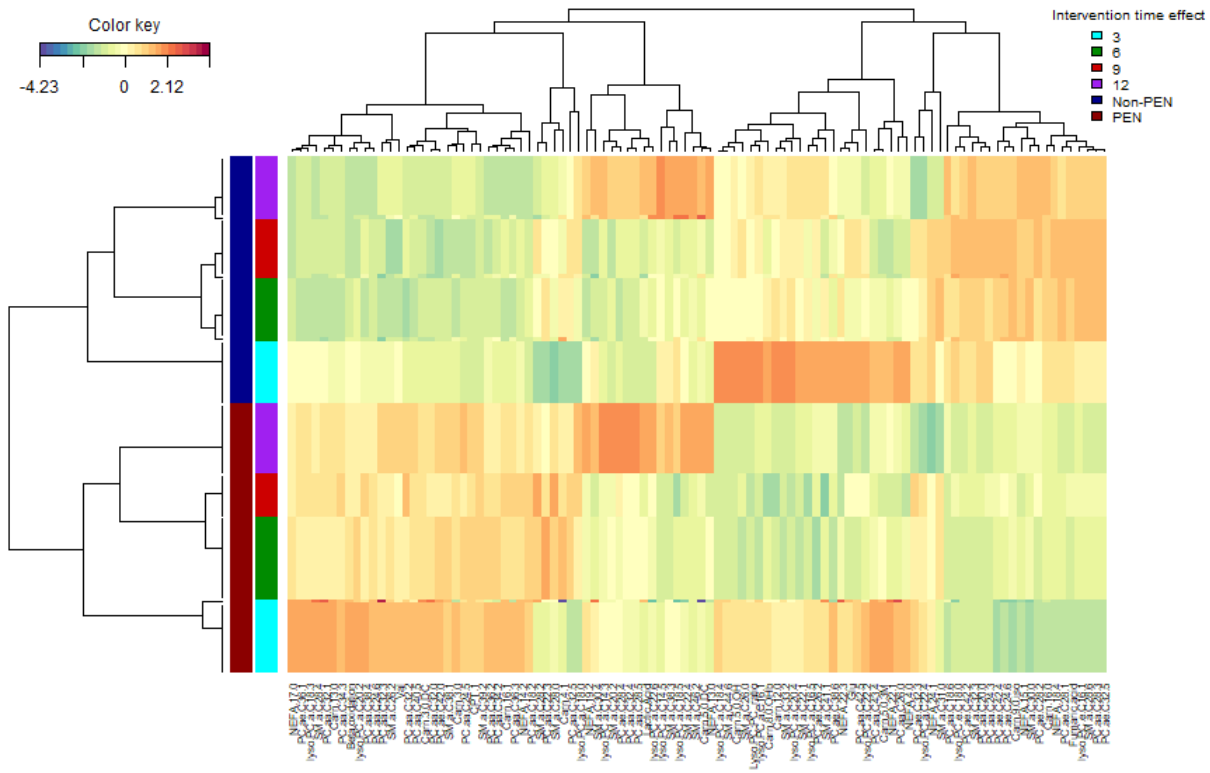

Figure 5. CIM plot obtained using splsda model with three principal components (PC), showing pairwise correlations between the metabolite species and the treatment groups (non-PEN/PEN) at the different time points of the study (3, 6, 9, and 12 months). The green and red colours indicate positive and negative correlations respectively, whereas yellow indicate small correlation values. The treatment groups are clustered on the left side of the CIM. Polar lipids and NEFA are represented by a formula X.Y where X: length of carbon chain, Y: number of double bonds, OH: indicates presence of hydroxyl group. Letters ‘a’ & ‘e’ indicate that the acyl chain is bound via an ester or ether bond to the backbone, respectively.

TABLES

Table 1 Baseline patient characteristics

		Total cohort (N=41)			Subgroup Tanner Stage 1-3 (N=20)		
Baseline patient characteristics		Non-PEN (N=19)	PEN (N=22)	p-value	Non-PEN (N=10)	PEN (N=10)	p-value
Gender (male/female)		11/8	12/10		5/5	4/6	
Age at diagnosis (years)		8.8 ± 3.6	11.8 ± 2.9	**0.005**	7.6 ± 3.0	10.7 ± 2.5	**0.02**
Age at study inclusion (years)		12.8 ± 3.1	15.0 ± 1.9	**0.007**	10.8 ± 2.7	13.8 ±1.5	**0.006**
Tanner Stage 1-3		10/19	10/22		10/10	10/10	
Time (years) of IBD until study inclusion		4.0 ± 2.9	3.2 ± 2.4	0.35	3.2 ± 2.1	3.1 ± 2.0	0.91
Positive family history		5/19	7/22		4/10	2/10	
Extra-intestinal involvement		4/19	6/22		4/10	5/10	
Disease location	L1 Terminal ileum	2/19	3/22		0/10	1/10	
	L2 Colon	7/19	6/22		5/10	4/10	
	L3 Ileocolonic	10/19	13/22		5/10	5/10	
	+ L4 (upper GI tract)	17/19	10/22		10/10	5/10	
Disease behaviour	B1 non-stricturing, non-penetrating	18/19	19/22		10/10	8/10	
	B2 stricturing	1/19	3/22		0/10	2/10	
	B3 penetrating	0/19	0/22		0/10	0/10	
	Perianal involvement	7/19	4/22		3/10	4/10	
Therapy at baseline	Azathioprine	11/19	10/22		5/10	5/10	
	5-Aminosalicylates	4/19	6/22		2/10	3/10	
	Infliximab	12/19	13/22		7/10	6/10	
	Methotrexate	2/19	4/22		2/10	2/10	
	Adalimumab	1/19	0/22		0/10	0/10	
Disease activity	Remission (wPCDAI <12.5)	17/19	20/22		10/10	8/10	
	Mild disease (wPCDAI ≥12.5 ≤40)	2/19	2/22		0/10	2/10	
Anthropometry	Height-for-age z-scores	0.09 ±0.79	-0.08 ±1.14	0.58	0.06 ± 0.72	-0.68 ± 0.96	0.07
	BMI-for-age z-scores	0.19 ±1.08	-0.38 ±1.12	0.11	-0.16 ± 1.04	-1.23 ± 0.84	**0.02**

Data are expressed as mean ± standard deviation. Significant differences are marked in bold. Abbr.: EEN, exclusive enteral nutrition; wPCDAI, mathematically weighted Paediatric Crohn's Disease Activity Index; IBD, inflammatory bowel disease.

Table 2: Anthropometric, bone, and muscle parameters as Z- scores at baseline and 12 months, and changes between baseline and month 12, mean (±SD)

	PEN			Non-PEN			
Z-Scores **Mean ± SD**	**0** **N=20* / 18**	**12** **N= 20* / 18**	**Delta 0-12** **N=20* / 18**	**0** **N=19* / 16**	**12** **N=19* / 16**	**Delta 0-12** **N=19* / 16**	**P-value (t-Test)** Diff 0-12 in PEN vs. non-PEN
Height	**-0.09** ±1.17	**0.10** ±1.03	**0.19** ±**0.30**	**0.09** ±0.79	**0.18** ±0.84	**0.08** ±0.21	**0.21**
BMI	**-0.34** ±1.15	**-0.06** ±1.24	**0.28** ±0.42	**0.19** ±1.08	**0.26** ±0.94	**0.06** ±0.41	**0.12**
TrbD4	**0.45** ±1.12	**0.27** ±1.31	**-0.18** ±1.08	**0.30** ±1.32	**0.45** ±1.26	**0.15** ±0.73	**0.30**
CtD65	**-0.17** ±2.18	**0.08** ±2.14	**0.25** ±0.75	**0.21** ±0.96	**0.13** ±0.80	**-0.08** ±**0.67**	**0.19**
CTth65	**-0.76** ±1.26	**-0.74** ±1.08	**0.02** ±0.5	**-0.71** ±0.49	**-0.65** ±0.62	**0.06** ±0.67	**0.84**
muscleCSA65	**-1.51** ±1.07	**-1.33** ±0.97	**0.18** ±0.63	**-1.05** ±0.84	**-1.15** ±0.76	**-0.10** ±0.70	**0.23**
muscleCSA65^{height}	**-1.41** ±1.08	**-1.02** ±1.11	**0.39** ±0.74	**-0.99** ±1.10	**-0.88** ±1.23	**0.11** ±0.55	**0.22**
Grip strength	**-1.35** ±1.11	**-1.28** ±1.09	**0.06** ±0.89	**-1.39** ±0.63	**-1.45** ±0.70	**-0.06** ±0.72	**0.26**

*N for height and BMI

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