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Published in:
Journal of Crohn's and Colitis

DOI:
10.1093/ecco-jcc/jjz208

Journal of Crohn's and Colitis, 2020, 595–607
doi:10.1093/ecco-jcc/jjz208
Advance Access publication December 24, 2019
Original Article

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Abstract

Background and Aims: Crohn's disease [CD] is characterised by chronic intestinal inflammation and dysbiosis in the gut. Riboflavin [vitamin B_2] has anti-inflammatory, antioxidant and microbiome-modulatory properties. Here, we analysed the effect of riboflavin on oxidative stress, markers of inflammation, clinical symptoms, and faecal microbiome in patients with CD.

Methods: In this prospective clinical intervention study, patients received 100 mg riboflavin [DSM, Nutritional Products Ltd] daily for 3 weeks. Clinical disease activity [Harvey-Bradshaw Index: HBI], serum biomarkers of inflammation and redox status [plasma free thiols], and faecal microbiome taxonomical composition and functionality [fluorescent *in situ* hybridisation: FISH; and metagenomic shotgun sequencing: MGS], were analysed before and after riboflavin intervention.

Results: In total, 70 patients with CD with varying disease activity were included. Riboflavin supplementation significantly decreased serum levels of inflammatory markers. In patients with low faecal calprotectin [FC] levels, IL-2 decreased, and in patients with high FC levels, C-reactive protein [CRP] was reduced and free thiols significantly increased after supplementation. Moreover, HBI was significantly decreased by riboflavin supplementation. Riboflavin supplementation led to decreased Enterobacteriaceae in patients with low FC levels as determined by FISH; however, MGS analysis showed no effects on diversity, taxonomy, or metabolic pathways of the faecal microbiome.

Conclusions: Three weeks of riboflavin supplementation resulted in a reduction in systemic oxidative stress, mixed anti-inflammatory effects, and a reduction in clinical symptoms [HBI]. FISH analysis showed decreased Enterobacteriaceae in patients with CD with low FC levels, though this was not observed in MGS analysis. Our data demonstrate that riboflavin supplementation has a number of anti-inflammatory and anti-oxidant effects in CD.

Key Words: Crohn's disease; riboflavin [vitamin B2]; clinical intervention study

1. Introduction

Crohn's disease [CD] is a chronic inflammatory disease of the gastrointestinal tract and is characterised by a relapsing-remitting disease course.[1] Its incidence is increasing globally, in particular in the recent decades and especially in regions adopting a Western lifestyle.[2] CD is accompanied by a high patient burden and impaired quality of life.[3] A complex interaction between inherited and environmental factors, the gut microbiome, and the host immune response are causative in the pathogenesis of CD.[4–7]

Thus, CD has a multifactorial aetiology, and is characterised by relapsing intestinal inflammatory events. Reducing these inflammatory events is an important therapeutic target to improve the quality of life of patients with CD. Such an approach of reducing inflammatory intestinal events may be accomplished by supplementation of the diet with anti-inflammatory food components. Riboflavin is such a component with anti-inflammatory potential.

Riboflavin is a water-soluble vitamin that plays a key role in several metabolic pathways, including human energy metabolism. Previous studies have demonstrated that riboflavin exerts anti-inflammatory and antioxidant effects in animal models of CD.[8–11] For instance, administration of either pure riboflavin or riboflavin-producing bacteria ameliorates chemically-induced colitis in mice.[8] Similarly, other experimental animal studies have demonstrated anti-inflammatory effects of riboflavin, such as a decrease in the production of pro-inflammatory cytokines, tumour necrosis factor-α [TNF-α], and interleukin-6 [IL-6], and a potentiating effect on the anti-inflammatory action of dexamethasone.[9]

It is currently unknown whether riboflavin alleviates inflammation and oxidative stress directly, by modulating the patient's immune system, or indirectly, by altering the composition of the gut microbiome. The latter seems of particular interest, since the gut microbiome of patients with CD is characterised by a reduced microbiota diversity compared with healthy individuals.[12] One of the most prominent effects on species level is a reduction in the abundance of the commensal bacterium *Faecalibacterium prausnitzii*.[13–19] This bacterial species has anti-inflammatory properties and is a potent producer of short-chain fatty acids [SCFAs], particularly butyrate.[15,17] In a pilot study in healthy individuals, it was demonstrated that a 2-week supplementation period of riboflavin resulted in an increase in the faecal abundance of *F. prausnitzii*.[11]

We designed a prospective clinical intervention study in patients with CD to further clarify the effect of riboflavin on multiple disease parameters. Because disease activity may affect the success rate of riboflavin interventions, we evaluated the effect of riboflavin supplementation in patients with either low or high faecal calprotectin levels separately. We hypothesised that riboflavin supplementation in patients with CD will show anti-inflammatory and antioxidant effects, resulting in a reduction of faecal calprotectin levels, C-reactive protein [CRP], and pro-inflammatory cytokines, and an improvement of systemic redox status, disease-specific symptoms, and quality of life [QoL], and that such effects may be mediated by changes in the faecal microbiome composition. Therefore, we analysed the effect of riboflavin on clinical disease scores, circulating inflammatory biomarkers, and systemic redox status, as well as on the faecal microbiota composition and functionality.

2. Materials and Methods

2.1. Study population

Patients aged 19–67 years were included from March 2016 until April 2017 from the IBD outpatient clinic of the University Medical Center Groningen [UMCG]. All patients had an established diagnosis of CD existing for at least 1 year, based on clinical, endoscopic, and histopathological criteria.

Patients were included and divided into two groups according to inflammatory disease activity, as determined by the faecal calprotectin [FC] level. The first group consisted of patients with low FC levels [defined as a faecal calprotectin level <200 µg/g] and the second group consisted of patients with high FC levels [defined by faecal calprotectin level >200 µg/g].

Exclusion criteria were as follows: swallowing disorders; pregnancy and lactation; use of antibiotics, probiotics, or specific prebiotic supplements in the 3 weeks preceding the riboflavin intervention; use of methotrexate drugs; colonoscopy or colon cleansing in the past 3 months; and severe CD activity (defined as a Harvey-Bradshaw Index [HBI] >12). In addition, patients using a vitamin B_2 supplement, or multivitamin complexes containing B vitamins [i.e., vitamin B complexes] in the 3 weeks preceding the riboflavin intervention were excluded from the study. Concomitant medication use for CD was allowed in all study groups. However, patients who reported changes in medication use during the study period as well as within 3 months preceding potential inclusion, were excluded from the study. No adverse events occurred in this study.

2.2. Ethical considerations

This prospective clinical intervention study has been approved by the Institutional Review Board [IRB] [in Dutch: 'Medisch Ethische Toetsingscommissie', METc] of the UMCG [IRB no. 2014/291] and registered on ClinicalTrials.gov [NCT02538354]. All patients provided written informed consent in accordance with the Declaration of Helsinki [2013].

2.3. Data collection and study design

At the time of inclusion, standard demographic characteristics, including age, sex, body mass index [BMI], smoking behaviour, and alcohol consumption, were recorded as well as CD-specific disease parameters [e.g., disease course, disease localisation, current CD maintenance therapy]. For each patient, the Montreal disease classification was used to determine the disease phenotype [including

age at diagnosis, localisation of the disease, and disease behaviour]. Moreover, CD-related surgical history was recorded. In addition, as a clinical measure of disease activity, the HBI was documented. All patients with CD were encouraged to maintain their normal dietary habits during the study period. Patients completed an extensive Food Frequency Questionnaire [FFQ] to obtain information on their habitual dietary intake [see Supplementary Methods, available as Supplementary data at *ECCO-JCC* online].

Patients were requested to collect faecal samples at home and store the samples in their home freezers, immediately after production. Two baseline samples [T0] were collected before the riboflavin intervention, to correct for day-to-day variation. Additional faecal samples were collected after 3 weeks of supplementation with riboflavin [T3]. Frozen faecal samples were transported to the UMCG on dry ice and stored at -80°C. Furthermore, the HBI and the Inflammatory Bowel Disease Questionnaire [IBD-Q] were completed and blood samples were collected and stored at -80 °C before and after riboflavin supplementation.

The period of riboflavin supplementation was based on the previously mentioned pilot study in healthy individuals, in which we observed a significant increase in *F. prausnitzii* abundance already after 2 weeks.[11] However, in the present study consisting of patients with CD, we investigated the effect of riboflavin on many different outcomes, including outcomes of disease activity and quality of life. Due to clinical logistics, the study period was set to be a period of 3 weeks of riboflavin supplementation.

2.4. Riboflavin capsules

Patients received daily riboflavin supplementation of the normal diet for a period of 3 weeks. The riboflavin supplement consisted of 100 mg of riboflavin [Riboflavin Universal, CAS no. 83-88-5] per capsule [DSM Nutritional Products Ltd, Basel, Switzerland]. Additional information on the capsule is included in the Supplementary Methods.

2.5. Laboratory parameters, serum cytokines and plasma free thiols [R-SH, sulphhydryl groups], and faecal calprotectin

Routine blood analyses were performed before and after the intervention, including CRP, erythrocyte sedimentation rate [ESR], platelets, white blood cell count [WBC], haemoglobin, liver function tests, and creatinine. In addition, serum riboflavin level [flavine adenine dinucleotide] was measured before and after supplementation.

To determine a potential effect on systemic inflammation and redox status, serum cytokines and plasma free thiols were quantified, respectively. Serum levels of multiple cytokines, chemokines, and markers for angiogenesis and vascular injury were measured before and after the riboflavin intervention period, using the electrochemiluminescence [ECL] multiplex assay (Meso Scale Discovery [MSD ®]), as previously described.[20] The MSD V-plex Proinflammatory panel 1 [IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α], Cytokine panel 1 [GM-CSF, IL-5, IL-7, IL-12/23p40, IL-15, IL-16, IL-17A, and TNF-β], Chemokine panel 1 [Eotaxin-1, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, MCP-1, and MDC], Angiogenesis panel 1 [VEGF, VEGF-C, VEGF-D, Tie-2, Flt-1, PIGF, bFGF] and Vascular injury panel 1 [SAA, CRP, VCAM-1, and ICAM-1] were analysed to detect a total of 37 inflammatory molecules. Plasma free thiol groups were measured as previously described, with minor modifications [detailed in Supplementary Methods].[21,22] Final concentrations were corrected for plasma albumin levels, since albumin is the most abundant human plasma protein and is the predominant source of thiols.[23]

Faecal calprotectin levels were quantified by enzyme-linked immunosorbent assay [ELISA] [Bühlmann Laboratories AG, Switzerland] as a routine measurement in the UMCG.

2.6. Patient-reported outcome measures: Harvey Bradshaw Index [HBI] and Inflammatory Bowel Disease Questionnaire [IBD-Q]

To assess the effect of riboflavin on patient-reported outcome measures, the Harvey-Bradshaw Index [HBI] and the Inflammatory Bowel Disease Questionnaire [IBD-Q] were used as measures of clinical disease activity and quality of life, respectively. The HBI and IBD-Q were determined before and after the intervention with riboflavin.

The HBI consists of a short questionnaire used to give a clinical reflection of disease activity, based on a number of clinical parameters: general well-being, abdominal pain, number of liquid stools per day, abdominal mass, and a number of CD-associated extra-intestinal complications.[24] Generally, in clinical practice, an HBI score <5 is defined as clinical remission.

The IBD-Q distinguishes physical domains [i.e., bowel symptoms and systemic symptoms] and psychosocial domains [i.e., emotional function and social function] by means of 32 disease-related questions. Response scores theoretically range from 32 to 224 points, where higher values correspond to an improved health status.

2.7. Microbiome analyses

2.7.1. Fluorescence *in situ* hybridisation

The faecal microbiota were measured by fluorescence *in situ* hybridisation [FISH]. Using this method, the abundance of the following bacteria were quantified in absolute counts: total bacteria [EUB338, Rhodamine], *F. prausnitzii* [Fprau645, FITC], Enterobacteriaceae [Ec1531, CY3], and the *Clostridium coccoides-Eubacterium rectale* group [mostly Lachnospiraceae] [Erec482, FITC]. The FISH methodology is described in the Supplementary Methods.[25]

2.7.2. Whole genome metagenomic shotgun sequencing

The taxonomical and functional [i.e., metabolic pathway] composition of the faecal microbiome were also characterised in higher resolution, by means of whole genome metagenomic shotgun sequencing [MGS]. From the frozen faecal samples, the microbial DNA was extracted using the Qiagen Allprep DNA/RNA Mini Kit [cat #380204]. The metagenomic shotgun sequencing of the microbial DNA was executed at the Broad Institute of Harvard University and the Massachusetts Institute of Technology [MIT] in Cambridge, MA, USA, using the HiSeq platform. The Nextera XT Library preparation kit was used for genomic library preparation. To remove adapters and trim the ends of the metagenomic reads, Trimmomatic [v.0.32] was used.[12,26]

The cleaned metagenomic reads were processed using the previously published bioinformatics pipeline.[12] First, the software tool MetaPhlAn2 was used to profile the taxonomic compositions expressed in relative abundances of the microbiome samples.[27] Second, the composition of functional pathways expressed in relative abundances was determined using the software HUMAnN2 [v.0.4.0] [http://huttenhower.sph.harvard.edu/humann2] and the multi-organism database MetaCyc (MetaCyc. MetaCyc Metabolic Pathway Database; available at: https://metacyc.org]) [accessed January 1, 2017]. This resulted in the identification of 295 different taxa and 341 microbial pathways in the faecal microbiome samples.

The taxonomic diversity [α-diversity] within the faecal microbiome samples was estimated using the Shannon diversity index by means of the vegan package in R [version 2.4.-1].[28] The interindividual diversity [β-diversity] was calculated via Bray-Curtis distances between samples, and were represented in principal coordinate analyses [PCoAs]. To test the proportion of explained variance in the inter-individual distances [i.e. Bray-Curtis distances] per clinical characteristic, the ADONIS function in the vegan package was used. Significance was calculated using 1000 permutations and a cut-off at test false-discovery rate [FDR] <0.1. Lastly, specific taxa and functional pathways were compared between before and after the intervention, by means of a paired Wilcoxon signed-rank test, with *p*-values adjusted for multiple testing using the Benjamini-Hochberg method for FDR. An FDR <0.1 was considered as statistically significant. For a detailed description of the statistics and R code, see Supplementary Methods.

2.8. Statistics

For detailed description of statistical analyses, see Supplementary Methods.

3. Results

3.1. Baseline characteristics of the study population

Initially, 79 patients with CD were enrolled in the study, of whom nine were excluded because of the following reasons: patients who developed an infection during the study and were treated with antibiotics [$n = 2$]; patients who developed an unrelated medical condition before the riboflavin supplementation [$n = 2$]; and patients who withdrew for personal reasons during the study period [$n = 5$]. For the faecal metagenomic sequencing analyses, patients with CD were excluded if the quality of the gut metagenomes was deemed insufficient [read depth below 10 million reads or contamination with human reads] [$n = 6$]. Eventually, the total study population analysed in this study consisted of 70 patients with CD, among whom 40 patients had low FC levels [<200 µg/g] and 30 patients had elevated FC levels [>200 µg/g]. The baseline cohort demographic and clinical characteristics are presented in Table 1. Adherence to the riboflavin supplement was confirmed by a significant increase in serum levels of riboflavin for the complete CD study cohort [$p < 0.001$]; [Supplementary Table S1, available as Supplementary data at *ECCO-JCC* online]. In addition, energy intake [kcal] and macronutrients were quantified at baseline for all patients with CD using the FFQ [Supplementary Table S2, available as Supplementary data at *ECCO-JCC* online]. There was no significant difference in energy intake or macronutrient intake between patients with low and high FC. As expected, patients with CD with high FC levels had consistently higher CRP levels and an elevated ESR [$p < 0.001$ and $p < 0.01$, respectively]. No adverse events were observed in this study.

3.2. Riboflavin supplementation improves systemic redox status

The effect of riboflavin on systemic redox status was assessed by determining the concentrations of albumin-adjusted free thiols in plasma [Supplementary Table S3 available as Supplementary data at *ECCO-J CC* online]. In the total CD study cohort, the concentration of free thiols significantly increased after 3 weeks of supplementation [Figure 1]. The largest effect on free thiols was observed for patients with CD with elevated FC levels: mean concentrations were significantly elevated after the intervention period [$p = 0.033$]. For patients with low FC levels, no significant increase was observed. In line, we did not observe major differential effects of riboflavin on albumin-adjusted plasma free thiols for several important disease phenotypes, such as ileocaecal resection status and primary disease localisation [Supplementary Figure S1 available as Supplementary data at *ECCO-JCC* online].

3.3. Riboflavin decreases serum levels of cytokines and inflammatory parameters

To assess the effects of riboflavin supplementation on inflammatory status in CD, an array of selected serum cytokines was measured before [T0] and after 3 weeks of riboflavin supplementation [T3] (see Table 2 and Supplementary Tables S4–S6 [available as Supplementary data at *ECCO-JCC* online] for the complete list of all analysed serum cytokines for the CD cohort, the low FC [<200 µg/g] subgroup and the high FC [>200 µg/g] subgroup). Distributions of a selection of analysed serum cytokines are illustrated in Figure 2 [CRP, and IL-2].

In the total study population, concentrations of interleukin-2 [IL-2] significantly decreased after 3 weeks of riboflavin supplementation [$p = 0.004$]. In the subgroup analysis, patients with CD with low FC levels showed a significant decrease in serum IL-2 concentrations [$p = 0.010$], whereas patients with high FC levels showed no difference after supplementation [$p = 0.124$]. However in these patients, serum CRP concentrations, as measured by the ECL assay, significantly decreased after the riboflavin supplementation period [$p = 0.010$]. TNF-α also decreased in the group of patients with high FC levels [$p = 0.044$]; however, this significant finding was lost after correction for multiple testing. No significant differences in serum cytokine concentrations were observed after 3-week riboflavin supplementation for IL-1β, IL-4, IL-6, and IL-10 [Table 2]. Of the routinely measured laboratory parameters, CRP, ESR, and platelet counts significantly decreased after 3 weeks of riboflavin supplementation in the total CD study population [$p = 0.017$; $p = 0.034$; $p = 0.011$, respectively] [Supplementary Table S1]. Platelet count was also reduced in the subgroup of patients with CD with low FC levels [$p = 0.021$]. Levels of CRP, derived from the standard laboratory measurements, were significantly decreased by riboflavin supplementation in patients with high FC levels [$p = 0.009$], but not for the patients with low FC levels at baseline. No significant reduction in FC levels was observed after the period of riboflavin supplementation.

3.4. Riboflavin supplementation reduces CD symptoms [HBI]

Clinical disease activity was measured at baseline [T0] and after the 3-week period [T3] of riboflavin supplementation [Table 3]. The HBI slightly improved after supplementation [T3] in the total IBD study cohort, which was a statistically significant decrease [$p < 0.001$]. Also, in subgroups, patients with either low FC or high FC levels showed a significant improvement of the HBI [$p < 0.001$; $p = 0.007$, respectively].

3.5. Riboflavin improves IBD-related Quality of Life [QoL]

Subjective QoL was quantified by the validated IBD-Q questionnaire [Table 4]. In the total study population, we observed a significant increase in response scores for both physical domains, i.e.,

Table 1. Baseline demographic and clinical characteristics of the study population [*n* = 70] consisting of patients with CD with low and high faecal calprotectin [FC] levels.

Characteristics	Total	FC <200 µg/g	FC >200 µg/g	*p*-value
	***n* = 70**	***n* = 40**	***n* = 30**	
Age [years]	41.9 [12.7]	44.2 [11.6]	38.8 [13.6]	0.080
Female gender	48 [68.6]	29 [72.5]	19 [63.3]	0.446
BMI [kg/m^2]	25.1 [5.0]	25.1 [5.3]	25.0 [4.7]	0.923
Active smoking	13 [18.6]	7 [17.5]	6 [20.0]	1.000
Ileocaecal resection	28 [40.0]	19 [47.5]	9 [30.0]	0.217
Montreal, location				**0.037***
L1 [ileal disease]	28 [40.0]	21 [52.5]	7 [23.3]	
L2 [colonic disease]	11 [15.7]	6 [15.0]	5 [16.7]	
L3 [ileocolonic disease]	31 [44.3]	13 [32.5]	18 [60.0]	
Montreal, behaviour				0.826
B1 [non-stricturing, non-penetrating]	34 [48.6]	19 [47.5]	15 [50.0]	
B2 [stricturing]	27 [38.6]	15 [37.5]	12 [40.0]	
B3 [penetrating]	9 [12.9]	6 [15.0]	3 [10.0]	
HBI				0.857
Remission [<5]	49 [70.0]	29 [72.5]	20 [66.7]	
Mild disease [5–7]	13 [18.6]	7 [17.5]	6 [20.0]	
Moderate disease [8–12]	8 [11.4]	4 [10.0]	4 [13.3]	
IBD medication				0.484
None	20 [28.6]	14 [35.0]	6 [20.0]	
5-ASA	9 [12.9]	6 [15.0]	3 [10.0]	
Thiopurines	16 [22.9]	7 [17.5]	9 [30.0]	
Anti-TNF	18 [25.7]	10 [25.0]	8 [26.7]	
Thiopurine + Anti-TNF	7 [10.0]	3 [7.5]	4 [13.3]	
Laboratory parameters				
Haemoglobin [mmol/l]	8.6 [0.9]	8.6 [1.0]	8.5 [0.9]	0.792
CRP [mg/l]*	1.8 [0.6;4.6]	0.9 [0.5;2.7]	3.6 [1.5;8.0]	**0.001***
ESR [mm/h]*	13.0 [5.0;23.5]	11.0 [4.0;18.5]	20.0 [8.5;30.5]	**0.005***
WBC [x 10^9/l]	7.1 [2.1]	6.7 [2.1]	7.6 [2.0]	0.090
Platelets [x 10^9/l]	287 [76]	273 [80]	307 [67]	0.060
AST [U/l]	23.5 [7.3]	23.9 [6.1]	23.0 [8.8]	0.628
ALT [U/l]*	18.5 [14.0;26.0]	18.5 [14.3;27.3]	18.5 [13.5;26.0]	0.717
Creatinine [µmol/l]	72.7 [13.2]	73.2 [14.0]	72.0 [12.2]	0.717
Riboflavin [nmol/l]	324 [60]	308 [56]	342 [62]	0.121

Data are presented as numbers (proportions, *n* [%]), mean [SD] or *median (interquartile range [IQR]) in case of skewed variables. Differences between groups were tested with independent samples t tests or MannWhitney U tests for non-normally distributed continuous variables, and chi square test or Fisher's exact test for nominal variables, as appropriate. Two-sided *p*-values <0.05 were considered as statistically significant. Significances are indicated in **bold**.

FC, faecal calprotectin; IBD, inflammatory bowel disease; BMI, body mass index; HBI, Harvey-Bradshaw Index; 5-ASA, 5-aminosalicylic acid; TNF, tumour necrosis factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell count; AST, aspartate transaminase; ALT, alanine transaminase; SD, standard deviation.

bowel symptoms and systemic symptoms [p <0.01 and p <0.001, respectively]. A similar result was found for patients with CD with low FC levels [bowel symptoms p <0.01, systemic symptoms p <0.001]. However, no significant differences in self-reported IBD-related QoL were observed in patients with CD with high FC levels.

3.6. Riboflavin supplementation decreased Enterobacteriaceae in patients with CD with low FC levels as determined by FISH, though MGS analysis did not show effects on the faecal microbiome composition or metabolic profile.

3.6.1. Fluorescence *in situ* hybridisation shows a decrease in Enterobacteriaceae

Riboflavin supplementation was associated with a significant decrease in the relative abundance of potentially pathogenic Enterobacteriaceae [including *Escherichia coli*] in the patients with low FC levels, but it was not found to affect the number of *F. prausnitzii* in the total study cohort nor in any of the subgroups [Supplementary Tables S7 and S8 and Supplementary Figure S2, available as Supplementary data at *ECCO-JCC* online].

3.6.2. Riboflavin supplementation did not affect faecal short-chain fatty acids [SCFAs] concentrations

Moreover, riboflavin supplementation did not change faecal concentrations of the short-chain fatty acids [SCFAs] acetate, propionate, and butyrate [Supplementary Table S9 and Supplementary Figure S3, available as Supplementary data at *ECCO-JCC* online]. However, we did detect a positive correlation between the relative abundance of *F. prausnitzii* and the concentrations of butyrate in the baseline faecal samples. Relative abundances of Enterobacteriaceae showed a negative correlation with the concentration of butyrate at baseline [Supplementary Table S10, available as Supplementary data at *ECCO-JCC* online].

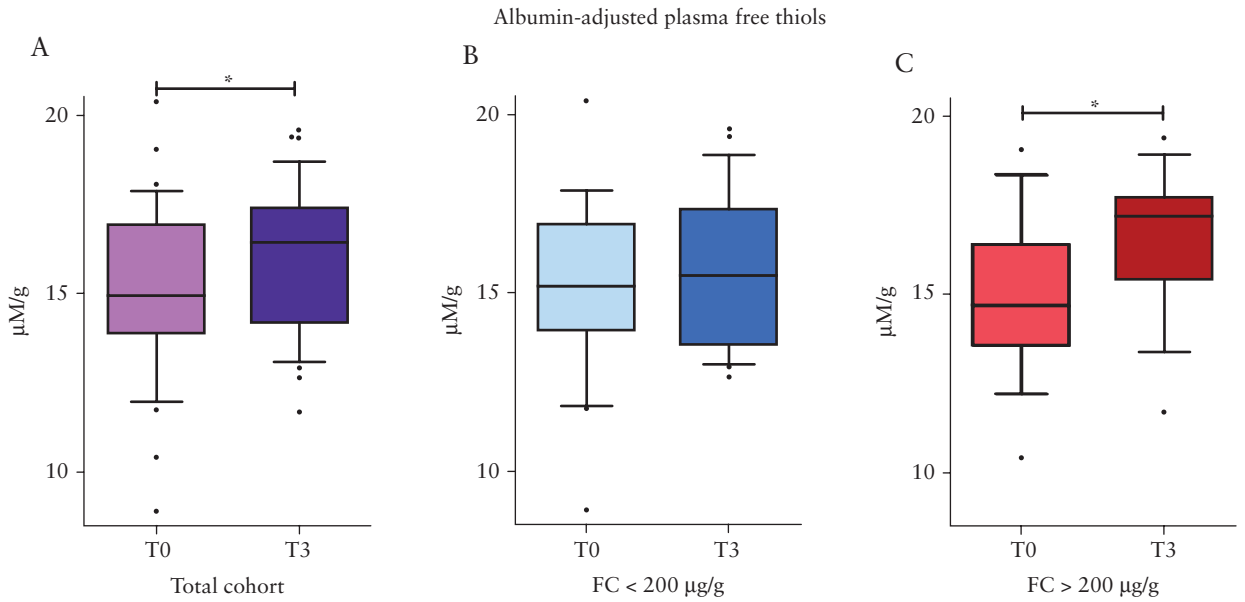


Figure 1. [A–C]. Riboflavin supplementation for 3 weeks [T0-T3] leads to an improved systemic redox status as reflected by increased plasma free thiol levels [adjusted for albumin, µM/g]. [A] Albumin-adjusted plasma free thiols significantly increase after 3 weeks of riboflavin supplementation, Total CD cohort [p <0.05]. [B] There is no significant change in albumin-adjusted plasma free thiol levels in patients with CD with low FC levels [<200 µg/g]. [C] Patients with CD with high FC levels [>200 µg/g] demonstrate significantly increased albumin-adjusted plasma free thiol levels [p <0.05]. *p <0.05 [two-sided]. CD, Crohn's disease; FC, faecal calprotectin.

Table 2. Effects of 3 weeks' riboflavin supplementation on biomarkers of inflammation: CRP, an array of pro-inflammatory cytokines and FC levels.

Total study population	**T0**	**T3**	***p*-value**
CRP	3.71 x 10^7 [9.30 x 10^6; 1.35 x 10^8]	3.20 x 10^7 [9.69 x 10^6 ; 9.32 x 10^7]	0.308
TNF-α	3.35 [2.61; 4.14]	3.03 [2.54; 3.53]	0.119
IL-2	0.18 [0.12; 0.24]	0.12 [0.07; 0.17]	**0.004***
IL-1β	0.05 [0.04; 0.13]	0.04 [0.02; 0.11]	0.189
IL-4	0.04 [0.02; 0.06]	0.03 [0.01; 0.05]	0.197
IL-6	1.00 [0.57; 1.64]	0.83 [0.52; 1.50]	0.336
IL-10	0.32 [0.22; 0.52]	0.38 [0.19; 0.45]	0.834
FC	155 [43; 479]	145 [46; 465]	0.337
FC <200 µg/g	**T0**	**T3**	***p*-value**
CRP	1.36 x 10^7 [5.45 x 10^6; 5.62 x 10^7]	1.01 x 10^7 [4.52 x 10^6; 7.50 x 10^7]	0.715
TNF-α	3.05 [2.46; 3.63]	2.84 [2.52; 3.31]	0.848
IL-2	0.18 [0.12; 0.23]	0.10 [0.07; 0.17]	**0.010***
IL-1β	0.05 [0.04; 0.08]	0.03 [0.01; 0.04]	0.056
IL-4	0.04 [0.02; 0.07]	0.03 [0.01; 0.05]	0.334
IL-6	0.77 [0.39; 1.06]	0.67 [0.36; 1.13]	0.520
IL-10	0.29 [0.19; 0.41]	0.28 [0.17; 0.45]	0.931
FC	55 [40; 128]	61 [40; 110]	0.846
FC >200 µg/g	**T0**	**T3**	***p*-value**
CRP	6.61x10^6 [3.66 x 10^6; 2.15 x 10^7]	5.47 x 10^6 [2.31 x 10^6; 1.55 x 10^7]	**0.010***
TNF-α	3.50 [2.83; 4.60]	3.31 [2.63; 4.00]	0.044
IL-2	0.18 [0.11; 0.26]	0.12 [0.09; 0.18]	0.124
IL-1β	0.06 [0.04; 0.13]	0.05 [0.03; 0.12]	0.955
IL-4	0.04 [0.03; 0.06]	0.03 [0.02; 0.05]	0.382
IL-6	1.32 [0.97; 1.78]	1.08 [0.71; 1.78]	0.157
IL-10	0.35 [0.25; 0.65]	0.38 [0.22; 0.58]	0.711
FC	515 [379; 1228]	505 [336; 1000]	0.191

All biomarkers are presented as median [interquartile range]. *p-values were calculated according to the Wilcoxon's signed rank test. Two-sided p-values <0.05 were considered as statistically significant. Significances [corrected for multiple comparisons] are indicated in **bold**.

CRP, C-reactive protein; FC, faecal calprotectin; TNF, tumour necrosis factor.

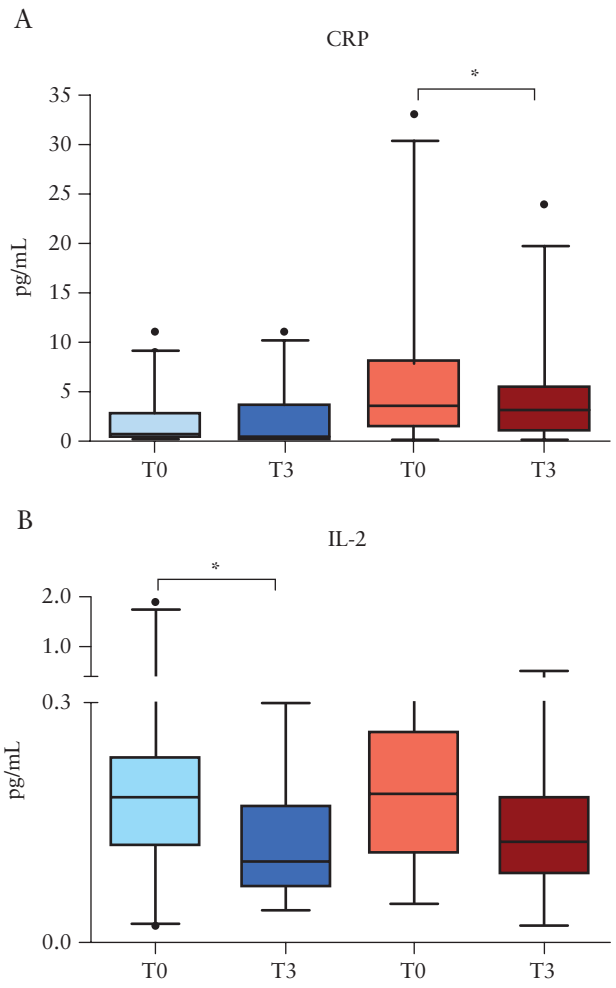


Figure 2. [A-B]. Serum levels of CRP and IL-2 significantly decrease within 3 weeks [T0-T3] of riboflavin supplementation. [A] Serum CRP levels [pg/mL] significantly decrease in patients with CD with high FC levels [>200 µg/g]. [B] Serum IL-2 levels [pg/mL] significantly decrease in patients with CD with low FC levels [<200 µg/g]. *p <0.05 [two-sided]. CRP, C-reactive protein; CD, Crohn's disease; FC, faecal calprotectin.

Table 3. Changes in Harvey-Bradshaw Index [HBI] after 3 weeks of riboflavin supplementation.

HBI	T0	T3	p-value
Total study population	3 [1; 5]	2 [1; 4]	**<0.001**
FC <200 µg/g	3 [1; 5]	2 [0; 4]	**<0.001**
FC > 00 µg/g	3 [2; 5]	2 [1; 4]	**0.007**

Response scores are presented as median [interquartile range] with corresponding p-values according to paired Wilcoxon's signed-rank test. Two-sided p-values <0.05 are considered statistically significant. Significances are indicated in **bold**.

3.6.3. Metagenomic shotgun sequencing shows that variance of microbiome is mainly determined as originating from one individual, rather than riboflavin supplementation

To further analyse possible modulating effects on the composition of the faecal microbiome, we next analysed the microbiome in higher resolution by metagenomic shotgun sequencing [MGS]. Here, the supplementation of riboflavin did not induce changes in the taxonomical diversity within the faecal samples of the patients with CD [total patients with CD: $p = 0.274$; low FC: $p = 0.491$; high FC: $p = 0.349$, Figure 3]. Also, when calculating the effect of riboflavin supplementation on the interindividual variance in the microbiome, riboflavin did not significantly affect the variance in the taxonomical composition [total CD cohort FDR: = 1.000; low FC levels FDR: = 0.910; high FC levels FDR: = 1.000, Figure 4], nor in the functional composition [total CD cohort FDR: = 1.000; low FC levels FDR: = 1.000; high FC levels FDR: = 1.000, Figure 5]. In contrast, when evaluating the effect of paired samples [i.e., samples originating from the same CD patient] on the interindividual variance, it showed that individual sample relatedness does affect the variance of the taxonomical composition significantly [total CD cohort FDR: = 0.002*; low FC levels FDR: = 0.002*; high FC levels FDR: = 0.002*, Figure 4], and of the functional composition [total CD cohort FDR: = 0.002*; low FC levels FDR: = 0.002*; high FC levels FDR: = 0.002*, Figure 5].

Riboflavin intake also did not induce changes in relative abundances of any species, nor in other taxonomical levels. In the patients with CD with low FC levels, the relative abundance of pathway-5189, encoding the biosynthesis of tetrapyrrole, was significantly decreased after the riboflavin supplementation [FDR = 0.06].

4. Discussion

In this study, subjective and objective CD disease parameters and the faecal microbiome were monitored in a cohort of 70 patients with CD, with and without elevated faecal calprotectin, during an intervention with oral riboflavin [vitamin B_2]. CD disease scores, circulating cytokines, systemic redox status, and the faecal microbiome constitution, were compared before and 3 weeks after riboflavin supplementation. Here, we show that riboflavin supplementation has a mixed effect on systemic biomarkers of inflammation, with a decrease of CRP, ESR, platelets, and IL-2, but in the absence of effects on the remaining biomarkers that were measured in this study. Moreover, a significant antioxidant effect was observed, as reflected by an increase in the concentration of plasma free thiols. In addition, clinical symptoms were reduced, as quantified by a reduction in the clinical disease activity index [HBI] and an improvement in the QoL. However, these clinical effects should be interpreted with caution as the present study was not placebo-controlled. As determined by FISH, there was a small decrease in Enterobacteriaceae in patients with low FC levels; however, when the microbiome was characterised by MGS, no significant alterations were observed in the faecal microbiota diversity, taxonomy, or metabolic pathway constitution, indicating that the observed anti-inflammatory effects of riboflavin supplementation might not be mediated through the faecal microbiome. In line, we did not observe major differential effects of riboflavin on study outcome parameters for several important disease phenotypes, such as ileocaecal resection status and primary disease localisation.

4.1. Effects of riboflavin on patient-reported outcomes

This study showed that clinical symptoms decrease after riboflavin supplementation, as quantified by a reduction in the clinical disease activity index [HBI] in all CD subgroups, and an improvement in the QoL as reflected by the IBD-Q in the total and low FC patient groups. However, the current study was not double-blind and placebo-controlled, which would have helped us to better understand the effects of riboflavin supplementation on patient-reported outcomes of disease activity [i.e. HBI scores and IBD-Q questionnaire results]. Despite this, we were able to demonstrate multiple effects of riboflavin on more

Table 4. Changes in quality of life [QoL] of Crohn's disease [CD] patients as measured by the Inflammatory Bowel Disease Questionnaire [IBD-Q] before [T0] and after riboflavin supplementation [T3].

Total study population	**T0**	**T3**	***p*-value**
Total score IBD-Q	173 [152; 193]	177 [160; 196]	**0.001**
Bowel symptoms	55 [48; 61]	58 [49; 64]	**0.002**
Systemic symptoms	22 [19; 27]	24 [19; 29]	**<0.001**
Emotional function	67 [60; 73]	66 [60; 74]	0.257
Social function	32 [26; 35]	32 [26; 34]	0.631
FC <200 µg/g	**T0**	**T3**	***p*-value**
Total score IBD-Q	175 [157; 200]	178 [162; 205]	**0.001**
Bowel symptoms	56 [48; 63]	59 [51; 66]	**0.005**
Systemic symptoms	22 [19; 27]	24 [19; 30]	**<0.001**
Emotional function	68 [61; 73]	67 [61; 82]	0.059
Social function	32 [27; 35]	33 [27; 35]	0.195
FC >200 µg/g	**T0**	**T3**	***p*-value**
Total score IBD-Q	171 [145; 188]	177 [143; 187]	0.253
Bowel symptoms	53 [47; 60]	57 [48; 61]	0.163
Systemic symptoms	22 [19; 28]	24 [18; 28]	0.127
Emotional function	63 [57; 70]	64 [57; 71]	0.714
Social function	30 [26; 33]	29 [23; 34]	0.361

Response scores are presented as median [interquartile range] with corresponding *p*-values according to paired Wilcoxon's signed-rank test. Two-sided *p*-values <0.05 are considered statistically significant. Significances are indicated in **bold**.

FC, faecal calprotectin.

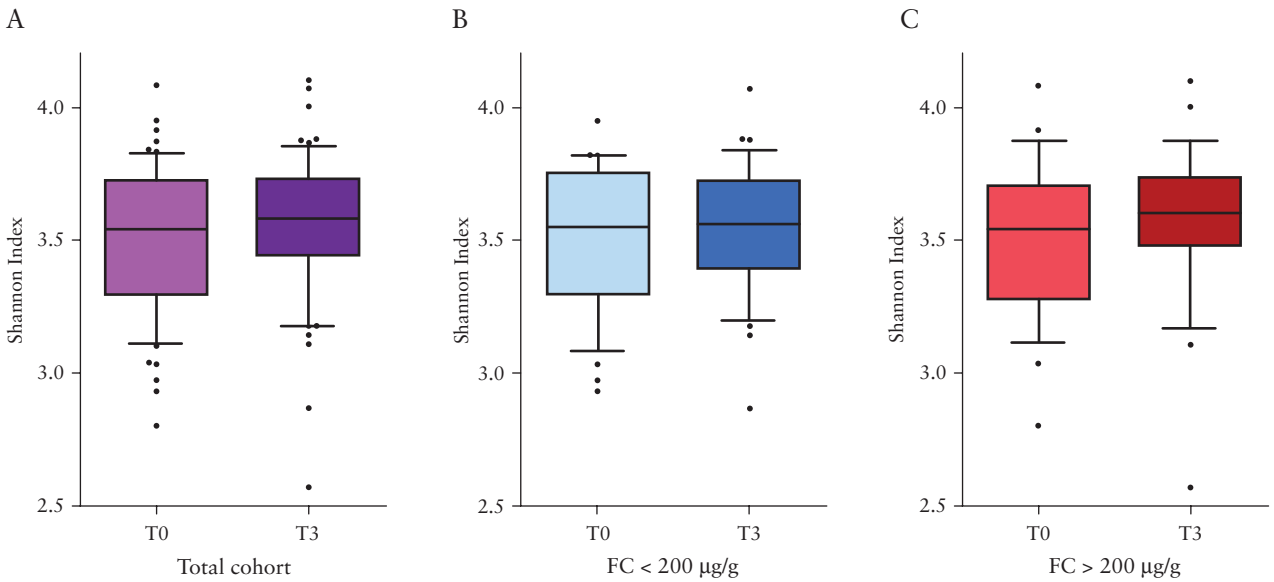


Figure 3. [A–C]. Boxplots representing the α-diversities at baseline [T0] and 3 weeks [T3] after intake of riboflavin, between patients with CD. [A]Total CD cohort. [B] Low FC group. [C] High FC group [*p* >0.05]. CD, Crohn's disease; FC, faecal calprotectin.

objective disease parameters, such as several biochemical markers for disease activity [i.e., serum cytokines and plasma free thiols].

4.2. Effects of riboflavin on biochemical markers of disease activity

4.2.1. Serum cytokine levels

In the total group of patients with CD, we observed a reduction in serum concentrations of the pro-inflammatory cytokine IL-2 after 3 weeks of riboflavin supplementation. This decrease in serum IL-2 levels was also observed in the CD subgroup with low FC levels at baseline. In addition, in the patients with CD with high FC levels at baseline, CRP was also shown to be decreased after 3 weeks of riboflavin supplementation. In a previous study, it was shown that IL-2 can only be secreted extracellularly after it has undergone oxidative folding [disulphide formation] in the endoplasmic reticulum, which is dependent on cellular flavin.[29] Although it is difficult to determine whether the observed alterations in cytokine concentrations originated from riboflavin supplementation, a previous study that profiled circulating cytokines in patients with CD, who were under maintenance therapy with infliximab, observed stability in pro-inflammatory cytokine concentrations over a course of 6 weeks, possibly indicating that our observed results might indeed be induced by the riboflavin supplementation.[30] Collectively, this

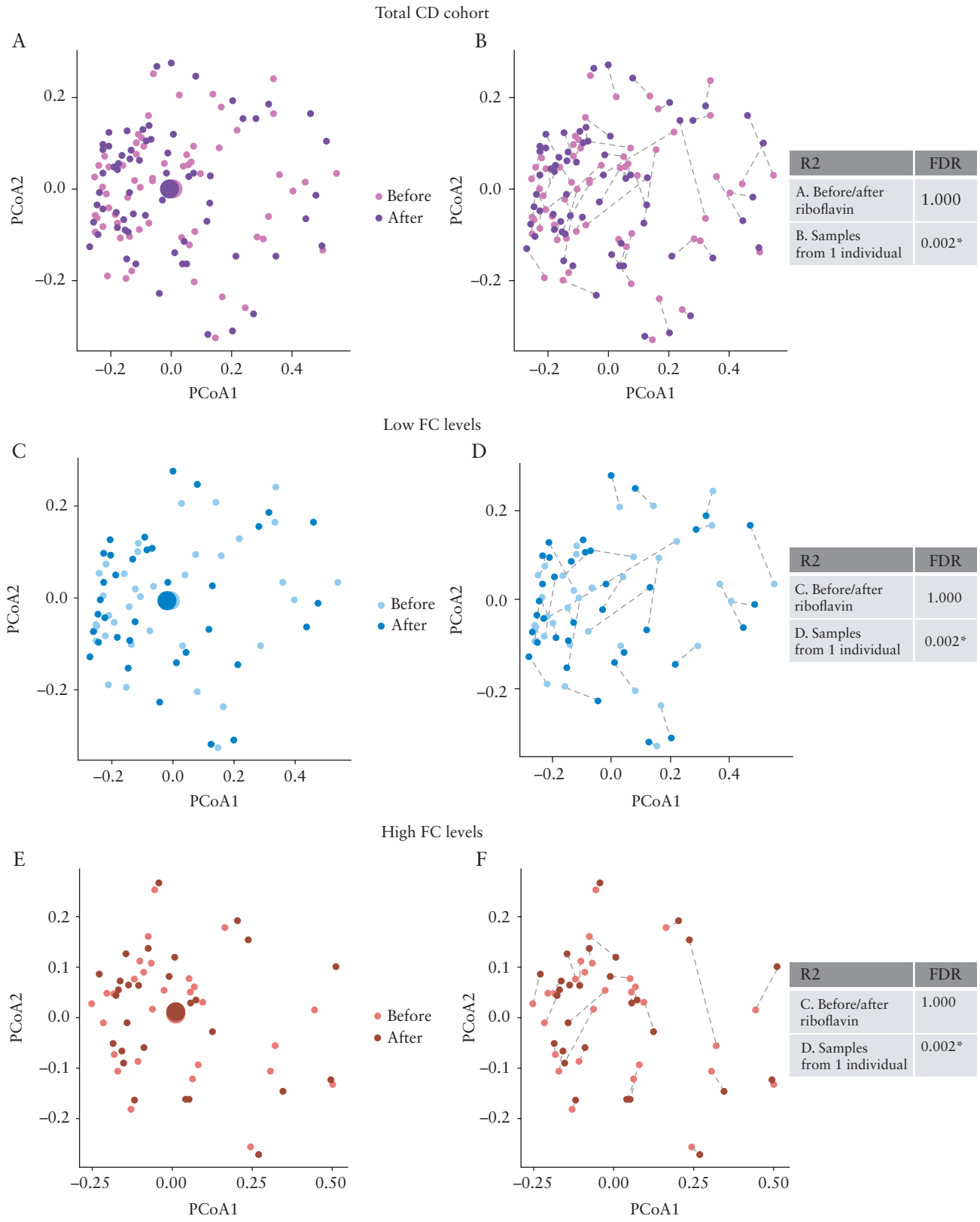
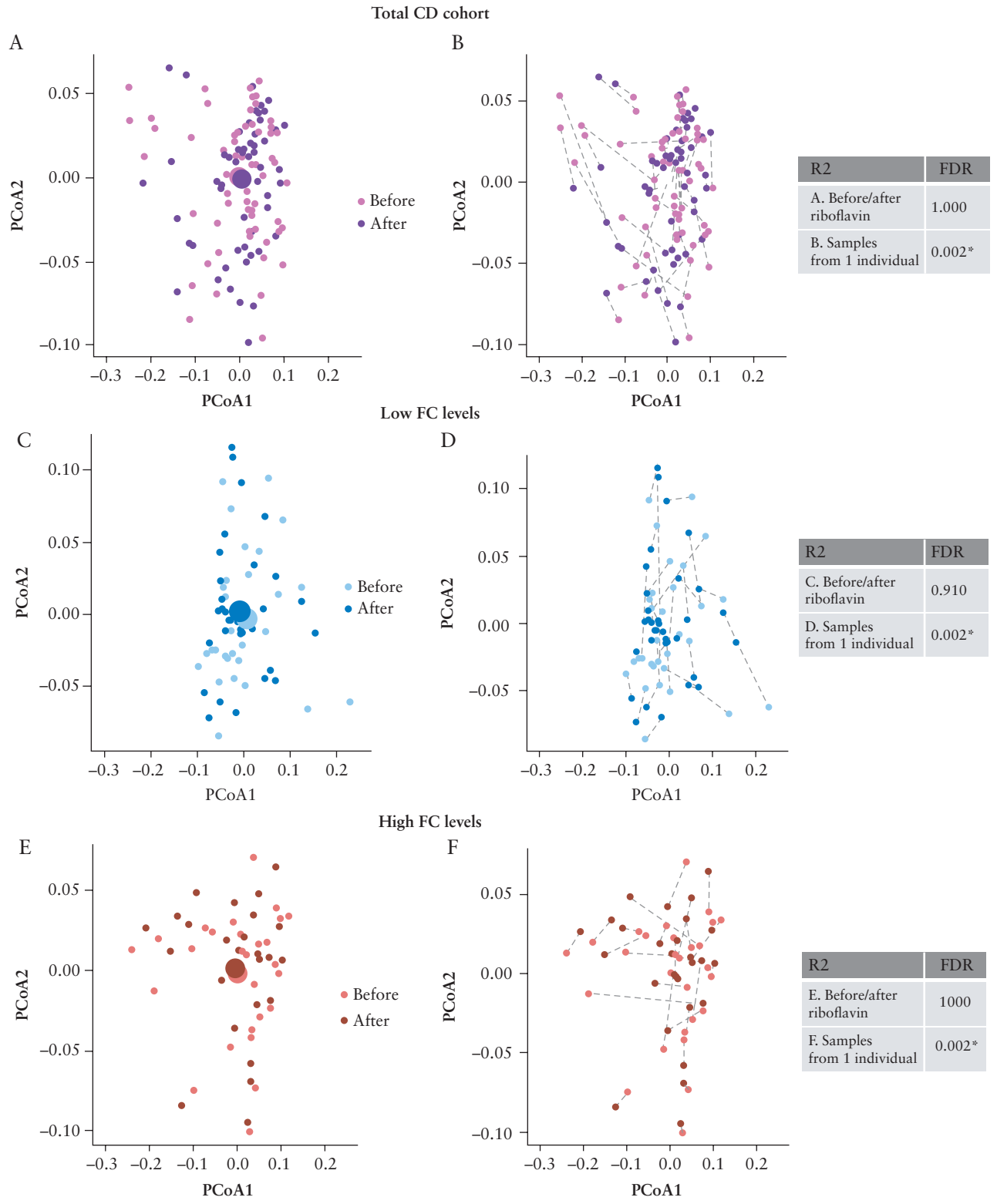


Figure 4. [A–F]. Principal coordinate analyses [PCoAs] of Bray-Curtis distances on species composition, calculated between T0 [before riboflavin] and T3 [3 weeks after riboflavin], on [A–B] the total patients, [C–D] the patients with a low baseline FC, and [E–F] patients with a high baseline FC. Each dot represents a patient with CD, with the lighter shade representing T0 and the darker shade representing T3. The dashed lines indicates that the faecal samples originate from the same CD individual [PCoA1: $p > 0.05$, PCoA2: $p > 0.05$]. CD, Crohn's disease; FC, faecal calprotectin.



R2	FDR
A. Before/after riboflavin	1.000
B. Samples from 1 individual	0.002*

R2	FDR
C. Before/after riboflavin	0.910
D. Samples from 1 individual	0.002*

R2	FDR
E. Before/after riboflavin	1000
F. Samples from 1 individual	0.002*

Figure 5. [A–F]. Principal coordinate analyses [PCoAs] of Bray-Curtis distances on predicted functional composition, calculated between T0 [before riboflavin] and T3 [3 weeks after riboflavin], on [A–B] the total patients, [C–D] the patients with a low baseline FC, and [E–F] patients with a high baseline FC. Each dot represents a patient with CD, with the lighter shade representing T0 and the darker shade representing T3. The dashed lines indicates that the faecal samples originate from the same CD individual [PCoA1: $p > 0.05$, PCoA2: $p > 0.05$]. CD, Crohn's disease; FC, faecal calprotectin.

might indicate that the observed effects on the analysed inflammatory markers may be ascribed to the anti-inflammatory potential of riboflavin, thereby reducing the inflammatory burden in CD.

4.2.2. Plasma free thiols

In the total group of patients with CD and in the patients with high FC at baseline, we observed an increase in plasma free thiols after 3 weeks of riboflavin supplementation, which is reflective of a reduction in systemic oxidative stress. In human metabolism, riboflavin is particularly known for its antioxidant properties, and has been documented to reduce ischaemic/reperfusion injury and lipid peroxidation, as well as to increase antioxidant enzyme activity, such as that of superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase, in animal models.[31,32] Also, in the pathogenesis of CD, it has been implied that oxidative stress plays an important role.[33–36] For a long time, plasma free thiols have been proposed as a measure of systemic redox status in various inflammatory conditions, but the value of this biomarker in CD has only recently been acknowledged.[37–39] In CD, it has been demonstrated that plasma free thiols are significantly decreased as compared with healthy individuals. Furthermore, there is considerable evidence that flavins lead to increased extracellular reducing capacity.[40,41] This indicates that increasing these levels of thiols, possibly by riboflavin, might alleviate oxidative stress and CD-related symptoms.

4.3. Potential mechanism of riboflavin effects—mediation via the faecal microbiome?

Our primary hypothesis was that riboflavin would increase the abundance of *F. prausnitzii* in the gut of patients with CD. Earlier, we showed that riboflavin [vitamin B_2] acts as redox mediator in the extracellular electron shuttling to oxygen of this bacterium, enabling its growth and survival at the aerobic-anaerobic interphase of the human gut.[40,42,43] Importantly, in a pilot study with healthy individuals, an increase in the relative abundance of *F. prausnitzii* was observed after a 2-week period of riboflavin supplementation.[11] However, in the present study, the results from MGS of the faecal microbiome led us to reject our hypothesis in patients with CD, since after a 3-week period of riboflavin supplementation, no alterations in either the microbiota diversity or in specific taxa, including *F. prausnitzii*, were observed. Only one gene encoding a single pathway involved in the biosynthesis of tetrapyrroles was decreased after 3 weeks of riboflavin, in patients with low FC levels at baseline. These results are in contrast with the aforementioned pilot study and the FISH analysis we performed on the faecal samples. In the FISH analysis, no effect on *F. prausnitzii* abundance was observed, though it did lead to a significant decrease in the number of potentially pathogenic Enterobacteriaceae [e.g., *Escherichia coli*] bacteria in the subgroup of patients with CD with low FC levels. We believe that the discrepancy in results between FISH and MGS methods might have multiple origins. For example, it might be that the current study was severely underpowered regarding the metagenomic sequencing, provided that study power was sufficient for the FISH analysis. Furthermore, we speculate that faecal sample heterogeneity may have significantly influenced the differences in results from both analytical tools, due to possible interindividual differences in sample collection, faecal consistency, sample storage, efficiency of DNA extraction, and possible noise in the applied bioinformatics tools regarding MGS analysis. Another important aspect regarding the FISH probe used for Enterobacteriaceae is its limited target specificity, since not all members of Enterobacteriaceae are measured.[44]

4.4. Strengths and limitations

The results of this study are important for several reasons. Currently, there are insufficient data to provide evidence-based dietary advice to patients with CD.[45–49] Previously, only a limited number of studies have evaluated the effect of a nutritional intervention [i.e., supplementation of pre- or probiotics] in CD. For example, the effect of the prebiotic fructo-oligosaccharides in CD was previously studied in a placebo-controlled trial, but in this study no clinical benefit was observed in patients with CD.[50] Moreover, the effect of oligofructose-enriched inulin [OF-IN] was evaluated on patients with CD in an double-blind, placebo-controlled study, in which a beneficial modulation of the gut microbiota was demonstrated.[51] Similarly, there are limited studies evaluating the effect of a vitamin intervention in CD. In an interesting randomised controlled study, the effect of a combination of vitamin E and vitamin C was assessed. In this study, a significant reducing effect was observed on oxidative stress indices.[52] More recently in a small study, a short vitamin D supplementation period resulted in an increase in the abundance of potential beneficial microbiota strains.[53] The present prospective study is the first clinical study to comprehensively investigate the effect of a riboflavin supplement in a well-described cohort of patients with CD. We have assessed the effect of riboflavin on different parameters, such as microbiota composition, biomarkers of inflammation and oxidative stress, and validated questionnaires of disease severity and quality of life.

One of the limitations of this prospective proof-of-concept study concerns our definition of CD disease activity. Unfortunately, there were no sufficient endoscopic data available for this cohort, which are preferentially used as a gold-standard measure of inflammatory disease activity. Instead, we used faecal calprotectin levels as an indirect, though reliable, surrogate marker for disease activity, and divided our patient cohort into subgroups of either quiescent or active disease, based on a faecal calprotectin cut-off level of 200 µg/g. The exact cut-off levels of FC presented in the literature are quite arbitrary. In our university hospital, a level <60 µg/g is considered indicative of no inflammatory activity, and a level >200 µg/g suggests mucosal inflammation. For completeness, we also separated groups in a more stringent manner: FC <60 µg/g vs >200 µg/g [high FC], and repeated our analysis. However, this did not affect our results and main conclusions.

4.5. Conclusions

In conclusion, this prospective clinical study demonstrates that riboflavin supplementation of the diet in patients with CD for 3 weeks results in mixed anti-inflammatory effects and reduced systemic oxidative stress and clinical symptoms [HBI]. Furthermore, riboflavin supplementation led to decreased Enterobacteriaceae abundance in patients with CD with low FC levels as determined by FISH, though MGS analysis did not show evident changes in the faecal microbiome. Our data demonstrate that riboflavin supplementation has a number of anti-inflammatory and anti-oxidant effects in CD.

Funding

This work was supported by the Top Institute of Food and Nutrition [TIFN] in Wageningen and the Center for Development & Innovation of the University Medical Center Groningen [UMCG] [no grant number, to JZHvM] and by the Junior Scientific Masterclass [JSM] of the University of Groningen [grant number: 17–57 to ARB]. The present study is partly financially supported by DSM Nutritional Products.

Conflict of Interest

RKW: unrestricted research grants from Takeda and Ferring Pharmaceutical Company. GD: unrestricted grants from Abbvie, Takeda, and Ferring Pharmaceuticals, advisory boards for Mundipharma and Pharmacosmos, and received speaker´s fees from Takeda, Pfizer, and Janssen Pharmaceuticals. It was not possible to obtain a conflict of interest statement from HAAA. All other authors have no conflict of interest to declare.

Acknowledgments

We would like to thank research coordinator Wilma Westerhuis-van der Tuuk [Department of Gastroenterology and Hepatology] for her contribution to the study [logistics of faeces samples collection].

Author's contributions

GD, HJMH, and JZHvM designed the study. GD and JZHvM acquired ethical approval. HMvD, MCV, EAMF, RKW, and GD identified eligible patients on the outpatient clinic. ARB, GD, BHJ, MAYK, and JZHvM collected clinical data and study material. HAAA, MSS, and HJMH performed the FISH analysis. MLCB performed the plasma free thiols measurement. ARB, MAYK, HAAA, AVV, RG, RKW, PdV, HvG, KNF, HJMH, GD, and JZHvM performed data curation and data analysis. ARB, JZHvM, and MAYK wrote the first draft of the manuscript. All authors contributed to results interpretation and critically reviewed the manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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