Prebiotic fructans have greater impact on luminal microbiology and CD3+ T cells in healthy siblings than patients with Crohn's disease: a pilot study investigating the potential for primary prevention of inflammatory bowel disease

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Funding

This work was supported by a clinical research fellowship granted by the charity Core (Guts UK) (CRH). FMF and PL received financial support from the Scottish Government Rural and Environment Science and Analytical Services.

Acknowledgments

The authors would like to acknowledge BENEO-Orafti, Teinen, Belgium who provided the oligofructose-enriched inulin. The authors would like to thank the patients and the siblings who generously participated in this study.

Keywords:

Prebiotics, Crohn's disease, microbiota, Pre-disease, first-degree relative, prevention

Abbreviations list

5-ASA	5-aminosalicylic acid
CD	Crohn's disease
CDAI	Crohn's disease activity index
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
GRR	Genotype relative risk
IBD	Inflammatory bowel disease
IQR	Interquartile range
NOD2	Nucleotide-binding oligomerization domain 2
OR	Odds ratio
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation

Abstract

Background & Aims

Siblings of people with Crohn's disease (CD) share aspects of the disease phenotype (raised faecal calprotectin, altered microbiota), which are markers of risk for their own development of CD. The aim was to determine whether supplementation with prebiotic oligofructose/inulin induces a prebiotic response and impacts the risk phenotype in CD patients and siblings.

Methods

Patients with inactive CD (n=19, CD activity index <150) and 12 of their unaffected siblings (with calprotectin >50µg/g) ingested oligofructose/inulin (15g/day) for three weeks. Faecal microbiota (qPCR), intestinal permeability (lactulose-rhamnose test), blood T cells (flow-cytometry) and calprotectin (ELISA) were measured at baseline and follow-up.

Results

Following oligofructose/inulin, calprotectin did not significantly change in patients (baseline mean 537 SD 535µg/g; follow-up mean 974 SD 1318µg/g, p=0.08) or siblings (baseline mean 73 SD 90µg/g: follow up mean 58 SD 72µg/g, p=0.62). Faecal *Bifidobacteria* and *Bifidobacterium longum* increased in patients and siblings; *Bifidobacterium adolescentis* and *Roseburia* spp. increased only in siblings. Compared with patients, siblings had a greater magnitude change in *Bifidobacteria* (+14.6% vs +0.4%, p=0.028), *B. adolescentis* (+1.1% vs 0.0% p=0.006) and *Roseburia* spp. (+1.5% vs -0.1% p=0.004). Intestinal permeability decreased significantly in patients after oligofructose/inulin to a level that was similar to siblings. Blood T cell abundance reduced in siblings but not patients following oligofructose/inulin.

Conclusions

Oligofructose/inulin supplementation did not significantly impact calprotectin, but the prebiotic effect was more marked in healthy siblings compared with patients with inactive CD and was associated with alterations in other CD risk markers. Future research should focus on dietary intervention, including with prebiotics, in the primary prevention of CD.

Introduction

Crohn's disease (CD) pathogenesis centres on an abnormal and prolonged T cell mediated immune response directed against aspects of the commensal gut microbiota that occurs in genetically susceptible individuals after, as yet mostly undefined, environmental insults. Consonant with these pathogenic factors, the phenotype of CD is characterized by altered microbiota, abnormal intestinal permeability, alterations in T cell phenotype and raised faecal calprotectin. Unaffected siblings have a relative risk (RR) of developing CD of up to 35,[1] and a significantly raised incidence rate ratio[2] compared with the background population. Meanwhile, unaffected siblings share some features of the CD phenotype including alterations in mucosal[3] and faecal microbiology,[4, 5] elevated faecal calprotectin,[4, 6, 7] increased histologic inflammation,[6, 8] alterations in peripheral blood T cell populations,[4] increased intestinal permeability,[9, 10] and increased antibody response to microbial antigens.[11] Recent data from >900 healthy first-degree relatives show that elevated faecal calprotectin is associated with future diagnosis of CD.[12]

Although there is much variation between studies in the microbial signature associated with IBD (likely due to variations in patient populations, study protocol, laboratory technique as well as cultural and geographic influences on diet and environmental exposures) some general patterns in the microbial alterations have been consistently demonstrated in IBD. These include reduced Firmicutes including *Roseburia* and *Faecalibacterium prausnitzii*, increased abundance of Proteobacteria and an overall lower diversity. Evidence regarding the alteration in *Actinobacteria* including *Bifidobacteria* is less consistent. Several of these characteristics of the gut microbiota have also been demonstrated in at-risk relatives of IBD patients as discussed above.[3-6] It remains unclear whether restitution of these microbial alterations in at-risk relatives can influence the natural history of disease development, however, in animal models of IBD

immunomodulatory and metabolic effects that reduce intestinal inflammation have been demonstrated with several of the bacterial species that are known to be diminished in IBD.

Prebiotics are defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health"[13] and include non-digestible polymers of fructose termed oligofructose and inulin. In animal models of IBD oligofructose consumption has been associated with reductions in intestinal permeability,[14] and modulation of immune cell numbers and cytokine production.[15] Furthermore, it has been demonstrated that the optimal effect of a high fibre prebiotic diet occurred when it was introduced prior to initiation of DSS colitis, implying a role for prebiotics in disease prevention.[16]

In human IBD, consumption of inulin-type fructans has been shown to improve Mayo score in UC and reduce faecal calprotectin.[17] In CD supplementation with oligofructose-enriched inulin has shown a prebiotic effect by increasing *Bifidobacteria* in the luminal microbiota.[18] Only eight patients in that study had active disease, which was significantly decreased after oligofructose-enriched inulin supplementation. However, several other clinical studies of prebiotics in CD have shown disappointing results,[19, 20] including a clinical trial in a large cohort of 103 patients, all of whom had active CD, which demonstrated neither a clinical effect nor an increase in Bifidobacteria in response to oligofructose/inulin.[21] This lack of effect of oligofructose/inulin in active CD is in contrast to the widely reported bifidogenesis associated with oligofructose/inulin consumption in healthy populations.[22, 23] Thus, it may be that the bifidogenic effect of oligofructose/inulin seen in healthy individuals is abrogated in active CD.

We therefore hypothesized that dietary supplementation with oligofructose-enriched inulin is associated with a prebiotic effect, decreased intestinal permeability, alterations in blood T cells

and decreased faecal calprotectin in patients with CD in remission and their healthy siblings and thus could be a potential intervention to prevent disease in populations of at-risk relatives.

Materials and Methods

This pilot study was a 3-week intervention to assess the effect of 15 g/d of oligofructose-enriched inulin in patients with CD and their healthy siblings. The primary outcome was change in faecal calprotectin concentrations in patients and siblings following three weeks of oligofructose/inulin supplementation. All participants provided written, informed consent prior to completing the study. This research was conducted according to the Declaration of Helsinki and was approved by Bromley Local Research Ethics Committee (ethics reference number 07/H0805/46).

Patients and siblings were aged between 16 and 35 years. Participants were excluded if they were unable to consent or if they had evidence of current infection with an enteric pathogen, use of antibiotics, probiotics or prebiotics within the last month, were pregnant or lactating, or had significant hepatic, renal, endocrine, respiratory, neurological or cardiovascular disease as determined by the principal investigator or a history of cancer with a disease-free state of less than two years. All participants were screened for raised calprotectin (>50 mg/g) using a rapid calprotectin test (Calpro AS, Lysaker, Norway) for entry into the study.

All patients had a diagnosis of CD for ≥3 months defined by histology or radiology and were currently in clinical remission, defined by clinical assessment, serum CRP ≤5 mg/L and a Crohn's disease activity index (CDAI) <150. Patients had stable medication regimes (i.e. no change in oral steroids within 4 weeks with a daily dose not exceeding 10 mg/d prednisone or equivalent, no change in oral 5-ASA treatment within 4 weeks, initiated on azathioprine or methotrexate not more recently than 4 months prior to the study with a stable dose for at least 4 weeks, no use of biologics within 3 months, no use of rectal 5-ASA or steroids within 2

weeks and no non-steroidal anti-inflammatory drugs within 2 weeks). Furthermore, patients with imminent need of surgery, short bowel syndrome or purely perianal CD were excluded.

Siblings were also screened to exclude irritable bowel syndrome using Rome III criteria.

Study protocol

All study visits were conducted at the Royal London Hospital, London, UK. A food frequency questionnaire was completed to assess consumption of inulin and oligofructose-containing foods during the preceding week.[24] This food frequency questionnaire has previously been validated against a 7-day semi-weighed food diary and found to be a reliable and reproducible tool for capturing short-term intake of inulin and oligofructose.[24] Participants underwent a limited flexible sigmoidoscopy without prior bowel preparation. Biopsies were taken from non-inflamed rectal mucosa and were sent for routine histological analysis to assess inflammatory activity. A faecal specimen was obtained for microbiological analysis and quantification of faecal calprotectin. A 5-h urine collection was performed following non-absorbable sugar dosing to measure intestinal permeability. Blood samples were taken for haematological (full blood count and erythrocyte sedimentation rate) and biochemical (urea, creatinine, electrolytes, liver function tests, CRP) markers, and for peripheral blood T cell analyses (leukocyte subsets and integrin expression).

Patients and their siblings consumed 15 g/d of oligofructose/inulin provided as sachets, (Orafti®Synergy1, BENEO-Orafti, Teinen, Belgium). This was a combination of chicory inulin (degree of polymerisation range 2-60) and oligofructose produced by partial enzymatic hydrolysis of chicory inulin (degree of polymerisation range 2-8), provided in a 50:50 ratio and purity of 90-94%. Participants completed daily symptom diaries during the three-week period and avoided prebiotic and probiotic supplements/foods (a list of example foods to avoid was provided). All participants were contacted weekly during the study period to ensure compliance

and to monitor any side-effects of oligofructose/inulin and for patients, to monitor for symptoms indicative of worsening disease activity or requirement for change in drug prescription.

At the final visit at 3-weeks, symptom diaries were collected, and the food frequency questionnaire was repeated. For patients, disease activity was calculated from the CDAI symptom diary over the preceding 7-days. Faecal, urine and blood samples were collected as at the baseline visit, supplementary figure S1.

Laboratory methods

Faecal microbiota

The gut microbiota were analysed using quantitative PCR (qPCR) using primers targeting gene sequences specific for 10 bacterial groups known to comprise the reported microbial signature in CD.[3, 4] Faecal microbial DNA extraction was carried out using the FastDNA spin kit for soil and the FastPrep-24 bead homogenizer (MP Biomedicals, Solon, Ohio, USA) following the manufacturer's instructions. A SYBR Green qPCR analysis of faecal microbial DNA was performed as described previously[25] with the following modifications: amplified bacterial 16S rRNA genes served as standard templates as previously described except for *E. coli* primers for which *E. coli* XL1-Blue was used. PCR reactions were performed in quadruplicate using a 7900HT Fast Real-Time PCR machine (Life Technologies). Data were analysed with SDS V.2.4 software (Life Technologies). Faecal water content was measured by lyophilization and concentrations of bacteria expressed as $\log_{10}$ 16S rRNA gene copies /gram dry faeces to correct for possible varying water content between patients and siblings. Normalized proportions of bacteria were obtained by dividing specific group quantities by universal quantities. For details of primers and PCR conditions see previous publication.[4]

Faecal calprotectin

Screening calprotectin measurements were performed using a lateral-flow rapid calprotectin test (Calpro AS, Lysaker, Norway). Faecal samples were also transferred on ice before aliquoting and freezing at -20^{0}C for subsequent analysis by ELISA kit (Calpro AS, Lysaker, Norway) according to the manufacturer's instructions using duplicate appropriately diluted samples. Faecal calprotectin concentration was determined relative to standard curves and expressed as μg/g of faeces.

Intestinal permeability

Measurement of intestinal permeability was undertaken using lactulose-rhamnose tests.[26] After overnight fasting, participants consumed 450 mL water containing 5 g lactulose, 2 g mannitol and 1 g L-rhamnose (BCM Specials, Nottingham, UK), followed by a 5 h fasting urine collection. Aliquots from the pooled urine sample were stored at −20°C until analysis. Urinary sugar separation and detection was carried out by liquid chromatography-tandem mass spectrometry. Following microcentrifugation, supernatant was transferred to a LC-2000 platform autosampler (Jasco, Easton, Maryland, USA) and urinary sugars separated by high performance liquid chromatography using an amino column in hydrophilic interaction liquid chromatography mode. Sugars were quantified by electrospray tandem mass spectrometry, using the API 3200 (AB Sciex, Framingham, Massachusetts, USA).

Lymphocyte analysis

Peripheral blood T cell analysis was carried out according to our previously published protocol.[4] Briefly, whole blood, collected in lithium-heparin Vacutainer tubes (BD Bioscience), was stored at room temperature for ≤4 h before labelling with fluorescently conjugated monoclonal antibodies to detect T cells (CD3+), naïve ($CD45RA^+$) and effector/memory ($CD45RA^-$) subsets of CD4 and CD8 T cells. Integrin α4β7 expression was assessed by labelling with anti-β7 (see supplementary methods). Data were acquired using a

LSRII flow cytometer (BD Bioscience) and collected FACSDiva software V.4.1.2 (BD Bioscience) using Flow-Count fluorospheres (Beckman Coulter) for absolute quantitation. Colour compensation was performed offline using Winlist V.6.0 (Verity Software House).

Genotype

Human DNA was extracted from whole blood using the phenol chloroform-isoamyl alcohol method.[27] Genotyping was performed using the Illumina Infinium Immunochip,[28] which includes 70 of the 71 CD disease risk loci described in Franke *et al.*[29] To increase detection of NOD2 mutations and capture the enhanced risk of NOD2 compound heterozygosity, three NOD2 (rs2066845/ G908R, rs2066844/R702W and rs5743293/3020insC) were individually assessed. Cumulative genotype relative risk (GRR) for each participant was therefore calculated across a total of 72 CD risk loci. A population distribution model of CD risk was generated using the REGENT R program[30] and previously published ORs.[29] Participants were categorized into reduced, average, elevated or high genotypic risk with reference to this model.[31]

Statistics

The primary outcome was the change in faecal calprotectin concentration in patients and siblings following three weeks of oligofructose/inulin supplementation. For patients, an SD for faecal calprotectin of 150 and a difference to detect of 180 µg/g (i.e. reduction from 235 to 55 µg/g) and for siblings, a SD for faecal calprotectin of 29 and a difference to detect of 35 µg/g (i.e. from 55 to 20 µg/g)[7] was used in the power calculation,[7] which indicated that a study size of 9 patients and 9 siblings would be required to be able to reject the null hypothesis with a statistical power of 90% and a significance level of 0.05.

Continuous and categorical variables were compared between patients and siblings using Student's T test and chi-squared test respectively. Where data were not normally distributed

(determined by visual inspection data distribution of histograms and by a significant Shapiro-Wilk tests) non-parametric techniques such as Mann-Whitney U test were used. Comparison between two time points within the same group was carried out using a related samples Wilcoxon signed ranks test or paired T test as appropriate. Correlations between continuous variables were assessed using the Pearson correlation coefficient.

Results

Participant characteristics

In total 19 patients and 14 siblings entered the study (due to technical problems with a rapid calprotectin test 3 patients and 4 siblings were entered into the study with unknown calprotectin), Supplementary figure S2. There were no significant differences in demographics between patients and siblings, nor were there differences in dietary intakes of inulin or oligofructose between groups at baseline or at the end of the intervention**, Table 1**. Eighteen of 19 patients and 11 of 14 siblings underwent flexible sigmoidoscopy. Rectal biopsies in all these participants confirmed normal histology.

Table 1 Participant demographic and clinical data

		Patients (n=19)	Siblings (n=14)	p-value
Age years mean (SD)		27.7 (6.9)	25.1 (5.1)	0.245*
Males, n (%)		12 (63)	8 (57)	0.727†
Body Mass Index, kg/m^2, mean (SD)		25.1 (5.0)	23.5 (3.0)	0.281*
Ethnicity, n (%)	White British	17 (90)	13 (93)	0.672†
	Asian/ Asian British	1 (5)	0 (0)	
	Black British/ mixed black/white	1 (5)	1 (7)	

Number of siblings, median (IQR)		1 (1)	1 (1)	0.869*
Smoking, n (%)	Never	13 (68)	8 (57)	0.672†
	Current	4 (21)	3 (21)	
	Previous	2 (11)	3 (21)	
Genotype category, n (%)	Reduced	5 (26)	3 (21)	0.422†
	Average	9 (47)	9 (64)	
	Elevated	2 (11)	2 (14)	
	High	3 (16)	0 (0)	
GRR value, mean (SD)		2.65	1.12	0.150*
Age at diagnosis, n (%)	Below 16 years	8 (42)	-	-
	16-40 years	11 (58)	-	
Disease location, n (%)	Ileal	6 (32)	-	-
	Colonic	3 (16)	-	-
	Ileocolonic	10 (53)	-	-
	Concomitant upper GI	1 (5)	-	-
	Concomitant perianal disease	3 (16)	-	-
Current drug use, n (%)	ASA	9 (47)	-	-
	Immunosuppressant	9 (32)	-	-
Right hemicolectomy, n (%)		9 (47)	-	-
Small bowel resection, n (%)		1 (5)	-	-
Disease behaviour, n (%)	Non-stricturing/ penetrating	10 (53)	-	-
	Stricturing	4 (21)	-	
	Penetrating	5 (26)	-	
	Baseline g/d, median (IQR)	2.9 (4.7)	3.7 (1.8)	0.585‡

Dietary inulin intake	End g/d, median (IQR)	3.3 (2.2)	2.5 (1.3)	0.202‡
Dietary oligofructose intake	Baseline g/d, median (IQR)	2.9 (4.5)	3.6 (2.0)	0.716‡
	End g/d, median (IQR)	3.3 (2.2)	2.5 (1.3)	0.190‡

*Student's T test

†Chi-squared test

‡Mann-Whitney U test

Oligofructose/inulin did not impact calprotectin in Crohn's disease or siblings

Faecal calprotectin as measured by ELISA was raised (>50 µg/g) in all patients (19, 100%) at baseline (mean 537 SD 535 µg/g), and values did not significantly change following oligofructose/inulin (mean 974 SD 1318 µg/g, p=0.08), **Figure 1.**

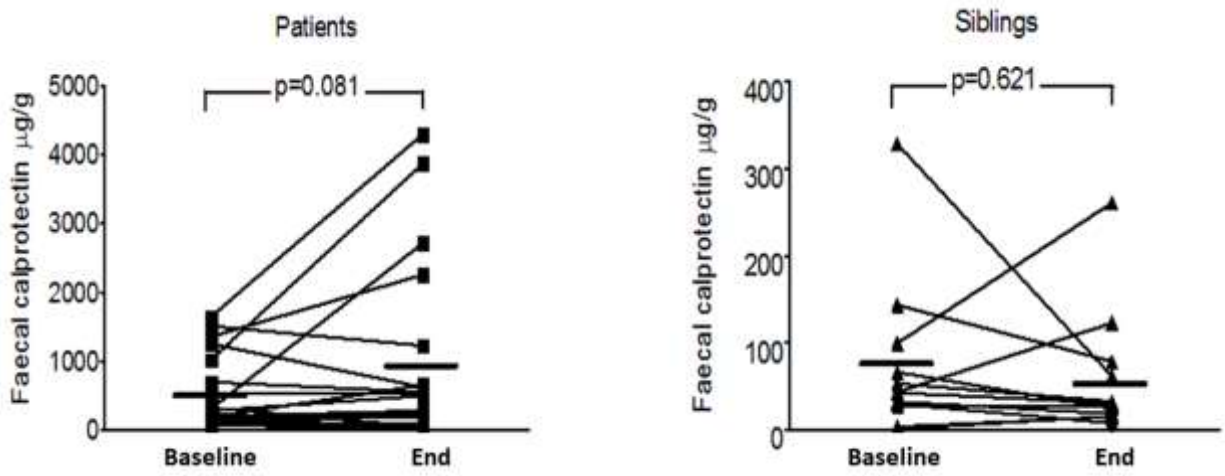


Figure 1: Faecal calprotectin in patients (n=19) and siblings (n=12) at baseline and following three weeks of oligofructose/inulin supplementation. Horizontal lines represent the group mean at each time point. Data were not available for 2 siblings. Wilcoxon-signed ranks test.

Faecal calprotectin was raised at baseline (>50µg/g) in six siblings out of 12 (50%, mean 73 SD 90 µg/g, data not available for 2 siblings), but also did not change significantly following oligofructose/inulin supplementation in the group as a whole (mean 58 SD 72 µg/g, p=0.62), nor in the subgroup of six siblings with raised calprotectin at baseline (baseline mean 138 SD 112 µg/g vs oligofructose/inulin mean 92 SD 97 µg/g, p=0.532).

In patients there was no correlation between baseline faecal calprotectin and the change in faecal calprotectin during the study period (Δfaecal calprotectin, median -15 SD 99 µg/g, correlation=0.352, p=0.140). In contrast, there was a significant negative correlation between baseline faecal calprotectin and Δfaecal calprotectin within siblings (Δfaecal calprotectin median 437, SD 1030 µg/g, correlation=-0.715, p=0.009), Supplementary Figure S3.

The prebiotic effect of oligofructose/inulin was greater in siblings compared with patients

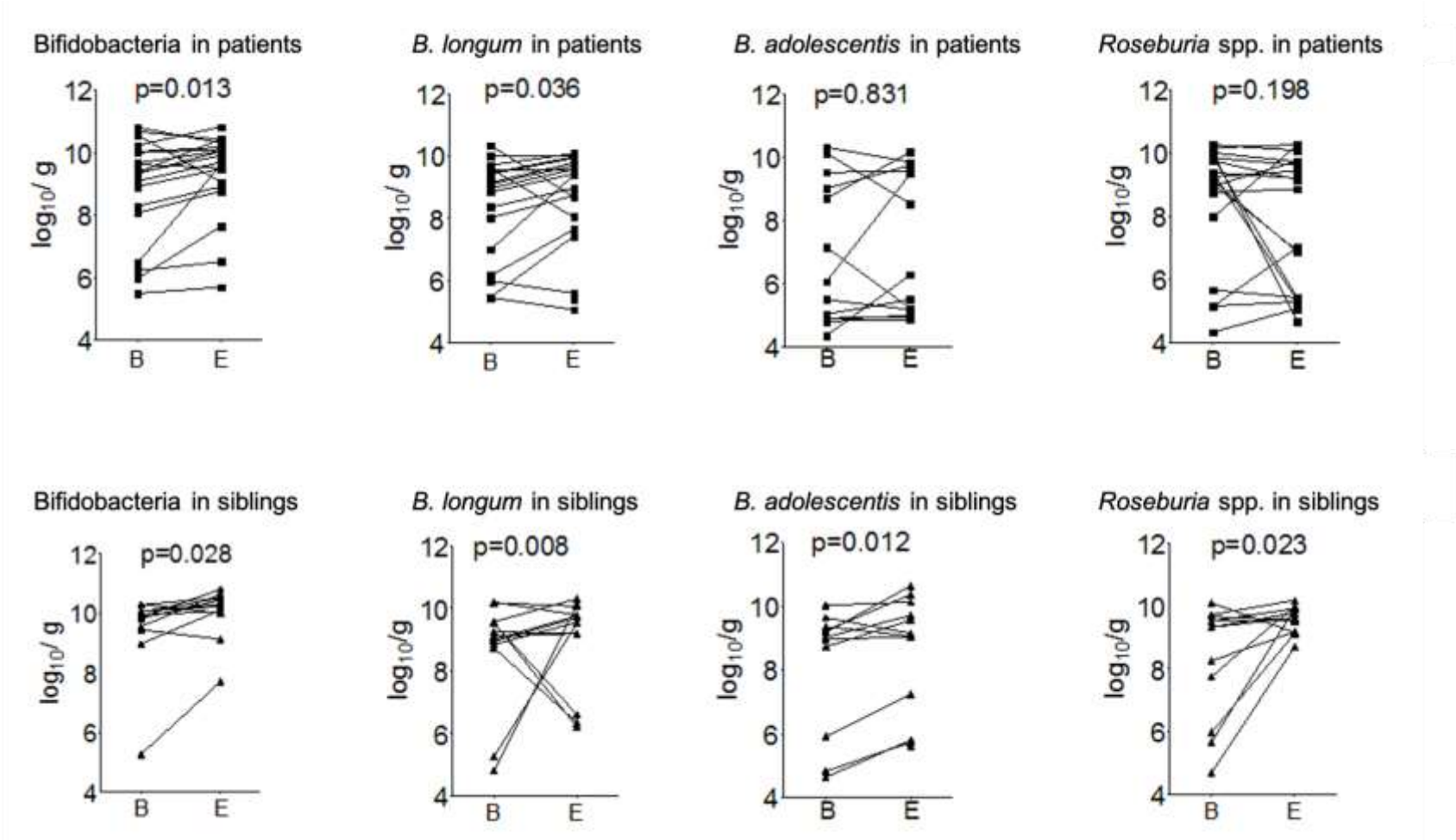
In both patients and siblings, concentrations of total Bifidobacteria and *B. longum* increased following oligofructose/inulin, whilst in siblings but not patients, *B. adolescentis* and *Roseburia* spp. also increased, **Table 2, Figure 2**. The concentration of total bacteria and of other bacterial groups and species analysed did not change significantly between baseline and following oligofructose/inulin in either siblings or controls.

Table 2 Comparison of the concentrations and proportions of gut microbiota between patients at baseline and following three weeks of oligofructose/inulin supplementation, in siblings at baseline and following oligofructose/inulin supplementation, and between patients and siblings at baseline and at the end of the oligofructose/inulin period. Data were not available for 2 siblings.

	Patients (n=19)				**Siblings (n=12)**				**P value for patients vs siblings**	
	Baseline	**Oligofructose / inulin**	**P value**		**Baseline**	**Oligofructose / inulin**	**P value**		**Baseline**	**Oligofructose / inulin**
Concentrations (log_{10} 16S rRNA gene copies/g), median (IQR)										
Universal	10.78 (0.61)	10.85 (0.74)	0.469		10.94 (0.48)	10.83 (0.60)	0.937		0.142	0.795
Bacteroides-Prevotella	8.88 (1.77)	9.18 (1.93)	0.091		10.50 (0.92)	10.37 (1.17)	0.530		**0.002**	0.110
Bifidobacterium	**9.36 (1.96)**	**9.69 (1.25)**	**0.013**		**9.91 (0.60)**	**10.25 (0.50)**	**0.028**		0.199	**0.035**
B. longum	**8.97 (3.41)**	**9.40 (2.14)**	**0.036**		**9.11 (3.48)**	**9.56 (2.75)**	**0.008**		0.706	0.412
B. adolescentis	5.17 (4.35)	5.16 (9.48)	0.831		**9.00 (4.22)**	**9.10 (3.84)**	**0.012**		0.106	**0.007**
Roseburia spp.	9.25 (1.88)	8.86 (4.32)	0.198		**9.33 (3.21)**	**9.60 (0.77)**	**0.023**		0.815	0.053
Faecalibacterium prausnitzii	7.11 (4.33)	8.16 (4.05)	0.091		9.06 (5.37)	9.26 (1.29)	0.347		**0.007**	**0.039**

Clostridial cluster XIVa	9.90 (1.13)	10.16 (0.70)	0.520		10.15 (0.70)	9.81 (1.17)	0.754		0.439	0.589
Lactobacillus	5.09 (1.31)	5.11 (2.68)	0.260		5.24 (0.70)	5.35 (0.91)	0.754		0.653	0.562
Escherichia coli	7.95 (2.31)	8.41 (2.41)	0.077		7.33 (1.01)	7.36 (0.93)	0.583		0.815	0.085
Percentage of universal (%)										
Bacteroides-Prevotella	3.96 (25.13)	5.56 (53.11)	0.084		46.99 (62.75)	29.13 (38.56)	0.308		**0.011**	0.252
Bifidobacterium	6.64 (12.09)	10.53 (25.22)	0.376		**10.35 (15.98)**	**32.53 (37.87)**	**0.034**		0.506	0.059
B. longum	2.95 (6.33)	2.74 (11.14)	0.421		1.32 (10.26)	4.83 (8.73)	0.060		0.843	0.704
B. adolescentis	0.00 (1.15)	0.00 (2.55)	0.795		**1.34 (3.19)**	**3.96 (10.46)**	**0.008**		0.152	**0.006**
Roseburia spp.	5.58 (10.89)	0.85 (7.06)	0.147		**3.12 (6.14)**	**6.08 (7.82)**	**0.023**		0.321	**0.028**
Faecalibacterium prausnitzii	0.04 (3.16)	0.53 (2.49)	0.126		1.48 (5.73)	3.51 (4.68)	0.272		**0.035**	**0.016**
Clostridial cluster XIVa	17.82 (31.54)	17.59 (11.50)	0.334		17.55 (27.98)	12.62 (16.28)	0.308		0.957	0.177
Lactobacillus	0.00 (0.00)	0.00 (0.02)	0.159		0.00 (0.00)	0.00 (0.00)	0.875		0.900	0.734
Escherichia coli	0.12 (2.84)	0.70 (5.46)	0.159		0.05 (0.29)	0.06 (0.06)	0.480		0.212	0.164

Figure 2: Change in concentration of bacteria from baseline (B) to the end of oligofructose/inulin supplementation (E) in patients (top row, n=19) and siblings (bottom row, n=12). Bacterial groups that were measured but were not significantly different between baseline and the end of the oligofructose/inulin intervention period are not shown. Data were not available for two siblings. Wilcoxon signed ranks tests.



At baseline there was no significant difference in concentrations or proportions of any of the Bifidobacteria species between patients and siblings. However, following oligofructose/inulin supplementation, siblings had significantly greater concentrations and proportions of Bifidobacteria and subspecies as well as the proportion of *Roseburia* spp. compared with patients, **Table 2**. Siblings had a significantly greater change compared with patients in proportions of Bifidobacteria (+14.6% vs. +0.4%, p=0.028), *B. adolescentis* (+1.1% vs. 0.0% p=0.006) and *Roseburia* spp. (+1.5% vs. -0.1% p=0.004), following oligofructose/inulin supplementation **Figure 3**.

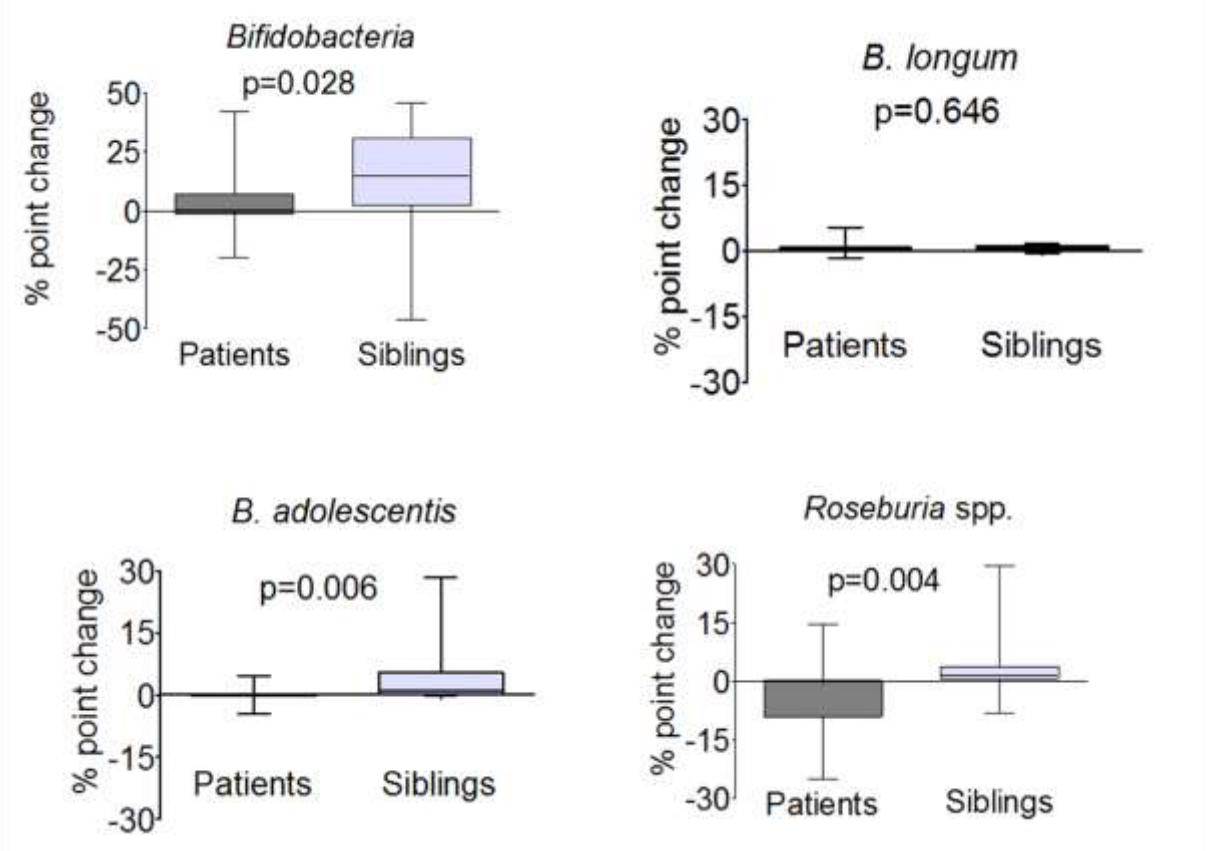


Figure 3 Box and whisker plot depicting the percentage point change in proportions of bacteria in patients (n=19) and siblings (n=12) from baseline and following three weeks of oligofructose/inulin supplementation. Data were not available for 2 siblings. Boxes represent the 25th and 75th centiles and the horizontal lines within the boxes are the medians. The whiskers delineate the maximum and minimum values. Mann-Whitney-U test.

The magnitude of the prebiotic effect was associated with baseline microbiota concentrations for some species. There was a significant negative correlation between baseline concentration of *Bifidobacteria* and the change in *Bifidobacteria* and between baseline concentrations of *B. longum* and the change in concentration of *B. longum* following oligofructose/inulin in both patients and siblings. In addition, baseline concentrations of *B. adolescentis* negatively correlated with the change in *B. adolescentis* and baseline concentrations of *Roseburia* spp. with the change in *Roseburia* spp. in siblings but not patients. For each analysis the slope of the correlation coefficient was greater for siblings than patients. However, there was no correlation in baseline concentrations of *F. prausnitzii* and change in *F. prausnitzii* concentrations following oligofructose/inulin, supplementary table S1.

Blood $CD3^+$ T cell frequency reduced following oligofructose/inulin in siblings but not in Crohn's disease

The frequency of $CD3^+$ T cells was not changed in patients between baseline (mean 1,010,411, SD 551 584 cells ml^{-1} blood) and following oligofructose/inulin supplementation (mean, 1,059,938, SD 724,583 cells ml^{-1} blood, p=0.565). However, in siblings there was a significant fall in $CD3^+$ T cells between the two time points (mean 1,591,879, SD 410,370 cells ml^{-1} blood vs mean 1,295,645 SD 137,488 cells ml^{-1} blood, p=0.03), **Figure 4**. Other T cell factors such as the relative proportion of $CD4^+$ and $CD8^+$ T cells, the proportion of memory T cells, the proportion of T cell subtypes that expressed β7 integrin and T cell expression of the CD69 activation marker were not altered by oligofructose/inulin supplementation.

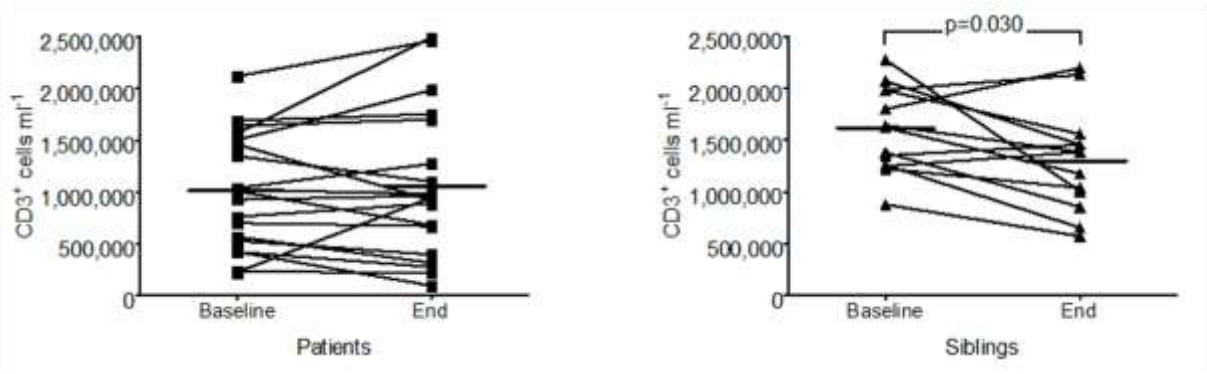


Figure 4: Frequency of CD3+ T cells between baseline and following three weeks of oligofructose/inulin supplementation in patients (n=19) and siblings (n=13). Horizontal lines represent the group mean at each time point. Data were not available for one sibling. Paired samples t-test.

Prebiotic effect was associated with alterations in blood T cell phenotype

In patients there were no correlations between the change in concentrations or proportions of any bacterial species with the change in the frequency of T cells. In siblings the change in proportion of *B. adolescentis* (median 0.54, IQR 8.6 %p) was significantly negatively correlated with the change in frequency of T cells (median -305,605, IQR 660,122 cells ml^{-1}, Pearson's R 0.673, p=0.023). The change in other bacterial concentrations was not correlated with the change in the frequency of T cells.

Intestinal permeability improved after oligofructose/inulin in Crohn's disease but not in siblings

Baseline intestinal permeability was significantly higher in patients compared with siblings (urinary lactulose-rhamnose ratio median 0.066 IQR 0.092 vs median 0.038 IQR 0.039, p=0.025). However, after 3 weeks oligofructose/inulin supplementation intestinal permeability was not significantly different between patients and siblings (median 0.041 IQR 0.038 vs median 0.038 IQR 0.037, p=0.630).

There was a significant reduction in intestinal permeability between baseline and following oligofructose/inulin supplementation in patients (urinary lactulose-rhamnose ratio from median 0.066, IQR 0.092 to median 0.041, IQR 0.038, p=0.049) but not in siblings (from median 0.038 IQR 0.039 to 0.032 IQR 0.033, p=0.583). This change was most significant in the 16 patients with ileal involvement, in whom intestinal permeability decreased significantly (from median 0.067, IQR 0.100 to median 0.038, IQR 0.037, p=0.009) whereas there was no change in the 3 patients with colonic CD, (from median 0.031, IQR N/A to median 0.1 IQR N/A, p=0.109).

No relationship between demographic, genetic and disease characteristics and the response to oligofructose/inulin

There was no association between smoking status, ethnicity, body mass index, age, previous surgery or immunosuppressant use with responses to oligofructose/inulin in terms of changes from baseline in: intestinal permeability; faecal calprotectin; blood T cells; or concentrations and proportions of *Bifidobacteria*, *Bifidobacteria* subspecies and *Roseburia* spp. There was no difference in the responses to oligofructose/inulin between participants categorized as elevated/high GRR compared with those categorized as average/ reduced GRR. There were no significant differences in response to oligofructose/inulin between males and females, except for the proportion of *Roseburia* spp. The change in proportion of *Roseburia* spp. (Δ*Roseburia spp.* proportions) was significantly lower males was (median 0% points, IQR 10%) than in females (median 1% points, IQR 4%, p=0.026). The proportions of *Roseburia* spp. at baseline did not differ between males (median 4%, IQR 11%) and females (median 6%, IQR 8%, p=0.768).

Disease activity and safety and tolerability of oligofructose/inulin

In patients, there was no significant difference in CDAI for the week preceding baseline (mean 65, SD 44) and the final week of oligofructose/inulin supplementation (71, SD 69, p=0.715).

One adverse event occurred in the patient group and none in the sibling group. The adverse event was a flare of CD in a 31-year-old male patient with ileal CD with baseline CDAI 51 and serum CRP <5 mg/L, but whose baseline faecal calprotectin was considerably raised 1016 μg/g. At the end of the 3-week oligofructose/inulin supplementation the patient's CDAI had risen to 222, CRP was 13 mg/L and faecal calprotectin had further risen to 3869 μg/g. The patient's symptoms and clinical parameters normalized with conventional treatment (oral prednisolone and azathioprine). As the patient reported the flare on the final day of oligofructose/inulin supplementation, they were not withdrawn from the study. No participant was withdrawn from the study due to side-effects associated with the oligofructose/inulin supplementation.

Discussion

This pilot study is the first to determine the effect of oligofructose/inulin on markers of CD activity in patients with inactive CD and on markers of risk of future CD in their unaffected siblings. Moreover, this is the first study to capture multiple dimensions of the response to oligofructose/inulin in humans, including microbiological and immunological responses as well as changes in intestinal permeability and faecal calprotectin. Oligofructose/inulin did not impact on faecal calprotectin in patients or siblings; however healthy siblings showed an enhanced prebiotic response to oligofructose/inulin compared with their CD siblings who were all in clinical remission. Healthy siblings also experienced a reduction in blood T cell numbers. Oligofructose/inulin in this dose was safe and well-tolerated.

Currently there are several reported and ongoing studies aiming to identify markers of risk of CD onset. The GEM (Genetic Environmental Microbial) project is a large prospective cohort and has recently reported (in abstract form) that calprotectin in healthy first-degree relatives of CD patients predicts future risk of CD.[12] These data, in turn motivate the search for interventions to reduce that risk. Prebiotics provide an intervention that impacts gut microbiota,

which is a key factor in CD pathogenesis, in a way that is inexpensive, well tolerated,[32] free from major side-effects and acceptable to patients[33] and their families. However, in this pilot study we did not demonstrate an effect of oligofructose/inulin on faecal calprotectin in patients with inactive CD and in healthy siblings. Thus, despite the important results reported by Lee *et al.*,[12] there is as yet no proven intervention that can modify calprotectin as a marker of CD risk.

Interestingly, there was a non-significant trend towards an increase in calprotectin in patients after oligofructose/inulin supplementation that was not apparent in the siblings. This trend implies divergent responses to prebiotics in IBD patients compared with healthy people. If this were confirmed in other cohorts it would have important implications for the implementation of prebiotics which may be investigated as primary prevention but potentially detrimental as treatment. Moreover, fermentable carbohydrates are associated with exacerbation of functional gastrointestinal symptoms in patients with IBD.[34] It may be that these functional effects are also associated with an exacerbation in inflammation, and indeed the authors reported increased faecal calprotectin after ingestion of fermentable carbohydrates including inulin-type fructans.[34]

The current study demonstrated a less pronounced prebiotic effect in patients with inactive CD compared with their siblings. This limited effect in inactive CD is in contrast to the lack of prebiotic effect previously shown with the same dose of inulin/oligofructose in patients with active CD.[21] Taken together these data indicate that the prebiotic response to oligofructose/inulin is absent in active CD, attenuated in inactive CD, significantly greater in at-risk siblings of CD patients and greatest in healthy subjects.[35] It may therefore be speculated that CD, particularly when active, limits the prebiotic effect of oligofructose/inulin. Thus, the therapeutic potential of prebiotics may be limited where inflammation is established, and intestinal damage has already occurred. In keeping with previous studies, the baseline abundance of bacteria significantly determined the prebiotic response and it may be that

inflammation-driven alterations to the microbiota determine the prebiotic capacity of oligofructose/inulin. Alternatively, the inflamed gut may present a milieu disadvantageous to specific species. For example, patients with CD have been shown to have lower colonic pH[36-39] which may inhibit complete fermentation of oligofructose/inulin and attenuate the selective benefit afforded to bacteria such as *Roseburia* spp. or *F. prausnitzii* that can exploit such oligosaccharides and produce anti-inflammatory metabolites such as butyrate.[40, 41] Bacteria converting lactate produced by Bifidobacteria into butyrate are also likely to be adversely affected by lower colonic pH.[42]

The bacteria that were stimulated by oligofructose/inulin ingestion may have anti-inflammatory properties[43] and as such would be predicted to be beneficial in IBD and in those at risk of IBD. For example, *Roseburia* spp. have been associated with reductions in colonic inflammatory macrophages and Th17 cells and downregulating IL-6 and STAT3 expression[44] and abundance of *Roseburia* spp. was predictive of response to the anti-integrin vedolizumab in both CD and ulcerative colitis.[45] Furthermore, in patients with ulcerative colitis, administration of *B. longum* with oligofructose/inulin (a symbiotic formulation) was associated with reductions in mRNA levels of human beta defensins 2, 3 and 4, as well as TNF-α and IL-1α. In addition, low *F. prausnitzii* has been associated with early recurrence of CD[46] and to predict relapse following withdrawal of anti-TNF treatment in patients in the STORI cohort.[47] In contrast, the patients in the current study also showed a non-significant increase in pro-inflammatory *E. coli*, a trend which was also seen (to a lesser extent) in siblings. Thus, prebiotics such as oligofructose/inulin may have the potential to stimulate bacteria with anti-inflammatory effects and these effects may be different between those with risk of IBD and those with established IBD.

Following oligofructose/inulin supplementation, blood T cell frequency significantly decreased in siblings but not in patients. Moreover, the decrease in blood T cell frequencies correlated with the change in *B. adolescentis* concentration. Specific components of the gut microbiota

may elicit greater immune responses as evidenced by enhanced IgA coating.[48] It may be that prebiotics have the capacity to shift the gut microbiota composition toward less immune-provoking species, which could attenuate inflammatory responses. Such changes in gut microbiota composition may lead to increased recruitment or greater retention of gut homing T cells into intestinal tissues, leading to a reduction in this population in the periphery. It has previously been demonstrated that both CD and the at-risk sibling phenotype are associated with reduced blood T cell frequency, mostly dependent on naïve $CD4^+$ T cell lymphopaenia.[4] However, in the current study it was not possible to demonstrate differences in these specific T cell subsets, possibly due to an inadequate sample size.

In the current study a reduction in intestinal permeability after oligofructose/inulin was seen in patients with CD, particularly in ileal CD, however intestinal permeability remained stable in siblings. The reduction in intestinal permeability after oligofructose/inulin was greatest in individuals (mostly patients) with high intestinal permeability at baseline, who also had attenuated prebiotic effect. This may indicate that the effect of oligofructose/inulin on intestinal permeability occurs independently of the prebiotic effect. In animal models of intestinal inflammation, oligofructose was associated with reductions in intestinal permeability.[14] Previous studies of prebiotics in healthy humans[49] and a variety of non-IBD patients[50-52] have shown variable effects of prebiotics on intestinal permeability. A possible explanation of this variability is that the phenotype of increased intestinal permeability may only be apparent in healthy individuals after exposure to environmental or dietary triggers. In one study the increased intestinal permeability phenotype in relatives was only apparent after ingestion of non-steroidal anti-inflammatory drugs.[53] Interestingly a recent study in healthy volunteers has indicated that *Bifidobacterium breve Bif195* can prevent intestinal injury caused by ingestion of acetylsalicylic acid,[54] indicating a possible role for prebiotics to strengthen the resilience of the intestine against environmental insults. Whether prebiotics exert their effect on intestinal

permeability through stabilization of the intestinal barrier in the face of environmental or dietary triggers warrants further investigation.

There was no association between demographic or disease related factors and the effect of oligofructose/inulin except for the effect on the proportion of Roseburia spp. which was lower in males. The effect of drugs on the gut microbiota has been detailed in several studies[55, 56] and therefore we excluded patients with recent treatment with antibiotics or non-steroidal anti-inflammatory drugs. However, in order to include a patient population representative of those in whom prebiotic treatment might be considered (i.e. as a supplementary maintenance therapy alongside conventional maintenance therapy), patients with stable immunosuppressant doses were included. There was no indication that the use of immunosuppressants affected the outcomes of interest, however this population was too small to be able to exclude this and the use of immunosuppressants, which inevitably only occurred in patients and not in siblings is a limitation of this study.

Dietary intake of oligofructose and inulin was not significantly different between patients and siblings either at baseline or at the end of the intervention period and was comparable to that in healthy UK populations[24] This is in keeping with previous data indicating that compared with healthy controls, patients with active CD consume lower amounts and patients with inactive disease consumed similar amounts of oligofructose/inulin.[57] However prebiotic supplements are infrequently employed by patients in the management of IBD [58] possibly due to a lack of effect, especially in active disease.

Strengths and limitations

The measurement of a wide range of different factors in the current study has allowed a more complete characterization of the response to oligofructose/inulin. Exploring features of CD in relatives as well as patients allows such factors to be resolved in the absence of the confounding

effect of disease and its treatment. Therefore, unaffected relatives provide a unique window into disease pathogenesis.[59] Similarly, the patients in the current study were well characterized and were in remission, ensuring that the effect of oligofructose/inulin could be discerned in the absence of significant inflammation. In addition, confining the relatives group to siblings avoids the potential confounder of large age differences found in parent-child comparisons. An additional strength of this study is that patients were on stable medications and none of the patients were treated with biological therapies, avoiding some potential confounding.

The inclusion of an unrelated healthy control group would allow determination as to whether healthy siblings respond to prebiotics in the same way as healthy controls. In addition, comparison with a placebo would allow effects to be attributed more definitively to oligofructose/inulin consumption and avoid potential confounding related to the use of immunosuppressants. The patients were screened for evidence of clinical disease activity, but despite this several of the patients had raised calprotectin indicating some heterogeneity in disease activity, and future studies in patients with biochemically or endoscopically confirmed remission may be required. Although a power calculation was performed, it was based on limited data and this study may have been underpowered and as it is uncontrolled it should be considered an important but preliminary step in understanding the effect of a dietary intervention in at-risk siblings. Finally, the microbiological technique used in this study was qPCR, and it is important to note that 16S rRNA sequencing techniques can provide much greater in-depth analysis of microbiome diversity, community structure and taxa composition, as well as avoid issues of sensitivity and specificity that can occur for some primers in qPCR. Future intervention studies in siblings should utilise such approaches.

Conclusion

Recent developments in prediction of CD motivate the search for interventions that can translate this knowledge into health benefit to prevent disease onset in at-risk individuals. In the current

study, oligofructose/inulin has been shown not to impact faecal calprotectin in healthy siblings of CD patients in the short term, but the prebiotic effect is more marked in at-risk siblings in contrast to inactive CD where it is attenuated. Prebiotics such as oligofructose/inulin should be investigated for their role in disease prevention in at risk populations.

Conflict of interest

None of the authors had any financial or personal relationships with the company or organization sponsoring the research at the time the research was done. **CRH** has received speaker fees from Takeda, Ferring, AbbVie, and Janssen, and consultancy fees from Pfizer. She has acted as local principal investigator for clinical trials for Janssen and GlaxoSmithKline. **SM**, **AJS** & **JOL** have nothing to disclose; **NEM** reports grants and personal fees from ImCheck Therapeutics SAS, outside the submitted work; **PL** & **FMF** reports grants from Scottish Government Rural and Environment Science and Analytical Services, during the conduct of the study; **KW** reports grants from Core (Guts UK), during the conduct of the study; grants from Clasado Biosciences, grants from Danone, grants from International Nut and Dried Fruit Board, grants from Almond Board of California, outside the submitted work; In addition, KW has a patent FODMAP by FoodMaestro with royalties paid to King's College London, Guys and St Thomas NHS Foundation Trust and FoodMaestro, and a patent for Volatile Organic Compounds as markers of response to diet in IBS with royalties paid to King's College London and University of Liverpool.

Author contributions to the manuscript

CRH: Conceptualization; Funding acquisition; Methodology; Investigation; Data curation; Formal analysis; Project administration; Writing - original draft. **NEM**: Investigation; Formal analysis; Writing - review & editing. **PL**: Validation; Resources; Data curation; Writing - review & editing. **FMF**: Validation; Resources; Data curation; Writing - review & editing. **SM**: conducted research; Writing - review & editing. **AJS**: Conceptualization; Funding acquisition; Methodology; Investigation; Resources; Supervision; Writing - review & editing. **JOL**: Conceptualization; Funding acquisition; Methodology; Investigation; Supervision; Writing -

review & editing. **KW**: Conceptualization; Funding acquisition; Methodology; Investigation; Project administration; Supervision; Writing - original draft.

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Figure legends

Figure 1

Faecal calprotectin in patients (n=19) and siblings (n=12) at baseline and following three weeks of oligofructose/inulin supplementation.

Horizontal lines represent the group mean at each time point. Data were not available for 2 siblings. Wilcoxon-signed ranks test.

Figure 2

Change in concentration of bacteria from baseline (B) to the end of oligofructose/inulin supplementation (E) in patients (n=19) and siblings (n=12).

Bacterial groups that were measured but were not significantly different between baseline and the end of the oligofructose/inulin intervention period are not shown. Data were not available for 2 siblings. Wilcoxon signed ranks tests.

Figure 3

Box and whisker plot depicting the percentage point change in proportions of bacteria in patients (n=19) and siblings (n=12) from baseline and following three weeks of oligofructose/inulin supplementation.

Data were not available for 2 siblings. Boxes represent the 25th and 75th centiles and the horizontal lines within the boxes are the medians. The whiskers delineate the maximum and minimum values. Mann-Whitney-U test.

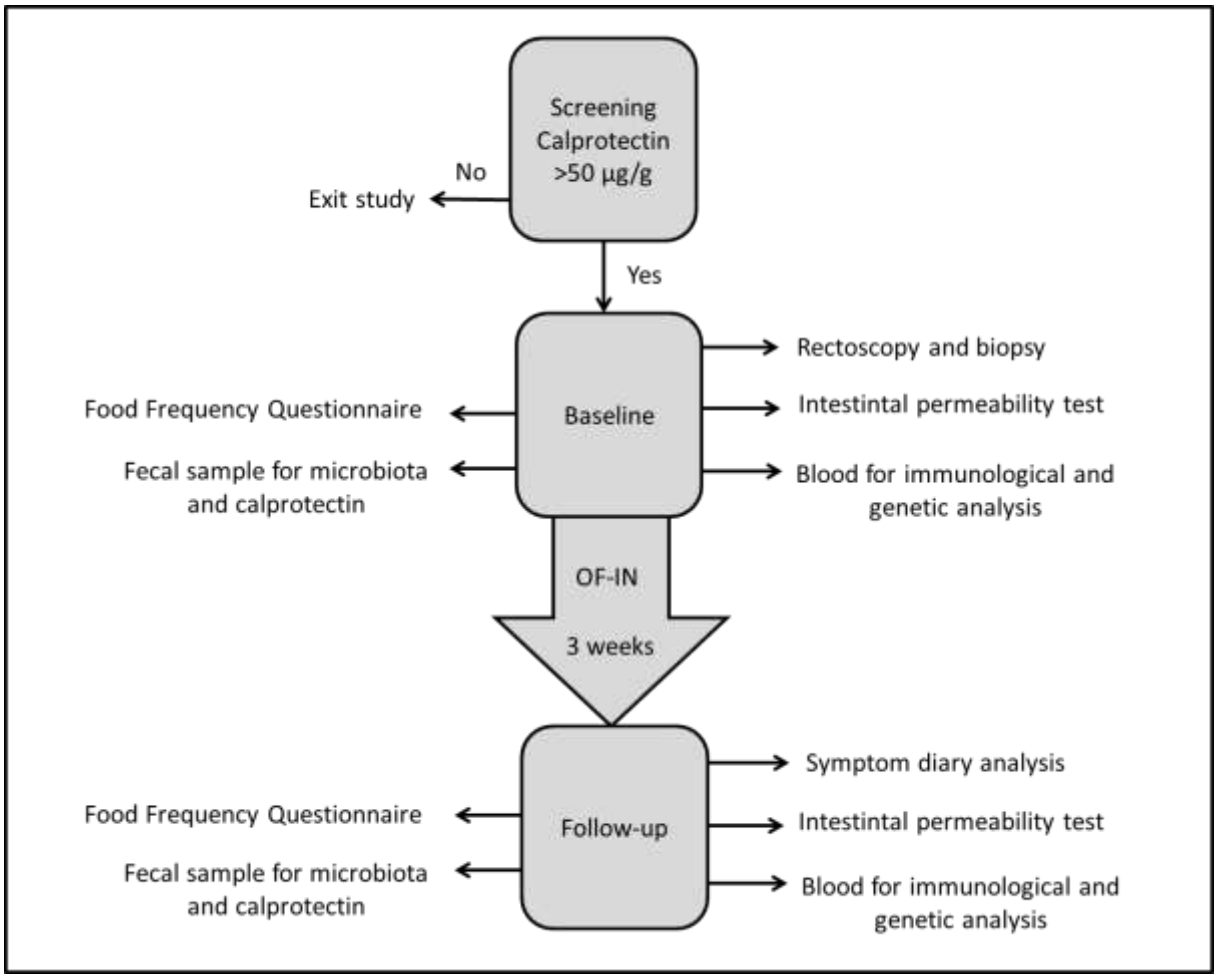
Figure 4

Frequency of CD3+ T cells between baseline and following three weeks of oligofructose/inulin supplementation in patients (n=19) and siblings (n=13).

Horizontal lines represent the group mean at each time point. Data were not available for one sibling. Paired samples T test.

Supplementary figure S1

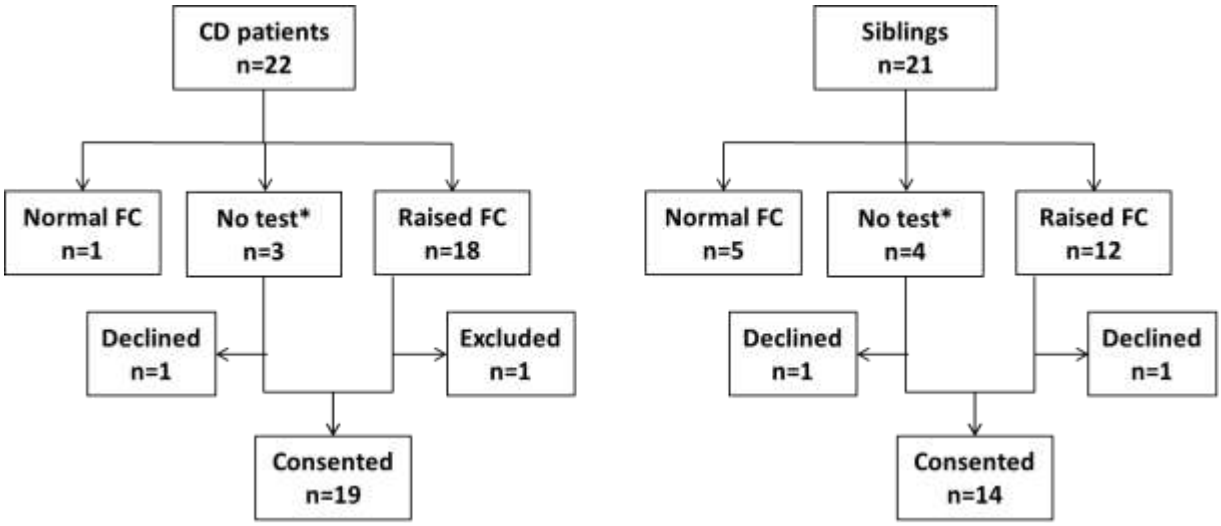
Diagram of study protocol



OF-IN, oligofructose/inulin 15 g/d

Supplementary figure S2

CONSORT diagram of the recruitment of participants to the study



Normal faecal calprotectin was defined as ≤ 50µg/g. *Due to problems with the supply of the rapid calprotectin test from the manufacturer, the rapid test was unavailable at certain times. Participants recruited during these periods were invited to enter the current study without faecal calprotectin screening.

Supplementary figure S3.

Correlation between baseline faecal calprotectin and change in faecal calprotectin between baseline and following three weeks of oligofructose/inulin supplementation (ΔFaecal calprotectin) in patients (n=19) and siblings, (n=12, data were not available for 2 siblings).

Supplementary Table S1

Correlation between baseline concentration of bacterial species and the change in concentration of that species between baseline and follow-up in patients and siblings after three weeks supplementation with oligofructose/inulin

	Patients				**Siblings**			
	Baseline concentration log_{10}/g (IQR)	Change in concentration log_{10}/g	Pearson's R	p	Baseline concentration, log_{10}/g (IQR)	Change in concentration (log_{10}/g)	Pearson's R	p
Bifidobacteria	9.36 (1.96)	0.54	-0.582	0.009	9.91 (0.57)	0.35	-0.834	0.001
B. adolecentis	5.17 (4.35)	0.03	-0.177	0.468	9.91 (0.57)	0.86	-0.846	0.001
B. longum	8.97 (3.41)	0.58	-0.770	<0.001	9.23 (1.81)	0.63	-0.839	0.001
Roseburia	9.25 (1.88)	0.19	-0.442	0.058	9.33 (2.38)	0.46	-0.972	<0.001

There was no correlation between baseline concentration and the change in concentration after oligofructose/inulin for *Faecalibacterium prausnitzii, Bacteroides-Prevotella*, Clostridial cluster XIVa, *Lactobacillus* or *Escherichia coli*.