Effects of a gluten-free diet on gut microbiota and immune function in healthy adult humans

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Abstract

Diet influences the composition of the gut microbiota and host's health, particularly in patients suffering from food-related diseases. Celiac disease (CD) is a permanent intolerance to cereal gluten proteins and the only therapy for the patients is to adhere to a life-long gluten-free diet (GFD). Herein, a preliminary study of the effects of a GFD on the composition and immune properties of the gut microbiota have been analysed in ten healthy subjects (30.3 years old) over one month. Faecal microbiota was analyzed by fluorescence *in situ* hybridization (FISH) and quantitative PCR (qPCR). The ability of faecal bacteria to stimulate cytokine production by peripheral blood mononuclear cells (PBMCs) was determined by ELISA. No significant differences in dietary intake were found before and after the GFD except for reductions (P=0.001) in polysaccharides. *Bifidobacterium, Clostridium lituseburense* and *Faecalibacterium prausnitzii* proportions decreased (P=0.007, 0.031 and 0.009, respectively) as a result of the GFD analysed by FISH. *Bifidobacterium*, *Lactobacillus* and *B. longum* counts decreased (P=0.020, P=0.001 and P=0.017, respectively), while *Enterobacteriaceae* and *E. coli* counts increased (P=0.005 and P=0.003) after the GFD assessed by qPCR. TNF-α, IFN-γ, IL-10 and IL-8 production by PBMCs stimulated with faecal samples was also reduced (P=0.021, P=0.037, P =0.002 and P =0.007, respectively) after the diet. Therefore, the GFD led to reductions in beneficial gut bacteria populations and the ability of faecal extracts to stimulate host's immunity. Thus, the GFD may constitute an environmental variable to be considered in treated CD patients for its possible effects on gut health.

Keywords: intestinal microbiota, gluten-free diet, coeliac disease, immunity

Introduction

Diet influences the composition and function of the gut microbiota and, thereby host's health, particularly in patients suffering from food-related diseases. Celiac disease (CD) is an inflammatory disorder of the small intestine caused by a permanent intolerance to gluten proteins in predisposed individuals. In these patients, gluten peptides trigger an abnormal immune response that causes the typical CD tissue lesion characterized by villous atrophy, crypt hyperplasia, and increased numbers of intraepithelial and lamina propria lymphocytes[1-2]. CD enteropathy is sustained by a Th1 immune response with production of pro-inflammatory cytokines (e.g. IFN-γ), as well as by an innate immune response mediated by interleukine (IL)-15 that activate intraepithelial lymphocytes and epithelial cell killing[3]. Increased production of pro-inflammatory cytokines by cells of the innate immune system (monocytes, macrophages and dendritic cells) is also thought to mediate the recruitment of lymphocytes into the lamina propria and epithelium, thus contributing to the disease[4]. The treatment with a gluten-free diet (GFD) usually leads to normalization of mucosal histology and remission of clinical symptoms. Nevertheless, compliance with this dietary therapy is very complex and patients often suffer from higher health risks and nutritional deficiencies [5-6]. The composition and metabolic activity of the intestinal microbiota is currently thought to be involved in a number of chronic inflammatory disorders. Most recent studies indicate that CD patients untreated and treated with a GFD have an unbalanced microbiota that can play a pathogenic role or constitute a risk factor for this disorder[7-8]. Nevertheless, part of the detected microbial changes could be due not only to the underlying disease but also to the dietary intervention by a GFD in treated CD patients. GFD has also been tested as dietary treatment for autism[9]. However, the possible effect of a GFD in the gut ecosystem remains largely unknown.

The objective of this work was to analyse the impact of a GFD on the composition and immune function of the microbiota in healthy subjects to gain further insights on interactions between diet and gut microbes, as well as on the possible effects of this therapy on gut health and quality of life of CD patients.

Experimental methods

Subjects

Ten volunteers (8 women and 2 men; mean age: 30.3 years; range: 23-40 years) were included in the study. All participants included in this study have no history of digestive pathology or sings of malnutrition. None of the volunteers were treated with antibiotics at least within the 2 months prior to the faecal sampling. Informed consent was obtained from the subjects, and the study was approved by the local Ethics Committee.

Dietary intervention and assessments

Subjects were submitted to a GFD by replacing gluten-containing foods by equivalent ones certified as gluten-free (20 ppm maximum gluten content) by the Spanish federation of coeliac association (FACE) over 1 month period. Food diary records were kept for 72h (2 weekdays and 1 weekend day) both before the start of the intervention and after 1 month to monitor dietary changes. At the front of the diary, detailed information on how to record food and beverages consumed using common household measures was provided. When completing the food diary records, subjects were instructed to record everything they ate or drank. Food diary records were returned to the dietician as soon as possible after completion when they were reviewed, and analyzed for energy, water and macronutrient contents based on the CESNID food-composition database of Spanish foods [(10)].

Faecal sampling

Faecal samples from each adult volunteer were collected before and after following a GFD and processed as describe elsewhere in duplicate [7, 11]. Briefly, 2 g (wet weight) were 10-fold diluted in phosphate-buffered saline PBS (130 mM sodium chloride, 10mM sodium phosphate, pH 7.2) and homogenized for 5 min in a Lab Blender 400 stomacher (Seward Medical London, UK). The homogenized samples were subjected to a low-spin centrifugation (2, 000 rpm, 2 min) to remove large particulate material, and aliquots of the obtained supernatants were used for either DNA extraction or hybridisation. Prior hybridisation, one volume of the supernatant was mixed with three volumes of fresh 4% paraformaldehyde (PFA), fixed at 4°C overnight, and stored in 50% ethanol–PBS at −80 °C until use for hybridisation[7, 11]. DNA extractions were done by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Fluorescence in situ hybridization (FISH) and flow cytometry detection (FCM)

Enumeration of bacteria present in faecal samples was carried by fluorescence *in situ* hybridization (FISH) using 16S rRNA-targeted oligonucleotide probes (MOLBIOL, Berlin, Germany), as previously described[7, 11]. The following probes were used: EUB 338, for detection of total bacteria[12], Ato291 for *Atopobium* group[13], Bif164 for *Bifidobacterium* genus[14], Lab158 for *Lactobacillus* and other lactic acid bacteria[15], Bac303 for *Bacteroides-Prevotella* group[16], Ecol1513 for *Escherichia coli*[17], Erec0482 for *Eubacterium rectale-Clostridium coccoides* group[18], CHis159 for *Clostridium histolyticum* group[13], CLis135 for *Clostridium lituseburense* group[19], Fprau645 for *Faecalibacterium prausnitzii*[20]. Cells were enumerated by combining in the same hybridization tube, one group specific FITC-probe with the EUB 338-Cy3 probe. The proportion of group cells was corrected by eliminating background fluorescence, which

was measured using the negative control NON 338 probe[7, 21]. Fixed cells were incubated in the hybridization solution (10 mM Tris-HCl, 0.9 M NaCl, pH 8.0 and 10% SDS) containing 4 ng/µl of each fluorescent probe at appropriate temperatures overnight, washed and resuspended in PBS solution for flow cytometric analysis[7,11].

Flow cytometry detections were performed using an EPICS® XL-MCL flow cytometer (Beckman Coulter, Florida, USA) as previously described[7, 22]. This instrument is equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and fluorescence detectors that detect appropriately filtered light at green (FL1, 525 nm) and red-orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200-300 events per second) to avoid cell coincidence. A total of 15, 000 events were recorded in a list mode file and analyzed with the System II V.3 software (Beckman Coulter). The proportion of each bacterial group was expressed as a ratio of cells hybridising with the FITC-labelled specific probe to cells hybridising with the universal EUB 338-Cy3 probe[22,7].

Quantitative real-time PCR (qPCR) analysis

qPCR was used to characterize the composition of the faecal microbiota by use of specific primers targeting different intestinal bacterial groups as described elsewhere [23, 24]. PCR amplification and detection were performed with an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, UK). Each reaction mixture of 25 µl was composed of SYBR® Green PCR Master Mix (SuperArray Bioscience Corporation, USA), 1 µl of each of the specific primers at a concentration of 0.25 µM, and 1 µl of template DNA. Bacterial concentration from each sample was calculated by comparing the Ct values obtained from standard curve. Standard curves were created using serial 10-fold dilution of pure cultures DNA corresponding to 10^2 to 10^9 cells as determined by microscopy counts using DAPI.

Isolation and stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood of four healthy volunteers (26-28 years old) as previously described[25]. Briefly, PBMCs were isolated by centrifugation over a Ficoll density gradient (Amersham Biosciences, Piscataway, NJ) and adjusted to 1×10^6 cells/ml in RPMI 1640 (Cambrex, New York, USA), supplemented with 10% foetal bovine serum (Gibco, Barcelona, Spain), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma). PBMCs were incubated in 24 well flat-bottom polystyrene microtitre plates (Corning, Madrid, Spain) and incubated at 37º C under 5% CO_2. 30 μl of faecal samples of each subject before and after following a GFD were use to stimulate PBMCs for 24 h. Purified lipopolysaccharide (LPS) from *E. coli* O111:B4 (Sigma, St. Louis, MO) was used at a concentration of 1 μg/ml as a positive control. Non-stimulated PBMCs were also evaluated as controls of basal cytokine production. All reagents were tested by the E-toxate test for LPS (Sigma) and shown to be below the limit of detection (2 pg/ml). Every sample used as stimulant was assayed in duplicated. Cell cultures supernatants were collected by centrifugation, fractionated in aliquots, and stored at -20ºC until cytokines were analysed.

Cytokines assays

Cytokine concentrations of supernatants were measured by enzyme-linked immunosorbent assay (ELISA). The pro-inflammatory cytokines TNF-α and IFN-γ, and the regulatory cytokine IL-10 were analysed by using the Ready SET Go! Kit (eBioscience, San Diego, CA), and the chemokyne IL-8 by using Diaclone ELISA commercial kit (Madrid, Spain), according to the manufacturer's instructions. The sensitivity of assays for each cytokine was as follows: 4 pg/ml for TNF-α and IFN-γ, 2 pg/ml for IL-10 and < 25 pg/ml for IL-8.

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Dietary composition (means and standard deviations) were calculated for crude (unadjusted) nutrients from the 72 h dietary registers and data were averaged for the analysis. Dietary variables were submitted to log-transformation and mean comparisons before and after the intervention were determined by applying the Student's *t* test. Microbial data are expressed as medians with interquartile ranges (IQR) and the differences in bacterial counts before and after gluten-free diet were determined by applying the Mann-Whitney *U* test. Results of cytokine production are expressed as means with standard deviations and differences were determined by applying the Student's *t*-test. In every case, *P*-values <0.050 were considered statistically significant.

Results and discussion

GFD influences the composition of the intestinal microbiota in healthy subjects

A preliminary study has been carried out to establish the effects of a GFD on the composition of the intestinal microbiota and to gain insights into the possible relation of this dietary therapy with gut health. The adult human subjects included in the study, 80% female (8/10) and 20% male (2/10), maintained a good health status during the intervention, and followed a conventional diet without any restriction except for gluten containing products. Dietary data before and after the intervention are shown in Table 1. No significant differences in dietary intake were found in energy and macronutrients as a result of the GFD except for significant reductions (P=0.001) in polysaccharide intake. Previous studies on the nutritional quality of the GFD also indicated that it is associated with reductions in the intake of polysaccharides together with energy compared with the standard gluten-containing diet of healthy individuals, according to our results[26]. The

composition of the faecal microbiota under the effects of the GFD determined by FISH is shown in Table 2. Individual baseline variability of bacterial populations expressed as median of differences in proportions of bacterial cells hybridising with group-specific probes related to total bacteria hybridising with EUB probe 338 was as follows: 2.38 (1.01-5.30) for *Atopobium* group, 1.86 (1.42-3.10) for *Bifidobacterium*, 1.54 (1.09-2.13) for *Lactobacillus* group, 2.16 (0.86-3.26) for *Bacteroides-Prevotella*, 1.29 (0.84-1.47) for *E. coli*, 6.54 (0.21-10.41) for *E. rectale-C coccoides,* 1.04 (0.41-2.48) for *C. histolyticum*, 1.63 (0.59-3.61) for *C. lituseburense* and 6.65 (0.62-6.72) for *F. prausnitzii. Bifidobacterium, C. lituseburense* and *F. prausnitzii* proportions decreased significantly (*P*=0.007, 0.031 and 0.009, respectively) as a result of the GFD (Table 2). *Lactobacillus* group proportions were also almost significantly reduced (*P*=0.058) after the GFD (Table 2). *Bacteroides-Prevotella, E. rectale-C. coccoides* and *C. histolyticum* group proportions were slightly reduced, while those of *E. coli* were increased after the GFD but not significantly (Table 2). Total counts determined by FISH using DAPI also showed significant reductions after the GFD from 10.25 to 9.98 log cell/g faeces (*P*=0.030). The composition of the faecal microbiota analysed by qPCR is shown in Table 3. Individual baseline variability of bacterial populations expressed as median of differences in log cells per gram of faeces (IQR) was as follows: 0.37 (0.17-0.65) for *Bifidobacterium*, 1.50 (0.21-1.68) for *Bacteroides*, 0.40 (0.08-0.82) for *C. coccoides*, 0.27 (0.13-1.05) for *C. leptum,* 0.27 (0.33-0.37) for *C. histolyticum,* 0.87 (0.20-1.13) for *Lactobacillus,* 0.48 (0.24-1.18) for *E. coli*, and 0.80 (0.15-1.37) for *Enterobacteriaceae*. The counts of *Bifidobacterium* and *Lactobacillus* groups decreased significantly after GFD (*P*=0.020 and *P*=0.001, respectively), while *E. coli* (*P*=0.003) and total *Enterobacteriaceae* counts significantly increased (*P*=0.005) as a result of the GFD, following a similar trend as that detected by FISH. Total counts were also reduced after the GFD when determined by qPCR from 9.85

to 9.71 log cell/g faeces (P=0.089) following the same trend as by FISH quantification. Therefore, introduction of a GFD implied a reduction in bacterial populations generally regarded as beneficial for human health such as *Bifidobacterium* and *Lactobacillus*, and an increase in those of opportunistic pathogens such as *E. coli* and total *Enterobacteriaceae*. These changes could be related to reductions in polysaccharide intake since these dietary compounds usually reach the distal part of the colon partially undigested, and constitute one of the main energy sources for beneficial components of the gut microbiota[27]. In addition, reductions in *Bifidobacterium* and *Lactobacillus* populations relative to Gram-negative bacteria (*Bacteroides* and *E. coli*) were previously detected in untreated CD children and particularly in treated CD patients with a GFD[7]. These findings indicate that this dietary therapy may contribute to reducing beneficial bacterial group counts and increasing enterobacterial counts, which are microbial features associated with the active phase of CD[7,28] and, therefore, it would not favour completely the normalization of the gut ecosystem in treated CD patients. The relative proportion of *F. prausnitzii* was also significantly reduced after the GFD in healthy adults following a similar trend as that detected in untreated or treated CD patients compared with controls[7]. A depletion of *F. prausnitzii* population in faecal mucus of active Crohn's disease patients has also been detected, leading to establish an inverse relation between the abundance of this population and inflammatory bowel disorders[29]. *Bifidobacterium* species composition was also analysed under the effect of the GFD by qPCR (Table 3). Individual baseline variability of bacterial populations expressed as median of differences in log cells per gram of faeces (IQR) was as follows: 0.71 (0.08-1.33) for *B. longum,* 0.68 (0.07-1.03) for *B. breve*, 1.16 (0.77-1.07) for *B. bifidum,* 0.86 (0.17-1.29) for *B. adolescentis,* 1.21 (0.63-1.72) for *B. catenulatum*, 0.30 (0.002-0.45) for *B. angulatum* and 0.35 (0.19-0.82) for *B. lactis*. The counts of *B. angulatum* were significantly increased (P=0.038) after the GFD, while those

of *B. longum* decreased (*P*=0.017), indicating that this species contributed to the reduction detected in total *Bifidobacterium* population (Table 3). The genome sequence of *B. longum* subsp. *longum* showed that more than 8% of the annotated genes were involved in carbohydrate and polysaccharide metabolism[30], which could explain the reduction of their levels after the GFD, paralleled to a reduction in polysaccharide intake.

GFD influences immunostimulatory activity of the intestinal microbiota

Changes in cytokine production patterns stimulated by faecal samples of healthy individuals after and before the GFD are shown in Fig. 1. Immunostimulatory properties of the colonic content of these individuals, representing an altered microbiota, were remarkably reduced after following a GFD, inducing a significantly lower production of the pro-inflammatory cytokines TNF-α (*P* =0.021) and IFN-γ (*P* =0.037), and the chemokine IL-8 (*P*=0.007). Thus, a GFD could contribute to reduce the pro-inflammatory signals in the gut by introducing modifications in the microbiota structure. In addition, the faecal samples of individuals under a GFD induced significantly lower production of the anti-inflammatory cytokine IL-10 (*P* = 0.002) than those of individuals on a standard gluten-containing diet. IL-10 inhibits the production of pro-inflammatory and Th1 cytokines such as TNF-α and IFN-γ[31]. Therefore, it could be expected that reductions in Th1-type cytokine production were accompanied by increases in the Th2-type and regulatory cytokine IL-10. However, when a stimulus increases IFN-γ production, IL-10 production can also be increased by counter-regulatory mechanisms and vice versa. In this case, it seems likely that GFD led to a generalized reduction of bacterial-induced cytokine production *in vitro* as a result of the generalized reduction caused by this dietary intervention in the total luminal bacterial load of the large intestine. Moreover, specific bacterial group changes could also be partially responsible for the differences detected in

cytokine induction since, for instance, *Bifidobacterium* genus and particularly some *B. longum* strains have been acknowledged for their ability to stimulate IL-10 production [25] and their levels were significantly reduced after the GFD. The immune suppressive effects associated with the GFD may be partly beneficial for CD patients, which are prone to a Th1-biased immune response, but may also imply a defect of their defence and regulatory mechanisms against harmful antigens and chronic inflammation.

Conclusions

The obtained results suggest that a GFD may influence the composition and immune function of the gut microbiota in healthy individuals without the influence of any underlying disease paralleled to reductions in polysaccharide intake. Although this is a preliminary short-term intervention study, bacterial deviations are similar as those detected previously in children after compliance with a long-term GFD. As intestinal bacteria constitute a constant challenge of antigens to their host that modulate immunity, the GFD should be considered as a possible environmental factor that may shape the microbiota composition and gut health in treated CD patients.

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coordinated the study, and wrote the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

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Figure legends

Figure 1. Cytokine production by PBMCs stimulated with faecal samples from healthy volunteers before and after the gluten-free diet and controls (non-stimulated PBMCs). Results are expressed as mean ± SD of duplicates measures determined in four independent experiments. Statistically significant differences were established by applying Student's *t*-test at $P<0.050$.

Table 1. Daily energy and nutrient intake before and after the gluten-free diet intervention.

Diet composition	Subject before GFD n=10		Subjects under GFD n=10	
	Mean	SD	Mean	SD
Energy (kcal)	1854.61	345.82	1784.06	301.93
Water (g)	2454.56	533.35	2764.96	464.18
Protein (g)	72.99	15.69	68.48	13.19
Energy from protein (%)	15.74	3.38	15.35	2.96
Fat (g)	78.69	21.12	71.95	19.00
Energy from fat (%)	38.19	10.25	36.30	9.58
Saturated fat (g)	23.21	11.17	22.42	6.55
Energy from saturated fat (%)	11.26	5.42	11.31	3.30
MUFA (g)	29.97	8.30	28.79	8.41
Energy from MUFA (%)	14.54	4.03	14.52	4.24
PUFA (g)	11.58	5.59	9.43	3.93
Energy from PUFA (%)	5.62	2.71	4.76	1.98
Cholesterol (mg)	262.36	181.37	266.76	115.07
CH (g)	212.41	55.42	218.87	69.05
Energy from CH (%)	45.81	11.95	49.07	15.48
Simple CH (g)	74.30	37.72	72.03	28.05
Energy from simple CH (%)	16.02	8.14	16.15	6.29
Polysaccharides (g) [a]	116.63	51.62	62.95	33.12
Energy from complex CH (%)	25.15	11.13	14.11	7.43
Dietary fiber (g)	19.52	10.78	17.56	9.13

Abbreviations: MUFA= Monounsaturated fatty acids, PUFA = Polyunsaturated fatty acids, CH = Carbohydrates

[a] Significant difference before and after the GFD was established by applying the Student's t test at $P< 0.050$.

Table 2. Composition of the faecal microbiota of healthy adults before and after following a gluten-free diet (GFD) analysed by fluorescent *in situ* hybridization and flow cytometry detection.

Microbial group	Adults under standard diet (n=10)		Adults under GFD diet (n=10)		Mann-Whitney *U* test
	†Median	IQR	†Median	IQR	**P*-value
Atopobium	6.09	3.46-9.60	3.35	1.8-4.87	0.122
Bifidobacterium	11.14	7.64-16.70	5.12	3.92-8.51	0.007*
Lactobacillus-Enteroccoccus	2.58	1.34-3.50	0.78	0.56-2.66	0.058
BacteroidesPrevotella	5.99	3.21-9.49	2.05	1.54-6.61	0.102
E. coli	6.08	3.46-10.56	8.42	4.21-12.21	0.501
E.rectale-C coccoides	6.56	4.19-20.15	4.52	1.81-9.77	0.122
C. histolyticum	7.87	3.99-12.01	6.93	3.72-10.47	0.753
C. lituseburense	5.39	3.44-8.02	3.43	1.41-4.21	0.031*
F. prausnitzii	13.77	8.37-17.98	5.08	4.41-7.20	0.009*

†Data are shown as medians and interquartile range (IQR) of group-specific bacterial proportions related to total population detected with the EUB universal probe as determined by FCM-FISH.

*Significantly different a $P < 0.05$ by applying the Mann-Whitney U-test.

Table 3. Composition of the faecal microbiota of healthy adults before and after following a gluten-free diet (GFD) analysed by qPCR.

Microbial group	Adults under standard diet (n=10)			Adults under GFD diet (n=10)			Mann-Whitney U-test *P-value
	Pr	†Median	IQR	Pr	†Median	IQR	
Bifidobacterium	10	8.85	7.84-9.24	10	7.79	7.43-8.45	0.020*
Bacteroides	10	8.61	8.03-9.20	10	8.21	7.25-9.21	0.450
C.coccoides	10	9.44	8.51-10.11	10	9.52	8.73-9.92	0.983
C. leptum	10	9.54	9.18-10.31	10	10.11	9.81-10.52	0.141
C. histolyticum	10	5.70	5.17-6.34	10	6.48	5.13-6.59	0.223
Lactobacillus	10	7.73	7.10-7.98	10	7.00	6.25-7.64	0.001*
E. coli	10	6.29	5.67-6.77	10	7.40	6.83-7.96	0.003*
Enterobacteriaceae	10	6.64	5.86-7.99	10	8.16	7.41-8.42	0.005*
***Bifidobacterium* species**							
B. longum	10	7.73	7.61-8.62	10	7.32	6.54-7.79	0.017*
B. breve	8	4.72	4.47-5.94	8	5.00	4.60-5.56	0.757
B. bifidum	10	6.75	6.73-6.76	10	6.75	6.75-6.85	0.208
B. adolescentis	7	5.71	4.83-8.65	8	6.73	5.39-7.80	0.975
B. catenulatum	10	6.81	6.05-8.44	10	6.66	5.88-8.13	0.538
B. angulatum	5	5.00	4.95-5.24	5	5.35	5.06-5.42	0.038*
B. lactis	6	5.82	5.21-6.58	5	4.89	4.62-5.63	0.201

†Data are shown as medians and interquartile range (IQR) of log of cell number per gram of faeces.

⌠Pr (Prevalence) reflects the number of positive amplifications from total samples analysed by qPCR.

*Significantly different a $P < 0.05$ by applying the Mann-Whitney U-test.

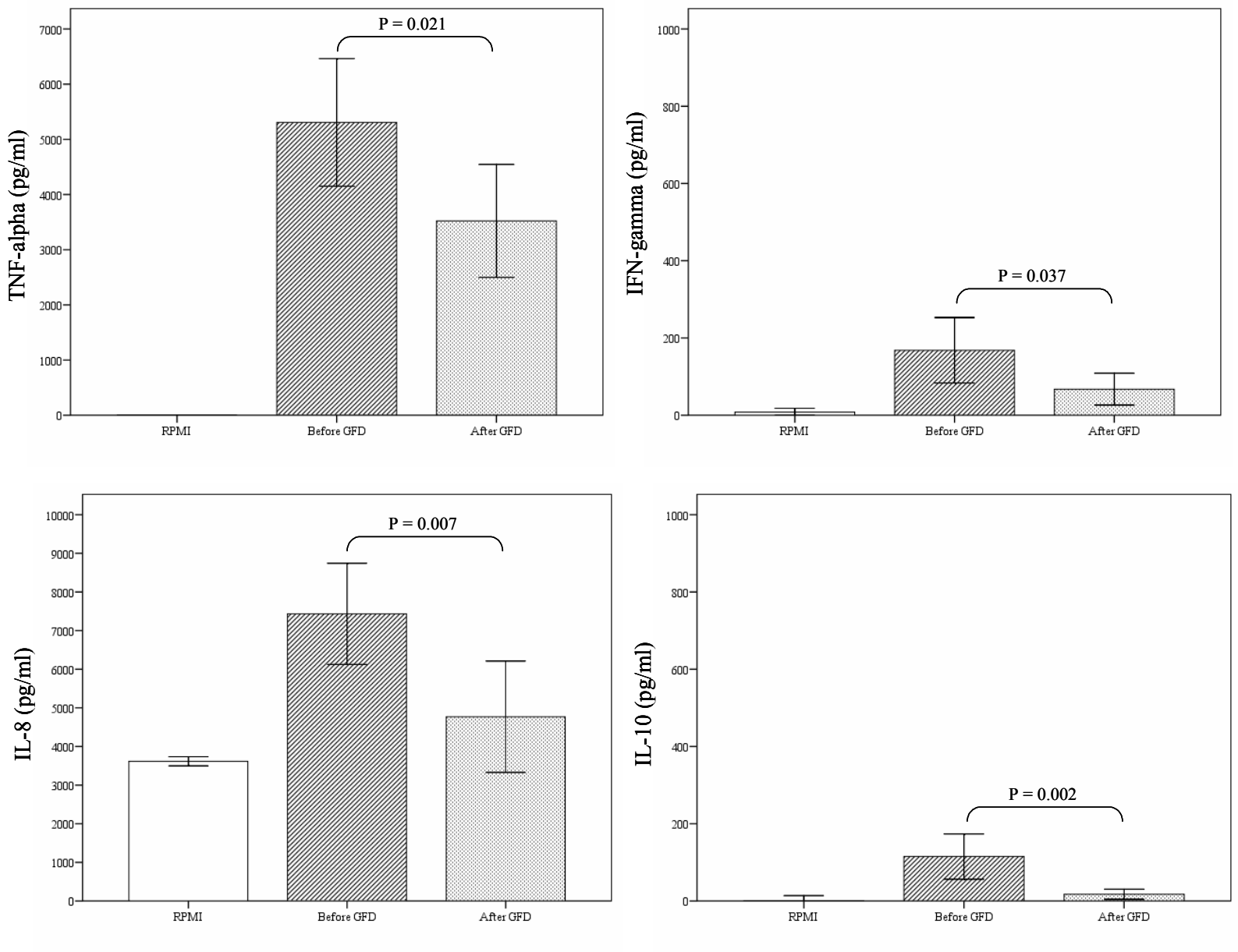

Figure 1. De Palma et al.