Metagenomic etiology and therapy in pediatric gastrointestinal inflammation

PhD thesis

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Budapest
2017

"Research is what I am doing when I don't know what I am doing."

Wernher von Braun

Table of Contents

List of Abbreviations 5

1 Introduction 8

1.1 Nutritional developmental origins of disease hypothesis 8

1.2 What biologic systems can be modulated by the nutrition? 8

1.3 The Human Microbiome Project 9

1.4 The microbiome matures during pediatric development 10

1.5 The microbiome is nutritionally responsive 11

1.6 The human microbiome as an active metabolizer 12

1.7 Rapid microbiome responses to marked diet changes 13

1.8 The microbiome possesses compositional stability 14

1.9 The microbiome possesses metabolic stability 14

1.10 The dynamically changing microbiota throughout life stages 14

1.11 The role of host immunity and infections in the modulation of gut microbiota 14

1.12 Postnatal (pediatric) nutritional exposures affect intestinal inflammation and associate with IBD susceptibility 16

1.13 Cellulose supplementation early in life ameliorates acute colitis in adult mice 17

1.14 Loss of omega-6 fatty acid induced pediatric obesity protects against acute murine colitis 18

1.15 Dysbiosis-associated gastrointestinal disorders and future therapeutic options 18

1.16 The manipulation of microbiota as a possible therapeutic option in pediatric GI disorders 19

1.17 CDIF and FMT 20

1.18 IBD and FMT 20

1.19 CDIF and IBD association 22

1.20 Other GI disorders and FMT 22

1.21 Non-GI disorders and FMT 22

2 OBJECTIVES 23

3 METHODS 24

3.1 Animals, diets and experimental design ... 24
3.1.1 Cellulose supplementation study ... 24
3.1.2 High fat supplementation study ... 26
3.2 Dextran sulfate sodium exposure ... 26
3.3 Tissue collection and histological analysis ... 27
3.4 Microbiota transplantation in mice ... 27
3.5 DNA extraction and analysis of microbiome ... 27
3.6 Flow cytometry ... 29
3.7 *In vivo* chemokine blocking ... 29
3.8 Human samples and Enzyme-linked Immunosorbent Assay (ELISA) ... 29
3.9 FMT protocol ... 30
3.9.1 Subjects with recurrent CDIF ... 30
3.9.2 Subjects with UC ... 31
3.9.3 Donor recruitment ... 32
3.9.4 FMT preparation ... 32
3.9.5 FMT treatment protocol in CDIF patients ... 33
3.9.6 FMT treatment protocol in UC patients ... 33
3.9.7 Sample collection ... 34
3.10 Human microbial data analysis ... 35
3.11 RNA sequencing and analysis ... 35
3.12 Histology ... 36
3.13 Additional statistical analysis ... 36
4 RESULTS ... 37
4.1 Cellulose supplementation studies in mice ... 37
4.1.1 Increased severity of DSS colitis on a synthetic, low fiber diet ... 37
4.1.2 Transient trophic and anticolitic effects of cellulose supplementation ... 38
4.1.3 Microbial diversity and composition changes following cellulose supplementation ... 41
4.2 ω-6 fat diet reversal and its effect in murine studies ... 46
4.2.1 Reversal from ω-6 fat diet protected against colitis ... 46

4.2.2 Protection against colitis was ω-6 and reversal dependent 47
4.2.3 ω6-reversal did not affect other models of colitis 48
4.2.4 The gut microbiome of ω6-reversed mice transmitted the colitis suppression. 48
4.2.5 The microbiome of ω6-reversed mice segregated from control 49
4.2.6 Cxcr5$^+$ CD4$^+$ T cells were suppressed in ω6-reversed mice 50
4.2.7 Cxcr5 ligand B-lymphocyte chemo-attractant (or Cxcl13) modulated colitis susceptibility 50
4.2.8 CXCL13 levels in the plasma of IBD patients 50
4.3 Clinical results of FMT 52
4.3.1 FMT treatment outcomes of CDIF patients 52
4.3.2 Clinical outcomes of patients with ulcerative colitis 52
4.3.3 Adverse events monitoring 57
4.4 Microbial results of FMT 58
4.4.1 Increased alpha-diversity following FMT in CDIF patients 59
4.4.2 Taxonomical changes upon FMT in CDIF 59
4.4.3 Microbial analyses of UC patients 60
4.4.4 Microbiome shifts upon FMT 61
4.4.5 Prolonged microbiome effects of serial FMT in two patients 62
4.4.6 Remarkable bacterial composition changes in pediatric CDIF after a single FMT 64
4.4.7 Normalization of microbiome composition (beta-diversity) upon FMT in CDIF patients 66
4.4.8 Microbiome shifts in CDIF following FMT are more pronounced than in UC 67
4.5 Mucosal transcriptome and epithelial cell proliferation changes after the FMT series 67
5 CONCLUSIONS 70
5.1 Cellulose supplementation early in life ameliorates acute colitis in adult mice 70
5.2 Loss of omega-6 fatty acid induced pediatric obesity protects against acute murine colitis 72

5.3 The manipulation of microbiota as a possible therapeutic option in pediatric gastrointestinal disorders ... 75

6 KEYNOTES ... 79

7 SUMMARY ... 80

8 ÖSSZEFOGLALÁS ... 81

9 REFERENCES ... 82

10 PUBLICATIONS ... 94

10.1 Related publications of the author ... 94

10.2 Additional unrelated publications of the author ... 95

10.3 Book chapters of the author ... 97

11 ACKNOWLEDGMENTS ... 98

List of Abbreviations

6MP: 6-mercaptopurine

ab: antibody

BMI: body mass index

CAP: College of American Pathologists

CD: Crohn's disease

CD: cluster differentiation

CDIF: *Clostridium difficile* infection

CLA: conjugated lineolic acid

CLAI: Clinical Laboratory Improvement Amendment

CXCR: C-X-C motif ligand chemokine receptor

D: donor

DAB: diaminobenzidine tetrahydrochloride

DCs: dendritic cells

DSS: dextran sulfate sodium

Dx: diagnosis

E: ethnicity

EIA: enzyme immunoassay

F: female

FFAR2: free fatty acid receptor 2

FMT: fecal microbiota transplantation

FOS: fructooligosaccharides

G: gender

GABA: gamma-aminobutyric acid

GALTs: gut-associated lymphoid tissues

GF: germfree

GI: gastrointestinal

GIT: gastrointestinal tract

GLP: Good Laboratory Practice

GOS: galactooligosaccharides

GPR43: G-protein coupled receptor 43

H: Hispanic

HC: high cellulose (12.5% cellulose)

HE: hematoxylin-eosin

HF (or ω6): high fat (ω6) diet (40% caloric content corn oil/linoleic acid)

HMP: Human Microbiome Project

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

ID: identification

IL: interleukin

IFX: infliximab

IND: Investigational New Drug

IRB: Institutional Review Board

LC: low cellulose (2.5% cellulose)

M: male

MGB axis: microbiota-gut-brain axis

MLNs: mesenteric lymph nodes

NEC: necrotizing enterocolitis

NF-κB: nuclear factor kappa-light chain enhancer of activated B cells

NH: non-Hispanic

NIH: National Institute of Health

OTUs: operational taxonomic units

P21/30/80/90/120, 30/80/90/120 days postnatal age

PBL: peripheral blood

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered solution

PCoA: principal coordinate analysis

PCR: polymerase chain reaction

PRED: prednisone

PSA: polysaccharide A

Pt: patient

qMRI: quantitative magnetic resonance imaging

R: race

R10 and R40: 10 or 40 days of reversal following high cellulose/ high $\omega 6$ diet

SCFAs: short chain fatty acids

SOPs: Standard Operating Procedures

TCMC: Texas Children's Microbiome Center

TNF-α: tumor necrosis factor α

Tx: therapy

UC: ulcerative colitis

W: White

y: years

1 Introduction

1.1 Nutritional developmental origins of disease hypothesis

There is a critical developmental period when nutrition may modify the predisposition to diseases later in life. The hypothesis "Developmental Origins of Health and Diseases" was established in the late 80's by Barker.(1) The retrospective studies showed that infant mortality (undernutrition *in utero* and low birth weight) permanently changes body metabolism and leads to an increased cardiovascular and metabolic disease risk later in life. The possible mechanism behind the hypothesis is programming. This process describes the mechanism whereby stimuli at a critical period of development may have long-lasting and irreversible effect on the body structure or function.

1.2 What biologic systems can be modulated by the nutrition?

Biological systems are most likely to respond to environmental stimuli when they are in flux. Developmental maturation and modification is a window opportunity to alter the biological systems. By definition, the biological system has to be responsive for environmental factors and nutritional stimuli. The biological system has to stay stable once adjusted to external stimuli. The biological system needs to be penetrant, and convey phenotype effects.

In my studies, we focused on the MICROBIOME, and how such a biological system may be modulated by the nutrition and to characterize the effect on host by these alterations.

1.3 The Human Microbiome Project

The Human Microbiome Project (2) was established in 2008 as a five-year project with a total budget of $115 million funded by the National Institute of Health (3) to characterize the human bacteria composition associated with health and disease.

The human microbiome is a pool of all microorganisms living in association with our body. The microorganisms contain ecological communities of commensal, symbiotic and pathogenic organisms. The number of bacteria is ten times more than the number of human cells; and the compounded genes are thousand times more in the bacteria than presented in the human genome.

An individual's microbiome is varying depending on genetic predisposition, the mode of delivery (natural birth or cesarean section), age and different environmental factors such as dietary and nutritional intake, physical activity, use of different drugs, and lifestyle. The homeostasis of gut microbiome is an important key to balance health. On the other hand, dysbiosis (alteration of abundance in bacterial taxa compared to healthy individuals) is associated with diseases and could lead to different disorders. Gut bacteria have a tremendous number of roles, including (1) modifying energy balance and metabolism, and (2) controlling pathogen colonization and resistance. The bacteria performing these roles – and the mechanisms they use – are actively being uncovered.

The improving diagnostic technique took over the culture-based procedures and provided comprehensive characterization of the microbial community. The advanced high-throughput technologies have changed the global view on microbiome dramatically. Metagenomics is the study of genetic materials recovered directly from environmental samples.

The process starts with the extraction of DNA by the biological sample, followed by amplification and sequencing of 16S ribosomal RNA genes. The 16S rRNA gene allows taxonomic identification from species to phyla level. The identification is made by the comparison to a reference-based library. The advanced bioinformatics techniques provided unique opportunity to map the microbial community and give microbial information about biological samples. Adding metabolic networks linked to the microbiome provides more information on function and offers functional profile.

Focusing on the intestinal microbiome, one large step forward came with the completion of the HMP. Being catalogued the different bacteria species from stools of 242 healthy individuals.(4; 5) The HMP has taught us that the gastrointestinal (GI) microbiome contains around 15,000 to 36,000 bacterial species, and specifically enriched in Bacteroidetes and Firmicutes. These two phyla cover nearly 90% of bacteria in the human gut microbiome. Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia are also presented in the human gut microbiome. However, there is a highly remarkable diversity between individuals at a lower level of taxonomy such as species or strain.

1.4 The microbiome matures during pediatric development

The GI microbiome changes during development. In the beginning, the fetal GI tract (6) is sterile. Colonization of the GIT begins immediately at birth and is directly influenced by mode of delivery, with infants delivered by Cesarean section having delayed colonization and decreased colonization with *Bacteroides fragilis* as well as *Lactobacillus*- and *Bifidobacterium*-like bacteria.(7) Controversial studies showed that the placenta harbors a unique microbiome.(8) First-pass meconium samples of newborns show extremely limited relative abundance of bacteria.(9; 10) The gut is rapidly colonized thereafter. After the first week of life, the microbiome appears to stabilize but varies depending on how the infant is fed. Breast-fed infants are colonized mostly by *Bifidobacteria* and *Ruminococci*, and lesser quantities of facultative anaerobes (such as *Streptococci*, *Staphylococci*, *Enterococci*, *Lactobacilli* and *Enterobacteria*).(11; 12) Formula-fed infants are colonized by a larger proportion of Firmicutes, Bacteroides and Proteobacteria. Recent studies have shown that formulas supplemented with prebiotics can increase the amount of *Bifidobacteria* and *Lactobacilli* in formula fed infants to similar levels found in breast-fed newborns.(13) In infancy, the microbiome continues to change, but by age 2 or 3, an “adult-like” microflora is established. However, a recent study from our collaborators provided evidence that healthy children and adult microbiome remarkably differs in composition and function.(14) Adult microbiome contains more Bacteroides species, while the relative abundance of Bifidobacterium species, Faecalibacterium species

and Lachnospiraceae are increased in children. This study proposes that microbiome may undergo a more prolonged development and more responsible for environmental effects.

1.5 The microbiome is nutritionally responsive

Is it really true that 'We are what we eat'? From a gut microbiome perspective, what we eat does indeed determine what bacteria we have more so than nationality, age, gender or body mass index (BMI) (Table 1). Humans can generally be divided into three 'enterotypes' based on the genera of bacteria in their gut.(15)

Table 1 Nutrition-associated modifications of the microbiota composition.

Dietary habits	Microbiota alteration
'Western' diet (animal protein, saturated fat)	*Bacteroides* enterotype
Polyunsaturated fat and alcohol	*Ruminococcus* enterotype
High carbohydrate diet	*Prevotella* enterotype
Ancient rural communities (high carbohydrates, plants)	↑Bacteroidetes (*Prevotella*)
Vegetarian lifestyle	↓Bacteroides, Escherichia
High fiber	↑Bacteroidetes, Actinobacteria
	↓Firmicutes, Proteobacteria
High fat	↑Firmicutes, Proteobacteria
	↓Bacteroidetes
High carbohydrate	↑Firmicutes
	↓Bacteroidetes

Humans consuming more modern diet riches in animal protein and saturated fat – the "Western diet" – harbor the *Bacteroides* enterotype. In contrast, those who consume high amounts of carbohydrates, similar to those from ancient rural communities, have the Prevotella enterotype. Finally, those with high polyunsaturated fat and alcohol intake belong to the *Ruminococcus*-dominated enterotype. The functional capacity of the human gut microbiome can be mapped to the enterotypes. For instance, the Bacteroides enterotype is characterized by genes involved in biotin, riboflavin, panthothenate and ascorbate biosynthesis were enriched in the *Bacteriodes* enterotype, associated with genes encoding enzymes involved in the degradation and fermentation of these nutrients. The Prevotella enterotype is enriched in thiamine and folate biosynthesis genes, while the Ruminococcus enterotype consists of heme biosynthesis.

1.6 The human microbiome as an active metabolizer

Gut microbiota are not passive responders to their host's diet; rather they metabolize a number of substances and function as a nutritive source for the host. For example, bacteria ferment complex carbohydrates into short chain fatty acids (SCFAs). SCFAs in turn boost the human metabolism level; increase energy intake and the bioavailability of toxins. Increased SCFAs decrease colonic pH, inhibit the growth of pathogens, and these nutritional components are the main energy source of colonic epithelial cells. All three SCFAs are reported to enhance colonic blood flow, to stimulate the proliferation of normal crypt cells. By increasing the frequency of the smooth muscle contractions of the colonic mucosa, acetate and propionate leads to an enhanced colonic motility. Butyrate is the main source (about 60 to 70%) of energy for the colonic epithelial cells. Butyrate also influences cell growth and differentiation. Propionate is associated with lipid metabolism

Additionally, pharmaceutical industries are exploiting the nutritive role of SCFAs, and provide prebiotics. Prebiotics are nonviable nutritional components that modify the colonic microbiota conferring benefits for the host health. These components escape digestion in the upper GIT and reach the colon, where they are fermented by the gut microbiota. Inulin-derived fructooligosaccharides (16) and galactooligosaccharides (GOS) change the composition and activation of the microbiota, especially lead to an increase in the amount of *Bifidobacteria* and *Lactobacilli*. These products have significant health benefits, including the influence of laxation, mineral absorption, lipid metabolism, and potential anticancer and anti-inflammatory effects.

Microbes also generate a number of essential vitamins for the host. For example, *Bacteroides fragilis* is known to produce Vitamin K_2, therefore a key component in bone and vascular health (increases bone mineral density and modulate the blood coagulation). Another nutrient, Vitamin B_{12} (cobalamin) is produced by *Lactobacillus reiteri*, and important for growth and the development of the nervous system. The absence of this bacteria and its product decreases the risk of different neurological and hematological disorders. Other micronutrients, such as biotin, folate, thiamin, riboflavin and pyridoxine (produced by *Bifidobacterium*), are not only reinforcing the immune system, but also have epigenetic effects to regulate cell proliferation. Choline metabolites are related to

Faecalibacterium prausnitzii and *Bifidobacterium*, and linked to glucose and lipid homeostasis, involved in obesity, diabetes and cardiovascular diseases.

Malnutrition and obesity exemplify the relationship between the gut microbiota and human nutrition.(17; 18) Undernutrition (kwashiorkor or marasmus) is linked to an altered microbiota with overall reduced gene content. Malnourished children had increased in *Lactobacillus* and *Bifidobacterium* species and decreased in Bacteriodales with nutritional therapy. They also showed signs of a more robust microbiome, including stools with reduced saccharides and an increase of carbohydrate, amino acid, nucleotide and fatty acid metabolism products. . Additionally, antibiotic supplemented therapeutic food can increase the percentage of weight gain and decrease the mortality than therapeutic food only.(19) According to the randomized, double-blinded, placebo-controlled trial, the recovery rate of severe acute malnutrition was 88.7% in the amoxicillin-complemented group and 90.9% in the cefdinir-complemented group, while placebo control reached only an 85.1% recovery rate. The mortality rate changed respectively (4.8% in the amoxicillin group, 4.1% in the cefdinir group and an increased 7.4% in the placebo group).

The obesity-associated microbiota changes are better characterized. Obese individuals have a significantly less diverse microbiome compared to non-obese controls. They are dominated by Actinobacteria (75%) and Firmicutes (25%) phyla, compared to Bacteroidetes (40%) in controls.

1.7 Rapid microbiome responses to marked diet changes

Changes in diet may lead to alteration in gut enterotypes. For instance, individuals switching from the Western diet to a vegetarian lifestyle showed decreased amount of *Bacteroides* and the related genus *Escherichia*. These changes in turn influence overall bowel health. Increased endotoxin linked to *Bacteroides* and *Escherichia* increases systemic inflammation, whereas high-fiber diets promoting Prevotella correlate with increased bowel transit and production of SCFAs that stimulate intestinal cells.

Supporting the central role of diet in shaping the microbiome, radical dietary change alters the microbiota composition. Wu and his research group showed that switching from a high

fat/low fiber diet to a high fiber/low fat diet led to detectable changes within 24 hours.(20) Both the composition of bacteria and bacterial gene expression patterns changed, indicating an acute shift in how the microbiome metabolized nutrients. However, only long-term diet changes may lead to actual switches among the three enterotypes.

1.8 The microbiome possesses compositional stability

The species composition may be highly variable among different people. However, within the same person, the gut microbiome composition varies little over time compared to other body sites such as oral cavity, skin and hair.(21)

1.9 The microbiome possesses metabolic stability

The second core principle is that even though microbial taxa may vary among individuals, the metabolic pathways catalyzed by the bacteria remain stable.(5) Hence, current techniques focused on identifying specific bacteria, such as shotgun sequencing of bacterial genomic DNA, may misrepresent the actual metabolic capacity of a microbiome. Instead, much larger scale systems biological approaches are needed in the future to confirm the conclusions of the Human Microbiome Project Consortium in respect to the high similarity in metabolic patterns representative of different microbial habitats of the human body.

1.10 The dynamically changing microbiota throughout life stages

The gut microbiome loses richness with age, correlating with altered dietary habits, chronic illnesses, and increased use of medications.(22)

1.11 The role of host immunity and infections in the modulation of gut microbiota

Gut microbes must maintain the barrier between host and all the environmental challenges present in the intestine. Stratification and compartmentalization is occurred to decrease the exposure of external stimuli and increase the efficacy of the immunity.(23)

The microbiota actively interacts with the innate and adaptive immune system. The infant gut microbiome affects the maturation of the host immune system and carefully adjust it in early days of life. The microbiota contributes to develop the gut-associated lymphoid tissues (GALTs). The GALTs are immune structures, which participate in lymphocyte functions and have an important role in tolerance or inflammation. The main function of the microbiota-induced development of the intestinal immune system is the promoted T helper 17 and T helper 1 cells (activated by the lamina propria dendritic cells [DCs] and macrophages) and the stimulated intestinal epithelial cells and DCs promoting IgA-producing B cells and plasma cell differentiation. (24; 25)

The decision-making process of the immune system (recognition of pathogenic or non-pathogenic bacteria) is orchestrated by the host-microbial interaction. The pathogenic bacteria will be destructed and eliminated, while the host intestine will tolerate the beneficial bacteria. The finely balanced interplay between the healthy beneficial microbiota and the exogenous and potentially harmful pathogens maintains the intestine homeostasis. This process is based on metabolites cellular and soluble elements associated to the microbiota and sensed by the immune system. These metabolites can be uniquely produced by the microbes (such as SCFAs), or evaluated (e.g. retinoic acid), or metabolized by the microbes (such as the host-presented bile acids).(18; 26) These specific metabolites and their activities -can present the current microbial composition and host-microbial communication.

Microbiota can produce nutritional components -short chain fatty acids (SCFAs), conjugated lineolic acid (CLA), polysaccharide A (PSA) gamma-aminobutyric acid (GABA) and histamine- which affect the host immune system via direct or indirect mechanisms. As an immunological standpoint, the end products of carbohydrate fermentation (by SCFAs) can bind to a G-protein coupled receptor 43 (GPR43) or free fatty acid receptor 2 (FFAR2); and affect inflammatory responses. (27) The receptor-binding mechanism leads to an over-expression of interleukin (IL)-10, and a decreased expression of IL-12 in the human monocytes. This interaction is a great example for the diet-GI bacterial metabolism –immune and inflammatory response complex. Furthermore, SCFAs play a role in the proliferation-occurred apoptosis of activated neutrophils via the decreased

tumor necrosis factor α (TNF-α), IL-8 and inhibited nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) pathway leading to an enhanced anti-inflammatory response.

Microbiota can also generate CLA, which are produced mostly by *Bifidobacterium breve*, *B. longum*, *Lactobacillus* and *Propionibacterium* and modulate the immune system with its anti-inflammatory properties. PSA (produced by *Bacteroides fragilis*) is also characterized by its immunomodulatory and anti-inflammatory activities. The neurotransmitters (GABA and histamine) affect the central nervous system produced by various microorganisms, especially *Lactobacillus brevis*.(28)

1.12 Postnatal (29) nutritional exposures affect intestinal inflammation and associate with IBD susceptibility

Inflammatory bowel diseases (IBDs) including Crohn's disease (CD) and ulcerative colitis (UC), have been recognized as disorders whereby environmentally sensitive developmental factors may play an important etiologic role (30). The peak incidence of IBD is in young adulthood. Therefore, there is a prolonged developmental period from conception to young adulthood for environmental influences to critically impact biological systems relevant for IBD pathogenesis (31). Important elements of IBD pathology are thought to be the intestinal microbiome, the gut mucosa, and the mucosa associated immune system (30; 32). Our research group recently shown in a mouse model that maternal supplementation of 4 micronutrients could significantly modify offspring colitis susceptibility in association with colonic mucosal gene expression and microbiome alterations (33; 34). However, the same diet did not induce obvious phenotype changes with respect to intestinal inflammation when given during pediatric development. These findings underscored that pertinent nutritional factors can exert persistent colitis modifying effects during critical periods of mammalian maturation.

1.13 Cellulose supplementation early in life ameliorates acute colitis in adult mice

As for IBD, the decreased consumption of dietary fibers has been recognized to potentially play an etiologic role (35). The rising incidence of IBD in the developed world associating with a decrease in dietary fiber intake was emphasized by Burkitt during the early 70's (36). In agreement with this observation, a recent study highlighted the importance of different nutritional habits and gut microbial diversity in children from two different continents and cultures (European and a rural African village) (37). The authors called the attention to the reduction of fiber intake and the associated decrease of microbial richness in European children, which may be relevant in respect to gastrointestinal diseases. Indirect epidemiologic data supports these conclusions where high fiber and fruit consumption are associated with a decreased risk of CD and high vegetable intake is associated with a decreased risk of UC (35).

Cellulose is an insoluble fiber and an abundant component of a vegetarian diet since it is present in most plant tissues (38). It has a proliferative effect on the colon, mostly in the distal areas in rodent models (39). The colonic trophic effects of cellulose do not depend on microbial fermentation (40), as opposed to the case of other fibers, such as guar gum (41). Although cellulose may be fermented in the colon, the secondary production of SCFAs is limited (42). Despite the limited fermentation of cellulose, it has been shown to substantially modify colonic microbial composition (43). These results implicate it as a potential prebiotic (non-digestible carbohydrate that favors the growth of desirable microflora in the large bowel). Since cellulose is a major component of vegetable and fruit fibers, the indirect nutritional epidemiologic data already discussed (35; 37) would support it as an important factor in IBD pathogenesis. However, the effect of cellulose on mammalian colonic mucosal microbiome (which may be more relevant for IBD pathogenesis than luminal bacteria (44; 45)), or colitis susceptibility has not yet been investigated especially from the developmental origins perspective.

Therefore, in this study, we examined the direct and prolonged effects of pediatric cellulose supplementation on large intestinal growth, chemically induced colitis susceptibility, and colonic mucosal microbial community composition in mice.

1.14 Loss of omega-6 fatty acid induced pediatric obesity protects against acute murine colitis

A large-scale prospective nutritional study linked ω-6 fatty acid consumption to the development of UC.(46) However, clear results from human epidemiologic observations (even in prospective and well controlled) are very difficult to obtain for obvious ethical and technical considerations.(47) Therefore, the timing and nature of environmental factors critical to IBD development have remained largely unknown, as have the molecular mechanisms, which may uncover the environmental contribution to disease induction.(48) Environmental influences may trigger critical pathogenic changes at any time within an individual prior to the onset of disease, which can occur during a broad age range from fertilization to adulthood in case of IBD. Here, we examined the complex effects of transient high ω-6 fat diet consumption during pediatric development on the young adult metagenome, and immune system in a murine model of IBD.

1.15 Dysbiosis-associated gastrointestinal disorders and future therapeutic options

The commensal microbiota is a major communicator of dietary modification towards the intestinal immune system of the host and can rapidly change its composition upon nutritional challenges. Disease-associated microbiome changes can be seen in different GI disorders. For example, dysbiosis is found in IBDs. IBD is linked to a reduced proportion of Bacteroidetes and Firmicutes (with an increased Firmicutes/Bacteroidetes ratio) and an increased proportion of Proteobacteria compared to healthy individuals.(49) Colitis is also associated with a decreased abundance of *Faecalibacterium prausnitzii*, which is characterized by a notable anti-inflammatory effect.(50) Premature colonization of the intestine and other trigger factors together can lead to necrotizing enterocolitis (NEC), which is characterized by an increased abundance of Proteobacteria and Gammaproteobacteria and a decrease in Bacteroidetes and Firmicutes.(51) The key role is the pathomechanism of NEC is supported by the fact that probiotic supplemented breast milk can decrease the chance of developing the disease.(52) Irritable bowel syndrome (IBS) is a functional bowel disorder described with abdominal pain and alternating defecation habit. Pediatric IBS subtypes are distinguished by microbial composition differences. IBS

is associated with an increased abundance of Gammaproteobacteria and pain frequency is linked to a greater proportion of *Alistipes* genus.(53)

Reduction in microbial diversity or richness is also related to the development of intestinal diseases (e.g., IBDs, NEC). However, alteration of diet does not specifically mean beneficial effect on the colon. Monotonous dietary intake may decrease mammalian vulnerability against intestinal inflammation in association with microbiota separation. (54) In conclusion, the understand of the unique human gut microbiome and its association with disorders will may help developing personalized medicine and therapeutics in the future. However, results of the microbiome studies are based mostly on findings from pharmacologically treated patients. Treatments highly modify microbiome, and more significantly than the disease itself. (55; 56) For example, enteric dysbiosis in untreated IBD patients appears to be rather modest and provided unexpected findings in recent pediatric studies.(57)

Microbial communities with different metabolic profiles on drugs will may modify the current treatment strategies. (58) Microbiome can modify drug action, metabolism and toxicity in the human gut and liver. Gut-associated microbes alter drug metabolism by modulating the drug-metabolizing enzymes activity, or change their level. Microbes also can directly produce enzymes that activate or degrade drug metabolites, or compete with molecules affecting xenobiotic metabolism. In the future, pharmacomicrobiomics will may be taken into consideration with pathologic and pharmacokinetic characteristics of individuals.

1.16 The manipulation of microbiota as a possible therapeutic option in pediatric GI disorders

The restoration of human microbial homeostasis may be highly beneficial for GI disorders, where dysbiosis present. The most complex bacteriotherapy is fecal microbiota transplantation (FMT). FMT is the process of stool transplantation from a healthy individual into a recipient. The history of FMT goes back to the 4th Century, when Ge Hong used human fecal suspension by mouth for food poisoning or severe diarrhea.(59) The first case series were reported in 1958 by Eiseman and colleagues, who treated

pseudomembranous colitis by fecal retention enema.(60) Immediate recovery and resolution of symptoms was observed within days in all patients. As our current knowledge, FMT is safe and highly effective for the treatment of recurrent *Clostridium difficile* infection (CDIF) in patients without complicating clinical conditions. Besides recurrent CDIF, there is an emerging interest to use FMT as a novel therapeutic option in patients with other GI disorders, such as IBD or other diseases.

1.17 CDIF and FMT

CDIF is emerging, despite the increased efforts towards prevention, improving diagnosis and therapy.(61) CDIF is the most commonly reported nosocomial pathogen in the United States (US). Epidemiologic data suggest that the CDIF-associated severe diarrhea is continuously increasing with a rise in hospitalization, morbidity and mortality.(62; 63) The latest reports showed CDIF to cause more than $1.5 billion excess medical costs annually in the US. (64) The incidence of pediatric CDIF is also increasing.(65) Recurrence of CDIF is 20-30%, which increases upon repeated infections in spite of new antibiotic regimens.(66) FMT has been shown to be the most effective treatment to date for CDIF.(67) The largest systemic review including 844 patients (76% diagnosed with CDIF) undergoing FMT showed 90.7% cure rates.(68) Similar cure rates have been shown for children,(69; 70) but microbiome analyses were performed only in a few cases.(71)

1.18 IBD and FMT

UC and CD are the two forms of IBD that affects about 50% of the estimated 1.8 million people suffering from IBD in the US.(72) About 20% of IBD cases present in children, where the disease frequently (~40%) becomes refractory to conventional medical therapy within 10 years of diagnosis, leading to colectomy.(73) FMT is an emerging unconventional treatment for UC.(74; 75) Almost half (46%) of adult UC patients would consider FMT as therapeutic option (76), and both adult and pediatric patients wish to have it available as a treatment modality.(77) A recent meta-analysis evaluated the efficacy of

FMT as a treatment option for IBD patients.(78) Eighteen studies were included with 122 patients (79 UC and 39 CD). The 9 cohort studies, 8 case studies and 1 randomized controlled trial demonstrated an overall 45% clinical remission over varying length of follow up time. According to disease severity, 37% of patients had active disease, 29% had moderate-to-severe disease, and 23% had mild disease. However, the review did not specify any correlation between FMT efficacy and IBD disease activity.

A pediatric trial tested 5 daily FMT in mild-to-moderate UC, where 6 out of 9 children (67%) achieved and maintained clinical remission 4 weeks after the last treatment.(79) However, a phase I pediatric study showed negative results following a single FMT via nasogastric tube.(80) The first randomized, placebo controlled trial of FMT as treatment for active UC was presented as an abstract at the Digestive Disease Week conference in 2014. Moayyedi *et al.* compared administration of FMT versus water-enema for 6 consecutive weekly treatments. They found no difference in the primary outcome of clinical remission after 6 weeks. However, 16 of the 27 patients in the active arm reported subjective improvement, and were allowed to continue receiving weekly FMT for an additional 6-12 weeks. With the extended therapy, 33% of patients achieved clinical remission.(81) Their consecutive publication indicated that FMT induces remission in a greater proportion of UC patients than placebo. Additionally, patients with a less than 1-year history of UC responded better to FMT than those with more prolonged disease course prior to the intervention.(82) Interestingly, another recently published placebo controlled trial did not find a clinically significant benefit from FMT in adult UC patients.(83) This protocol differed significantly from Moayyedi, et al. by administering only 2 FMT treatments within 3 weeks by naso-duodenal delivery and they used autologous stool as placebo. The different outcomes from the two controlled studies may be related to the difference between the placebo used, the mode of delivery, the number of FMT given in addition to differences in recipient disease activity at the time of FMT initiation.

1.19 CDIF and IBD association

Interestingly, *Clostridium difficile* is an opportunistic pathogen that presents at high rates in patients with underlying chronic diseases and long-term hospitalization. For instance, the prevalence of CDIF at disease onset was 8.1% in a pediatric population with underlying IBD at Texas State, USA.(84) This prevalence is much higher than the general population.

1.20 Other GI disorders and FMT

There is an increasing attention on the association between intestinal microbiome, obesity and obesity-related metabolic disorders. The presented altered microbiome is associated with low-grade inflammation of the gut.

Researchers described dysbiosis in patients with irritable bowel syndrome. The composition of microbiota represented the symptoms associated with the disease (constipation vs. diarrhea type IBS). (85) Additionally, 70% of the patients experienced improvement of their symptoms following FMT.(86) Furthermore, long-term resolution was seen almost half of the patients.

The strong association between diet, host metabolism and gut microbiome demonstrates homeostasis and misbalance in cases with metabolic disorders. The transplantation of microbiome composition from a lean subject to an obese improved insulin resistance by the increased presence of SCFA-producing bacteria.(87)

1.21 Non-GI disorders and FMT

There is an increasing interest in the role of intestinal dysbiosis and non-gastrointestinal disorders, and to treat patients with FMT. The gut microbiota regulates intestinal function, the immune and nervous systems of the intestine, and influence the brain by the microbiota-gut-brain (MGB) axis. This concept opens the door for a possibly novel therapeutic option for the treatment of neuropsychiatric disorders, chronic fatigue disease and autism.

2 OBJECTIVES

1. There is a direct link between nutrition, microbiome and host response.

2. Postnatal exposure to different nutrients (such as cellulose or fat) has transient or persistent effect on intestinal homeostasis and predispose to the development for intestinal inflammation.

3. Complex bacteriotherapy, such as FMT provides treatment and/or the resolution of symptoms for patients suffering in CDIF and IBD.

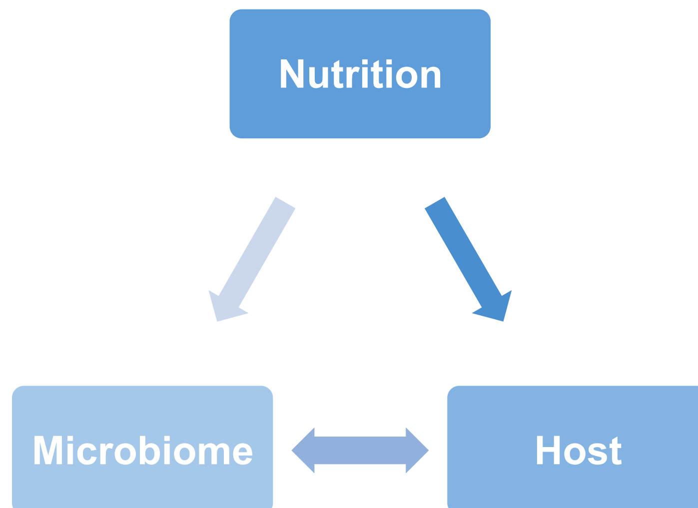

Figure 1 The Nutrition-Microbiome-Host Triangle.

3 METHODS

3.1 Animals, diets and experimental design

3.1.1 Cellulose supplementation study

Our initial observations were made on C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME, USA) receiving (regular chow [12.5% dietary fiber]; 2920X, Harlan-Teklad, Madison, WI, USA) or synthetic low cellulose (2.5% cellulose, LC; #102460, Dyets Inc., Bethlehem, PA, USA) from 30 to 90 days of age. In the consecutive experiments, 21-day old (postnatal days 21, P21) C57BL/6J male mice were provided free access to regular chow (12.5% dietary fiber) within the same room of our animal facility for 9 days. At P30, the mice were randomly allocated to receive synthetic low cellulose or high cellulose (12.5% cellulose, HC; #102532) diet for 50 days (Table 2).

Table 2 The composition of the synthetic low cellulose (LC: 2.5% cellulose) and the high cellulose (HC: 12.5% cellulose) diets.

		LC	HC	LC	HC
Ingredient	kcal/gm	grams/kg		kcal/kg	
Casein	3.58	200		716	
DL-Methionine	4	3		12	
Sucrose	4	341.46		1366	
Cornstarch	3.6	242.5	172.5	873	621
Dyetrose	3.8	90	60	342	228
Corn Oil	9	51		459	
Cellulose	0	25	125	0	
Mineral Mix #200000	0.47	35		16.45	
Vitamin Mix #300050	3.92	10		39.2	
Choline Bitartrate	0	2		0	
Ethoxyquin	0	0.04		0	

Conventional chows regularly contain ~12.5% fiber (including cellulose). At P80, the HC animals were reversed to control (low cellulose) diet for 10 (HCR10 = P90), or 40 days (HCR40 = P120) (Figure 2). For the microbiome studies, 10 animals in two cages were used for each dietary group. Those were allocated to receive either low cellulose (control, LC, 10 animals in 2 cages), or high cellulose diets (HC, 10 animals in 2 cages). At P80, 2 animals from each cage were crossed between the groups to eliminate cage bias. Five control animals at P80 and 5 at P90 were euthanized for tissue collection. There was no significant separation between these groups based upon a principal coordinates analysis

(PCoA) of 16S rRNA sequence information; therefore, these were grouped together as a single control. As for the high cellulose group, 2 animals were crossed between the two cages at P80. One cage was continued on high cellulose diet until P90 (HC), and the other cage was reversed to the control diet (low cellulose, LC) until P90 (HCR10), when those were euthanized for tissue collection. The discovery cohorts included 3-3 mice in two independent studies (P90 LC and HC; and P90 LC and HCR10) with the same experimental conditions.

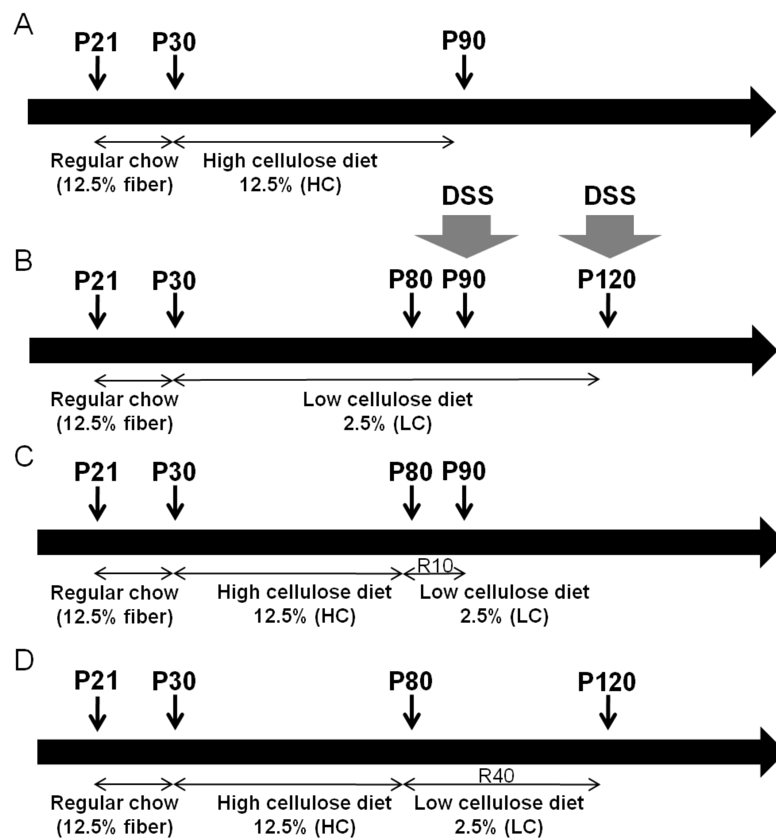

Figure 2 Schematic description of the cellulose feeding protocols. A: 12.5% high cellulose (HC) group without reversal for the purposes of colonic length and microbiota analysis. B: 2.5% low cellulose (LC) group. C: 10-day reversal group (HCR10). D: 40-day reversal group (HCR40). For the B, C and D groups DSS was administered at postnatal day 90 (P90) and P120, respectively. (DSS: dextran sulfate sodium; R10 and R40: 10 or 40 days of reversal following high cellulose diet; P21/30/80/90/120, 30/80/90/120 days postnatal age).

3.1.2 High fat supplementation study

P21 male C57BL/6 mice received standard rodent diet (2920X) until P30. Then, mice were randomly assigned to artificial diets: low 12% caloric content corn oil/linoleic acid: control diet, C (DYET# 102460); or to high ω-6 (40% caloric content corn oil/linoleic acid: ω6 or HF) (DYET# 102459) from P30 to P80. Following this 50-day period, the animals were reversed to C for 10 (ω6-R10) or 40 (ω6-R40) days. High milk fat, high cholesterol (MF) diet (DYET# 112734) was used with the same feeding protocol to examine fat-dependent effects. Fat to body weight ratio was interrogated on P90 and P120 by quantitative magnetic resonance imaging (qMRI). 6-week-old Swiss-Webster germfree (GF) mice were derived by cesarean section under GF conditions at Taconic Farms Inc. (SWGF; Hudson, NY, USA) and delivered with specific GF shipping for the microbiota transfer experiments.

All mice were housed in our specific pathogen free animal facility during the experiment. All applicable institutional and governmental regulations concerning the ethical use of animals were followed. The protocol was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

3.2 Dextran sulfate sodium exposure

Susceptibility to colitis was tested by administering 2% (wt/vol) (for the synthetic chow experiments) or 3% (for the original regular chow vs. synthetic chow observations) dextran sulfate sodium (DSS; MW=36000-50000, MP Biomedicals, LLC, Solon, OH, USA) in the drinking water at P90 or P120 *ad libitum* for 5 days followed by regular water for an additional 9 days. DSS of this molecular weight induces diffuse colitis from cecum to distal large bowel (88). The animals were weighed daily and colonic length measurements were performed at the end of the experiments following CO_2 asphyxiation. Weight loss during DSS administration in mice has been shown to correlate well with molecular and histological outcome measures of colitis severity (27; 31; 89). Therefore, we decided to follow weight loss, colon length, and histological severity of intestinal inflammation as the primary outcomes measuring colitis severity in our DSS experiments.

3.3 Tissue collection and histological analysis

At the end of the feeding periods, mice were sacrificed by CO_2 asphyxiation between 11:00 AM and 2:00 PM without any previous food restriction. The colons were placed on ice, transected longitudinally, cleansed from feces, washed with ice cold normal saline, followed by the collection of colonic mucosa with a microscope slide (90) (excluding the cecum). The mucosal scrapings were flash frozen on dry ice, and stored at -80°C as earlier described (31).

For histological analysis, additional colonic samples were transected and processed for standard hematoxylin-eosin (HE) staining following fixation in 10% formaldehyde. Histological severity of intestinal inflammation was determined by a blinded pathologist based upon tissue damage grade and the presence of inflammatory cells. (54)

Morphometric analyses (crypt length, surface area) were performed with Olympus DP2-BSW program on transected proximal (post-cecal) colonic specimens.

3.4 Microbiota transplantation in mice

Cecal contents were pooled from ω6-R40 or C120 littermates. Cecal extracts were suspended in phosphate-buffered solution (PBS, 2.5 ml per cecum) and were administered orally by gavage (0.1 ml per mouse) immediately to sterilely packed 6-week-old SWGF male GF mice (Taconic, Hudson, NY, USA) as described by Vijay-Kumar et al.(91) Transplanted mice were fed with-control diet for 5 days, and then 3% of DSS was given to induce acute colitis. Body weight was measured daily. The animals were euthanized and tissues were collected as above.

3.5 DNA extraction and analysis of microbiome

Colonic mucosal samples were submitted to the Texas Children's Microbiome Center (TCMC, Houston, TX, USA) for DNA extraction and sequencing. Community DNA was extracted from each specimen using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), following the HMP modifications (2) to the manufacturer's instructions. The resulting DNA was quantitated using both a NanoDrop-

1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and Qubit fluorometer (Life Technologies Corporation, Carlsbad, CA, USA). Barcoded universal primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 926R (5'-CCGTCAATTCMTTTRAGT-3') were utilized to amplify the V3-V5 region of the 16S rRNA gene. The discovery study utilized a different set of PCR primers (28F [5'-GAGTTTGATCNTGGCTCAG-3'] and 519R [5'-GTNTTACNGCGGCKGCTG-3'] amplifying the V1-V3 region of the 16S rRNA). Each library construct was then processed and purified for 454 sequencing. Sequencing was performed on the Roche GS FLX 454 sequencer (454 Life Sciences, Branford, CT, USA).

Sequence data were parsed by barcode and quality filtered using QIIME (version 1.3.0) (92), as implemented in the Genboree Microbiome Toolset (93). Sequences shorter than 200 bp lengths, having average quality scores less than 20, harboring ambiguous base calls, or having mismatches to their barcode or sequencing primer were excluded from further analysis. Both the barcodes and sequencing primers were trimmed away, and the remaining sequences from the control and treatment groups were pooled and assigned to operational taxonomic units (OTUs) at a similarity cut off of 97% using Cd-hit (94). The data set was screened for potential chimeras using the ChimeraSlayer algorithm (95), and all potential chimeras were excluded from downstream analysis. Identities were assigned to each OTU using the Ribosomal Database Project Classifier (96).

Prior to the calculation of diversity metrics or comparison across treatments, the sequence libraries were randomly subsampled to achieve even sampling depth (subsampled depth = 3634 sequences per library). All results presented here were determined using our subsampled sequence libraries. Community similarity was evaluated across treatments among treatments using principle coordinates analysis (PCoA) of OTU data. PCoA plots were generated using weighted Unifrac distances, as calculated in QIIME.

Sequence libraries were also evaluated for potential differences in composition using two-tailed Mann-Whitney U-tests and false discovery rate correction for multiple comparisons in Genespring GX (version 12.0) (Agilent Technologies, Santa Clara, CA, USA). A stratified approach was used, first identifying OTUs that differed significantly with respect to the treatment groups and controls, then exploring these differences further in

the context of the R10 and R40 treatment, with the goal of identifying changes that may be transient or longer-lasting.

3.6 Flow cytometry

To conduct lymphocyte population analysis, mesenteric lymph nodes (MLNs) and spleens (SPLs) underwent mechanical disruption, erythrocyte lysis and preparation for flow cytometry as described previously.(97) Lymphocytes were labeled for the markers cluster differentiation (CD) 4 and C-X-C motif ligand chemokine receptor (CXCR) 5 (BD Biosciences, San Diego, CA, USA). Flow cytometry was performed using a FACSCanto instrument (BD Biosciences, San Diego, CA, USA), and the data was analyzed using FlowJo software (Treestar, Ashland, OR, USA).

3.7 *In vivo* chemokine blocking

C57BL/6J mice were treated with 0.2 mg of neutralizing C-X-C motif ligand chemokine (CXCL) 13 antibody (ab) (#MAB470; R&D Systems) or isotype control ab (#MAB006; R&D Systems, Minneapolis, MN, USA). Both mouse abs were delivered by intraperitoneal injection in 1 mg/ml concentration dissolved in PBS. To examine the effect of CXCL13 ab on DSS-induced colitis model, abs were administered on the first, third and fifth day (3 times) after initiating 3% DSS.

3.8 Human samples and Enzyme-linked Immunosorbent Assay (ELISA)

33 pediatric patients were recruited prior to endoscopy following informed consent though the institutional review board (IRB) approved tissue bank of the Pediatric Inflammatory Bowel Disease Consortium Registry at the Baylor College of Medicine (H-17654). Sera of 12 UC, 11 CD and 10 healthy controls (C) were used for quantification of circulating CXCL13 levels. Pediatric controls included children who underwent colonoscopic evaluation for diagnoses of hematochezia, diarrhea, or abdominal pain, but whose endoscopy was grossly and histologically normal. BD Vacutainer® CPT™ Cell

Preparation Tube with Sodium Citrate was used to isolate peripheral blood mononuclear cells (PBMCs) from peripheral blood (PBL). PBLs were centrifuged at 1000x g for 10 min and stored at -80°C. QuiAmp DNA mini kit (Qiagen, Valencia, CA) was utilized on the isolated PBMCs to retract DNA. Plasma concentrations of CXCL13 were quantified by Quantikine ELISA methodology according to the manufacturer's instructions (#DCX130; R&D Systems, Minneapolis, MN, USA). All samples were measured in duplicate. Color intensity of the assay was measured by a standard ELISA microplate reader. Color intensity correlates with the amount of bound CXCL13. Quantikine kit standards were used for generation of standard curves.

3.9 FMT protocol

3.9.1 Subjects with recurrent CDIF

Ten pediatric patients (5 females and 5 males; 2-16 years of age [y], average 9.3 y) with recurrent CDIF received FMT under an IRB approved protocol (Table 3). The experimental nature of FMT was highlighted during consenting.

Table 3 Characterization of patients received fecal microbiota transplantation. Patients highlighted had no major underlying diseases and were evaluated for further analysis.

Pt	G	R	E	Age (y)	Significant complicating disease	Prior CDIF treatment
1	M	W	NH	15	ulcerative colitis	vancomycin
2	**F**	**W**	**NH**	**16**		**vancomycin and metronidazole**
3	F	W	H	15	cerebral palsy	vancomycin
4	**M**	**W**	**NH**	**4**		**vancomycin**
5	**M**	**W**	**NH**	**2**		**vancomycin**
6	F	W	NH	2	heart transplant	vancomycin
7	M	W	NH	6	Crohn's disease	Vancomycin
8	F	W	NH	2	infantile spasm	vancomycin
9	**M**	**W**	**NH**	**15**		**vancomycin**
10	F	W	NH	16	mild ulcerative colitis	vancomycin

(E: ethnicity; F: female; G: gender; H: Hispanic; M: male; NH: non-Hispanic; Pt: patient; R: race; W: White; y: years)

Three patients had IBD (2 UC and 1 CD), 1 had heart transplant, and 2 had significant neurologic impairment as underlying conditions (Table 3). All patients received at least 1 course of metronidazole (10-14 days) and vancomycin orally. They all had recurrent/ongoing symptoms in spite of at least 2 courses of antibiotics. Eight out of ten patients received vancomycin until one day prior to FMT; one patient had finished vancomycin therapy 2 weeks before FMT, and one 3 weeks prior to FMT.

3.9.2 Subjects with UC

Subjects were recruited from the patients treated by the Pediatric Gastroenterology, Hepatology, and Nutrition Section at Baylor College of Medicine/Texas Children's Hospital. Only patients whose clinical, endoscopic and histologic findings supported the diagnosis of UC were recruited. Only steroid, thiopurine, or biologic agent dependent patients were included following informed consent (i.e. "immunotherapy" dependent). Enrollees had to test negative for *Clostridium difficile* toxin by polymerase chain reaction (PCR), or enzyme immunoassay (EIA), and agree to withdraw all medications prior to and during the trial. They also had to agree to a pre-treatment surgical consultation and acknowledge the potential need for colectomy, if disease exacerbations cannot be controlled by conventional medical therapy.

We analyzed the clinical and microbial data of 3 patients from our first pilot study (Table 4) and 6 patients from our second Investigational New Drug (IND)-linked phase 1 clinical trial (Table 5).

Table 4 Patient characteristics and clinical outcomes after sequential FMT from our first pilot study.

Patients (Age, Gender)	Disease Behavior	Mayo Score	Tx after Dx	Mayo Score	FMT #	Remission during FMT (in Days)	Mayo Score	Remission after last Medication (Days)	Remission after last FMT (Days)	Tx following Flare
		At Dx		Before FMT			After FMT			
1 (16y M)	Pancolitis	2	IFX	0	30	65/70 (93%)	0	261	126	IFX
2 (15y M)	Pancolitis	2	6MP	1	25	58/58 (100%)	0	159	80	PRED FMT
3 (14y F)	Pancolitis	3	PRED	0	22	36/36 (100%)	0	105	79	PRED FMT Colectomy

(Dx: diagnosis; F: female; IFX: infliximab; M:male; 6MP: 6-mercaptopurine; PRED: prednisone; Tx: therapy)

Table 5 Patient characteristics, premedication and donor characterics in our IND phase 1 clinical study.

Study ID	G	R	E	Age (y)	Treatment prior to FMT	FMT specimens	Age at diagnosis (y)	Time between diagnosis and first FMT (months)
P001*	M	W	NH	16	Prednisone	D001	13	41 (*29)
P002	F	W	NH	18	Prednisone	D002	17	7
P003[#]	F	W	H	15	Prednisone	D001	13	18 ([#]6)
P004	M	W	NH	20	Prednisone	D001	16	43
P005	M	W	NH	17	Prednisone	D001	17	3
P006	M	W	NH	13	Prednisone	D001	11	22
D001	M	A/I	NH	37				
D002	F	A/I	NH	30				

(D: donor; E: ethnicity; F: female; G: gender; H: Hispanic; ID: identification; IFX: infliximab; M: male; 6MP: 6-mercaptopurine; NH: non-Hispanic; PRED; prednisone; Pt: patient; R: race; W: White; y:years)

3.9.3 Donor recruitment

Healthy adult stool donors (between 18 and 45 years of age) were recruited by the research staff following informed consent. Donors were asked to volunteer for the screening (pass a health questionnaire, serologic and stool tests and regularly supply stool samples according to the study protocol.

3.9.4 FMT preparation

The stool preparations were performed in the TCMC. This facility operates under Good Laboratory Practice (GLP) and is part of the clinical enterprise in the Department of

Pathology, accredited by the College of American Pathologists (CAP) and certified by Clinical Laboratory Improvement Amendment (CLIA). Standard Operating Procedures (SOPs) on fecal specimen preparation and for decontamination procedures for biosafety cabinets and equipment were followed before and after fecal preparation. Freshly collected stool specimens from the healthy adult donor (within 2 hours of passing) were delivered on ice for processing. Specimens were aliquoted to ~50 g aliquots, and cold sterile normal saline solution (NSS) was added prior to homogenization in a strainer bag with 500-µm pore size (Seward Laboratory Systems Inc., Port Saint Lucie, FL) using the Smasher Laboratory Blender/Homogenizer (AES CHEMUNEX Inc., Cranbury, NJ). Sterile glycerol was added to filtered homogenized stool specimens containing the fecal microbiome at a final concentration of 10% according to Hamilton et al.(98) Stool preparations were immediately stored at -80˚C until transplantation or analysis. All stool preparations were labeled with an expiration date 8 weeks from the date of preparation. At each FMT treatment, fecal preparations were rapidly thawed at 35˚C in a water bath and used within 15 minutes. Sterile NSS was used as a diluent to reach the final volume of 250 ml from 50mg of original stool prior to delivery.

3.9.5 FMT treatment protocol in CDIF patients

Patients received filtered, frozen-thawed fecal preparation from a single, screened, standardized (D002, 9 patients), or a self-designated donor (D005-father of patient 6) through colonoscopy, followed by enema or nasogastric consecutive FMT, if clinically indicated. The donor screening and process was approved by the US Food and Drug Administration and published previously.(99) The CDIF patients received the therapy only one time (or a second time if there was no clinical improvement in their condition.

3.9.6 FMT treatment protocol in UC patients

UC patients received a sequential therapy of more FMTs:

Initial colonoscopy and FMT treatment (Day 1): At the time of colonoscopy, an assessment for macroscopic colitis using the Mayo classification was performed. Biopsies were

obtained from the rectosigmoid and cecum in an ascending fashion for routine histopathology and research purposes. Following mucosal sampling, subjects underwent FMT with 250 ml of thawed stool preparation, 1/3 of which was endoscopically administered into the terminal ileum and 2/3 into the right colon as targeted site as found feasible by Brandt and colleagues.(100)

Subsequent FMT Treatments: The duration of FMT therapy was planned to be 12 weeks.

Days 2 through 14: Subjects came to the ambulatory clinic daily for clinical symptom evaluation and fecal retention enema administration (60-250 ml rectally [as tolerated] with retention for at least 30 minutes).

Days 15 through 28: Enemas were given 3 times a week on weeks 3 and 4 of the protocol.

Days 29-84 (2 to 3 months): Enemas were given weekly for a total of 3-8 weeks (less than 8 secondary to the cessation of the protocol according to the FDA mandate).

During the IND study (P001-P006, this protocol does not involve the first 3 patients from the pilot study), monthly enema was provided up to a year.

As supportive care, patients were allowed to take 4 mg (2 tablets of over the counter Imodium) loperamide by mouth 15-30 minutes prior to enema treatments to help retain the preparation. This dose of loperamide is appropriate for the age group. Loperamide is over-the-counter and FDA approved for the treatment of inflammatory bowel disease associated diarrhea.

Response and progression was monitored by PUCAI during the protocol. The clinical symptoms survey was performed prior to each enema delivery and a disease progression table was recorded for each enrolled patient.

3.9.7 Sample collection

Stool samples of CDIF patients were collected the day prior FMT and 8-9 weeks following treatment on a follow-up visit. Select patients provided additional samples.

Samples from UC patients were collected on the day prior to FMT, two weeks after the last weekly enema, and at 6 months after the initiation of FMT.

3.10 Human microbial data analysis

The fecal microbiome was characterized using 454 pyrosequencing of the bacterial 16S rRNA gene on samples. Stool samples were processed by the TCMC for DNA extraction, sequencing and microbial data analysis (same as mentioned above in our mice protocol).

Given variation in sequencing depth, the 16S rRNA gene libraries were sub-sampled to an equal depth (i.e., 1900 sequences per library) prior to the evaluation of richness or calculation of diversity indices, including the Shannon diversity index and unweighted UniFrac distance measures. The results from the microbiome characterization of the donor were compared to the patient's microbiome prior and after transplantation.

3.11 RNA sequencing and analysis

RNA samples from colon biopsies were QCed by spectrophotometry (NanoDrop-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and microfluidic electrophoresis (Experion Automated Electrophoresis System, Bio-Rad Laboratories, Hercules, CA). PolyA-selected libraries were prepared from total RNA samples with TruSeq RNA Sample Preparation Kits (Illumina, San Diego, CA). Cluster generation was performed with Illumina TruSeq SR Cluster Kits v3 - cBot – HS, in a cBot Cluster Generation System and 100 bp paired-end-sequencing using Illumina TruSeq SBS Kits on an Illumina HiSeq 2000 Sequencing System resulting in mean sequencing depth of 160 million (101-213 million) reads per sample. CASAVA software (Illumina) was used to convert raw read data to fastq format. Sequencing reads were trimmed for quality ($q < 20$) and adapters and then aligned to the human genome (GRCh37/hg19) using Tophat2[9]. Cufflinks[10] was used for estimation of transcript abundances based on Fragments Per Kilobase of exon per Million fragments mapped (FPKM). Differential expression analysis was carried out using Cuffdiff, which calculates a test statistic based on the log ratio of a gene's expression in two conditions against the log of one. Multiple testing correction at a false discovery rate < 0.05 was applied to identify differentially expressed genes.

3.12 Histology

The biopsy specimens were examined by a GI pathologist, who was blinded to the previous reported histology reports. The specimens were fixed in 10% neutral buffered formalin immediately following endoscopy. The tissue samples were routinely processed and paraffin embedded. Paraffin sections (3 micron) were cut and HE stained with eight tissue sections on one slide. Duplicated 3-micron sections were stained for histone (H3) via immunohistochemistry using a polyclonal rabbit anti-human histone (06-570; Millipore, Billerica, Massachusetts). After pretreatment with HIER1 (Bond Epitope Retrieval Solution 1) for 30 minutes at 100 degree Fahrenheit, the specimens were incubated with the primary antibody dilution 1:800 at room temperature for 15 minutes. The detection system used was the Novocastra Bond Polymer Refine Detection System (biotin-free, peroxide conjugated) from LEICA, Newcastle upon Tyne, United Kingdom with diaminobenzidine tetrahydrochloride (DAB) as the chromogen and HE as the counterstain.

3.13 Additional statistical analysis

Nonparametric, two tailed Mann-Whitney-tests was utilized for richness calculation; parametric, two-tailed Student's T-test and correlation calculations were used in the analyses of OTU number changes and group comparisons. The statistical significance was declared at $p<0.05$. Error bars represent standard error of the mean (60). A parametric, two-sided Student's two-sample t-test was used in the analysis of shared similarities and distances. Spearman correlation coefficient and level of significance was calculated in Prism4.03 between Mayo score and length of clinical remission. The statistical significance was declared at $p<0.05$ with Bonferroni corrections.

4 RESULTS

4.1 CELLULOSE SUPPLEMENTATION STUDIES IN MICE

4.1.1 Increased severity of DSS colitis on a synthetic, low fiber diet

Chemical colitis in C57BL/6J mice is usually induced by transient exposure to 3-5% DSS (88; 101). When we initiated 3% DSS treatment in animals that received a synthetic, low cellulose (2.5% cellulose, LC) diet, they lost excessive weight necessitating early euthanasia (Figure 3A). Therefore, mice on the LC diet had significantly increased susceptibility to chemically induced colitis compared to animals on regular chow (12.5% dietary fiber). Based on our findings, we utilized 2% DSS treatment to induce colitis in our further experiments involving low cellulose diets.

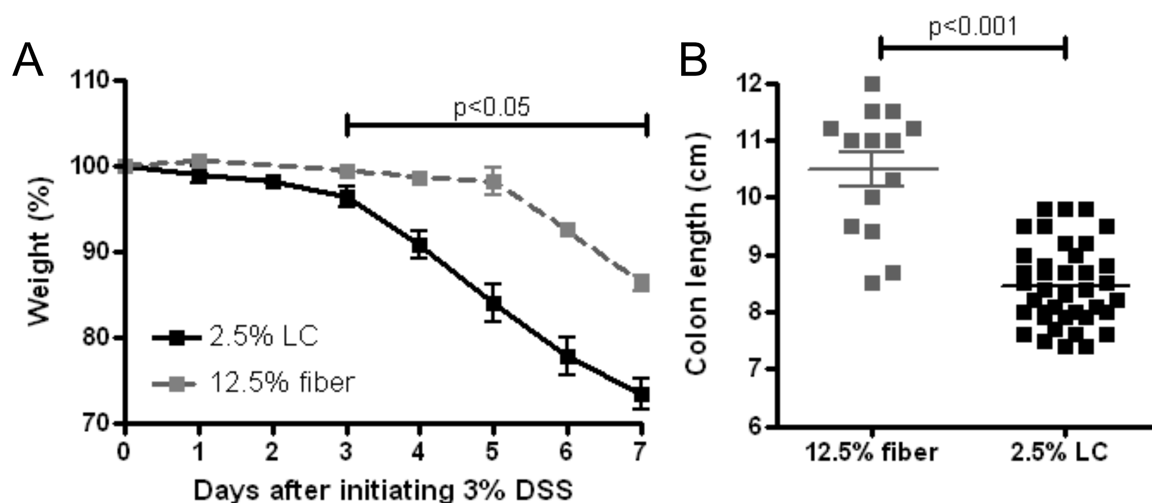

Figure 4 Increased severity of colitis and decreased colonic lengths in P90 mice fed by a synthetic diet [low cellulose (2.5%, LC) as fiber] compared to regular chow (12.5% dietary fiber). Upon 3% DSS challenge (A, n=8-10), the animals on the LC diet lost significantly more weight than on regular chow. On the seventh day, the experiment had to be terminated for severe weight loss in the LC group. In independent experiments where the mice did not receive DSS (B, n=14-40), colonic lengths of mice fed the synthetic diet were significantly shorter than in animals fed regular (12.5% fiber) chow.

Upon comparing large intestinal lengths between mice receiving regular chow and our synthetic (LC) diet, we recognized that colons were significantly shorter on the artificial diet (Figure 3B) without any previous exposures (such as DSS). Since dietary fibers and cellulose specifically have been shown to promote intestinal lengthening (trophic effects) in rodents (39; 102), we postulated that the low cellulose content of the synthetic diet may have been responsible for the observed shorter baseline colon lengths (leading to smaller colonic surface area) and increased susceptibility to DSS colitis (secondary to increased DSS to relative colonic surface, i.e. higher concentrations of the chemical irritant at the mucosa).

4.1.2 Transient trophic and anticolitic effects of cellulose supplementation

Dietary fibers have been observed to decrease colitis severity in acute and chronic rodent models (103-105). However, the means by which this effect is accomplished, and whether all fiber types have such anti-inflammatory properties, remains questionable. We were particularly interested if dietary cellulose supplementation by itself could decrease DSS colitis severity and how persistent such effects may be. Consequently, the cellulose content of our synthetic diet was selectively increased (high cellulose, HC) and fed to the animals during the pediatric period (P30-P80 (106)). Thereafter, the mice were reversed back to the LC diet for 10 (high cellulose reversed 10 days: HCR10 = P90 total), or 40 (HCR40 = P120 total) days to examine the persistence of any anticolitic effect induced by pediatric cellulose supplementation. The pediatric period is highly relevant with respect to the nutritional and developmental origins of IBD since the disorders most commonly present in young adulthood (31). Colons were significantly longer in mice fed a high cellulose diet even after 10 days of reversal compared to controls (Figure 5A). Remarkably, the large intestines were similar in length compared to control following 40 days of reversal (Figure 5C). The severity of DSS (2%) colitis correlated inversely with baseline colonic lengths in the feeding groups. Mice reversed for 10 days from the high cellulose diet (HCR10) were protected compared to the controls (LC) (Figure 5B). Colitis susceptibility was similar between the 40 day reversal (HCR40) and LC groups (Figure 5D). The severity of histological inflammation upon DSS challenge also decreased ($p=0.016$) in the HC

dietary group compared to controls (LC) supporting the findings on weight loss and colonic shortening (Figure 6).

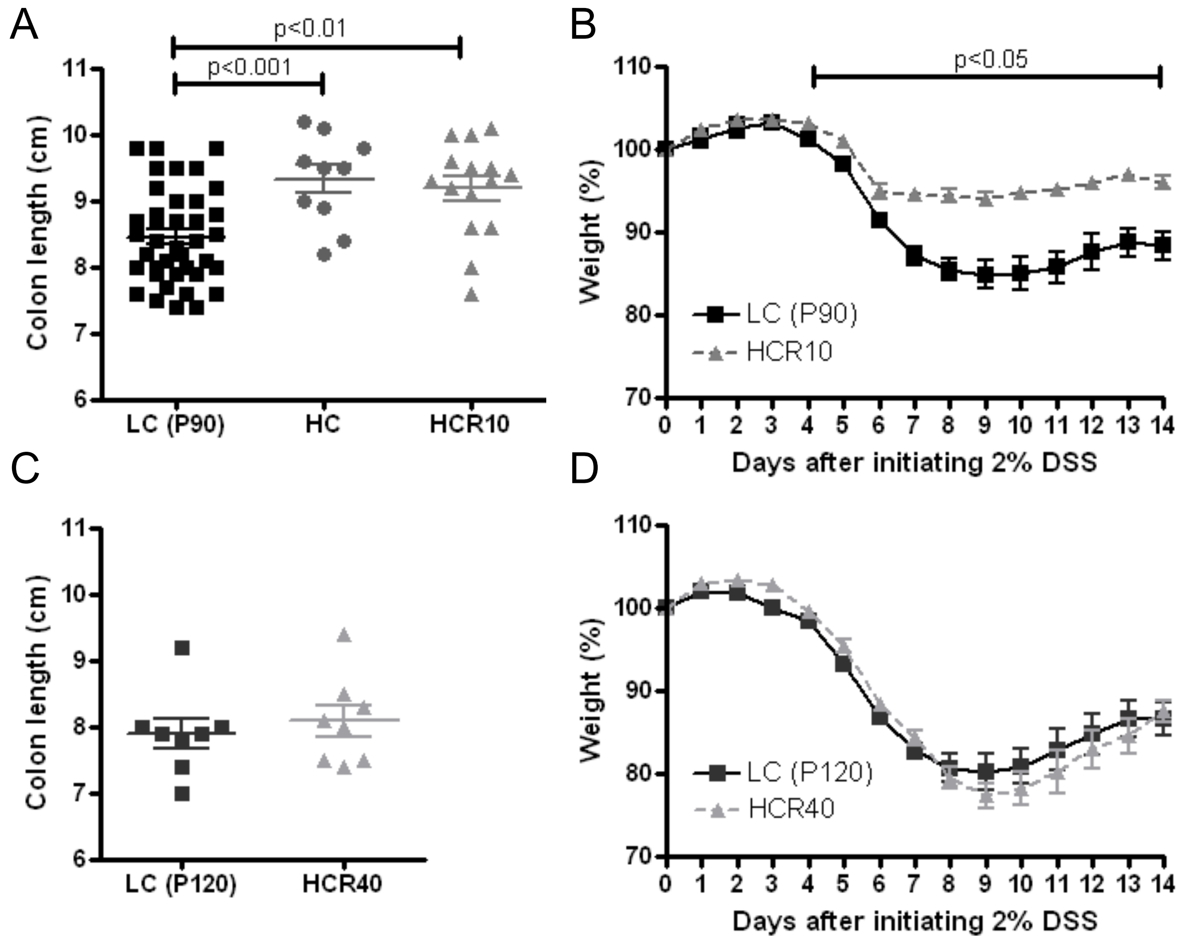

Figure 5 Transient colonic trophic effect and protection against dextran sulfate sodium (DSS) colitis upon cellulose supplementation. A. Colons were significantly longer in animals receiving 12.5% cellulose supplemented diet (HC). This trophic effect persisted after 10 days of reversal (HCR10) from the 12.5% cellulose to 2.5% cellulose diet (LC P90) [n=40-10-15]. B. Other mice were exposed to 2% DSS (as opposed to 3% in Figure 1, due to the increased sensitivity of mice to DSS on the low cellulose diet) in their drinking water at P90 for 5 days then received regular water. Mice reversed for 10 days (HCR10) from the high cellulose (12.5%) diet were protected (less weight loss) against DSS compared to controls (LC P90) [n=7-10 per group]. The histological score confirmed our results: high cellulose supplemented group had decreased severity of colitis upon DSS challenge (See Figure S2). C. The cellulose supplementation induced trophic effect was lost after 40 days of reversal (HCR40) from the 12.5% cellulose to 2.5% cellulose diet (LC

P120) [n=8-8]. D. Colitis susceptibility was also similar between the 40 day reversal (HCR40) and control groups (LC P120) [n=7-10 per group].

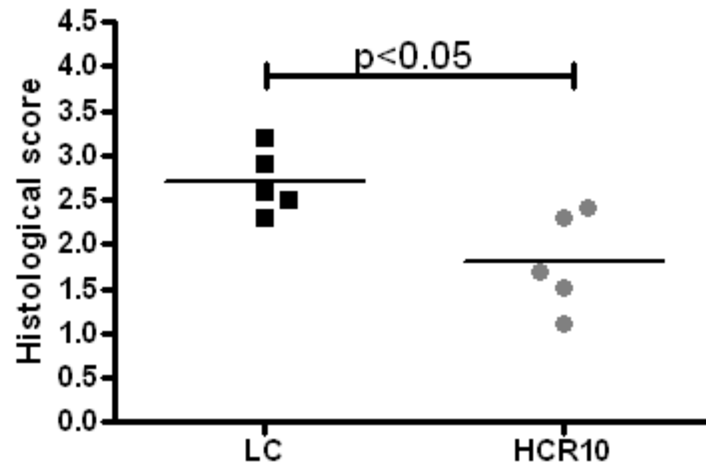

Figure 6 Histological severity of colitis in a validation experimental group. Colitis severity was significantly (U test p=0.016) decreased in the 12.5% high cellulose (HCR10) group compared to controls (low cellulose, LC). N=5. Cellulose supplementation was given between postnatal day 30 to 80 , then DSS treatment was administered for 5 days. We did follow-up altogether with the DSS-treatment for 14 days.

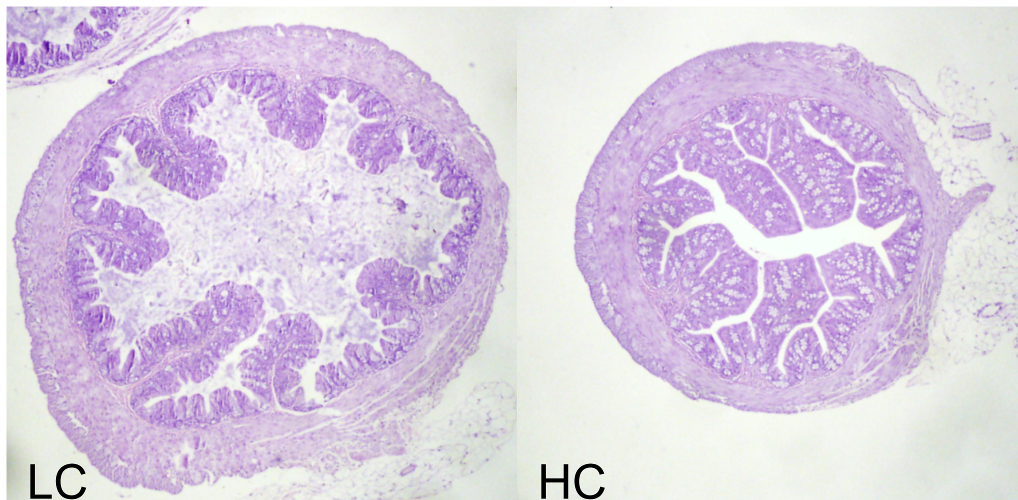

Figure 7 Transected proximal colons from LC (left) and HC (right) mice at P90. Note the significantly (p<0.001; 7-8 measurements, average length 1180 µm vs. 393 µm) elongated

crypts in HC compared LC. The surface area increase secondary to this morphological change was calculated to be 20% in HC compared to LC (magnification x40).

Morphometric analyses on representative proximal colonic samples from HC and LC (Figure 5A) mice showed that HC leads to significant ($p<0.001$) elongation of crypts leading to a 20% increase in transectional surface area (Figure 7). The overall average colonic surface area increase upon cellulose supplementation was calculated to be around 20%.

4.1.3 Microbial diversity and composition changes following cellulose supplementation

Mucosa associated microbes may be more important in gut homeostasis and inflammation than luminal ones (45). The effect of different fibers on the metabolic activity and composition of the gut microbiome was observed both in fecal samples and in *in vitro* models (37; 107; 108). Nevertheless, the consequence of dietary fiber (including cellulose) supplementation on the colonic mucosal microbiome in mammals has not been studied, especially with high-throughput methodologies. Therefore, we examined if dietary cellulose supplementation may affect the colonic mucosal microbiome. The persistence of effects on the gut microbiome 10 days following a decrease in cellulose consumption was also studied in a discovery and a validation cohort.

A variety of microbial taxa were detected within the colonic mucosa of mice on the HC, LC, and HCR10 diets. Although several could be identified to genus level, the majority could not be identified confidently beyond the family level. Several bacterial families were detected in increased quantities in the colonic mucosa of mice on the HC diet compared to LC (Figure 8A). This fiber-dependent augmentation of microbial richness was persistent up to 10 days of reversal to the low cellulose diet (HCR10). A tendency toward increased OTU richness was also observed in the HC group compared to LC, but was diminished by 10 days reversal (Figure 8B). The HC OTU richness was on average 242 OTUs, while HCR10 and LC had a same average of 226.

Figure 8 Cellulose related increase in microbial richness. A. High cellulose (12.5%, HC) diet stimulated a significantly increased number of detectable families compared to low cellulose (2.5%, LC). This separation persisted 10 days following reversal (HCR10). B. The OTU counts of species were slightly increased in the HC group compared to LC, but not in the reversal group (HCR10). p values represent two tailed non-paired T test.

PCoA showed a distinct separation between the HC and LC group communities (Figure 9). HCR10 samples clustered between the LC and the HC microbiome, indicating partial reversal of the cellulose-induced separation following 10 days on low cellulose diet. Cage effects did not influence community separation within the groups.

The most abundant family level taxon changes mirrored the PCoA results (Figure 8), in which the HC and LC groups appeared to be distinctly different from one another, and the HCR10 group was intermediate to the two others, but perhaps closer to the LC group.

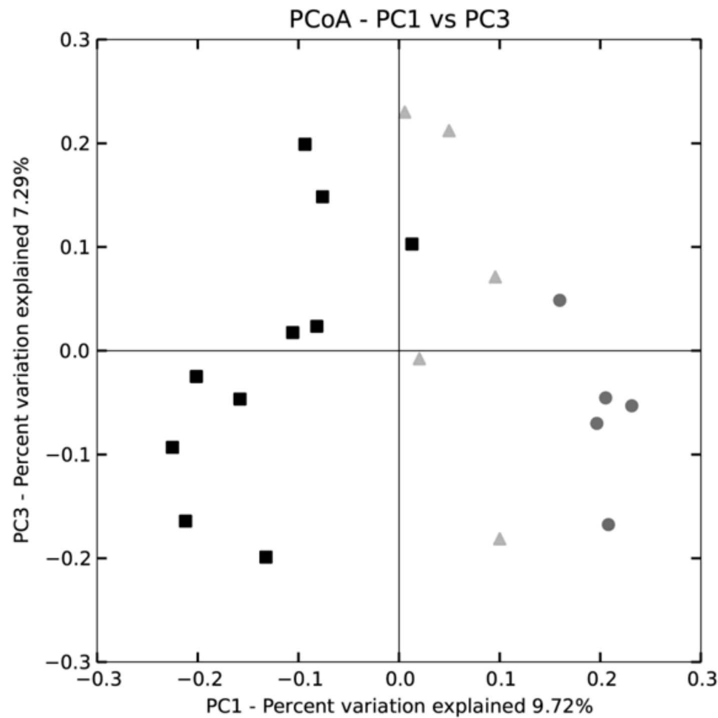

Figure 9 Cellulose induced shifts in the composition of the gut microbiome. Principle coordinates analysis (PCoA) shows separation of the colonic mucosal microbiome on high cellulose diet (● high cellulose [12.5%]; n=5). This separation decreased by 10 days of reversal (▲ 10 days reversal from high cellulose diet [HCR10]; n=5) compared to controls (■ control [2.5% cellulose] diet; n=10). Individual animals did not show separation depending on cage origin.

Two phyla (Actinobacteria and Tenericutes) were significant increased and diminished in abundance, respectively in the HC group compared to control (LC). Only members of the phylum Actinobacteria showed persistently decreased abundance after 10 days of reversal (Table 6). An example of this can be seen in Figure 10, where an Olsenella-like OTU (i.e. OTU 898) demonstrated significantly decreased abundance in the HC and HCR10 groups relative to controls (LC).

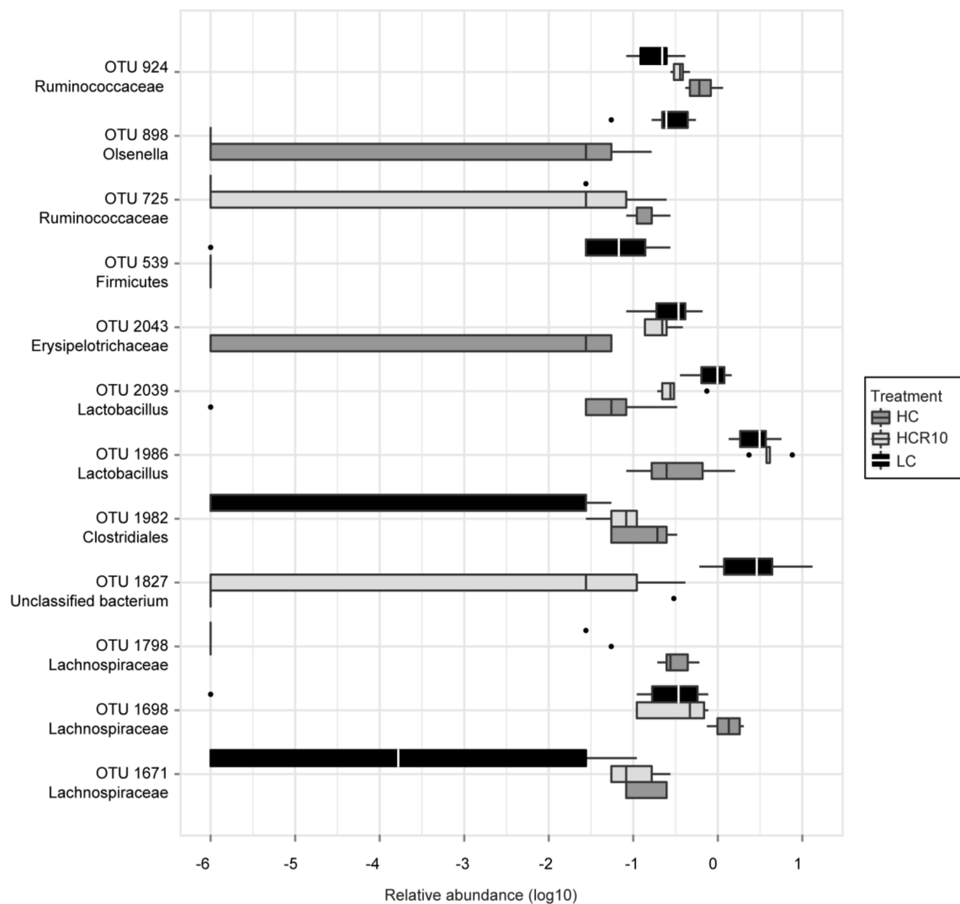

Figure 10 Boxplots depicting shifts in OTU abundance following diet reversal. A stratified approach was used to identify transient changes in OTUs. Each of the OTUs depicted differed significantly between the HC and the LC treatment, as well as between the HCR10 and the HC groups, or between the HCR10 and the LC groups.

Our discovery cohort indicated a decrease in the family level abundance differences between the HC and the LC groups following 10 days of reversal to the LC diet from HC (Figure 11). The validation experiments conformed these results. There were 10 distinguishable families with significant abundance difference between the HC and the LC groups. This number decreased to 6 families by 10 days of reversal (HCR10) with overall higher p values than in the HC-LC comparisons (data not shown). Persistent family abundance differences in the HC and the HCR10 groups, when compared to the LC group, were in Coriobacteriaceae (decrease), Peptostreptococcaceae and Clostridiaceae (increase) (Table).

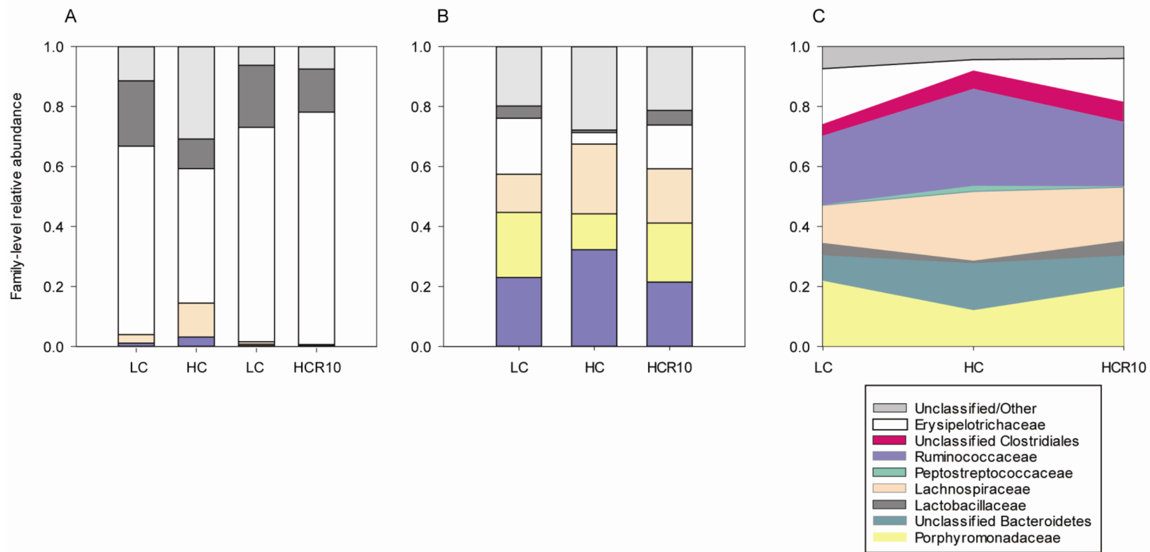

Figure 11 A. Relative abundance of the most common families within the colonic mucosal samples of the discovery group. Altered relative abundance of bacterial families was presented upon high cellulose (HC) diet compared to control (Low cellulose, LC). This alteration diminished following 10 day reversal (HCR10). B. Relative abundance of the five most common (and the unclassified) families within the colonic mucosal samples. The HC group is more different from the LC than the 10 day reversal dietary group (HCR10). C. Abundance variation in the 10 most abundant bacterial families between the dietary groups. A trend for reversal in abundance can be seen in most of the taxa by 10 days of reversal (HCR10) from the high cellulose diet (HC) in relationship to the low cellulose (LC) group.

Table 6 The effects of cellulose supplementation on colonic mucosa associated bacterial phyla and families. Actinobacteria was significantly modified in abundance secondary to increased amounts of dietary cellulose (HC: high [12.5%] cellulose vs. LC: low [2.5%] cellulose), and separated at 10 days of reversal (HCR10) from the LC group as well. There were three families (Coriobacteriaceae, Peptostreptococcaceae and Clostridiaceae) with significant and persistent abundance difference on HC and HCR10 compared to controls (LC) following the temporary cellulose supplementation. p values represent two tailed non-paired T test, U values represent two tailed non-parametric Mann-Whitney U-test (ns: not significant, na: not applicable).

				HC vs. LC		HCR10 vs. LC	
	LC	**HC**	**HCR10**	**p**	**U**	**P**	**U**
PHYLUM							
Actinobacteria	0.365988	0.115575	0.088057	0.010551	0.0193	0.002612	0.0007
FAMILY							
Coriobacteriaceae	0.365988	0.071547	0.055036	0.002391	0.0007	0.001153	0.0007
Peptostreptococcaceae	0	1.733627	0.093561	2.77E-08	na	0.008005	na
Clostridiaceae	0.156852	1.056687	1.172262	0.001377	0.0013	0.047661	ns

4.2 ω-6 FAT DIET REVERSAL AND ITS EFFECT IN MURINE STUDIES

4.2.1 Reversal from ω-6 fat diet protected against colitis

Mice fed for 50 days with high ω-6 fat diet exhibited significantly higher body fat compared to controls. This body composition difference was lost following 40 days of reversal (ω6-R40; Figure 12A). In the meantime, dietary reversal from high ω-6 fat diet induced prolonged protection against DSS-induced colitis. The ω6-R40 animals displayed lower body weight loss (Figure 12B) and lower histologic severity of colitis than control (Figure 12C-E). These findings were replicated in independent experiments.

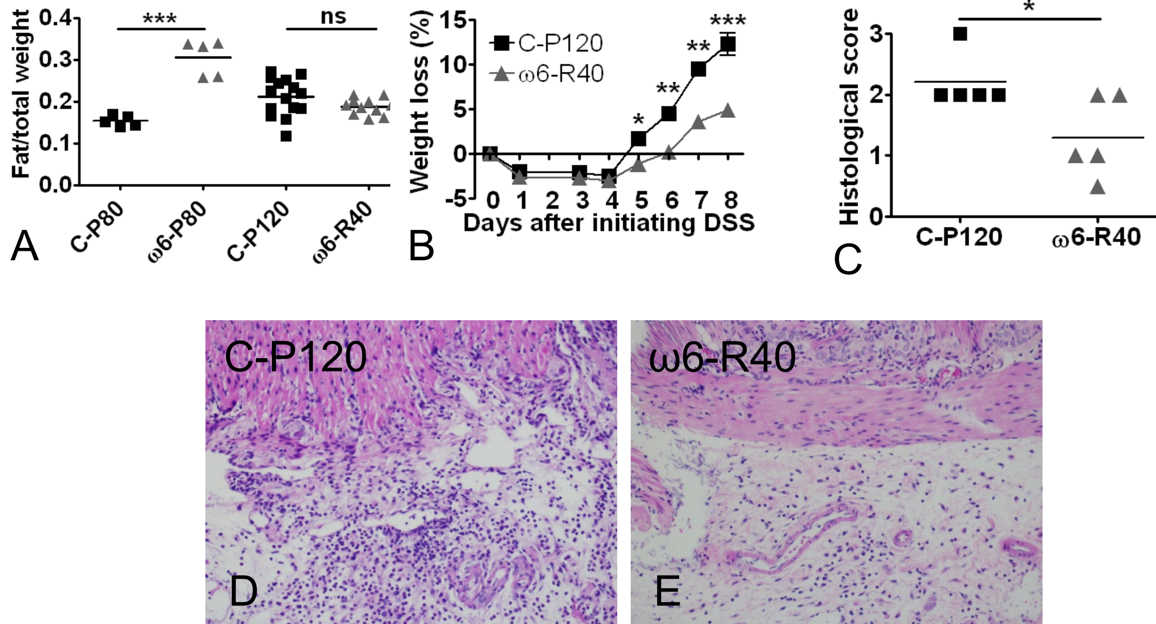

Figure 12 ω-6 fat diet dependent protection against colitis. (A) qMRI measurements showed significantly increased fat composition following ω-6 fat dietary intake in mice (ω6-P80) compared to controls (C-P80), which diminished after 40 days of reversal (C-P120, compared to ω6-R40). (n=14-8-15-11, respectively). (B) However, 5 days following 5-day-DSS-exposure (2%), the ω6-R40 group lost less weight compared to control (C-P120) (n=5-5). (C-E) ω6-R40 group had milder tissue damage upon DSS challenge compared to controls. (C) Histological severity of colitis was higher in control compared to ω6-R40. (D-E) A greater degree of tissue damage and inflammatory cell infiltration was observed in control colons compared to ω6-R40 (magnification x200). Error bars represent standard error of the mean (60). *$p<0.05$, **$p<0.01$, *** $p<0.001$, unpaired *t* test.

4.2.2 Protection against colitis was ω-6 and reversal dependent

We were interested to investigate whether the colitis protective effect was specific to ω-6 or whether the transient pediatric consumption of any type of fat would induce a similar phenotype. Using a high milk fat (MF) diet instead of ω-6 fat, we observed the opposite response. The transiently MF fed mice lost more weight upon DSS challenge than controls (Figure 13A). Thereafter, we sought to establish whether exposure to high ω-6 is sufficient to protect against colitis, or if dietary reversal to control diet is necessary to establish the phenotype. Without dietary reversal, mice had a similar degree of weight loss as controls upon DSS challenge at both P90 (60 days of ω-6 feeding) and P120 (90 days of ω-6 feeding) (Figure 13B-C).

The velocity and extent of weight loss was similar in both the ω-6 and milk-fat reversal groups (Figure 13D).

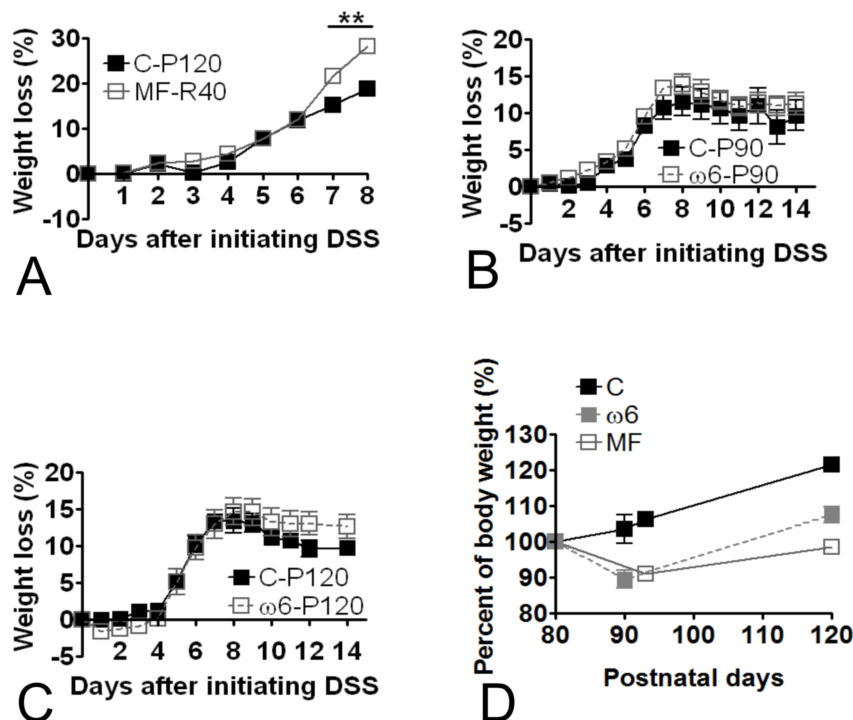

Figure 13 Protection against colitis is ω-6 fat dependent and associated with reversal. (A) Using milk fat (MF) diet instead of ω-6 fat diet, the reversal group (MF-R40) developed more severe colitis compared to control (C-P120). (n=5-5) (B-C) ω-6 high fat dietary group and control mice showed similar response to DSS without reversal. There was no

significant difference in body weight changes between the dietary groups (P90; n=7 or P120; n=5). (D) Weight changes following dietary reversal (at p80) showed similar velocity of weight loss in both of the different fat supplemented groups (MF or ω-6) within 10-13 days being on control diet followed by weight gain. Error bars represent standard error of the mean (60). **p<0.01, unpaired *t* test.

4.2.3 ω6-reversal did not affect other models of colitis

We examined the effects of the transient high ω-6 feeding in both IL-10-/- mice,(109) and in the adoptive transfer model of CD4+, CD45RB^{hi}+ T cells into *Rag1-/-* mice.(110) The nutritional intervention did not have any significant effect on these chronic models of colitis (data not shown).

4.2.4 The gut microbiome of ω6-reversed mice transmitted the colitis suppression

We were interested to elucidate whether the gut microbiome of ω6-R40 mice may be functionally modified to transfer protection against chemically induced colitis. We collected fecal material from transiently high ω-6 fat fed and control mice and transferred it into germfree mice (fecal microbiota transplantation). The transplanted animals were then exposed to DSS to examine the effects of the dietary intervention induced microbiome changes on acute colitis. The recipients of the ω6-R40 microbiome had a 100% survival rate compared to a 60% survival in controls (Figure 14A). Colons retained their lengths and exhibited less severe colitis even in the survived ω6-R40 mice compared to controls (Figure 14B-C).

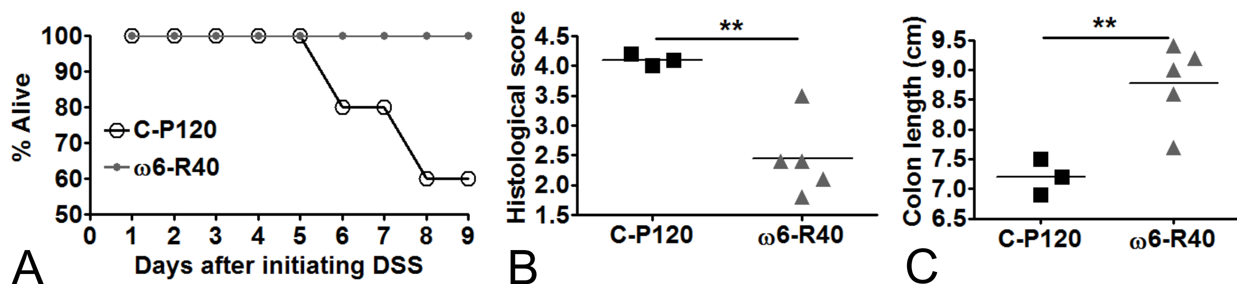

Figure 14 Microbial changes following transient ω-6 fat diet. (A-C) Germfree murine recipients of feces from the reversed high fat dietary group (ω6-R40) were protected against colitis. After initiation of DSS treatment, (A) increased mortality in the control group was

observed (C-P120) compared to the ω6-R40 group. (B) Histological colitis severity was less and (C) colons length remained longer in ω6-R40 compared to C-P120.

4.2.5 The microbiome of ω6-reversed mice segregated from control

Analysis of the gut microbiome revealed separation between the dietary groups. Unweighted principal component analysis of colonic mucosal microbiomes showed a segregation of mice transiently fed with high ω-6 fat diet from controls even after 40 days of dietary reversal (Figure 15D). Upon examination of the 20 most abundant genera in the microbiomes (Figure 15E), only the *Enterococcus* genus showed a significant decrease in ω6-R40 group compared to control (p=0.0079). At the species level, the abundance of *Hespellia porcina*, *Enterococcus faecalis*, *Clostridium innocuum* and *Oscillibacter* species decreased in the reversal group (ω6-R40) compared to control (p=0.0317, 0.0159, 0.0159 and 0.0159 respectively) (table not shown).

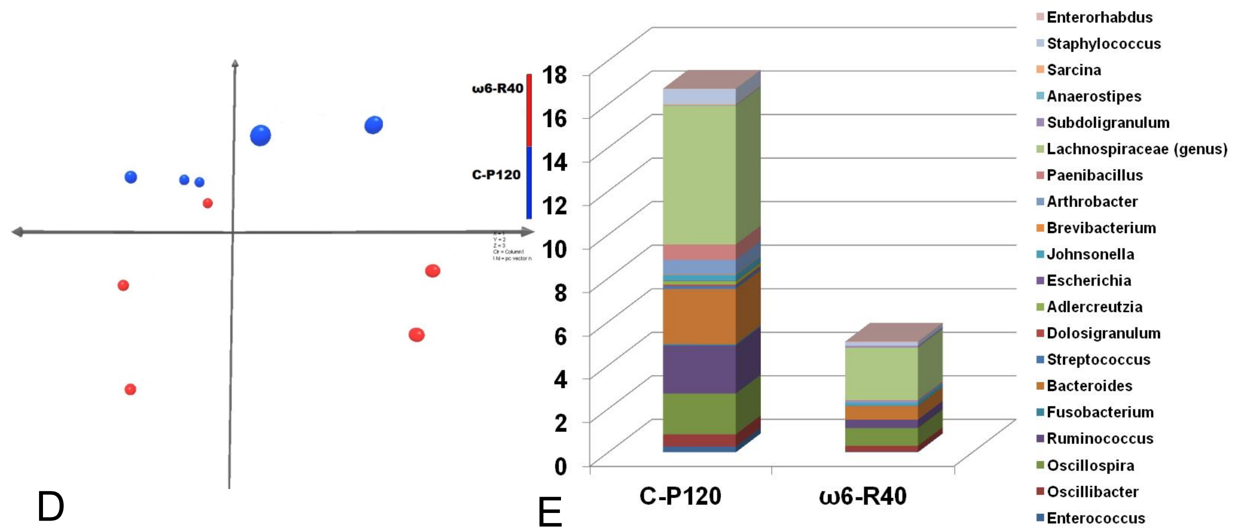

Figure 15 (D) Unweighted principal component analysis (PCA) of the colonic mucosal microbiomes showed separation between the experimental groups, which persisted after 40 days of reversal from high ω-6 fat diet. (n=5-5). (E) Genera (most abundant 20) level microbiome separation between the ω6-R40 and control groups. Only *Enterococcus* abundance was statistically significantly lower in the ω6-R40 group. **p<0.01, unpaired *t* test.

4.2.6 Cxcr5$^+$ CD4$^+$ T cells were suppressed in ω6-reversed mice

We next explored how the colonic epithelium/mucosa was influenced by a transient consumption of high ω-6 fat diet. DNA methylation specific amplification microarrays (MSAM) and gene expression microarrays on isolated colonic mucosa were employed to interrogate persistent diet induced epigenetic changes.(33) Even though these high throughput approaches did not yield statistically significant results (data not shown), we observed a small reduction of *Cxcr5* expression in the colonic mucosa of ω6-reversed mice. We hypothesized that *Cxcr5* transcripts may have originated from mucosa associated leukocytes derived from mesenteric lymph nodes (MLNs) rather than from epithelial cells (which generally comprise >90% of the cell population in our colonic mucosal scrapings). Flow cytometric analysis of different T cell subsets confirmed a significant reduction in the numbers of Cxcr5$^+$ CD4$^+$ T cells in the MLNs of ω6-R40 animals in independent experiments (Figure 16A-B).

4.2.7 Cxcr5 ligand B-lymphocyte chemo-attractant (or Cxcl13) modulated colitis susceptibility

The observed decrease in the number of Cxcr5$^+$ CD4$^+$ T cells implicated the importance of *Cxcr5* and its ligand B-lymphocyte chemo-attractant (or Cxcl13) in murine acute colitis. Therefore, we examined the effect of Cxcl13 antibody (ab) treatment in the severity of DSS-induced colitis. Cxcl13 ab treated mice had a tendency to loose less weight during DSS challenge (Figure 3C). Additionally, we observed increased colonic length and decreased colitis severity in the Cxcl13 ab treated mice compared to isotype controls (Figure 16D-E), indicating that the inhibition of *Cxcr5* signaling is protective against murine acute colitis.

4.2.8 CXCL13 levels in the plasma of IBD patients

Finally, we sought to establish whether the CXCR5-CXCL13 pathway may be activated in human IBD based on our murine experiments. CXCL13 was measured in the blood of untreated pediatric patients with UC, CD, and controls. Plasma samples were collected at the time of diagnostic endoscopy. Plasma concentration of CXCL13 was

significantly elevated in children with treatment naive CD with a similar trend in UC, compared to controls (Figure 16F).

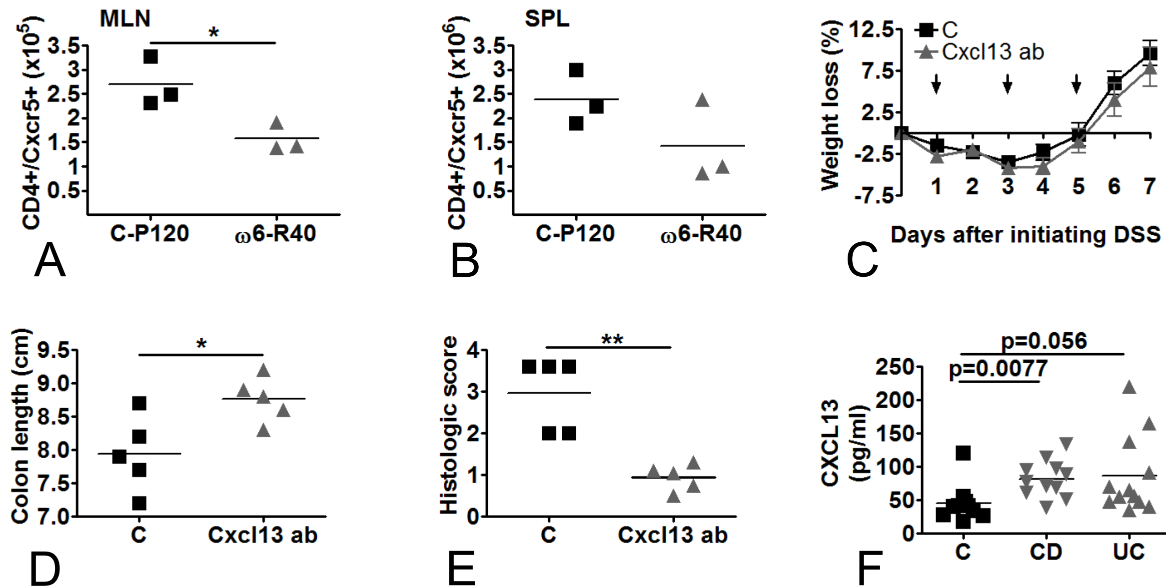

Figure 16 Cxcr5-Cxcl13 immunologic pathway revealed to play an important role in DSS-induced colitis upon transient ω-6 fat diet. (77) Flow cytometry showed significantly decreased number of $Cxcr5^{+}$ $CD4^{+}$ T cells in the mesenteric lymph nodes (MLN), but not in the spleens (SPL) of ω6-R40 compared to C-P120 (n=3-3 animals, 2 MLNs per mice, altogether 6 lymph nodes per group). (C-E) Cxcl13 antibody (ab) treatment protected mice against DSS-induced colitis (n=5-5). Arrows indicate intraperitoneal ab treatment. Cxcl13 ab treated mice had a tendency for decreased weight loss during DSS-challenge. This finding was associated with longer colons and decreased histologic severity of colitis in the Cxcl13 ab treated group compared to controls. (F) Significantly increased CXCL13 concentration was measured in serum of treatment naïve human pediatric CD patients, with a similar trend in UC. Error bars represent standard error of the mean (60). *$p<0.05$, **$p<0.01$, unpaired *t* test. CD: Crohn's disease; UC: ulcerative colitis.

4.3 Clinical results of FMT

4.3.1 FMT treatment outcomes of CDIF patients

All 4 CDIF patients without underlying disease (P2, P4, P5 and P9) had resolution of their symptoms for more than 2 months following a single FMT.

Out of the 6 patients with complicating clinical conditions, only 2 (P6 and P8) had obvious clinical benefit from *C. difficile* directed antibiotic therapies prior to FMT. These patients responded well to a single FMT, including the youngest solid organ transplant patient receiving FMT to date (on concurrent immunotherapy with tacrolimus and mycophenolate mofetil). However, both patients were treated with antibiotics for upper respiratory infections within the 2-month follow up period and developed recurrent CDIF. Both received repeat intra-gastric FMT with full resolution of their symptoms for over 2 months.

The UC patient (P1) required FMT 3 times to clear CDIF, but still had colectomy during his subsequent clinical course in spite of being free from *C. difficile*. Our second patient with UC (P10; maintenance mesalamine therapy only) had rapid resolution of her symptoms after FMT for 9 weeks, then experienced a *C. difficile*-positive flare of her UC and was started on steroids and 6-mercaptopurine along with oral vancomycin for 2 weeks. She responded well to these interventions and subsequently tested negative for *C. difficile* 2 weeks after stopping vancomycin.

There was no obvious clinical benefit from FMT in the CD patient in-spite of having a negative *C. difficile* test 2 months after the treatment.

One patient (P3) was diagnosed with poorly managed constipation and continued *C. difficile* carriage during her second enema FMT.

4.3.2 Clinical outcomes of patients with ulcerative colitis

Response and progression of UC patients was monitored by PUCAI during the protocol. Clinical remission was defined as PUCAI≤15. Endoscopic disease severity (Mayo score) was recorded at the initiation of the trial (colonoscopy) and at the 14-week time

point (flexible sigmoidoscopy). Table contains the PUCAI score and endoscopic Mayo score system.

Table 7 Pediatric ulcerative colitis activity index (PUCAI) and endoscopic Mayo score.

PUCAI Score	**Endoscopic Mayo Score**
Abdominal pain: 0-10	Normal or inactive colitis: 0
Rectal bleeding: 0-30	Mild colitis: mild friability, erythema, decrease in vascularity: 1
Stool consistency: 0-10	Moderate colitis: friability, marked erythema, vascular pattern absent, erosions: 2
Number of stools per 24 hours: 0-15	Severe colitis: ulcerations and spontaneous bleeding: 3
Nocturnal stool: 0-10	
Activity level: 0-10	

Serial FMT transiently supported immunotherapy withdrawal in pediatric UC patients in our small pilot study. The FMT enabled all 3 patients to be symptom-free for at least 4 weeks following FMT and supported the withdrawal of immunotherapy (no treatment other than mesalamine) for more than 105 days in all. The number of FMT treatments significantly correlated with the time of being immunotherapy-free ($r=0.998$; $p=0.04$).

Following our promising pilot study, we developed an IND approved phase 1 clinical trial providing FMT for six patients. Only one patient (P001) remained in the trial for the first six months. All others had to be withdrawn from the study according to exclusion criteria for worsening symptoms. The single patient (P001) with prolonged remission (PUCAI≤15) throughout the course of the study had endoscopic remission at enrollment (Mayo 1), and 2 weeks after the last weekly enema (Mayo 0; 14 weeks of the trial). However, subjects with pseudopolyps, and/or moderate to severe endoscopic disease (5 patients, Mayo 2-3) developed moderate to severe symptoms within 2 days to 8 weeks and were withdrawn from the study. The length of clinical remission was negatively correlated with mucosal disease activity at the initiation of FMT ($r=-0.845$, Spearman: $p=0.033$; Figure 17).

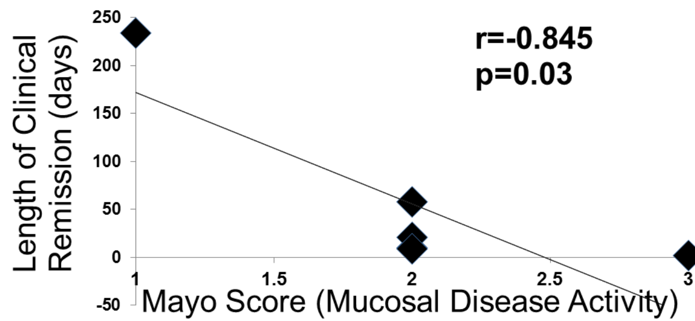

Figure 17 Mucosal disease activity and therapeutic response to FMT in the phase 1 FMT study. The length of clinical remission negatively correlated with mucosal disease activity at the initiation of FMT (r=-0.845, two-tailed Spearman: p=0.03). Endoscopic Mayo score was arbitrarily increased by 1 if pseudopolyp/s were detected.

Two of the patients withdrawn from the study had colectomy for steroid dependent disease within one year from the time of dropout. One patient's clinical course was complicated by cytomegalovirus (CMV), which was diagnosed at the colonoscopy prior to the first FMT. Following failure of FMT, she had limited response to ganciclovir, 6-mercaptopurine (6MP) and infliximab (IFX), entered a phase II adult trial of an experimental drug. Currently, she is in clinical remission. One patient had poor weight gain on 6MP and low dose intermittent steroids with mild clinical disease activity and has been recently advanced to IFX therapy. One geographically distant referral patient was lost to follow-up.

The single patient with positive response to the treatments remained in remission on monthly FMT via enema up to 6 months into the protocol. Unfortunately, he was unable to receive more treatments secondary to the trial being placed on hold for a serious adverse event in another patient (see below). Nevertheless, he remained in clinical remission off of any therapy for more than 3 months, with a normal fecal calprotectin (110.9 mcg/g, ULN=162.9 mcg/g) at 2 months after the last FMT. Later, he developed bloody diarrhea, which responded to weaning steroids and oral mesalamine therapy.

A summary of all our patients' treatment can be seen in Table 8. Endoscopic and histological photos captured before and after treatment is shown in Figure 18.

Table 8 The number of days in remission negatively correlated with the initial Mayo score of patients enrolled into our pilot trial (P1-3) and phase I study (P001-006). Altogether 178 FMT were delivered in our center. Patients received various numbers of FMT. Endoscopic evaluation (colonoscopy and flexible sigmoidoscopy) with Mayo scoring was completed on the first day of the protocol and at 14 weeks (2 weeks after the weekly enemas). Patients in remission or with mild disease activity (Mayo score 0-1) stayed in remission and completed the 14-week evaluation with Mayo score=0. Patients who had moderate disease activity (Mayo score 2-3) or presented with pseudopolyp developed UC symptoms and were withdrawn from the study (* and # labels the patients who were enrolled in both the pilot study and this trial one year apart)

Study ID	**Treatment # (rescue enemas)**	**Mayo score before FMT**	**Mayo score after 14-week FMT**	**Days spent in remission**
P001*	32 (1)	1	0	204
P002	10	2		10
P003#	17 (1)	1 (P)		21
P004	9	2		9
P005	2	3		2
P006	24 (5)	1 (P)		58
P1	*30*	*0*	*0*	*261*
*P2**	*25*	*1*	*0*	*159*
P3#	*22*	*0*	*0*	*105*

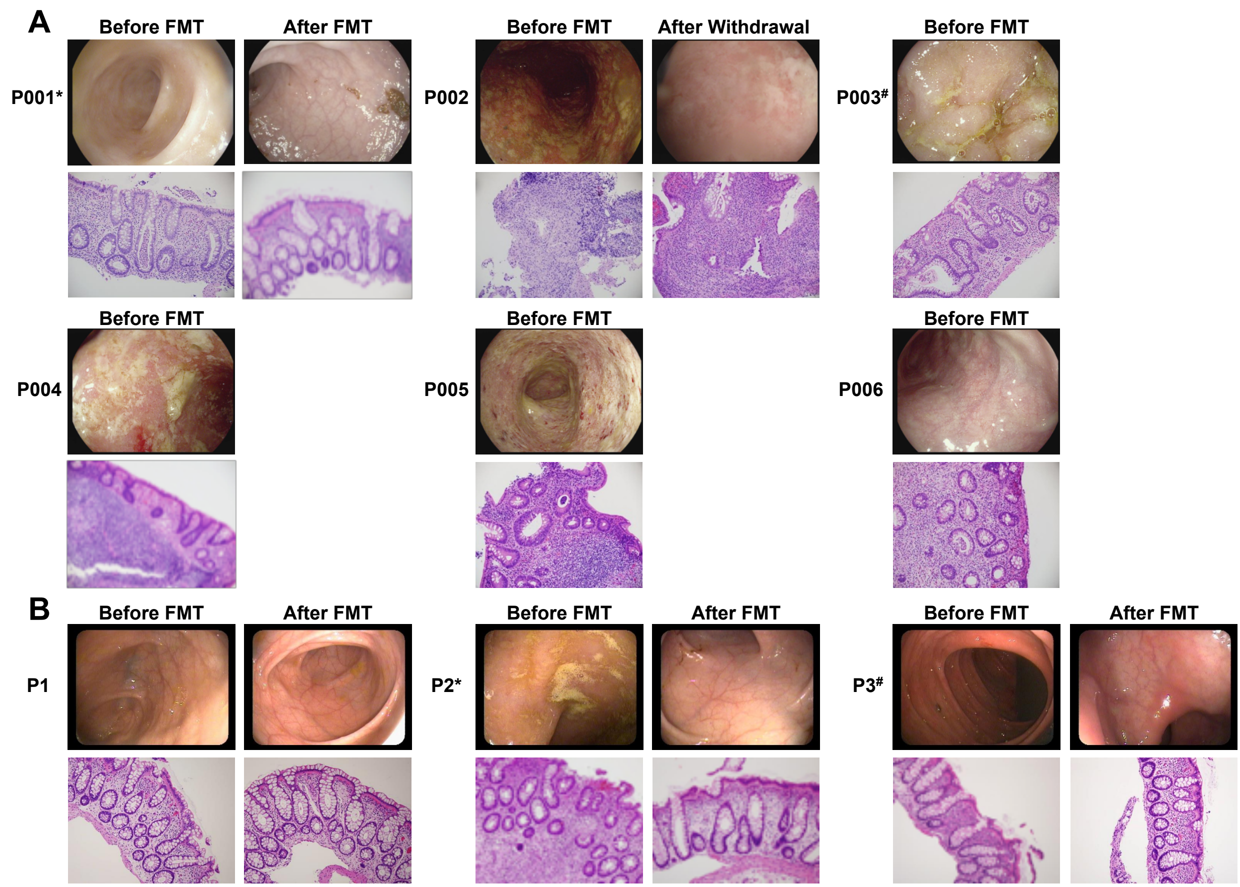

Figure 18 The endoscopic and histological picture of patient colons from this phase 1 (A) and our prior pilot (B) studies. For clinical details see Table 8.

4.3.3 Adverse events monitoring

The risks of FMT in pediatric patients are unknown, but based on the experience in adults with bacterial colitis FMT is usually well tolerated and the risks are believed to include: possible transmission of a bacterial or viral disease, allergic reaction, worsening of CD related symptoms (bleeding, pain, bowel perforation, or rupture, infection/sepsis), and possible but unlikely death. There is one report of a 61 year old male with CD, refractory *C. difficile* colitis and a history of multiple episodes of *E. coli* bacteremia, who developed *E. coli* bacteremia within 24 hours of FMT.(111) Given the time course it seems likely that the FMT contributed to this development of bacteremia; therefore bacteremia is included as a potential risk. Adverse events are possible, but uncommon with nasogastric tube use, including vomiting and rarely aspiration. The largest systematic review summarized 67 published studies (overall 844 patients -76.3% with recurrent CDIF and 13.2% with IBD).(68) The cure rate was 90.7% in CDIF; and beneficial effects were reported in 78.4% of cases with IBD. 7 publications involved pediatric patients. The following adverse events were reported: fever, rectal discomfort, abdominal pain, nausea, bloating and headache.

During the phase 1 IND study of pediatric ulcerative colitis patients, 101 FMT were delivered. Seventeen adverse events occurred (
Table and Table). Adverse events included: fever, cough, vomiting, runny nose, throat ache, nausea, abdominal pain (solicited) and altered laboratory parameters including decreased sodium, decreased hemoglobin, increased AST and ALT (unsolicited). The probable and possibly related AEs included fever, abdominal pain and decreased sodium level. Additional AEs, such as cough, runny nose and throat ache were considered unrelated to FMT, and were attributed to a possible upper respiratory infection. Nausea was present on the first day in two cases unrelated to FMT, probably secondary to anesthesia.

During the study, there was one serious adverse event (SAE; Grade 3: fever and abdominal pain), which required hospitalization. This SAE was possibly related to the treatment. Based on further evaluation with endoscopy, we concluded that the SAE was probably induced by the fecal preparation that was received by the patient with severe colonic mucosal disease activity. This may have been an allergic response, or non-specific transient, self-resolving inflammatory exacerbation leading to fever. Nevertheless, the DRC and the FDA placed the trial on hold, and no further FMT was given to any patient.

Table 9 The frequency of adverse events during the IND clinical trial with pediatric ulcerative colitis patients.

	Total #	Percentage (%)
Adverse Events	17	100
Adverse Event Group		
Solicited	13	76.5
Unsolicited	4	23.5
Adverse Event Grade		
Grade 1 (mild)	12	70.6
Grade 2 (moderate)	3	17.6
Grade 3 (severe)	2	11.8
Grade 4 (life threatening)	0	0
Adverse Event Relatedness		
Unrelated	7	41.2
Unlikely	5	29.4
Possible	4	23.5
Probable	1	5.9
Definite	0	0

Table 10 Category, severity and relativeness of adverse events of patients during the IND approved protocol.

Study ID	Protocol Day	AE Event	AE Group	AE Grade	AE Relatedness
IMT_UC_ P006	Week 12	Cough	Solicited	Grade 1 (mild)	Unrelated
	Week 12	Running nose	Solicited	Grade 1 (mild)	Unrelated
	Week 12	Throat ache	Solicited	Grade 1 (mild)	Unrelated
	Week 12	Vomit	Solicited	Grade 1 (mild)	Unrelated
IMT_UC_ P007	Week 1 Day 6 (Day 6)	Fever	Solicited	Grade 2 (moderate)	Unrelated
IMT_UC_ P009	Week 2 Day 2 (Day 9)	Fever	Solicited	Grade 1 (mild)	Possible
	Week 2 Day 2 (Day 9)	Cough	Solicited	Grade 1 (mild)	Unrelated
	Week 2 Day 2 (Day 9)	Vomit	Solicited	Grade 1 (mild)	Unrelated
	Week 1 Day 1 (Day 1)	Nausea	Solicited	Grade 1 (mild)	Unlikely
IMT_UC_ P010	Week 1 Day 1 (Day 1)	Nausea	Solicited	Grade 1 (mild)	Unlikely
IMT_UC_ P011	Week 1 Day 1 (Day 1)	Nausea	Solicited	Grade 1 (mild)	Unlikely
	Week 8	Fever	Solicited	Grade 2 (moderate)	Possible
	Week 8	Fever	Solicited	Grade 3 (severe)	Probably
	Week 8	Decreased sodium	Unsolicited	Grade 2 (moderate)	Possible
	Week 8	Decreased hemoglobin	Unsolicited	Grade 1 (mild)	Unlikely
	Week 8	Increased AST	Unsolicited	Grade 1 (mild)	Unlikely
	Week 8	Increased ALT	Unsolicited	Grade 1 (mild)	Unlikely
	Week 8	Abdominal pain	Solicited	Grade 3 (severe)	Possible

4.4 Microbial studies of FMT

Based on encouraging findings of clinical remission and the current literature about gut microbiome and its role in intestinal inflammation, we initiated microbial studies on stool samples of the same pediatric patients with CDIF and UC during the FMT. This

approach is unique in testing FMT as primary (not adjuvant) treatment modality for pediatric UC and FMT; and how this treatment influences the gut microbiome by time.

4.4.1 Increased alpha-diversity following FMT in CDIF patients

We examined the samples of those CDIF patients who did not have underlying clinical conditions and uniformly responded well to FMT (P2, P4, P5 and P9). Species richness increased significantly (p=0.0025) following FMT in the recipients, even surpassing that of the donor (Figure 19A). Bacterial Shannon's diversity similarly had a significant (p=0.0010) increase upon FMT in the pediatric recipients. The diversity was beyond that of the donor (Figure 19B). The post FMT group had species richness and diversity similar to that of the healthy group.

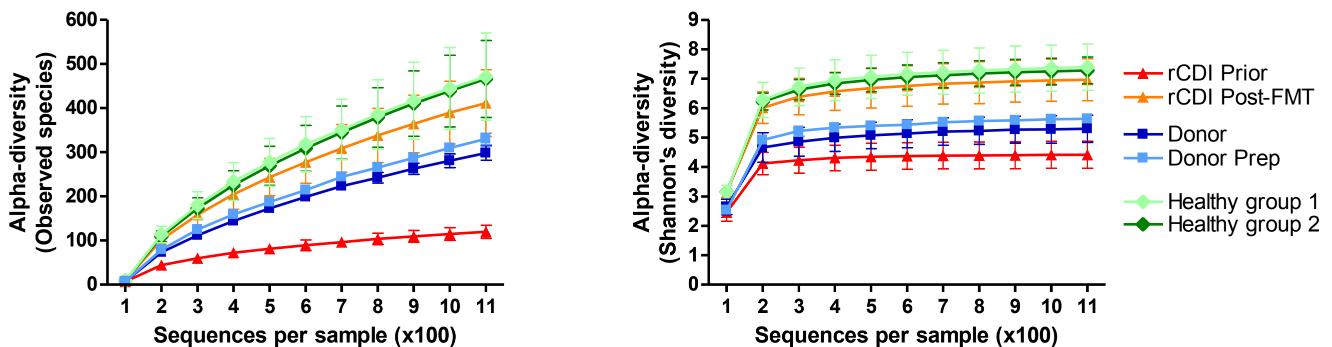

Figure 19 Microbiome responses to FMT. Alpha-diversity showed exponentially increased number of observed species (richness, p=0.0025) and diversity (Shannon's, p=0.0010) following FMT. The CDIF post-FMT group had increased richness (left panel) and diversity (right panel) similar to those seen in the healthy control groups. Statistical analysis revealed no significant difference between the healthy control group and the CDIF post-FMT group for richness (p=ns) and diversity (p=ns). The CDIF post-FMT group also had significantly increased diversity levels when compared to the donor group (p=0.0010).

4.4.2 Taxonomical changes upon FMT in CDIF

At the taxonomic level, *Bacteroidetes* increased in abundance following FMT in children without disease, as opposed to the IBD patients who did not respond clinically to the FMT (Figure 20). Specifically, several OTUs most closely related to the genus

Bacteroides were found to be more abundant in the post FMT group. The OTU most closely related to *Bacteroides uniformis* had the most significant (p=0.0049) increase in abundance after FMT treatment. The other significantly increased OTUs post FMT were those belonging to the family Lachnospiraceae.

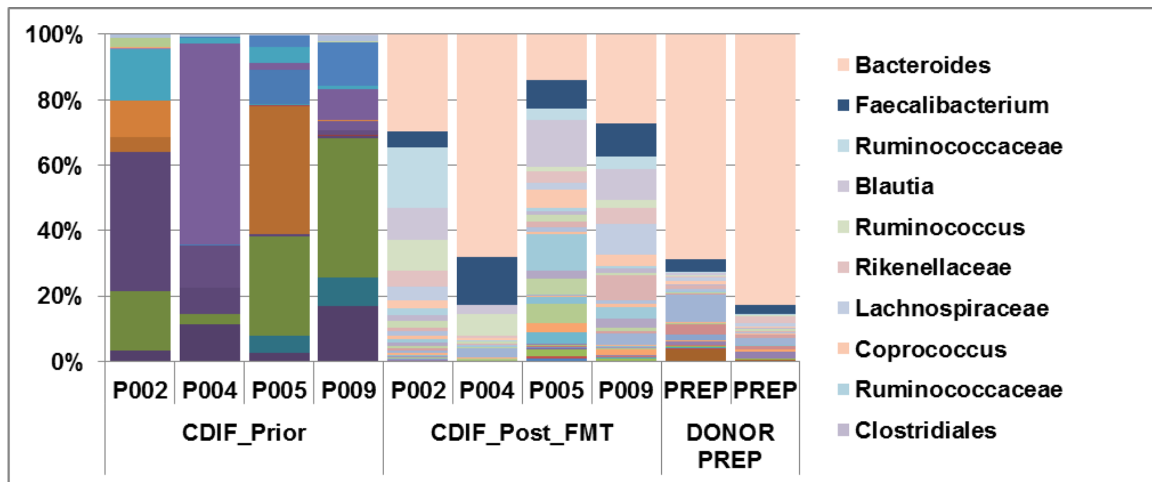

Figure 20 Bar-graph of taxonomical changes in CDIF patients prior and after FMT compared to the donor microbial composition.

4.4.3 Microbial analyses of UC patients

Principal coordinate analysis (PCoA) of fecal bacterial community composition data generated from 16S rRNA sequencing revealed that UC samples prior to FMT did not clearly separate from the donor. However, those did cluster differently from healthy pediatric microbiomes (Figure 21). Interestingly, P001 (Figure 21*) separated the farthest from the donor in spite of having received FMT from the same donor one year prior to this study enrollment (he was part of our discovery trial of reference (99)).

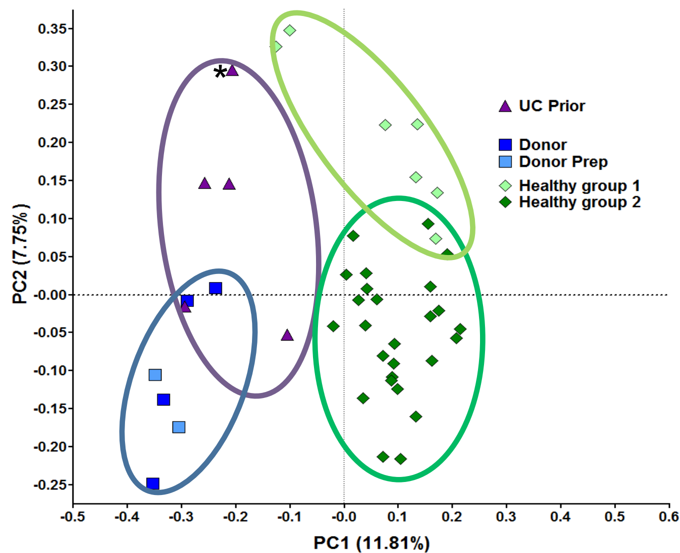

Figure 21 Principal coordinates analysis (PCoA) of (unweighted Unifrac distances of bacterial community composition as depicted by 16S rRNA gene sequencing of fecal samples upon serial FMT. Prior to FMT, the microbiomes of UC patients separated from those of healthy children but did not separate clearly from donor. Surprisingly, patient (P001*) who received FMT previously from the same donor shared the least degree of similarity with the donor as compared to the other UC patients.

4.4.4 Microbiome shifts upon FMT

We had the ability to study microbiome responses to intense FMT over a 6-month period in one patient only (Figure 22). The fecal microbiome of P001 shifted towards that of healthy children. Nevertheless, the patient's microbiome remained distant from both the donor and other healthy children living in the same geographical area, even 6 months into therapy.

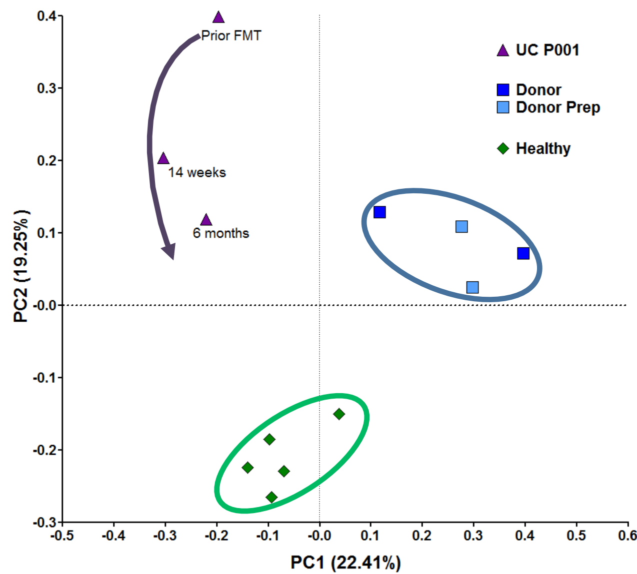

Figure 22 Fecal microbiome composition in our patient with prolonged remission (P001) moved towards both the donor and healthy pediatric microbiomes during the 6 months of the FMT series.

4.4.5 Prolonged microbiome effects of serial FMT in two patients

Two patients participated both in our discovery FMT trial(99) and this study, with more than a year separating the two. Both patients received their FMT series from the same donor during an overlapping time period. This provided an unprecedented opportunity for us to examine the prolonged effects of an extended course of FMT (series of 25 and 22 FMT, respectively). Upon serial FMT, these patients experienced normalization of their microbiomes, characterized by large shifts toward a state resembling healthy pediatric composition (Figure 23, arrows). In contrast, upon suspension of FMT the microbiomes of the UC patients shifted back to their original state in a matter of 10-12 months.

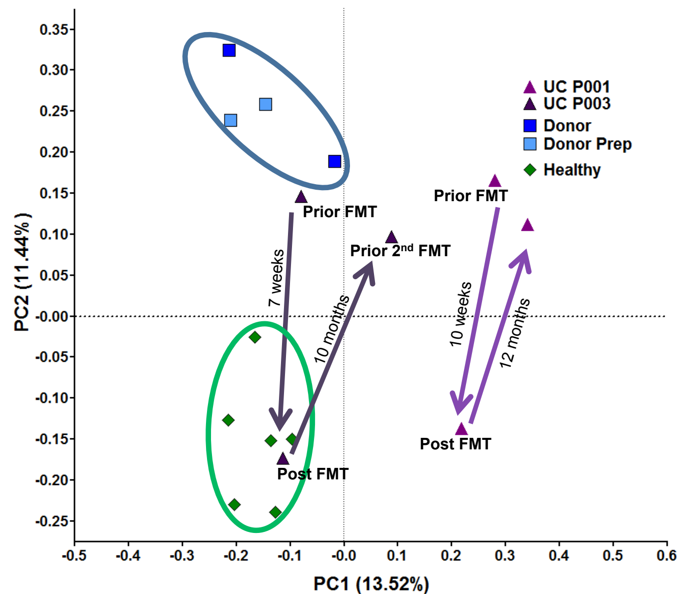

Figure 23 PCoA revealed shift towards the microbiome profiles of healthy children upon FMT in patients with long-term follow-up. Within 7 to 10 weeks (P003 and P001, respectively) FMT patients clustered close to microbiome of healthy individuals. However, the lack of treatment (within 10 to 12 months) resulted in a shift back to original state in both patients.

We also examined the prolonged microbiome responses in detail from both of the UC patients at the level of the operational taxonomic unit (OTU) level and compared those to those which were observed in donor stool (Figure 24). Two weeks after 25 FMTs had been administered, 17% of the OTUs detected in P001 at baseline (prior to FMT) remained. Meanwhile, 12% of the OTUs detected were from the donor. Surprisingly, 71% of the OTUs were novel or unknown origin (i.e. not detected either in the recipient prior to FMT, or in the donor). Twelve months after the last FMT, these numbers shifted. Thirty two percent (32%) were original patient OTUs (detected prior to FMT treatment), 13% were of likely donor origin, and 55% were novel or unknown origin. In addition to shifts in the potential origin of OTUs, there were notable changes in OTU level richness with an increase observed at 2 weeks, but a decline observed 12 months after the last FMT. Similar changes occurred in P003. Two weeks after last FMT in P003, the abundance of OTUs by origin was the following: 28% preserved from prior to FMT, 13% originated from donor and 59% were novel OTUs. In this case the fraction of OTUs did not shift significantly at

10 months after last FMT (32%, 10% and 58%, respectively). In the meantime OTU richness changes were very similar to those observed in P001 (Figure 24).

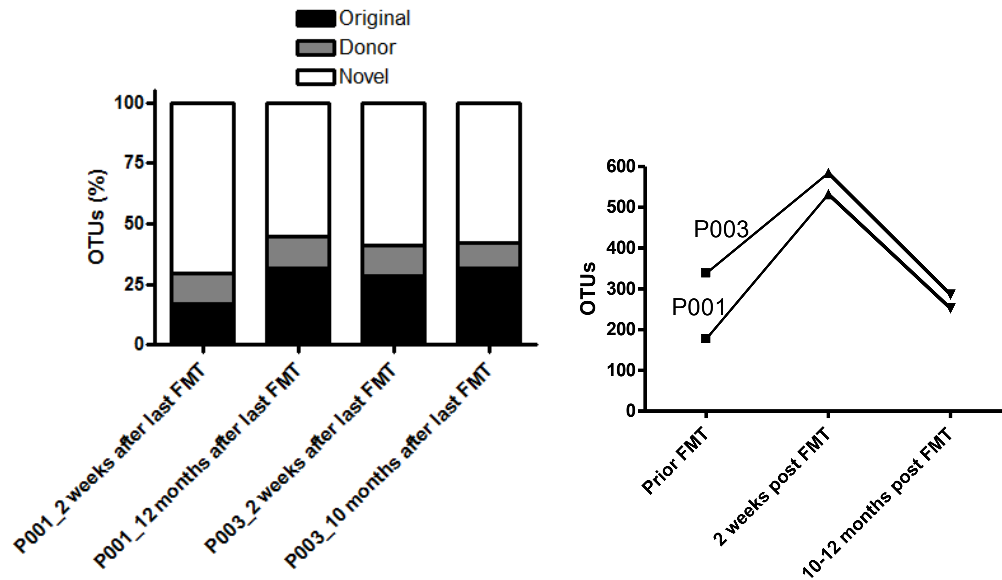

Figure 24 Proportions of OTUs by known origin following FMT (left panel). Surprisingly, the highest proportion of OTUs observed in both patients observed were of novel or unknown origin, arguing for reconstitution of the recipient microbiomes as opposed to transplantation of donor bacteria. In the meantime, about 10% of donor bacteria appear to engraft in a prolonged fashion in pediatric UC patients following FMT. OTU number changes upon serial FMT (right panel). Two weeks following FMT, the OTU numbers increased from 177 to 532, and from 338 to 584, respectively. However, cessation of treatments resulted a drop in OTU numbers close to the original state (to 252 and 286 OTU, respectively).

4.4.6 Remarkable bacterial composition changes in pediatric CDIF after a single FMT

We next focused our attention to bacterial operational taxonomic unit (OTU) changes secondary to FMT. OTU richness significantly increased in the CDIF group (Figure 25; p=0.0286) 8 weeks after FMT. We compared the OTU changes secondary to FMT in CDIF patients to a small cohort of pediatric UC patients who received an intense course of FMT (22-30 treatments) over 6-12 weeks from the same universal donor as our CDIF patients.

Interestingly, there was only a tendency toward increased OTU numbers in UC in response to serial FMT (Figure 25). This finding indicated an increased microbiome change upon a single FMT in CDIF compared to that from serial FMT in UC.

We also examined OTU persistence, and donor engraftment in CDIF recipients secondary to FMT. We determined OTU persistence in healthy children over 2 months to facilitate the interpretation of FMT-induced microbiome variation. Healthy children retained an average of 40% of their OTUs between repeated stool collections over an 8-week period (Figure 25). Compared to healthy children, the OTU profiles of CDIF patients retained a highly significantly (Figure 25; $p=3.8x10^{-10}$) lower proportion of their original OTUs following FMT. Remarkably, only 1% of the pre-FMT OTUs were detected 8-9 weeks after a single stool transplant upon resolution of CDIF. OTU retention was significantly lower in CDIF than in the UC patients (Figure 25; 1% vs. 18%; p=0.0024). As for donor engraftment, CDIF patient retained 26% donor OTUs at 8-9 weeks, which were not detected in their stool prior to FMT. Donor engraftment after a single FMT was significantly higher in the CDIF patients ~2 months after the treatment than in UC patients only 2 weeks after a series of FMTs from the same donor (Figure 25; 26% vs. 11%; p=0.00015). The proportion of novel (neither present in pre-FMT recipient, nor the donor) OTU was remarkably high in both CDIF and UC, but this did not show significant difference between the two patient groups (Figure 25; 73% vs. 71%; p=ns).

There was no correlation between the proportion of preserved OTUs and the age of individuals in the CDIF, UC, or control groups.

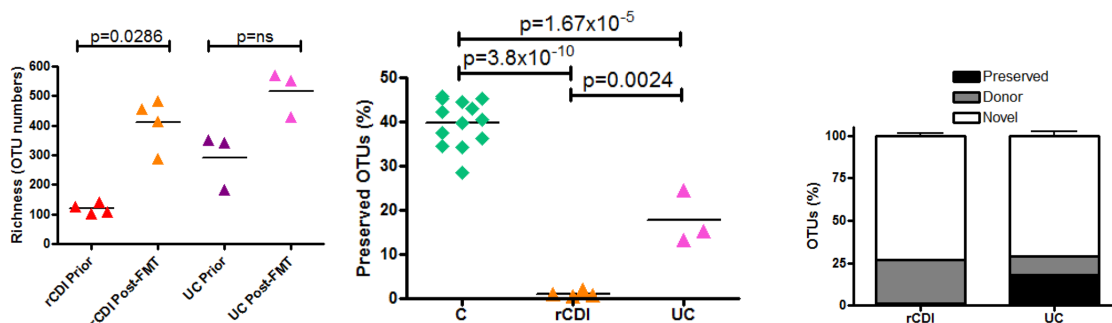

Figure 25 OTU numbers (richness, left panel) increased post-FMT in rCDIF (recurrent CDIF) compared to pre-FMT (p=0.0286). However in UC patients, there was only a tendency towards increase in OTUs upon FMT (p=ns). OTU preservation (middle panel)

was significantly lower in both rCDIF and UC patients compared to controls ($p=3.8x10^{-10}$ and $p=1.67x10^{-5}$ respectively). rCDIF patients also had significantly lower OTU preservation than UC (p=0.0024). Additionally, donor engraftment (26% average) 2 months after FMT was significantly higher ($p=1.49x10^{-4}$) in rCDIF than in 2 weeks after a series of FMT in UC (11% average; right panel). In the meantime, there was large number of novel OTUs detectable in both rCDIF (73% average) and UC patients (71% average) after FMT, which did not differ significantly (p=0.458) between the two groups.

4.4.7 Normalization of microbiome composition (beta-diversity) upon FMT in CDIF patients

Using the same universal donor in 9/10 cases provided a unique opportunity for us to examine the microbial changes upon a single fecal transplant. Prior to FMT all CDIF patient clustered distantly from the donor and healthy children on PCoA, with the exception of one patient with overflow diarrhea and *C. difficile* carriage. On average the stool communities of pre FMT patients shared 9.8% similarity with the universal donor and 6% similarity with healthy children, based on unweighted Unifrac distances. Post-FMT, patients shared 30% similarity with the healthy children and 31% similarity with the universal donor, both of which were significant findings ($p=5.37\times10^{-9}$ and $3.65\times^{10-99}$, respectively). When comparing the other healthy group's gut microbiome to CDIF patients, the two groups were 7.5% similar preFMT and 29.8% similar postFMT ($p=4.3\times10^{-36}$).

The CDIF patients without any underlying clinical condition responded to FMT with major microbiome shifts after 8 weeks. Recipient microbiomes clustered between healthy children and the microbiomes of the single standardized donor following FMT (Figure 26). One of these patients (patient 4) was found to be positive for *C. difficile* 4 months after FMT during an upper respiratory infection, without gastrointestinal complaints. He was considered an asymptomatic carrier. Interestingly, asymptomatic carriage of *C. difficile* did not significantly modify microbiome composition in this child.

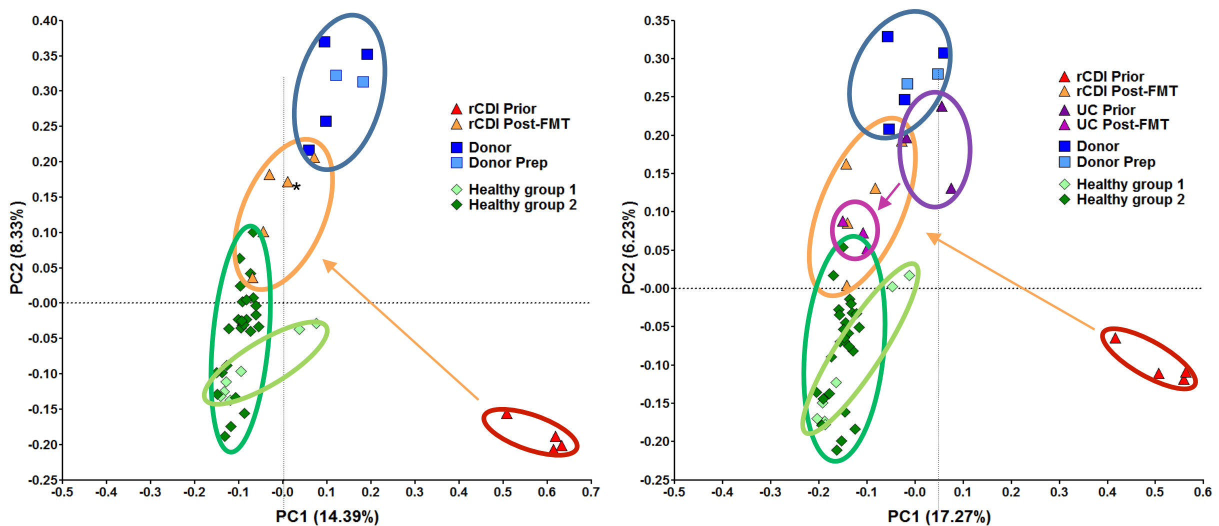

Figure 26 Principal Coordinate Analysis (PCoA) revealed a major shift (orange arrow) in rCDIF (recurrent CDIF) patient microbiomes upon FMT to a similar composition of healthy children and donor samples. Patient 4 (*) was found to be an asymptomatic carrier for *C. difficile* 4 months after FMT. Apparently, this carrier state clustered with recipient microbiomes at 2 months after FMT. When compared to UC patients receiving a series of FMT, microbiome shifts were greater in rCDIF (orange arrow) than in UC (pink arrow). [n=4 rCDIF, 3 UC, 3 individuals in healthy group 1 and 13 healthy group 2]

4.4.8 Microbiome shifts in CDIF following FMT are more pronounced than in UC

When comparing fecal microbiome responses to FMT, we found a more pronounced composition shift in the CDIF patients than in UC (Figure 26). Importantly, CDIF patients who received just one FMT treatment had larger shift compared to UC patients who received serial FMT.

4.5 MUCOSAL TRANSCRIPTOME AND EPITHELIAL CELL PROLIFERATION CHANGES AFTER THE FMT SERIES

Examined the samples of our pilot study subjects, RNA sequencing demonstrated that 742 genes decreased in expression (>1.5 fold decrease in expression, false discovery rate [FDR]<0.05) in the rectal mucosa of the patients (n=3) 2 weeks following FMT (detailed data not shown). Only 12 genes increased significantly in terms of relative expression. Down-regulated genes were compared by gene ontology enrichment analysis to a control set of human genes lacking evidence of changes in gene expression following

FMT (FDR>0.97). Genes linked to leukocyte activation and mitotic cell cycle progression were down-regulated. More specifically, 7 biological processes were highly significantly (FDR<10^{-5}) enriched by more than 2-fold in association with the down-regulated genes, compared to the control genes (Figure 27).

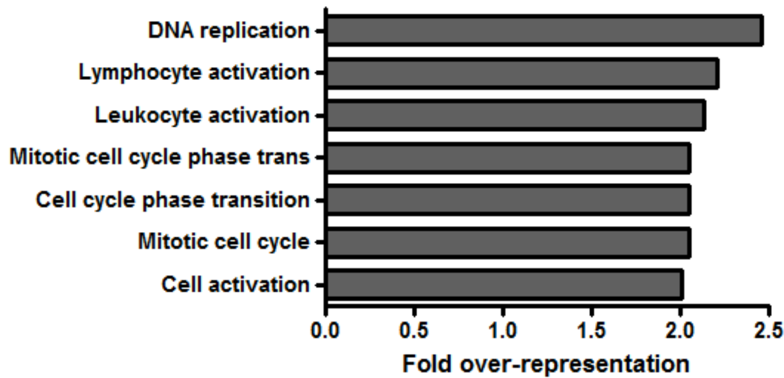

Figure 27 Biological processes with more than 2 fold over-representation (FDR <10^{-5}) in the down-regulated genes compared to control following serial FMTs in UC patients.

Based on these findings, we decided to functionally assess the consequences of FMT in the colonic mucosa of the patients in respect to mitotic activity changes. We found that 2 out of the 3 patients showed evidence that epithelial mitoses had been reduced by more than 50 percent (Figure 28).

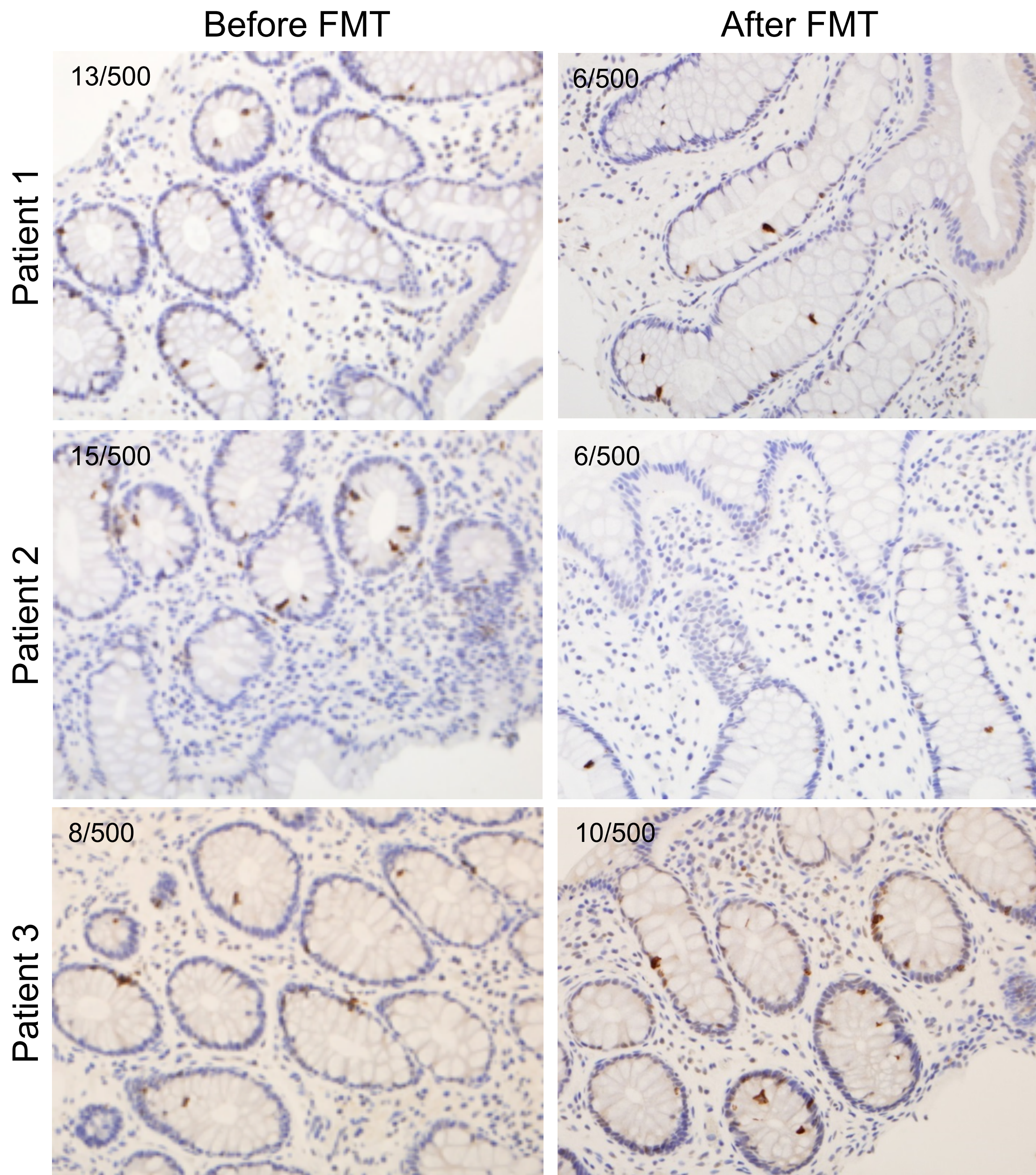

Figure 28 Epithelial Cell Mitosis Suppression Following FMT. In 2 out of 3 patients, the numbers of epithelial mitoses (blinded examination of 500 epithelial cells) decreased by more than 50% following high intensity FMT. Mitoses were highlighted by histone (H3) immunohistochemistry. Magnification 40x

5 CONCLUSIONS

5.1 Cellulose supplementation early in life ameliorates acute colitis in adult mice

Murine pediatric cellulose supplementation induces transient trophic, anticolitic and microbiome-modifying effects according to our study. The cellulose-stimulated lengthening of the colons persisted for 10 days, which was associated with protection against DSS. Additionally, we found that the cellulose-dependent trophic and anticolitic effects were transient by being lost 40 days after reversal of cellulose supplementation. This later result highlights the remarkable capacity for the large bowel towards anatomical reconstitution in mammals during young adulthood (between P90 and P120). The means by which DSS provokes colitis is still of debate, but recent studies indicate that it induces hyperosmotic stress leading to NF-kB activation through the posttranslational methylation of protein phosphatase 2A.(112) Regardless, toxin-induced colitis is bound to depend on the toxin-amount/mucosal-surface-area ratio. Lengthier colons with larger mucosal surface in the same species are likely to be less vulnerable to the toxin if the animal consumes the same amount. The average surface area increase induced by cellulose supplementation in this dietary model was calculated to be around 30%. Consequently, our study suggests that cellulose supplementation during animal development induces growth of the large intestine, thereby leading to an increased mucosal surface that decreases the toxin to surface-area ratio and ultimately provides protection against noxious insults.

Dietary fibers have been shown to modify both microbial composition and colitis susceptibility in mammals. Therefore, we studied the consequences of dietary cellulose on the colonic mucosal microbiome composition as well. Cellulose supplementation induced a significant microbiome separation by PCoA. Cluster differences were less pronounced following 10 days of reversal to low cellulose, but those were not diminished completely. This observation was true for the increased richness of the microbiome during high cellulose supplementation and 10 days of reversal as well. These findings support the presumption of De Filippo and colleagues (37) that increased dietary fiber consumption may be an important reason for the enhanced stool microbiome richness in rural African children compared to European children. Given that the animals in this work were maintained in the same facility room and even transferred between cages, our results also

indicate that cellulose supplementation dynamically increases the inter-species tolerance within mammalian commensal microbiota thereby inducing increased gut microbial richness within the same geographic environment. This finding contradicts the conclusions of De Filippo and colleagues (37), who suggested that a fiber-rich diet provides means to maintain overall enteral bacterial species richness by sustaining a diverse microbial environment within a geographical location. Rather, cellulose consumption (i.e. fiber-rich diet) can increase intestinal microbiota richness within the same microbial environment and can readily support mucosal protection.

At the phylum level, Actinobacteria decreased persistently during high cellulose supplementation and following 10 days reversal. This finding coincides with observations in *TLR2-/-* mice, which can be more susceptible to colitis than WT.(101) Actinobacteria were present in greater abundance in colonic mucosa of *TLR2-/-* mice similar to human studies on IBD associated microbiome.(56; 113) The decreased abundance of Actinobacteria in our current work was associated with a milder form of DSS colitis, supporting a potential colitic effect for this phylum in mammals.

Three families were modified in a prolonged fashion (in both HC and HCR10) upon cellulose supplementation: Coriobacteriaceae (a family within the phylum Actinobacteria) decreased, and Peptostreptococcaceae and Clostridiaceae increased compared to controls. The abundance of Peptostreptococcaceae and Clostridiaceae increased in human studies examining biopsies and stool samples from chronic pouchitis patients.(114; 115) Intestinal mucosal ulceration and the severity of chronic pouchitis were associated with increased numbers of different species within Peptostreptococcaceae and Clostridiaceae. Based on these findings, it is difficult to interpret the significance of the changes affecting these taxa in our experiments where pre-inflammation (no DSS exposure) microbiome composition was examined. Similar debates about microbiome associations of IBD (i.e. primary change, or secondary effect of inflammation) have been raised.(116)

The overall decline of family level composition variation upon 10-day reversal from high cellulose diet may indicate that the persisting gut morphological changes (i.e. longer colons with larger mucosal surface) may be more important towards colitis protection than the remaining shifts in community composition of the colon in this feeding model. This

conclusion is supported by a recent publication highlighting the importance of Allobaculum (a member of the Erysipelotrichaceae) and Paludibacter (a member of the Porphyromonadaceae) as key contributors to DSS colitis sensitivity.(117) We detected reduced abundances of Erysipelotrichaceae and Porphyromonadaceae on high cellulose diet (Figure 6), which could contribute to colitis protection (with less severe colitis). However, most of these changes diminished after 10 days reversal (both in our discovery and validation experiments) suggesting a less important influence of the microbiome on the colitis of the HCR10 mice than their longer large intestinal phenotype.

5.2 Loss of omega-6 fatty acid induced pediatric obesity protects against acute murine colitis

Western diets (usually containing high fat, especially ω-6 fatty acid) have been proposed to increase the risk of IBDs.(35) Dietary ω-6, and its metabolites have been shown to have pro-inflammatory properties and to exaggerate immunological responses in animal models.(118) The increased intake of essential ω-6 fatty acid, such as linoleic acid, has been associated with ulcerative colitis in a prospective human trial.(46) These findings are supported in studies on rodent models of colitis.(119) In the meantime, the effects of transient pediatric exposure to high ω-6 fatty acid on successive colitis susceptibility have not been examined. Early adulthood is the peak for IBD presentation, which indicates the potential importance of pediatric dietary exposures (including increased ω-6 consumption) in the developmental origins of the diseases. In this study, we examined how ω-6 induced transient obesity during pediatric development may impact colitis in young adult mice. On the contrary to our expectations, we found that reversal from ω-6 induced pediatric obesity protected mice from acute colitis in young adulthood. The colitis protection was exclusive to the acute/chemical injury model, since chronic colitis severity was not modulated by the same dietary intervention in both IL-10-/- mice,(109) and in the adoptive transfer model of CD4+, CD45RB^{hi}+ T cells into *Rag1-/-* mice.(110)

The acute colitis protection was specific to ω-6 (corn oil), at least in comparison with saturated fat (milk fat). This finding is consistent with recent work showing that saturated fatty acids (such as palm oil, which is similar to milk fat in fatty acid

composition) augmented small intestinal inflammation in a CD relevant murine model independently of obesity.(120) Additionally, Devkota, et al. found that saturated fat, but not polyunsaturated fat, triggers colitis in genetically susceptible mice in association with the bloom of *Bilophila wadsworthia.*(121) Indeed, clinical studies indicate that the type of dietary fat consumed rather than obesity itself is risk factor for the development of IBD.(35; 122; 123) However, with the experiments in this work, it cannot be ruled out that other immunomodulator substances both in corn oil, or milk fat may have played a role in the observed phenotype modifications. It is important to highlight that the phenotype observed in our work was also dependent on the cessation of increased ω-6 consumption, since obese mice during high ω-6 feeding were not protected from DDS induced colonic inflammation. Importantly, the velocity and extent of weight loss was similar in both the ω-6 and milk-fat reversal groups. Therefore, the colitis protection by transient ω-6 supplementation cannot be explained simply on the basis of weight loss. Rather, ω-6 supplementation during mammalian pediatric development appears to specifically induce a less colitis prone microbiome and host immunophenotype in a prolonged fashion.

Genetic work indicates that the development of IBD can be influenced by diverse [over 163 susceptibility loci (124)], potentially interactive genetic polymorphisms,(125) each of which contribute to the diseases with low penetrance. Monozygotic twin and other epidemiologic studies underscore the involvement of primarily non-genetically modulated, environmentally responsive, intercalating biologic systems.(48) The disease specific disruption of a single biological system, such as the microbiome, appears to be relatively limited in IBD,(56; 126) in agreement with the genetic observations. Rather, the individually small, but interactive dysregulation of multiple biologic systems within the host-microbiome network (127) may lead to the onset of IBD. Therefore, if one wishes to explore the consequences of a potentially critical environmental change in respect to IBD development, then a parallel multi-omic approach is warranted.(30) We set out to examine metagenomic, colonic mucosal and immunologic associations of transient ω-6 feeding induced acute colitis protection. Importantly, the colitis protection could be transmitted by the fecal microbiome into germ free mice underscoring the participation of the microbiome in the dietary intervention induced phenotype. The different genetic background of

germfree recipients in comparison to the donor mice (used in all the other experiments) highlights the translational relevance of the microbiome findings by overarching the boundaries of single mouse strains. *Enterococcus* was specifically decreased in the colonic mucosal microbiome of the ω6-R40 group (Figure 2E). Recently *Enterococcus* has been shown to trigger colonic inflammation in mouse model experiments.(128; 129) Additionally, increased abundance of *Enterococcus* was found in patients with ulcerative colitis, and it correlated with disease activity and severity.(130) These observations support the potential importance of the ω-6 fat reversal induced decrease of mucosal *Enterococcus* to participate in colitis protection.

In respect to the colonic mucosa, we did not detect significant epigenetic (DNA methylation) and transcriptional changes associated with the examined dietary intervention. However, a trend for decreased mucosal *Cxcr5* expression was found in the reversed animals. Count to threshold values for *Cxcr5* were very low suggesting that the signal may have been originated from mucosa associated leukocytes derived from mesenteric lymph nodes (MLN) rather than from epithelial cells [which generally comprise >90% of the cell population in the colonic mucosal scrapings (131)]. Multi-parameter flow cytometric analysis of T cell subsets revealed a significant reduction of $Cxcr5^{+}$ $CD4^{+}$ T cells in the MLN of the high ω-6 reversed animals. Cxcr5 and its ligand B-lymphocyte chemo-attractant (or Cxcl13) has been shown to orchestrate lymphoid organogenesis and the migration of CD4+ T cells from the mantle zone to the more central regions in lymphoid follicles. The expression of *Cxcr5* on CD4+ T cells is induced by the interaction of OX40 (or CD134) and its ligand OX40L, thereby serving as an upstream event for the Cxcr5 dependent T cell migration.(132; 133) Constitutive OX40/OX40L interaction stimulates autoimmune-like diseases, such as interstitial pneumonia and spontaneous colitis.(134) Inhibition of the OX40/OX40L interaction and secondary *Cxcr5* expression in CD4+ T cells decreased the severity of DSS-induced colitis in mice.(135) CXCR5+, IL-21 producing CD4+ T lymphocytes have been shown to play an important role in IBD.(136) All these publications indicated that CXCR5-CXCL13 pathway may play in an important role in controlling mammalian intestinal inflammation. Therefore, we examined the effects of anti-Cxcl13 (the ligand of Cxcr5) immunotherapy in modulating DSS colitis. Anti-

Cxcl13 treatment was protective. Consequently, we examined CXCL13 levels in treatment naïve IBD patients. The plasma concentration of CXCL13 was significantly elevated in children at the diagnosis of CD with a similar trend in UC, compared to controls. This result was consistent with our prediction that the CXCL13-CXCR5 pathway may be important in the pathology of IBD. The observed elevation of serum chemokines was not mirrored by gene expression changes at the intestinal mucosal level. Our recent work with treatment naïve pediatric UC colon biopsy samples,(137) and the observations of others in untreated ileal mucosal samples of pediatric IBD patients (127) has not detected differences in *CXCR5*, or *CXCL13* expression compared to controls. These results coincide with our findings in the murine model system of the present work, indicating that the CXCL13-CXCR5 pathway is more relevant for leukocyte than epithelial signaling in respect to IBD development. The lack of colitis protection in the chronic murine models suggests that this specific chemokine signaling is more important in modulating the onset and flares of disease rather than the maintenance of chronic inflammation in IBD.

Chemokines have been recognized as novel therapeutic targets for IBD, but CXCL13 specifically was not implicated.(138) Our results may set the stage for developing CXCL13 directed biologic treatments for the disease group in the future. Interestingly, tofacitinib is an emerging Janus kinase inhibitor utilized in IBD therapy,(139) which has been observed to suppress CXCL13 concentration in synovia of rheumatoid arthritis patients. This finding supports our predicament that the CXCR5-CXCL13 pathway may be a target for future biologic treatments in IBD.(140)

5.3 The manipulation of microbiota as a possible therapeutic option in pediatric gastrointestinal disorders

Similarly to others, we found FMT to be safe and highly effective for the treatment of pediatric recurrent CDIF in patients without complicating clinical conditions. According to the microbial data, we found robust switch in CDIF patients' microbiome following FMT.

As the ulcerative colitis study, our results indicate that FMT is safer and more effective in patients who start the treatments with endoscopic remission (endoscopic Mayo

score 0-1 without pseudopolyps). Indeed, if we included our inception cohort results (99) when examining the relationship between mucosal disease status and response to FMT during withdrawal of immunotherapy, we found an even more significant correlation between endoscopic disease activity at treatment initiation and clinical remission in pediatric UC patients (r=-0.878, two-tailed Spearman: p=0.003; Figure 29).

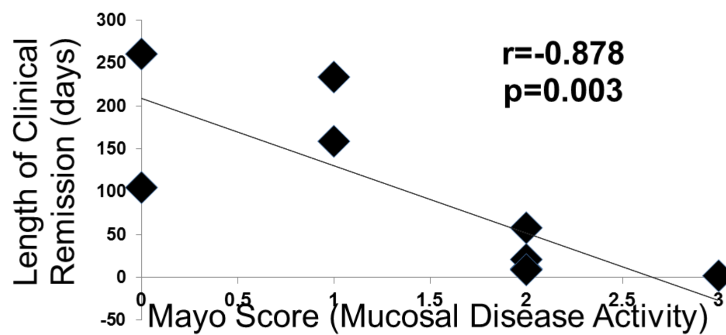

Figure 29 The length of clinical remission negatively correlated with mucosal disease activity at the initiation of FMT (r=-0.878, two-tailed Spearman: p=0.003).

We hypothesize that this may be related to an inhibition of the dynamic interaction between the gut microbiome and the colonic mucosa during active disease. Such inhibition would limit the success of microbiome based therapeutic interventions in UC during disease flares secondary to host unresponsiveness. Indeed, gene expression and gut microbiome associations have been shown to be decreased in clinically active UC patients compared to controls.(56)

Our phase I withdrawal study is unique in many ways compared to prior trials investigating FMT in UC:

1. Our protocol is the only immunotherapy withdrawal study of FMT in UC to date. This characteristic created a strong internal control for therapeutic efficacy of FMT within each patient compared to prednisone treatment.
2. All of our patients were children or young adults without any co-morbidity, making the recipients potentially ideal candidates for this experimental treatment.

3. The majority of our patients had short disease duration (3-43 months) prior to FMT, which according to Moayyedi et al.(82) may have led to an increase in FMT efficiency.
4. 83% (5 out 6) of the recipients received stool from a single donor, limiting biologic variation on the donor side.
5. The intensity of FMT therapy was considerably higher than other FMT trials to date.

Overall, fecal transplantation was generally well tolerated and safe. The one SAE of fever leading to hospitalization is an event that has been recognized in adult IBD patients receiving FMT and has been observed to be self-resolving in the majority of the cases.(78) Our clinical impression is that worsening disease during the course of FMT is likely to be a sign for treatment failure, and cessation of therapy should be seriously considered in such instances.

Correlative metagenomic studies showed shifts in our pediatric UC patients towards healthy pediatric microbiome composition with FMT. However, cessation of FMT resulted in return to the original composition along with disease recurrence in those who transiently benefitted from our treatment protocol. Interestingly, the microbiome of the single patient with therapeutic success in this study separated the farthest from the donor. This observation is in agreement with the publication by Suskind et al.(141) in which they observed the highest clinical benefit from FMT in pediatric CD patients where they used donors with the most distant microbiome composition form the recipient.

In conclusion, our findings underscore the importance of mucosal disease staging prior to considering FMT as a primary treatment modality for UC. While our patients PUCAI score placed them in the "mild" range, the endoscopic staging did not agree, further emphasizing importance of relying on mucosal disease staging and not just subjective measurements like PUCAI. Patients with medically induced mucosal healing, or mild endoscopic disease may benefit the most from FMT. Additionally, pretreatment microbiome analysis could identify the most ideal donor candidates from a preferentially existing donor pool, based on microbiome composition distance from the recipient. Our

observations further the notion that donor stool with the most distant microbiome composition form the recipient may provide the most optimal outcome during IBD directed FMT therapy.

6 KEYNOTES

1. There is a direct link between nutrition, microbiome and host response.

2. Postnatal exposure to different nutrients (such as cellulose or fat) has transient or persistent effect on intestinal homeostasis and predispose to the development for intestinal inflammation.
   Even temporary microbial and intestinal changes following cellulose and high ω-6 supplementation were linked to transient protection against DSS induced colitis in mice.

3. Complex bacteriotherapy, such as FMT provides treatment and/or the resolution of symptoms for patients suffering in CDIF and IBD via microbiome alteration.
   We established an FMT protocol to provide treatment for pediatric patients with CDIF and UC. The complex microbial changes were associated with the resolution of GI symptoms in our patient cohort.

7 SUMMARY

To better understand the fascinating association of nutrition, intestinal microbiota and host response, I examined the complexity of microbial responses altered by nutrition in mouse models. This work includes the first in depth analysis of effects of dietary cellulose supplementation on the composition of the mammalian colonic mucosal microbiome. The results indicate a transient effect of this nutritional intervention even following a prolonged high cellulose exposure. The developmental origins-based nutritional approach (testing the prolonged effects of a temporary nutritional exposure during the pediatric period) demonstrated that the colonic trophic effects of cellulose are also transient. These findings underscore the significant constitutional and morphological adaptive capacity of the colonic microbiome and the large intestine, respectively. Temporary microbial and intestinal changes were associated with transient protection against experimental colitis in mice. Such massive adaptive capabilities demonstrate the dynamic responsiveness of the mammalian gut towards luminal physical/compositional changes. Furthermore, transient high ω-6 diet during pediatric development in mice induced prolonged protection against murine colitis, which associated with persistent colonic mucosal microbiome and lymphoid organ composition changes.

This novel nutrition-microbiota-host paradigm fits with the well-known clinical observation that the modulation of microbiome by complex fecal microbiota transplantation (FMT) treats antibiotic-resistant *Clostridium difficile* infection (CDIF) and associated with the improvement of inflammatory bowel disease (IBD) symptoms. Our bench-side research developed into a bedside clinical trial, by providing FMT for pediatric patients with recurrent CDIF and ulcerative colitis (UC). Overall, FMT was generally well tolerated and safe. The complex microbial changes were associated with the resolution of GI symptoms in our patient cohort.

These findings may bare important implications for the nutritional developmental origins of IBD and promote the future development of novel preventive and therapeutic solutions based on dietary guidance and intestinal microbiome modulation.

8 ÖSSZEFOGLALÁS

A gyermekkorban végbemenő külső behatások hosszantartó változásokat hoznak létre a szervezetben, melyek később betegségek kialakulásához vagy éppen ellenkezőleg, betegségekkel szemben védelmet jelenthetnek. Ez a kritikus időszak gyermekkorban különösen jelentős figyelmet érdemel. A különböző táplálkozási hatások megváltoztatva a mikrobiomot a gyulladásos betegségek kialakulását/lefolyását befolyásolhatják. A táplálkozás-microbiom-host interakció szélesebb körű megértéséhez egér modellben vizsgáltuk különböző táplálkozási tényezők (cellulóz és ω-6 zsírsav) és azok hatását a mikrobiomra, illetve ezen valtozások hatását a később kialakuló intesztinális gyulladásra.

Tézisemben a gyermekgyógyászatban előforduló gasztrointesztinális megbetegedésekkel foglalkozom. Ebben a fejlődési időszakban a táplálkozási hatások fontos szerepet játszanak a microbiom megváltozásában és ez később gyulladásos bélbetegséghez vezethet, illetve a microbiom direkt megváltoztatásával a betegség megelőzhetővé válhat.

Állatkísérleteink eredményeinek függvényében lehetőségünk nyílt, hogy mikrobiom vizsgálatainkat kiterjesszük egy újszerű klinikai terápia alkalmazására. Klinikai vizsgálataink során komplex mikrobióta széklettranszplantációt (FMT) biztosítottunk *Clostridium difficile* fertőzésben (CDIF) és colitis ulcerosában (UC) szenvedő gyerekeknek. A dolgozatomban az FMT komplex hatását vizsgálom a mikrobiomra és a gasztointesztinális megbetegedésekre.

Az eredményeink és további vizsgálatok lehetővé teszik a táplálkozási tényezők komplex hatásának szélesebbkörű megértését, az FMT hatását a szervezetre és lehetőséget nyújthatnak további mikrobiológiai terápiák kialakításában korai gyermekkorban. A táplálkozási szokások megváltoztatása, illetve a mikrobiom direkt változtatása (FMT) a gyulladásos bélbetegségek újszerű megelőzését és terápiáját teheti lehetővé.

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10 PUBLICATIONS

10.1 Related publications of the author

Kellermayer R, Nagy-Szakal D, Harris RA, Luna RA, Pitashny M, Schady D, Mir SAV, Lopez, ME, Gilger MA, Belmont J, Hollister EB, Versalovic J. (2015) Serial Fecal Microbiota Transplantation Alters Mucosal Gene Expression in Pediatric Ulcerative Colitis. American Journal of Gastroenterology, 110(4): 604-6. **IF:9.2**

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11 ACKNOWLEDGMENTS

The last few years that I have spent working on my thesis is a time that I will always remember fondly, filled with a lot of amazing and happy memories. The pathway of discovery is full of challenges, however this leads the way to an exciting and extraordinary journey. These life experiences have been very closely linked to a lot of fantastic people who work at the 1st Department of Pediatrics (Semmelweis University, Budapest, Hungary) and Texas Children's Hospital (Baylor College of Medicine, Houston, Texas, USA).

First of all, I would like to thank my supervisors Adam Vannay and Richard Kellermayer for supervision, encouragement and for all of their mentorship in shaping my project from start to finish.

I would like to express special appreciation and gratitude toward my advisor Adam Vannay for his support both during the first year of my PhD and at the completion. I learned many basic scientific methods and the process of complex thinking during his mentorship. I appreciate the support of Tivadar Tulassay and Andras Arato who provided a perfect working environment for me to extend my clinical and scientific knowledge. Special thanks also goes to all my coworkers at the Research Laboratory of 1st Department of Pediatrics, Semmelweis University.

I would like to express my utmost gratitude towards Richard Kellermayer, who has been the most supportive, encouraging, brilliant, creative and selfless mentor I could ever wish for. His encouraging attitude helped carry and support me through the beginning of my scientific carrier, and provided me a solid foundation for success. Being a clinical doctor and basic scientist, he taught me a complex and comprehensive way of thinking about scientific problems in a hospital environment, always focusing on the patients. I appreciate the opportunity he gave me to play an integral role in a cutting-edge study in which we were able to take bench-side research to a bed-side clinical trial, providing fecal microbiota transplantation for pediatric patients with recurrent Clostridium difficile infection and

ulcerative colitis. I also truly appreciate the faith and support of all the patients who were enrolled into our clinical studies.
I would like to specifically thank to the leaders of research at Baylor College of Medicine who supported my work: James Versalovic, Marc Gilger, Milton Finegold, Nina Tatevian, Emily Hollister, Joseph Petrosino, Rob Shulman, C Wayne Smith, Antone Opekun, Ruth Ann Luna and Douglas Burrin.
I am also incredibly grateful for the support from the Department of Pediatric Gastroenterology, Hepatology and Nutrition at Texas Children's Hospital, and my amazing coworkers, and best friends: Faith Ihekweazu and Sabina Mir.
I owe special thanks to Gabor Veres, who supported me from the beginning throughout my medical student years, and involved me in undergraduate research. This provided me with a strong base for my clinical and scientific carrier.

Last, but not least, I am forever grateful for my family, especially my beloved parents (Katalin and Ferenc Nagy Szakal) and my sister, Fanni Nagy Szakal who were always beside me during the difficult times and great discoveries. I would like to express my gratitude for all the support I received from my extended family, my loving grandmas (Zsoka Mihalyi and Ferencne Nagy Szakal), my always-curious brother-in-law (Matyas Buzogany), my god parents (Valeria and Lajos Polka); and all my friends in Budapest, Houston and in New York. You have cherished with me every great moment and supported me whenever I needed it.
I would like to dedicate my thesis to my grandfather, Gyorgy Mihalyi, who was a polyhistor of his era and shared with me his love of mathematics and science from the very beginning, and taught me to enjoy the challenge of discovery.
Finally, I would like to wish to all my readers SERENDIPITY, "the occurrence and development of events by chance in a happy and beneficial way". I was taught this word on my first day in Houston by my amazing mentor, and it has helped carry me throughout my postdoctoral research years.