Fecal markers in Multiple Sclerosis Sex makes the difference

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Research article

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Abstract

Background. Multiple Sclerosis (MS) is primarily considered as a neuro-inflammatory CNS disease. Yet, experimental data suggest a role for gut microbiota and microbial products like short chain fatty acids (SCFA) in the pathogenesis of MS. Very recently a high-ranked publication reported beneficial effects of propionate, a SCFA, in MS patients. Based on experimental and preliminary human data, we hypothesized that not only the gut microbiota but also microbial products and fecal inflammatory markers might be altered in MS.

Methods. In a pilot study, we investigated fecal markers (short chain fatty acids, calprotectin) as well as clinical markers in patients with relapsing-remitting MS (RRMS) under different therapeutic regimes and compared the results to age-matched control subjects.

Results. We observed a non-significant reduction in fecal SCFA in RRMS patients compared to control subjects. Fecal calprotectin concentrations did not differ significantly between MS patients and control subjects. We observed a significant reduction in fecal SCFA concentrations in women compared to men.

Conclusions. We conclude that the observed sex-associated difference in fecal SCFA concentrations might be a contributing factor in the pathogenesis of MS, especially when taking into account the female predominance in MS. We suggest investigating the role of SCFA in MS in a longitudinal study (starting in drug-naïve patients) in larger cohorts of MS patients with defined therapeutic regimes. Such a study would allow to distinguish between drug effects and disease-immanent effects and might help to identify a potentially modifiable sex-associated contributing factor in MS.

Trial registration. Registered by the local Ethics Committee (Reg.Nr. 81/18, Ethikkommission der Aerztekammer des Saarlandes, Saarbruecken, Germany).

Background

Multiple Sclerosis (MS) is a B and T cell mediated neuroinflammatory disease. Autoreactive Th1 and Th17 $CD4^+$ T helper cells are found alongside reduced regulatory T cells (Tregs), which leads to a proinflammatory setting (1, 2). Experimental autoimmune encephalomyelitis (EAE) mouse model is the most widely used animal model of MS. EAE onset has been shown to be linked to microbial stimuli: colonization with commensal bacteria of formerly germ-free mice led to immediate EAE development (3) while mice that were kept under germ-free condition did not develop EAE.

Bacterial products, such as short-chain fatty acids (SCFA), show beneficial effects by counteracting demyelination (4). SCFA include acetate (C2), propionate (C3), butyrate and isobutyrate (both C4) as well as valerate/pentanoate and isovalerate (both C5). Among these, acetate, propionate and butyrate are most abundant in the gut. SCFA are produced by gut microbiota by fermentation of dietary fibers (DF) in the intestinal lumen. Acetate and propionate derive predominantly from members of the phylum *Bacteroidetes* (such as *Prevotellaceae*) while butyrate origins only from a few members of the *Firmicutes*

phylum (such as *Faecalibacterium*). Branched SCFA such as isobutyrate and isovalerate derive exclusively from protein and amino acid fermentation (5). Valerate occurs in lesser concentrations compared to the more abundant acetate, propionate and butyrate and is considered to derive from different dietary components (6).

Very recently, a high-ranked publication reported beneficial effects for propionate as add-on therapy in drug-naïve MS patients (7).

In mouse models, SCFA have been shown to possess pro- and anti-inflammatory effects (8, 9). SCFA can cross the intestinal epithelium and the blood brain barrier (BBB) (9, 2). SCFA exert various effects on immune cells: e.g. SCFA modulate inflammatory pathways such as the NF-kB pathway by G-protein coupled receptors (GPR) and exert epigenetic effects in T lymphocytes by inhibiting histone deacetylase activity (HDAC) (10) leading to higher levels of Tregs (11). In turn, Tregs suppress overly active T-cell mediated immune responses (such as autoreactivity). They are involved in maintaining the BBB as well as regulating activity of central nervous system (CNS) microglia (quoted after (12)). Valerate has been shown to strongly increase IL-10 levels in T cells and in regulatory B cells (Bregs); a strong immunosuppressive mediator (6).

Furthermore, SCFA have been shown to possess beneficial effects on EAE pathology. Suppression of demyelination and enhancement of remyelination has been shown for butyrate in a mouse model (4). The same study found a positive effect on oligodendrocyte differentiation by butyrate. Different studies found significant amelioration of EAE due to valerate and propionate (6, 13).

In analogy, very recently, a high-ranked publication reported beneficial effects for propionate as add-on therapy in drug-naïve MS patients (7) via enhanced Treg-differentiation.

Hence, SCFA, which derive from the microbiota, are likely to be relevant for MS pathology. While there are a number of animal studies, only few studies focused on microbiota and microbial products in MS patients. Nevertheless, an altered gut microbiota composition and reduced concentrations of SCFA are discussed as potential triggers of MS (14). MS patients are known to have significant gut microbiota composition aberrations in comparison to healthy controls (15–17). A decrease in fecal SCFA concentrations was described in a Chinese cohort of MS patients (18). The same study found a correlation between increased *Streptococcus* abundance, Th17 cells and an inverse correlation to Tregs. The Treg frequency directly correlated with fecal SCFA concentrations. Similar findings were made in an US cohort: patients suffering from a secondary progressive MS (SPMS) showed SCFA blood concentrations which were 50–65% reduced in comparison to healthy controls (19).

Calprotectin (a protein derived from leukocytes that migrate in the gut lumen) is most reflects intestinal inflammation sensitively and can be quantitatively analyzed in the feces (20). Elevated fecal calprotectin concentrations have not only been described in inflammatory bowel diseases, but also in neurological disorders such as Parkinson's disease (21, 22). The aim of this pilot study was to expand the knowledge of SCFA production in RRMS patients in a German cohort. Considering the potential effects of SCFA on

the (intestinal) immune system this study also investigated fecal calprotectin as a marker of intestinal inflammation in RRMS patients receiving different immunomodulatory or immune therapies.

Methods

76 subjects were assessed between 2018 and 2019 in the neurological departments of Gesundheitszentrum Glantal and Saarland University Hospital, Homburg/Saar, Germany. Inclusion criteria were majority (18–80 years), diagnosis of a relapsing-remitting MS (RRMS) according to McDonald's criteria (2017), capability to give written informed consent. Healthy controls underlay the same inclusion criteria except for MS diagnosis. Exclusion criteria were pregnancy, contractually incapable persons, uncontrolled psychiatric diseases, coexistent neurodegenerative or intestinal bowel disease (acute or chronic), coexistent infection within the past four weeks and intake of antibiotics within the past eight weeks. The same exclusion criteria plus absence of MS and other autoimmune diseases applied to controls.

The enrolled RRMS patients received betaferones, glatirameracetate, teriflunomid, dimethylfumarate, fingolimod or natalizumab. Betaferones, glatirameracetate, teriflunomid and dimethylfumarate were hereinafter subsumed under basic therapy, natalizumab and fingolimod are considered as escalation therapy.

All enrolled subjects underwent medical history, clinical examination including expanded disability status scale (EDSS), Constipation Scoring System (CSS (23)) and neuropsychological examination with Mini-mental status test (MMST), fatigue impact scale (FIS-d; German version) and Beck's depression inventory (BDI). All subjects were provided with a stool sample kit with instructions to collect fecal samples at home as previously reported (24).

Data was processed with IBM SPSS 24®. Normality was tested with Shapiro Wilk test (SW). Since none of our metric data was distributed normally, data are reported as median (X, range Y-Z) with minimum and maximum. Mann-Whitney-U-Test (MWU) and Kruskal-Wallis-test (KW) were used to compare differences between groups. Pearson's correlation coefficient was used to examine metric variables for correlations, Spearman's correlation coefficient was used to test correlation between metric and ordinal scaled variables. Eta correlation coefficient was used to test correlation between metric and nominal variables. Statistical significance is assumed for $p < 0.05$.

Fecal SCFA and calprotectin were quantitatively analyzed as described previously (21, 24).

Cohort

76 subjects were enrolled in this study: 41 patients with RRMS, 35 healthy controls. There was a female predominance of 1,2:1 (2,4:1 in the MS group, 0,6:1 in the healthy controls). Age ranged from 22 to 72 years with a median of 48 years. Median age was equal in patients (48 years, from 22 to 68 years) and controls (48 years, from 23 to 72 years). 18 RRMS patients had a mild/moderately active MS, 23 RRMS

patients had an active/highly active MS. Median EDSS was 2.5 with a range from zero to 7.0 points. For further information regarding disease activity, medication etc. see Supplementary Table 1.

Results

6.1 EDSS, CSS, CRP

EDSS was significantly lower in the group with mild/moderate MS (median: 1.5, range 0.0 to 4.5) compared to the active/highly active MS (median: 3, range 1.0-7.0) (p=0.004). There was no sex difference with regard to EDSS scores. Median CSS was 1 (range 0-17). Relevant constipation was assumed for a cut-off value of 15 points. There was no difference between men and women with regard to CSS scores. MS patients showed significantly higher scores in the CSS (median 2, range 0-17) compared to controls (median 0, range 0-16) (p=0.006). Patients with active/highly active MS (median 4, 0-17) showed significantly higher CSS scores compared to patients with mild/moderate disease activity (median 0, 0-17) (p <0.001).

Table 1 Constipation scores of RRMS patients sorted after applied therapy. Fingolimod and Natalizumab as escalation therapy were associated with significantly higher constipation scores (p=0.001).

Therapy	N	Median	Minimum	Maximum
no therapy	3	0.0	0	8
betaferones	5	1.0	0	9
glatirameracetate	6	0.0	0	1
teriflunomide	1	00	0	0
dimethylfumarate	6	1.0	0	6
fingolimod	**6**	**4.5**	**0**	**17**
natalizumab	**14**	**4.5**	**0**	**14**
healthy control	35	0.0	0	16

In order to screen for systemic inflammation that might be a confounder for the investigated fecal markers, we analyzed CRP concentrations in blood. As CRP concentrations were not part of the initial study protocol, date were not available for all of the enrolled subjects (CRP concentrations were available for 27/41 RRMS patients and 29/35 control subjects). None of the subjects included in this study showed a clinically relevant increased CRP concentration. CRP was slightly higher in MS patients (median: 1.3mg/l, range 1,0-9.6 mg/l) than in healthy controls (median: 1.0mg/l, range 1.0-14.0 mg/l) (p=0.926).

We observed no sex-associated difference. There was no difference in CRP concentrations between RRMS patients under basic and RRMS patients under escalation therapy.

Group comparison of MMST, FIS and BDI did not reveal relevant or new aspects.

6.2 Calprotectin

Fecal calprotectin levels were equally distributed between RRMS patients (median: 19 µg/g, range 19-141 µg/g) and healthy controls (median: 19µg/g, range 19-328µg/g).

There was no difference for fecal calprotectin concentrations between men and women. Median fecal calprotectin concentrations were descriptively higher in patients with RRMS without immunotherapy (median 39 µg/g, range 19-58 µg/g) in comparison to those who received a therapy (median 19 µg/g, 19-141 µg/g) and healthy controls (median 19µg/g, range 19-328 µg/g) (p=0.220, respectively p=0.198). There was no difference in fecal calprotectin concentrations between basic and escalation therapy or between different drugs.

6.3 Short-chain fatty acids

There was no statistical difference between fecal SCFA concentration between RRMS patients and controls (Table 5). Descriptively, median fecal butyrate concentration was reduced by 77% in RRMS patients in comparison to controls (p=0.219).

Median fecal acetate concentration was also descriptively reduced by 71.7% in RRMS patients with active/highly active MS in comparison to RRMS patients with mild/moderate MS (p=0.554).

RRMS patients with basic therapy had similar fecal SCFA concentrations as healthy controls while patients with escalation therapy and RRMS patients without therapy had both descriptively reduced median fecal SCFA concentrations (Table 6).

There was a significant sex-related difference in all fecal SCFA concentrations except for the branched-chain SCFA isovalerate and isobutyrate: men had significantly higher SCFA concentrations than women (Tab. 2, Fig. 2).

Table 2 Median fecal SCFA concentrations (in mmol/g) in men and women and significance of sex specific difference

Sex		Acetate	Propionate	Butyrate	IsoButyrate	Valerate	IsoValerate
Female	N	42	42	42	42	42	42
	Median	**6.47**	**1.44**	**1.37**	**0.88**	**0.53**	**0.55**
	Minimum	0.07	0.10	0.02	0.00	0.00	0.01
	Maximum	160.26	43.07	41.49	6.13	5.64	5.49
Male	N	34	34	34	34	34	34
	Median	**68.89**	**19.40**	**15.63**	**2.10**	**1.58**	**1.94**
	Minimum	0.67	0.22	0.05	0.02	0.01	0.02
	Maximum	193.06	99.47	52.54	11.35	19.76	17.03
Sig. (p) in MWU		**0.012**	**0.002**	**0.003**	**0.061**	**0.004**	**0.068**

When analyzed separately for RRMS patients and healthy controls, there was only a trend for female RRMS patients towards lower fecal SCFA concentrations whereas the control group showed a significant sex-related difference for acetate, propionate and butyrate (Fig. 3).

Table 3 Significance for sex related difference within controls

SCFA	acetate	propionate	butyrate	isobutyrate	valerate	isovalerate
p	0.006	0.005	0.017	0.232	0.068	0.322

Even though none of the enrolled subjects showed a clinically relevant elevation of CRP concentrations, CRP concentrations were positively correlated with calprotectin concentrations (corr. 0.304, p=0.023 Pearson).

The fecal concentrations of all investigated SCFA showed a statistically significant correlation with the age.

Table 4 Pearson correlation of age and fecal SCFA concentration and significance

SCFA	acetate	propionate	butyrate	isobutyrate	valerate	isovalerate
Pearson Correlation	0.335**	0.268*	0.335**	0.279*	0.316**	0.275*
sig. (2-tailed)	0.003	0.019	0.003	0.015	0.005	0.016

Discussion

7.1 Discussion of results

Animal models suggest that fecal SCFA (and gut microbiota) might play a role in the pathogenesis of MS. Hitherto, there are only sparse data concerning alterations in the gut's immune system in MS patients. In this study, we investigated fecal markers related to intestinal inflammation in RRMS patients and healthy controls.

There was a non-significant reduction in fecal SCFA concentrations in RRMS patients, especially for butyrate. Fecal calprotectin concentrations did not show any difference between RRMS patients and controls. Blood CRP concentrations, although not clinically elevated in any of the investigated subjects, correlated positively with fecal calprotectin concentrations which highlights the role of intestinal inflammation in MS. Constipation scores were significantly higher in RRMS patients and correlated with the disease activity. Immunosuppressive drugs, especially natalizumab and fingolimod, lead to significantly higher constipation scores. Notably, constipation is not a known side effect of these two drugs.

Our finding of descriptively reduced fecal SCFA concentrations in RRMS patients is in accordance with other studies in this field: Park et al. showed, that SCFA blood levels were roughly 50-65% reduced in MS patients (19). Fecal SCFA levels have already been reported to be reduced in MS in a Chinese cohort (18). Additionally, changes in the gut microbiota of MS patients have been published (16, 17, 15). Recently, the general relevance of SCFA for MS has been investigated in a clinical study by Duscha et al., who observed an enhancement of Treg-differentiation, a reduction of autoinflammation and amelioration of the disease course following oral administration of propionate (7). Nevertheless, it should be taken into account that oral administration of SCFA is unlikely to exert the same effects as SCFA produced by gut microbiota in the colon: orally administered SCFA are absorbed in the small intestine and act systemically, while SCFA produced in the colon by the gut microbiota mainly exert local effects and are unlikely to affect blood SCFA concentrations in a relevant way.

While Berg-Hansen et al. found elevated concentrations of calprotectin in the cerebrospinal fluid of MS patients in 2009 (25), fecal calprotectin concentrations have not been reported in MS patients so far. In spite of the assumption that there might be an intestinal inflammation in MS, we did not find elevated fecal calprotectin concentrations in our RRMS cohort. This might be due to the fact that we investigated fecal calprotectin in RRMS patients who were mostly under immunotherapy. Immunotherapies aimed at counteracting the inflammatory process in the CNS might affect systemic and enteric inflammation (either by direct mechanisms or via the gut brain axis) and consequently also fecal calprotectin concentrations. For natalizumab, the enteric anti-inflammatory effect is already known and therapeutically used in therapy of Crohn's disease (26). Assuming that MS therapies affect gut-associated immunity, this effect might modulate the gut microbiota (as shown by Storm-Larsen et al. for dimethylfumarate (27)) and subsequently SCFA production as well. Since SCFA are known to enhance

gut motility, an altered gut microbiota (and a subsequent reduction in fecal SCFA) might predispose to constipation. Indeed, we observed higher CSS scores in RRMS patients compared to age-matched controls. Future studies are needed to clarify whether an altered intestinal motility in MS is associated with an altered gut microbiota. In addition, longitudinal studies are necessary to distinguish between disease-immanent and therapeutic effects on the gut microbiota and intestinal inflammation in MS.

We are not able to draw conclusions on fecal calprotectin in drug-naïve MS patients as the vast majority of our RRMS cohort was under therapy. We suggest investigating intestinal inflammation in drug-naïve subjects patients and in MS relapses. Only such studies will be able to clarify the role of SCFA in the pathogenesis of MS.

There could also be a yet unknown pathogenic factor which leads to an altered gut microbiota and consecutively to a reduction of SCFA which finally initiates intestinal inflammation. Hypothetically, MS could be a second event caused by a lack of SCFA which leads to a lack of Tregs and overly active T cell immunity.

On the other hand, we cannot rule out the possibility of a confounder in constipation scores by MS itself. It is possible that several patients have lesions in vagal nuclei or in afferents to those, which might account for constipation. Additionally, reduced physical activity due to high EDSS might also account for higher constipation scores in these patients.

A surprising result of our study was the marked sex-associated difference in SCFA concentration between women and men. Fecal SCFA concentrations differed significantly between men and women – even within healthy controls. Sex-specific differences for the microbiota have been described (28), but there is still a lack of studies investigating sex-specific differences for fecal SCFA. Apart from branched-chain SCFAs, all fecal SCFA concentrations in our study were significantly reduced in women. The relevance and the reproducibility of this finding has to be determined. In addition, confounding factors like diet need to be considered. Fecal SCFA concentrations have already been subject of multiple studies, e.g. anorexia (29), obesity, diabetes and as a marker of risk for cardiometabolic disease (30). Neither Chen et al., Jangi et al. nor Miyake et al. reported a sex-specific effect on fecal SCFA. None of the above mentioned studies regarding gut microbiota and fecal SCFA concentrations reported a sex-specific difference for SCFA – maybe because this aspect was not explicitly examined. We found one gastroenterological study from 2013, in which Jakobsdottir et al. compared patients with microscopic colitis and celiac disease. Despite the fact that serum and fecal SCFA are not directly comparable, they also reported a significant sex-related difference of serum SCFA concentrations (31): women showed significantly reduced blood concentrations of SCFA. Mean acetate concentrations were about 12% reduced in women. In our study, median fecal acetate was 90.6% reduced in women. Yet, another Chinese study by Chen et al. could not reproduce a sex-related difference when analyzing serum SCFA levels (32).

The exact reasons for the higher female susceptibility for MS is not yet known. As SCFA are thought to modulate the immune system, SCFA might be pathophysiologic relevant for MS, especially if studies in drug-naïve MS patients should be able to reproduce this finding. We hypothesize, that reduced fecal SCFA

concentrations in female subjects might be an additional risk factor for MS contributing to the higher susceptibility in comparison to men.

Limits of the Study

As the investigated RRMS patients were under different treatment regimes, we also analyzed subgroups of RRMS patients which were defined by the therapeutic regime. Yet, the number of subjects per subgroup was rather small and the study population did not represent the full spectrum of available MS therapies. Future studies should also focus on drug-naïve patients in order to identify therapy-related effects. As there is evidence that the gut microbiota is altered in MS, future studies should include the analysis of the gut microbiota Another relevant aspect might be to investigate alterations in the gut microbiota, microbial markers like SCFA and calprotectin related to MS relapse.

Since our control group was age-matched, but not sex-matched, this might be a confounder due to male predominance in the control group.

Conclusion

We suggest that intestinal inflammation stays a “hot topic” in MS. Based on the available evidence, we expected to find a negative correlation between calprotectin and SCFA. While there was a trend towards reduced SCFA concentrations in MS patients compared to controls, this difference was non-significant, most likely due the heterogeneity of our MS cohort concerning treatment. Yet, we observed a strongly sex-linked difference of fecal SCFA independent from MS. Future studies dealing with microbiota analyses in MS and in general should consider sex-associated differences as a potential confounder. With regard to the known female predominance, SCFA might be a pathogenically contributing factor in MS.

List Of Abbreviations

BBB – blood brain barrier

BDI – Beck’s depression inventory

Bregs – regulatory B cells

CNS – central nervous system

CSS – Constipation Scoring System

DF – dietary fiber

EAE – experimental autoimmune encephalomyelitis

FIS – Fatigue impact scale

GPR – G-protein coupled receptor

HDAC – histone deacetylase

KW – Kruskal Wallis Test

MMST – Mini Mental Status Test

MS – Multiple sclerosis

MWU – Mann Whitney U test

RRMS – relapsing remitting multiple sclerosis

SCFA – short-chain fatty acids

SD – standard deviation

ST – student's t-test (unpaired)

SW – Shapiro Wilk Test

Tregs – regulatory T cells

Declarations

4.1 Ethics approval and consent to participate

The study protocol was reviewed and approved by the local Ethics Committee (Reg.Nr. 81/18, Ethikkommission der Aerztekammer des Saarlandes, Saarbruecken, Germany). All subjects provided written informed consent to participate.

4.2 Consent for publication

Not applicable.

4.3 Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

4.4 Competing interests

The authors declare that they have no competing interests.

4.5 Funding

The study did not receive any funding.

4.6 Authors contributions

AB conceived essential aspects of the data analyses, performed these analyses and drafted the manuscript. MA assisted in creating the study protocol, enrolled and examined subjects (study-related procedures), created a database for analysis, contributed to statistical analyses and revised the manuscript. MF and MU created the study protocol, supervised the study and revised the manuscript. AS performed laboratory analyses and revised the manuscript. KF and SW provided critical feedback to the study design and the manuscript.

4.7 Acknowledgements

Not applicable.

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Figures

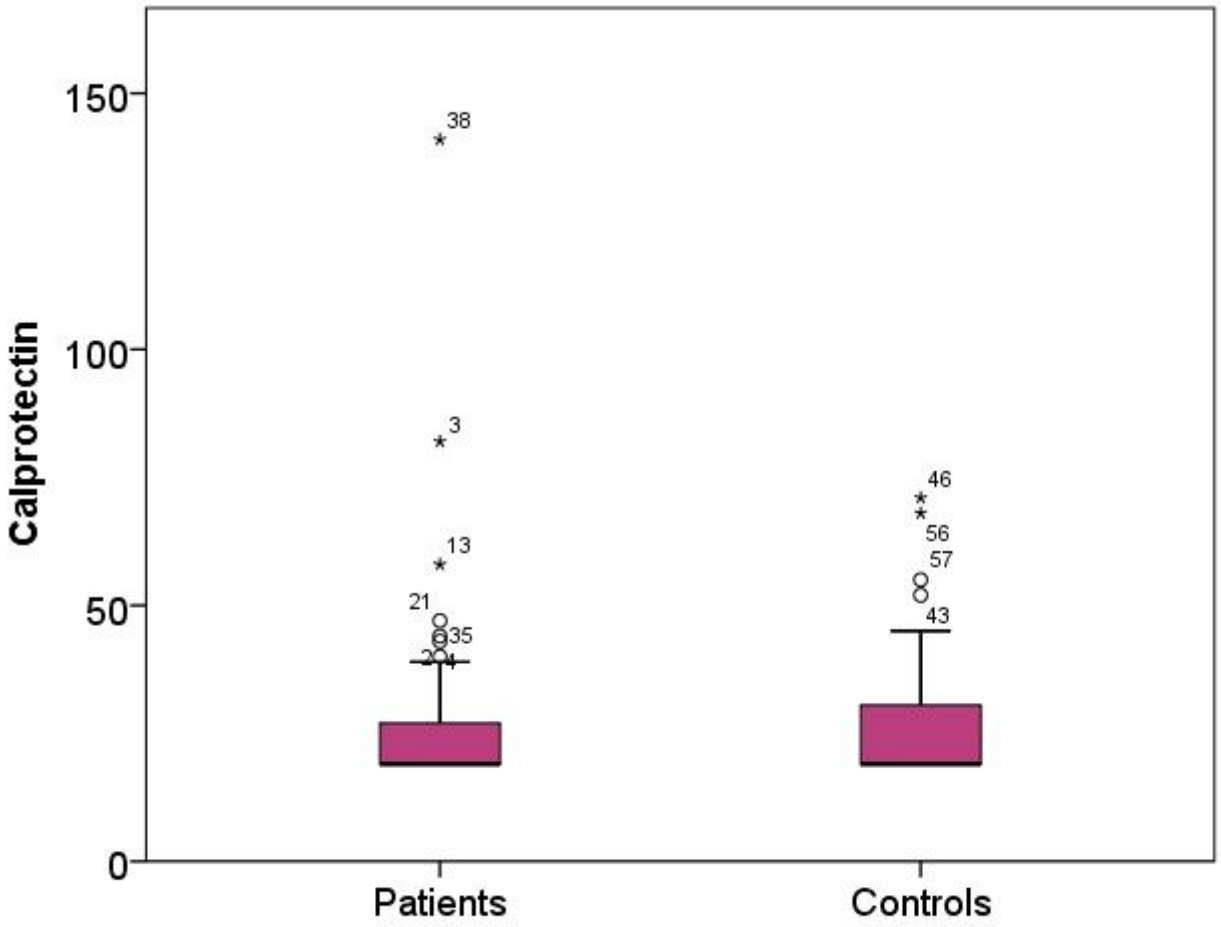

Figure 1

Fecal calprotectin in patients and controls visualized as boxplot. The control group contains an outlier with a fecal calprotectin concentration of 328µg/g which is not depicted for better visualization and scaling.

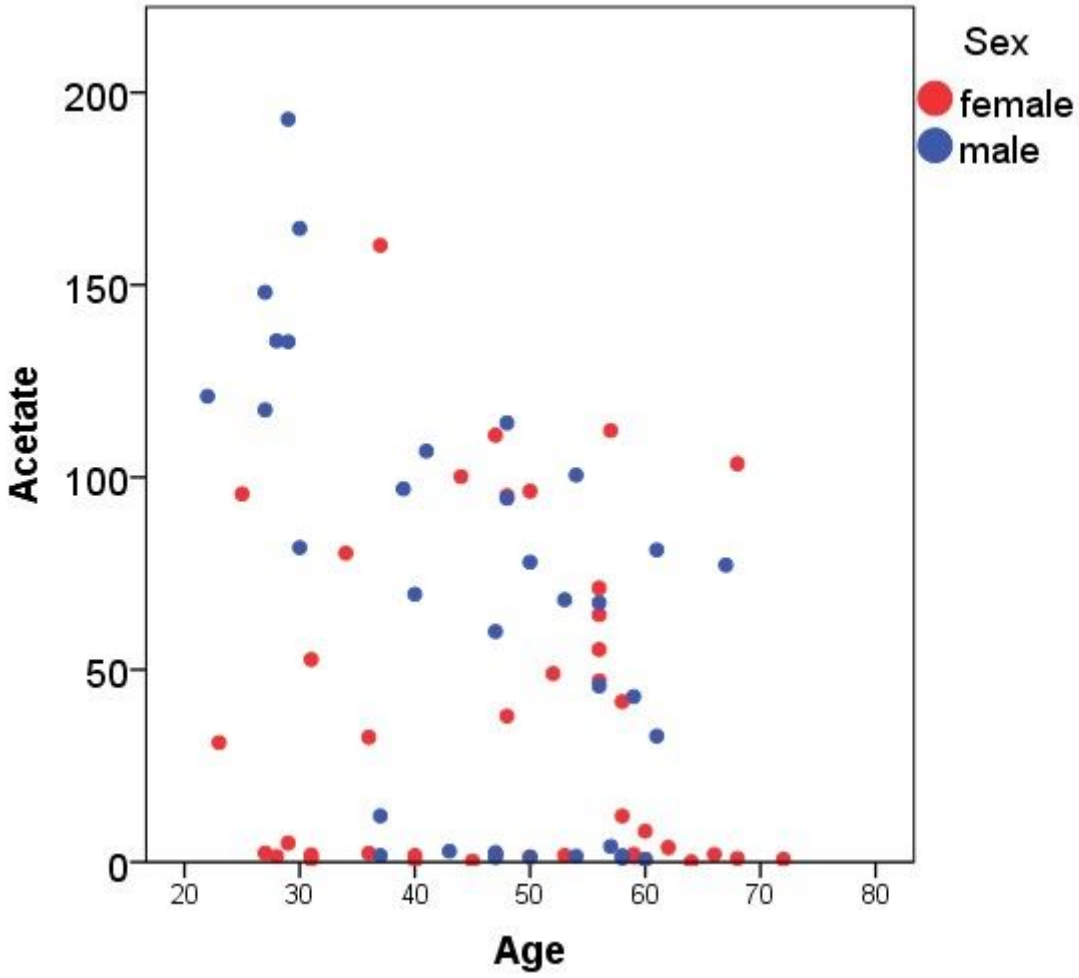

Figure 2

Fecal Acetate (in mmol/g) plotted for age (in years) and sex

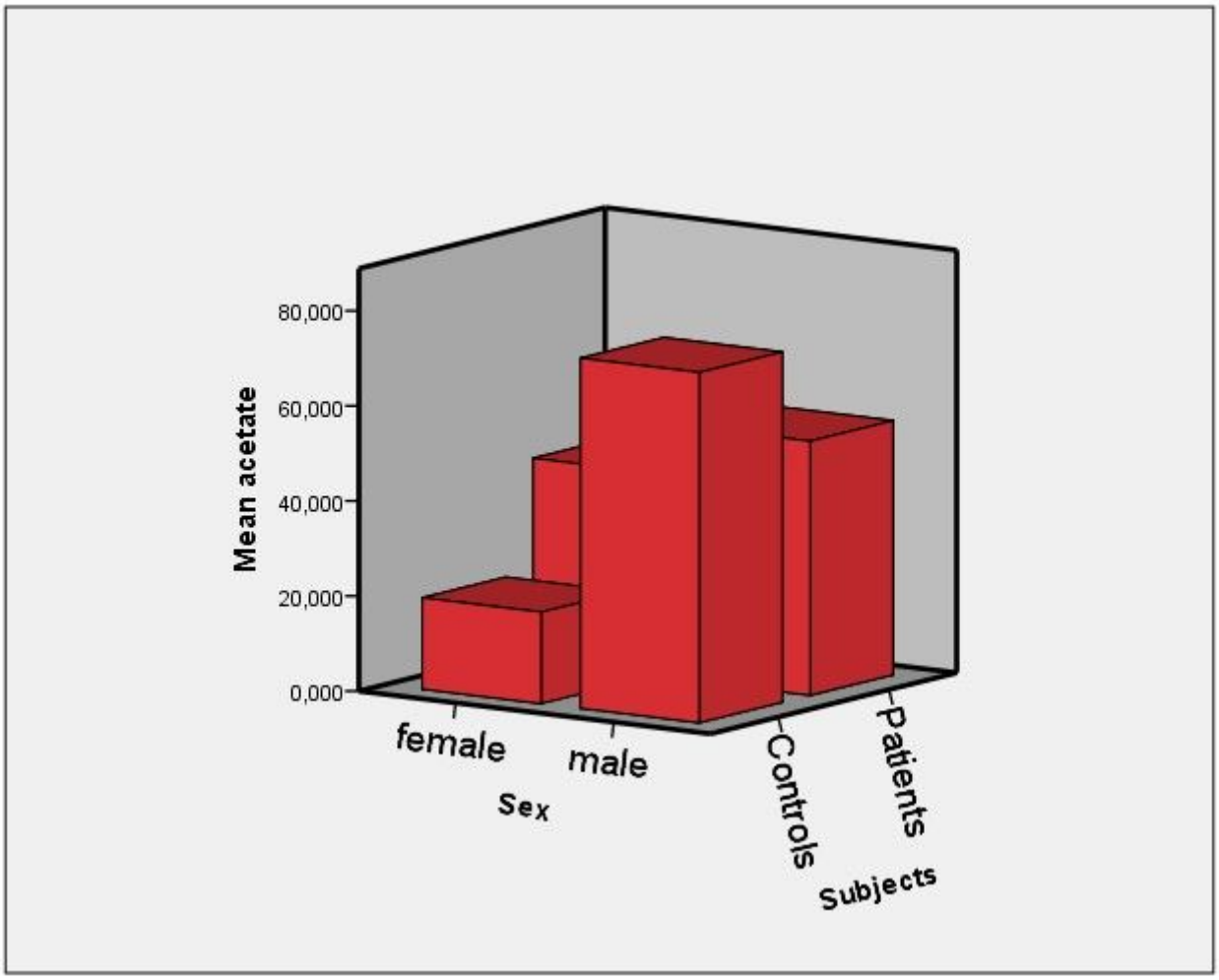

Figure 3

Mean fecal acetate (in mmol/g) split up for sex and case/control

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1MedianfecalSCFAconcentrations.pdf
- SupplementaryTable2MedianfecalSCFAconcentration.pdf