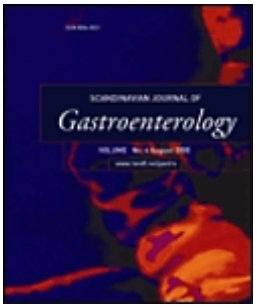

Intestinal inflammatory profile shows increase in a diversity of biomarkers in irritable bowel syndrome

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Keyword:	chemokine, cytokine, fructose intolerance, functional bowel disease, growth factor

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Dear Editor

We are grateful to the critical response from the reviewer. We have added response to the 7 Comments from the reviewer . In the revised version the changes made in 2-7 are in yellow background.
We hope that the revised version can be published in SJG

On behalf of the authors

Jon Florholmen
professor

Reviewer: 1

1. The English needs to be corrected/edited. There are several grammatical mistakes, especially on the singular/plural form of the subject/predicate.
Response: The English language has been improved in the revised manuscript by a competent native English person.

2. I am worried about the confounding factors in the immunological status of the subjects. Were all the immunological illnesses excluded? How "severe chronic disease" is defined on page 6, row 112.

Response: Severe chronic diseases were excluded including severe immunological diseases such as rheumatoid, Bechterew, SLE etc. The severe diseases were based diagnosis in the patients medical record and on well-documented and accepted international criteria. We fully agree with the critic and the content of severe disease is more detailed described in the revised version.

3. Are all the inclusion and exclusion criteria explained in the paper?

Response: We agree that the mandatory inclusion and exclusion criteria are suboptimal presented. In short, the logistics were as followed: there was a screening of patients performing a coloscopi and recruited were patients that fulfilled Rome II criteria for IBS diagnosis. Then the next step was the ordinary management of patients with IBS: exclusion of other GI diseases. This included as described, *blood tests, stool samples, breath tests, endoscopy, histological examination, X-ray, or ultrasound investigations to exclude organic disease or other malabsorption diseases including lactose intolerance*
These extra investigation were not mandatory except for blood test, and performed when only judged as actual by the clinician. Therefore, one exclusion criteria was other GI diseases. In addition as described *Patients with post-infectious IBS were excluded. If patients did meet Rome II criteria for diagnosis of IBS and other GI diseases and severe chronic disease were excluded, they were invited to participate in the study as the IBS group.* In the revised version standard screening laboratory test including transglutaminase was performed,
The revised version is changed to a more optimal and readable describing inclusion and exclusion criteria.

4. The authors write that the individual diagnostic workup was not mandatory. What were the exclusion criteria? How could they be excluded if the laboratory analyses were not

mandatory? Why was the "individual diagnostic workup" done if it was not at all used in analyzing the results?
-Only fructose intolerance test was performed. What about other intolerances (like lactose) and allergies - again if the tests were not mandatory?

Response: The logistics are described and commented above, According to the question of food allergy and lactose intolerance. the patients were carefully asked for food allergy and lactose intolerance and were excluded if typical symptoms. In cases of uncertainty food allergy blood tests and lactose intolerance test were performed. In the revised version this is implemented

5. Dietary intake (content and timing) was not evaluated (e.g. fiber intake vs bloating). It could be a confounding factor.

Response: The patients were told to use their ordinary food intake but a more defined diet intake including fiber content would definitely given more comprehensible data. If this lack represents a confounding factor and/or a causal factor is questionable. Anyhow, this is implemented in the Discussion of microbiota potential effect on our inflammation markers- see point 7.

6. FRD should be in the abbreviation list or explained in the text. It is a bit surprising that there were no mixed type of IBS.

Response: FRD added both in the abbreviation list. As described in the text: *All IBS patients had a combination of diarrhea and constipation so subgrouping could not be performed*

7. The possible role of diet and intestinal microbiota on the inflammation markers could be shortly discussed.

Response: added in Discussion, last paragraph

Intestinal inflammatory profile shows increase in a diversity of biomarkers in irritable bowel syndrome

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Running title: Immune profile in IBS

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Author conflict of interest/study support

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Abbreviations

bFGF, basic fibroblast growth factor; FRD, fructose reduced diet; GM-CSF, granulocyte-macrophage colony-stimulating factor; IBS. irritable bowel syndrome; IL, interleukin; IP, interferon gamma individual protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet derived growth factor-BB; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TH, T helper cell, TNF, tumor necrosis factor.

Key words: chemokine, cytokine**,** fructose intolerance, functional bowel disease, growth factor

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ABSTRACT

Background: It has been proposed that irritable bowel syndrome (IBS) is as a low-grade mucosal inflammatory disease.

Objective: To characterize the intestinal inflammatory profile in IBS patients with or without fructose intolerance.

Design: Patients referred to colonoscopy with IBS complaints were screened for participation. IBS patients diagnosed according to the Rome II criteria and with no organic gastrointestinal disease were included in the study. One subgroup was patients included in a fructose-reduced diet study for 2 months with effects based on VAS symptom scores. Healthy controls were subjects under investigation of colorectal cancer screening with no IBS or other gastrointestinal diseases. All patients included had normal histology from rectum. Mucosal cytokines, chemokines and growth factors were measured by multiplex technology.

Results: Of 27 inflammatory markers tested in the mucosal tissue, 13 were significantly increased and none was significantly decreased in IBS as compared to controls. Significantly increased were the proinflammatory cytokines tumor necrosis factor, the typical TH1 markers IFNγ, IL-1β, IL-2 and RANTES, the typical TH2 markers IL-5 and IL-9, the TH17 marker IL-17, TNF, the pleiotropic IL-15, and the growth factors bFGF and GM-CSF. In IBS patients with fructose intolerance only IL-5 was significantly increased compared to patients without fructose intolerance.

Conclusion: A dysregulated mucosal inflammatory profile with an increased level of TH1, TH2 and TH17 markers, and growth factors were observed in bowel mucosa in of IBS patients when compared to healthy controls.

1. Introduction

Irritable bowel syndrome (IBS) is characterized as a functional disease as there are no well-documented causal pathophysiological mechanisms. Typically, in IBS there is a hypersensitivity with low response threshold to various external stimuli, first documented by Ritchie J in 1973 [1]. This hypersensitivity of afferent neurons is activated by stimuli such as distension of hollow organs and chemical mediators such as proinflammatory and lipotoxic molecules (for review, see reference [2]). The underlying mechanisms behind visceral hypersensitivity in IBS is unknown, but the most referred hypothesis is that the visceral hypersensitivity in IBS is caused by an aberrant neuroimmune interaction (for review, see [2-7]).

One of the first reports suggesting IBS as a low-grade mucosal inflammatory disease was by Collins in 1992 [8], but this hypothesis is still controversial [9]. Two features support this hypothesis: IBS is frequently seen both after a gastrointestinal infection [10] and in inflammatory bowel disease in remission [11]. However, the documentation of visceral hypersensitivity as a neuroimmune dysregulation in IBS is poor. At the level of mucosal immune cells, several reports describing increased number of T lymphocytes [12-14], mast cells [15,16], and degranulated mast cells [14,17]. Somewhat contradictory, decreased levels of T cells and mast cells have been reported [18] in post infectious and classical IBS. Moreover, various results have been reported for cytokines and chemokines at mucosal level: increased transcript levels of IL-1β [19] has been reported, decreased transcript levels were observed for IL-10 [20,21] and for the chemokines IL-8, CXCL-9 and MCP1 [22], an imbalance between TH1 and TH2 cytokines was observed [23], whereas no changes were observed for the proinflammatory TNF alpha, IL-6 and IL-beta [22]. Finally, in contrast to these reports, no significant differences between classical, non-postinfectious IBS and healthy control were found neither at the mucosal levels of immune cells nor at transcript levels

[24,25]. Taken together, there are contradictory reports concerning the type of immune dysregulation involved if IBS can be explained as a low-grade inflammatory bowel disease.

The aim of this study was to identify the inflammatory profile of various relevant cytokines, chemokines and growth factors in colon biopsies from patients with IBS, including the subgroup with self-reported fructose intolerance, compared to healthy controls.

2. Materials and methods

2.1. Subject groups

The patients were recruited from three cohorts: from patients referred to colonoscopy due to IBS symptoms, from the FINN study (Fructose malabsorption In North Norway, [25] 26, Berg 2013), and patients in colorectal cancer screening. The recruited patients were first interviewed for a complete medical record to ensure they fulfilled Rome II criteria for IBS diagnosis. The inclusion criteria were patients who fulfilled Rome II criteria and were willing to participate. They then underwent an individual diagnostic workup including, but not mandatory, blood tests, stool samples, breath tests, endoscopy, histological examination, X-ray, or ultrasound investigations to exclude organic disease or other malabsorption diseases including lactose intolerance and food allergy. A standard screening laboratory test including test for celiac disease was performed. According to the question of food allergy and lactose intolerance, the patient were carefully asked for food allergy and lactose intolerance and were excluded if typical symptoms. In some cases with uncertainty, food allergy blood tests and lactose breath test were performed. The exclusion criteria were patients with other gastrointestinal diseases, including post-infectious IBS, use of laxatives due to constipation, patients with severe medical disorders such as diabetes mellitus, cancer, severe cerebral, lung or heart diseases and finally patients with severe immunological diseases such as rheumatoid arthritis, SLE etc. A subgroup of the IBS patients were further included in the FINN study. They performed a diagnostic test for self-reported dietary fructose intolerance (12-week fructose reduced diet (FRD) followed by 1 week high-fructose provocation test) [27]. Finally, patients with no IBS symptoms according to Rome II criteria represented the healthy control group. All patients performed colonoscopy, 20 biopsies were obtained from rectum and stored at – 70°C until analysis. Biopsies were also obtained for ordinary histological examinations (haematoxylin and eosin staining). The IBS group and the healthy control group were

included in the study if no endoscopic nor histological sign of pathology, and no other gastrointestinal disease including post-infectious IBS and severe medical disorders. Moreover, in all subjects, VAS registrations for pain and bloating (0-100 mm, 0 mm for no symptoms and 100 mm for maximal symptom score) were performed, and the number of stools and registration of stool quality on a scale from 1-7 (Bristol scale [27]).

2.2. Analyses of cytokines, chemokines and growth factors

The mucosal profile of various relevant cytokines, chemokines and growth factors were determined by multiplex technology. Homogenization of tissue was performed with the following protocol: A mix of 495 µL CytoBuster Protein Extraction Reagent (Novagen, San Diego, CA) and 5 µL Protease Inhibitor cocktail set 1 (Calbiochem, Darmstadt, Germany) was added to 50 mg of tissue sample and homogenized with Xiril Dispomix. After completion, the samples were incubated for five minutes on ice and thereafter centrifuged at 2,500 x *g* for 20 minutes at 4 °C. The supernatants were transferred to Nunc tubes and stored at -70°C. The samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA, USA) containing the following interleukins, chemokines and growth factors: Interleukin (IL)-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein (IP-10), monocyte chemotactic protein (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet derived growth factor-BB (PDGF-BB), regulated upon activation T cell expressed and secreted (RANTES), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF). The samples were analyzed on a Multiplex Analyzer (Bio-Rad Laboratories) according to instructions from the manufacturer.

2.3. *Ethics and registrations*

The study was approved by the Regional Committee of Medical Ethics North Norway (ID: 136/2006), and the study was registered in clinicaltrials.gov NCT00555191

2.4. *Statistical analysis*

Baseline characteristics and individual mediator measurements were explored using parametric tests; in most cases it was necessary to perform a logarithmic transformation to obtain a normal distribution. Chi-square test was used for contingency tables. A principal component analysis was performed on the mediator readings from all subject groups. Further details in the result section.

3. Results

3.1. Subject groups

A total of 42 IBS patients were included, 14 patients performing fructose reduced diet (FRD) where 8 patients had fructose intolerance based on the criteria of effect of fructose restricted diet (< 2 g fructose/meal) and positive provocation test on fructose-rich meals [27] and 6 patients had no fructose intolerance. All IBS patients had a combination of diarrhea and constipation so subgrouping could not be performed. The demographics are shown in table 1.

3.2. *Mucosal intestinal inflammatory profiles in IBS patients versus healthy controls*

Of the 27 mediators measured, 13 was significantly ($p<0.05$) increased and none were decreased in the IBS patients compared to controls (Table 2).

3.3. *Cytokines*

Most of the proinflammatory cytokines were increased at significant levels such as the TH1 cytokines INFγ , IL-1β, IL-2, the pleiotropic IL-15, the TH17 cytokine IL-17, TNF, but not IL-6, IL-7 or IL-8. The TH2 cytokines IL-4 and IL-9, but not IL-5, was significantly increased compared to the healthy control group (table 2).

3.4. Chemokines

Of the chemokines analyzed, only RANTES and Eotaxin were significantly increased when compared to the control group (table 2).

3.5. Growth factors

Of the various growth factors analyzed, bFGF, PDGFBB, and GM-CSF were significantly increased, but not the other growth factors when compared to the control group (table 2).

3.6. Intestinal immune profile in subgroups of IBS

IL-5 was significantly increased in IBS patients with self-reported fructose intolerance only (0.4 pg/mL [0.2 – 0.6] n=6) compared to patients without fructose intolerance (0.1 pg/mL [0.0 – 0.3] n=8) P= 0.02 (data not shown for the rest).

3.7. Principal component analysis

Principal component analysis of the dataset extracted 3 components with a cumulative explained variance of 55% (KMO 0.69; Bartlett's test of sphericity Chi-square = 1835; df = 351; P = 4.9 E-199). The rotated solution showed that PC1 (30 % variance explained) had highest factor loadings on the mediators TNF, IL-4, IFNγ, IL-17 and IL-2 corresponding to a pro-inflammatory cytokine axis. PC2 (13 % variance explained) had highest factor loadings on IL-8, IL-6, IL-9, FGF basic, and MIP-1α corresponding to a pro-inflammatory chemokine axis. Finally, PC3 (11 % variance explained) had highest loadings on VEGF, IL-10, IL-12 (p70), IL-7, and IL-5 the direction of which is not entirely clear. Figure 1 shows factor

loadings of PC1 and PC2 for the individual observations in the study groups. Many IBS subjects are located among the normal controls. However, some degree of immune activation may exist in subgroups of IBS.

4. Discussion

In this study, we have found increased mucosal levels of proinflammatory TH1 cytokines IFN-γ, IL-1b, 1L-2; the TH1 chemokine RANTES, the TH 17 cytokine IL-17cytokine, the TH2 and allergy -associated cytokines IL-5, TNF and IL-9; the growth factors FGF basic and GM-CSF, when compared to healthy control without IBS symptoms. Our study indicates that in IBS with no sign of infiltrating leucocytes at HE histology examinations represents a disease of immune dysregulation whereas an apparent minor immune dysregulation may be due to the fructose intolerance.

The mucosal levels of some of the mucosal cytokines TH1, TH2 and TH17 were significantly increased in IBS compared to the healthy control group. These cytokines which is generally increased in IBD, although at much higher levels, has to some extent been studied in IBS [28,29]. Thus, various differences to mucosal levels in healthy controls have been reported in some publications [19,23,30] but not in others [21,24,25]. Also of interest to note was the mucosal TNF as one of the main mediators of IBD [28], was increased in this study but not in other studies [20,22]. This may be due to methodological differences, especially considering the low-level increase registered. The TNF-alpha G/A polymorphism at position 308 has been observed in IBS patients [31] and may have pathophysiological role in IBS.

The two TH2 cytokines IL-5 and IL-9 were increased compared to the healthy control group. Both cytokines secreted from mast cells that has been proposed to be one of the main mediators of hypersensitivity in IBS [32]. Our IL-5 and IL9 data are in agreement with previous reports in IBS [33,34]. Therefore, all these data taken together indicate that in IBS a mucosal TH2 dysregulation exists, which mediates a low-grade inflammation with activation of inflammatory cells such as mast cells [3,32].

The mucosal level of the TH1 chemokine RANTES and eotaxin was significantly increased in IBS. First, the eotaxin result fits well with the documentation of mast cell

activation in IBS [16] and the mast cell activation of eotaxin [35]. Moreover, our data agrees with our findings of increased levels of the TH1 cytokines and TNF as cytokines with strong effect on activation of chemotaxis. In another report, CXCL-9 and MCP1 were even lower in IBS than in the healthy controls [22]. These discrepancies are hard to explain. The chemokines are responsible for the leukocyte migration by creating a chemical gradient from the vascular endothelium to the infected cells and by selective expressions of different chemokine receptors on the leukocytes [36]. It is interesting to speculate that a lack of a more complete and global chemokine response may be one explanation of the lack of a tissue invasion of leucocytes measured by conventional diagnostic procedures.

Of the various growth factors measured, there were significant increases in the growth factors bFGF, PDGFBB and GM-CSF. As far as we know there are no previous reports of these two mucosal growth factors in IBS. Other growth factors such as neural growth factor has been shown to be overexpressed in intestinal mucosa in IBS [37]. In general, growth factors play a role to maintain the mucosa integrity [38] are proposed to have anti-inflammatory effects. In chronic inflammation such as in IBD, these mediators play a role in the mucosal healing.

In IBS patients with self-reported fructose intolerance, only IL-5 was significantly increased compared to IBS patients without fructose intolerance indicating a possible allergy-like mechanism. The pathophysiological mechanisms behind this condition is unknown, but it has been proposed to be due to reduced intestinal absorption capacity of fructose [39]. Whether an allergic mechanism could be involved, has to be further investigated.

In general, IBS is most likely a heterogeneous disease where the main proposed contributing pathophysiological factors are neuroendocrine abnormalities, low grade inflammation, failure of the gut barrier, increased fecal bile content, abnormal visceral hypersensitivity and psychosocial stress. Moreover, stress as a causal factor of immune

dysregulation in the gut has been known for long in animals [40] and in humans (for review, see [41]. The heterogeneity is supported by the various contradictory reports concerning the mucosal immunophenotype between our and other reports (for review, see [4,7,42,43]. The pathophysiological mechanism behind IBS can either be abnormal interactions between neurons/nerves and immune cells [43], also referred to as the "neuroimmune" synapse [6]. Moreover, though many of the IBS subjects in the present study show a pro-inflammatory tendency, a substantial part of the IBS subjects falls within the "normal control" sphere (figure 1), which underlines the impression that IBS is a disorder of heterogenic pathogenesis. Finally, we should be aware that the suggested immunological disturbancies in IBS is still least controversial and described by some as a possible myth [44].

The strength of this study is the broad mucosal characterization of cytokines, chemokines and growth factors that would be potentially involved in IBS as an immunological active disease. The weakness of the study that should initiate further studies is that whole biopsy extracts is relatively crude. A mucosal immunologic characterization at cellular level would give more comprehensive data. Moreover, IBS is most likely a heterogeneous disease concerning the potential etiological factors and the various clinical phenotypes. Therefore, further studies are needed focusing on subgrouping defined by subgroup phenotypes of IBD, as well as precipitating factors such as infection, stress and antibiotics. Moreover, potentially, the inflammatory biomarkers could be explained by changes in the fecal microbiota, and microbiotic phenotypes related to diet including fiber intake [45,46]. This awaits further studies.

In conclusion, a dysregulated mucosal immune profile with a mixed TH1, TH2, TH17 and growth factor profile is observed in IBS patients when compared to healthy control. This indicates that IBS is a low-grade inflammatory bowel disease with an apparent incomplete

chemotactic stimulus that may preclude recruitment of leucocytes from the general circulation.

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Table 1

Demographic and baseline variables for patients included.

	IBS-All N=42	IBS-FT n=6	IBS-FI n=8	Controls N=20	P
Age (median [range])	48.5	50 [40 72]	48 [25 62]	59 [35 79]	Ns
Female per cent	68 %	63 %	100 %	35 %	0.019#
Abdominal pain/discomfort (mm)	55.2	61	50	0	Ns*
Bloating (mm)	56	63	51	0	Ns*
Stool frequency (median [range])	1.8	1.6	1.7	1.0	Ns*
Boston scale stool consistency (mean [range])	4,6	5.2	3.9	4.0	Ns*

FI: Fructose intolerance. FT: fructose tolerance. IBS: Irritable bowel syndrome. # Controls vs IBS groups; * IBS subgroups compared.

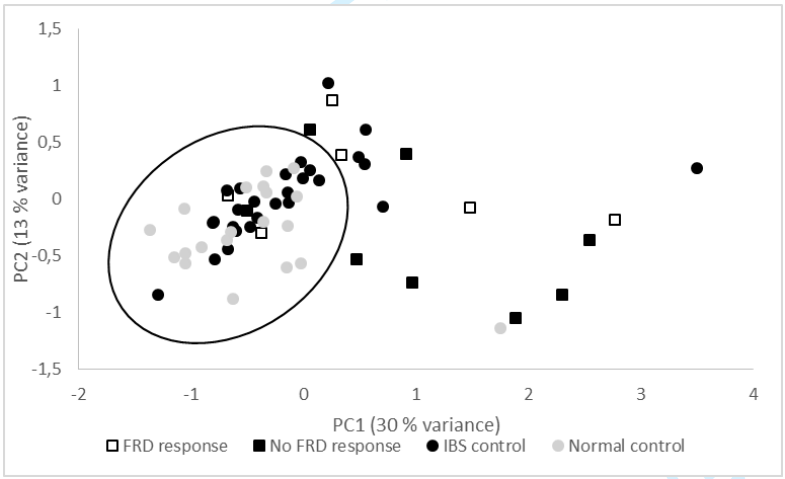
Table 2. Mucosal levels of cytokines, chemokines and growth factors in patients with IBS and in healthy controls.

Mediator	IBS (N = 42)	Normal control (N = 20)	P
IFNγ	18 (16 – 20)	14 (12 – 17)	0.010
IL-1β	6.7 (5.6 – 7.9)	4.1 (3.1 – 5.5)	0.004
IL-1RA	1299 (1012 – 1668)	818 (533 – 1254)	0.057
IL-2	0.64 (0.58 – 0.71)	0.39 (0.35 – 0.44)	<0.0005
IL-4	0.91 (0.79 – 1.04)	0.71 (0.62 – 0.82)	0.032
IL-5†	0.29 (0.25 – 0.33)	0.33 (0.22 – 0.43)	0.435
IL-6	1.5 (1.1 – 2.0)	1.1 (0.9 – 1.2)	0.136
IL-7	10.7 (9.7 – 11.9)	10.6 (9.2 – 12.2)	0.893
IL-8	24 (16 – 35)	15 (11 – 19)	0.101
IL-9	8.2 (7.2 – 9.2)	6.5 (5.7 – 7.5)	0.040
IL-10	5.5 (4.7 – 6.4)	4.6 (3.8 – 5.5)	0.165
IL-12p70	5.8 (4.9 – 6.9)	5.2 (4.5 – 6.1)	0.404
IL-13	0.85 (0.79 – 0.91)	0.81 (0.73 – 0.90)	0.448
IL-15	2.3 (2.1 – 2.6)	1.7 (1.5 – 1.8)	<0.0005
IL-17†	3.2 (2.6 – 3.7)	2.0 (1.6 – 2.4)	0.001*
TNF	160 (143 – 178)	130 (115 – 147)	0.025
Eotaxin	6.7 (5.6 – 7.9)	4.1 (3.1 – 5.5)	0.004
IP-10	590 (437 – 798)	401 (301 – 533)	0.119
MCP1MCA	75 (66 – 86)	71 (60 – 85)	0.647
MIP-1α	3.3 (2.6 – 4.2)	3.0 (2.3 – 4.0)	0.645
MIP-1β	74 (62 – 89)	64 (53 – 77)	0.321
RANTES	2011 (1378 – 2934)	891 (545 – 1454)	0.016
bFGF	674 (596 – 761)	446 (384 – 518)	<0.0005
G-CSF	6.4 (5.0 – 8.2)	6.4 (5.2 – 8.0)	0.966
GM-CSF	4.4 (3.8 – 5.1)	2.4 (1.7 – 3.1)	<0.0005
PDGFBB	11.7 (9.9 – 13.7)	8.2 (6.1 – 11.1)	0.032
VEGF	160 (130 – 196)	150 (117 – 192)	0.722

Values are mean (95% CI of mean) expressed in pg/mL of tissue supernatant. Logarithmic transformation was applied to obtain Gaussian distribution in all cases except those marked

with dagger. P-values were generated by independent samples t-test; equality of variances were observed in all cases except IL17 where the corrected t was used (*).

Figure 1. Principal component analysis of 27-plex mediator measurement in rectal mucosa from subjects with IBS w/wo fructose intolerance, IBS controls, and normal controls. Three PC's were extracted accounting for a cumulated 55% of the variance. The plot shows individual factor loadings of the two first components in a rotated solution. PC1 (30% variance) represents an adaptive pro-inflammatory component with high contribution from mediators like TNF, IFNγ, IL-17, IL-4, and IL-2. PC2 (13% variance) represents an early response pro-inflammatory component with high contribution from mediators like IL-8, IL-6, IL-9, bFGF, and MIP-1α.



Intestinal inflammatory profile shows increase in a diversity of biomarkers in irritable bowel syndrome

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Abbreviations

bFGF, basic fibroblast growth factor; FRD, fructose reduced diet; GM-CSF, granulocyte-macrophage colony-stimulating factor; IBS. irritable bowel syndrome; IL, interleukin; IP, interferon gamma individual protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet derived growth factor-BB; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TH, T helper cell, TNF, tumor necrosis factor.

Key words: chemokine, cytokine**,** fructose intolerance, functional bowel disease, growth factor

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ABSTRACT

Background: It has been proposed that irritable bowel syndrome (IBS) is as a low-grade mucosal inflammatory disease.

Objective: To characterize the intestinal inflammatory profile in IBS patients with or without fructose intolerance.

Design: Patients referred to colonoscopy with IBS complaints were screened for participation. IBS patients diagnosed according to the Rome II criteria and with no organic gastrointestinal disease were included in the study. One subgroup was patients included in a fructose-reduced diet study for 2 months with effects based on VAS symptom scores. Healthy controls were subjects under investigation of colorectal cancer screening with no IBS or other gastrointestinal diseases. All patients included had normal histology from rectum. Mucosal cytokines, chemokines and growth factors were measured by multiplex technology.

Results: Of 27 inflammatory markers tested in the mucosal tissue, 13 were significantly increased and none was significantly decreased in IBS as compared to controls. Significantly increased were the proinflammatory cytokines tumor necrosis factor, the typical TH1 markers IFNγ, IL-1β, IL-2 and RANTES, the typical TH2 markers IL-5 and IL-9, the TH17 marker IL-17, TNF, the pleiotropic IL-15, and the growth factors bFGF and GM-CSF. In IBS patients with fructose intolerance only IL-5 was significantly increased compared to patients without fructose intolerance.

Conclusion: A dysregulated mucosal inflammatory profile with an increased level of TH1, TH2 and TH17 markers, and growth factors were observed in bowel mucosa in of IBS patients when compared to healthy controls.

1. Introduction

Irritable bowel syndrome (IBS) is characterized as a functional disease as there are no well-documented causal pathophysiological mechanisms. Typically, in IBS there is a hypersensitivity with low response threshold to various external stimuli, first documented by Ritchie J in 1973 [1]. This hypersensitivity of afferent neurons is activated by stimuli such as distension of hollow organs and chemical mediators such as proinflammatory and lipotoxic molecules (for review, see reference [2]). The underlying mechanisms behind visceral hypersensitivity in IBS is unknown, but the most referred hypothesis is that the visceral hypersensitivity in IBS is caused by an aberrant neuroimmune interaction (for review, see [2-7]).

One of the first reports suggesting IBS as a low-grade mucosal inflammatory disease was by Collins in 1992 [8], but this hypothesis is still controversial [9]. Two features support this hypothesis: IBS is frequently seen both after a gastrointestinal infection [10] and in inflammatory bowel disease in remission [11]. However, the documentation of visceral hypersensitivity as a neuroimmune dysregulation in IBS is poor. At the level of mucosal immune cells, several reports describing increased number of T lymphocytes [12-14], mast cells [15,16], and degranulated mast cells [14,17]. Somewhat contradictory, decreased levels of T cells and mast cells have been reported [18] in post infectious and classical IBS. Moreover, various results have been reported for cytokines and chemokines at mucosal level: increased transcript levels of IL-1β [19] has been reported, decreased transcript levels were observed for IL-10 [20,21] and for the chemokines IL-8, CXCL-9 and MCP1 [22], an imbalance between TH1 and TH2 cytokines was observed [23], whereas no changes were observed for the proinflammatory TNF alpha, IL-6 and IL-beta [22]. Finally, in contrast to these reports, no significant differences between classical, non-postinfectious IBS and healthy control were found neither at the mucosal levels of immune cells nor at transcript levels

[24,25]. Taken together, there are contradictory reports concerning the type of immune dysregulation involved if IBS can be explained as a low-grade inflammatory bowel disease.

The aim of this study was to identify the inflammatory profile of various relevant cytokines, chemokines and growth factors in colon biopsies from patients with IBS, including the subgroup with self-reported fructose intolerance, compared to healthy controls.

2. Materials and methods

2.1. Subject groups

The patients were recruited from three cohorts: from patients referred to colonoscopy due to IBS symptoms, from the FINN study (Fructose malabsorption In North Norway, [25] 26, Berg 2013), and patients in colorectal cancer screening. The recruited patients were first interviewed for a complete medical record to ensure they fulfilled Rome II criteria for IBS diagnosis. The inclusion criteria were patients who fulfilled Rome II criteria and were willing to participate. They then underwent an individual diagnostic workup including, but not mandatory, blood tests, stool samples, breath tests, endoscopy, histological examination, X-ray, or ultrasound investigations to exclude organic disease or other malabsorption diseases including lactose intolerance and food allergy. A standard screening laboratory test including test for celiac disease was performed. According to the question of food allergy and lactose intolerance, the patient were carefully asked for food allergy and lactose intolerance and were excluded if typical symptoms. In some cases with uncertainty, food allergy blood tests and lactose breath test were performed. The exclusion criteria were patients with other gastrointestinal diseases, including post-infectious IBS, use of laxatives due to constipation, patients with severe medical disorders such as diabetes mellitus, cancer, severe cerebral, lung or heart diseases and finally patients with severe immunological diseases such as rheumatoid arthritis, SLE etc. A subgroup of the IBS patients were further included in the FINN study. They performed a diagnostic test for self-reported dietary fructose intolerance (12-week fructose reduced diet (FRD) followed by 1 week high-fructose provocation test) [27]. Finally, patients with no IBS symptoms according to Rome II criteria represented the healthy control group. All patients performed colonoscopy, 20 biopsies were obtained from rectum and stored at – 70°C until analysis. Biopsies were also obtained for ordinary histological examinations (haematoxylin and eosin staining). The IBS group and the healthy control group were

included in the study if no endoscopic nor histological sign of pathology, and no other gastrointestinal disease including post-infectious IBS and severe medical disorders. Moreover, in all subjects, VAS registrations for pain and bloating (0-100 mm, 0 mm for no symptoms and 100 mm for maximal symptom score) were performed, and the number of stools and registration of stool quality on a scale from 1-7 (Bristol scale [27]).

2.2. Analyses of cytokines, chemokines and growth factors

The mucosal profile of various relevant cytokines, chemokines and growth factors were determined by multiplex technology. Homogenization of tissue was performed with the following protocol: A mix of 495 µL CytoBuster Protein Extraction Reagent (Novagen, San Diego, CA) and 5 µL Protease Inhibitor cocktail set 1 (Calbiochem, Darmstadt, Germany) was added to 50 mg of tissue sample and homogenized with Xiril Dispomix. After completion, the samples were incubated for five minutes on ice and thereafter centrifuged at 2,500 x *g* for 20 minutes at 4 °C. The supernatants were transferred to Nunc tubes and stored at -70°C. The samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA, USA) containing the following interleukins, chemokines and growth factors: Interleukin (IL)-1β, IL-1 receptor antagonist (IL1-ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (bFGF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein (IP-10), monocyte chemotactic protein (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet derived growth factor-BB (PDGF-BB), regulated upon activation T cell expressed and secreted (RANTES), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF). The samples were analyzed on a Multiplex Analyzer (Bio-Rad Laboratories) according to instructions from the manufacturer.

2.3. *Ethics and registrations*

The study was approved by the Regional Committee of Medical Ethics North Norway (ID: 136/2006), and the study was registered in clinicaltrials.gov NCT00555191

2.4. *Statistical analysis*

Baseline characteristics and individual mediator measurements were explored using parametric tests; in most cases it was necessary to perform a logarithmic transformation to obtain a normal distribution. Chi-square test was used for contingency tables. A principal component analysis was performed on the mediator readings from all subject groups. Further details in the result section.

3. Results

3.1. Subject groups

A total of 42 IBS patients were included, 14 patients performing fructose reduced diet (FRD) where 8 patients had fructose intolerance based on the criteria of effect of fructose restricted diet (< 2 g fructose/meal) and positive provocation test on fructose-rich meals [27] and 6 patients had no fructose intolerance. All IBS patients had a combination of diarrhea and constipation so subgrouping could not be performed. The demographics are shown in table 1.

3.2. *Mucosal intestinal inflammatory profiles in IBS patients versus healthy controls*

Of the 27 mediators measured, 13 was significantly ($p<0.05$) increased and none were decreased in the IBS patients compared to controls (Table 2).

3.3. *Cytokines*

Most of the proinflammatory cytokines were increased at significant levels such as the TH1 cytokines INFγ , IL-1β, IL-2, the pleiotropic IL-15, the TH17 cytokine IL-17, TNF, but not IL-6, IL-7 or IL-8. The TH2 cytokines IL-4 and IL-9, but not IL-5, was significantly increased compared to the healthy control group (table 2).

3.4. Chemokines

Of the chemokines analyzed, only RANTES and Eotaxin were significantly increased when compared to the control group (table 2).

3.5. Growth factors

Of the various growth factors analyzed, bFGF, PDGFBB, and GM-CSF were significantly increased, but not the other growth factors when compared to the control group (table 2).

3.6. Intestinal immune profile in subgroups of IBS

IL-5 was significantly increased in IBS patients with self-reported fructose intolerance only (0.4 pg/mL [0.2 – 0.6] n=6) compared to patients without fructose intolerance (0.1 pg/mL [0.0 – 0.3] n=8) P= 0.02 (data not shown for the rest).

3.7. Principal component analysis

Principal component analysis of the dataset extracted 3 components with a cumulative explained variance of 55% (KMO 0.69; Bartlett's test of sphericity Chi-square = 1835; df = 351; P = 4.9 E-199). The rotated solution showed that PC1 (30 % variance explained) had highest factor loadings on the mediators TNF, IL-4, IFNγ, IL-17 and IL-2 corresponding to a pro-inflammatory cytokine axis. PC2 (13 % variance explained) had highest factor loadings on IL-8, IL-6, IL-9, FGF basic, and MIP-1α corresponding to a pro-inflammatory chemokine axis. Finally, PC3 (11 % variance explained) had highest loadings on VEGF, IL-10, IL-12 (p70), IL-7, and IL-5 the direction of which is not entirely clear. Figure 1 shows factor

loadings of PC1 and PC2 for the individual observations in the study groups. Many IBS subjects are located among the normal controls. However, some degree of immune activation may exist in subgroups of IBS.

4. Discussion

In this study, we have found increased mucosal levels of proinflammatory TH1 cytokines IFN-γ, IL-1b, 1L-2; the TH1 chemokine RANTES, the TH 17 cytokine IL-17cytokine, the TH2 and allergy -associated cytokines IL-5, TNF and IL-9; the growth factors FGF basic and GM-CSF, when compared to healthy control without IBS symptoms. Our study indicates that in IBS with no sign of infiltrating leucocytes at HE histology examinations represents a disease of immune dysregulation whereas an apparent minor immune dysregulation may be due to the fructose intolerance.

The mucosal levels of some of the mucosal cytokines TH1, TH2 and TH17 were significantly increased in IBS compared to the healthy control group. These cytokines which is generally increased in IBD, although at much higher levels, has to some extent been studied in IBS [28,29]. Thus, various differences to mucosal levels in healthy controls have been reported in some publications [19,23,30] but not in others [21,24,25]. Also of interest to note was the mucosal TNF as one of the main mediators of IBD [28], was increased in this study but not in other studies [20,22]. This may be due to methodological differences, especially considering the low-level increase registered. The TNF-alpha G/A polymorphism at position 308 has been observed in IBS patients [31] and may have pathophysiological role in IBS.

The two TH2 cytokines IL-5 and IL-9 were increased compared to the healthy control group. Both cytokines secreted from mast cells that has been proposed to be one of the main mediators of hypersensitivity in IBS [32]. Our IL-5 and IL9 data are in agreement with previous reports in IBS [33,34]. Therefore, all these data taken together indicate that in IBS a mucosal TH2 dysregulation exists, which mediates a low-grade inflammation with activation of inflammatory cells such as mast cells [3,32].

The mucosal level of the TH1 chemokine RANTES and eotaxin was significantly increased in IBS. First, the eotaxin result fits well with the documentation of mast cell

activation in IBS [16] and the mast cell activation of eotaxin [35]. Moreover, our data agrees with our findings of increased levels of the TH1 cytokines and TNF as cytokines with strong effect on activation of chemotaxis. In another report, CXCL-9 and MCP1 were even lower in IBS than in the healthy controls [22]. These discrepancies are hard to explain. The chemokines are responsible for the leukocyte migration by creating a chemical gradient from the vascular endothelium to the infected cells and by selective expressions of different chemokine receptors on the leukocytes [36]. It is interesting to speculate that a lack of a more complete and global chemokine response may be one explanation of the lack of a tissue invasion of leucocytes measured by conventional diagnostic procedures.

Of the various growth factors measured, there were significant increases in the growth factors bFGF, PDGFBB and GM-CSF. As far as we know there are no previous reports of these two mucosal growth factors in IBS. Other growth factors such as neural growth factor has been shown to be overexpressed in intestinal mucosa in IBS [37]. In general, growth factors play a role to maintain the mucosa integrity [38] are proposed to have anti-inflammatory effects. In chronic inflammation such as in IBD, these mediators play a role in the mucosal healing.

In IBS patients with self-reported fructose intolerance, only IL-5 was significantly increased compared to IBS patients without fructose intolerance indicating a possible allergy-like mechanism. The pathophysiological mechanisms behind this condition is unknown, but it has been proposed to be due to reduced intestinal absorption capacity of fructose [39]. Whether an allergic mechanism could be involved, has to be further investigated.

In general, IBS is most likely a heterogeneous disease where the main proposed contributing pathophysiological factors are neuroendocrine abnormalities, low grade inflammation, failure of the gut barrier, increased fecal bile content, abnormal visceral hypersensitivity and psychosocial stress. Moreover, stress as a causal factor of immune

dysregulation in the gut has been known for long in animals [40] and in humans (for review, see [41]. The heterogeneity is supported by the various contradictory reports concerning the mucosal immunophenotype between our and other reports (for review, see [4,7,42,43]. The pathophysiological mechanism behind IBS can either be abnormal interactions between neurons/nerves and immune cells [43], also referred to as the "neuroimmune" synapse [6]. Moreover, though many of the IBS subjects in the present study show a pro-inflammatory tendency, a substantial part of the IBS subjects falls within the "normal control" sphere (figure 1), which underlines the impression that IBS is a disorder of heterogenic pathogenesis. Finally, we should be aware that the suggested immunological disturbancies in IBS is still least controversial and described by some as a possible myth [44].

The strength of this study is the broad mucosal characterization of cytokines, chemokines and growth factors that would be potentially involved in IBS as an immunological active disease. The weakness of the study that should initiate further studies is that whole biopsy extracts is relatively crude. A mucosal immunologic characterization at cellular level would give more comprehensive data. Moreover, IBS is most likely a heterogeneous disease concerning the potential etiological factors and the various clinical phenotypes. Therefore, further studies are needed focusing on subgrouping defined by subgroup phenotypes of IBD, as well as precipitating factors such as infection, stress and antibiotics. Moreover, potentially, the inflammatory biomarkers could be explained by changes in the fecal microbiota, and microbiotic phenotypes related to diet including fiber intake [45,46]. This awaits further studies.

In conclusion, a dysregulated mucosal immune profile with a mixed TH1, TH2, TH17 and growth factor profile is observed in IBS patients when compared to healthy control. This indicates that IBS is a low-grade inflammatory bowel disease with an apparent incomplete

chemotactic stimulus that may preclude recruitment of leucocytes from the general circulation.

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Table 1

Demographic and baseline variables for patients included.

	IBS-All N=42	IBS-FT n=6	IBS-FI n=8	Controls N=20	P
Age (median [range])	48.5	50 [40 72]	48 [25 62]	59 [35 79]	Ns
Female per cent	68 %	63 %	100 %	35 %	0.019#
Abdominal pain/discomfort (mm)	55.2	61	50	0	Ns*
Bloating (mm)	56	63	51	0	Ns*
Stool frequency (median [range])	1.8	1.6	1.7	1.0	Ns*
Boston scale stool consistency (mean [range])	4,6	5.2	3.9	4.0	Ns*

FI: Fructose intolerance. FT: fructose tolerance. IBS: Irritable bowel syndrome. # Controls vs IBS groups; * IBS subgroups compared.

Table 2. Mucosal levels of cytokines, chemokines and growth factors in patients with IBS and in healthy controls.

Mediator	IBS (N = 42)	Normal control (N = 20)	P
IFNγ	18 (16 – 20)	14 (12 – 17)	0.010
IL-1β	6.7 (5.6 – 7.9)	4.1 (3.1 – 5.5)	0.004
IL-1RA	1299 (1012 – 1668)	818 (533 – 1254)	0.057
IL-2	0.64 (0.58 – 0.71)	0.39 (0.35 – 0.44)	<0.0005
IL-4	0.91 (0.79 – 1.04)	0.71 (0.62 – 0.82)	0.032
IL-5†	0.29 (0.25 – 0.33)	0.33 (0.22 – 0.43)	0.435
IL-6	1.5 (1.1 – 2.0)	1.1 (0.9 – 1.2)	0.136
IL-7	10.7 (9.7 – 11.9)	10.6 (9.2 – 12.2)	0.893
IL-8	24 (16 – 35)	15 (11 – 19)	0.101
IL-9	8.2 (7.2 – 9.2)	6.5 (5.7 – 7.5)	0.040
IL-10	5.5 (4.7 – 6.4)	4.6 (3.8 – 5.5)	0.165
IL-12p70	5.8 (4.9 – 6.9)	5.2 (4.5 – 6.1)	0.404
IL-13	0.85 (0.79 – 0.91)	0.81 (0.73 – 0.90)	0.448
IL-15	2.3 (2.1 – 2.6)	1.7 (1.5 – 1.8)	<0.0005
IL-17†	3.2 (2.6 – 3.7)	2.0 (1.6 – 2.4)	0.001*
TNF	160 (143 – 178)	130 (115 – 147)	0.025
Eotaxin	6.7 (5.6 – 7.9)	4.1 (3.1 – 5.5)	0.004
IP-10	590 (437 – 798)	401 (301 – 533)	0.119
MCP1MCA	75 (66 – 86)	71 (60 – 85)	0.647
MIP-1α	3.3 (2.6 – 4.2)	3.0 (2.3 – 4.0)	0.645
MIP-1β	74 (62 – 89)	64 (53 – 77)	0.321
RANTES	2011 (1378 – 2934)	891 (545 – 1454)	0.016
bFGF	674 (596 – 761)	446 (384 – 518)	<0.0005
G-CSF	6.4 (5.0 – 8.2)	6.4 (5.2 – 8.0)	0.966
GM-CSF	4.4 (3.8 – 5.1)	2.4 (1.7 – 3.1)	<0.0005
PDGFBB	11.7 (9.9 – 13.7)	8.2 (6.1 – 11.1)	0.032
VEGF	160 (130 – 196)	150 (117 – 192)	0.722

Values are mean (95% CI of mean) expressed in pg/mL of tissue supernatant. Logarithmic transformation was applied to obtain Gaussian distribution in all cases except those marked

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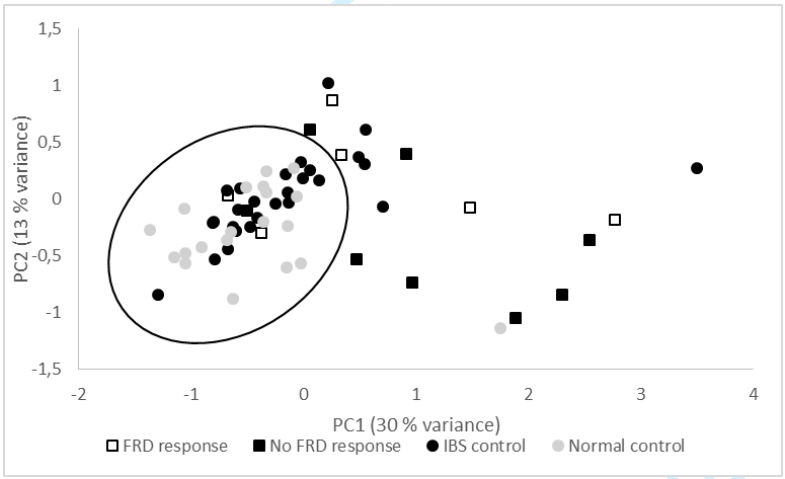
Figure 1. Principal component analysis of 27-plex mediator measurement in rectal mucosa from subjects with IBS w/wo fructose intolerance, IBS controls, and normal controls. Three PC's were extracted accounting for a cumulated 55% of the variance. The plot shows individual factor loadings of the two first components in a rotated solution. PC1 (30% variance) represents an adaptive pro-inflammatory component with high contribution from mediators like TNF, IFNγ, IL-17, IL-4, and IL-2. PC2 (13% variance) represents an early response pro-inflammatory component with high contribution from mediators like IL-8, IL-6, IL-9, bFGF, and MIP-1α.



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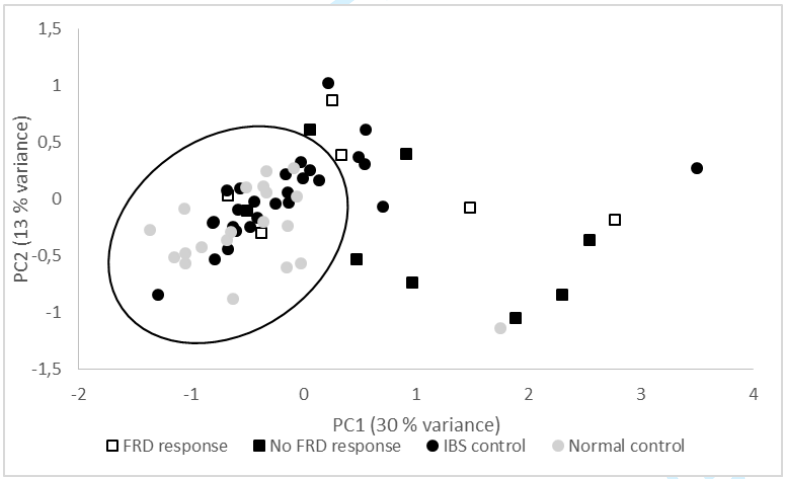

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