Monocyte chemotactic protein-1 and nitrotyrosine in irritable bowel syndrome

ALEXANDRA CHIRA[1], CORNELIA BRAICU[2], LIVIUTA BUDISAN[2], ROMEO IOAN CHIRA[3], IOANA BERINDAN-NEAGOE[2,4,5] and DAN LUCIAN DUMITRASCU[1]

[1]Second Medical Clinic, Department of Internal Medicine, 'Iuliu Hatieganu' University of Medicine and Pharmacy, 400006 Cluj-Napoca; [2]Research Center for Functional Genomics, Biomedicine and Translational Medicine, 'Iuliu Hatieganu' University of Medicine and Pharmacy, 400337 Cluj-Napoca; [3]First Medical Clinic, Division of Gastroenterology, Department of Internal Medicine, 'Iuliu Hatieganu' University of Medicine and Pharmacy, 400006 Cluj-Napoca; [4]MEDFUTURE - Research Center for Advanced Medicine, University of Medicine and Pharmacy Iuliu-Hatieganu, 400337 Cluj-Napoca; [5]Department of Functional Genomics and Experimental Pathology, The Oncology Institute 'Prof. Dr. Ion Chiricuta', 400015 Cluj-Napoca, Romania

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Abstract. Irritable bowel syndrome (IBS) is one the most frequent and common functional gastrointestinal disorders that has a multifactorial etiopathogenesis. Multiple biomarkers have been tested in search for a reliable and specific biomarker, but there is not yet a specific biomarker for IBS. The aim of this study was to evaluate two biomarkers of different putative pathways of the pathogenesis of IBS: the monocyte chemotactic protein-1 (MCP-1) and nitrotyrosine, in order to establish their role as potential biomarkers. We enrolled 42 consecutive IBS patients diagnosed by Rome III criteria and 35 consecutive healthy controls. Serum concentrations for the two biomarkers (MCP-1 and nitrotyrosine) were determined using commercial ELISA kits. Serum levels of MCP-1 were not statistically significantly higher in IBS patients than in controls (204±130 vs. 174±73 pg/ml; P=0.311). Nitrotyrosine levels were statistically significantly lower in IBS patients than in controls (30±12 vs. 353±14 nM; P=0.050). MCP-1 levels were higher in IBS patients with metabolic syndrome versus IBS patients without metabolic syndrome (239±153 vs. 168±120 pg/ml; P=0.948) and in controls with metabolic syndrome (174±56 pg/ml). MCP-1 serum levels were statistically significantly higher in IBS patients with metabolic syndrome than in controls (239±153 vs. 157±89 pg/ml; P=0.037), suggesting multiple factors being involved, particularly the diet and its relation with the metabolic syndrome, and it suggests that MCP-1 could be a marker of subclinical atherosclerosis. Low-grade inflammation might be related to oxidative stress, which plays an underestimated role in the pathogenesis of IBS.

Correspondence to: Dr Romeo Ioan Chira, First Medical Clinic, Division of Gastroenterology, Department of Internal Medicine, 'Iuliu Hatieganu' University of Medicine and Pharmacy, 3-5 Clinicilor Str., 400006 Cluj-Napoca, Romania
E-mail: romeochira@yahoo.com; ioan.chira@umfcluj.ro

Abbreviations: BMI, body mass index; IBS, irritable bowel syndrome; IBS-C, IBS with constipation; IBS-D, IBS with diarrhea; IBS-M, mixed IBS; MCP-1, monocyte chemotactic protein-1; NT, nitrotyrosine; PI, post-infectious; SD, standard deviation

Key words: biomarkers, irritable bowel syndrome, MCP-1, nitrotyrosine, oxidative stress

Introduction

Irritable bowel syndrome (IBS) is one of the most frequent and common functional gastrointestinal disorders (1,2). IBS is defined by the association of pain or abdominal discomfort with a disturbed bowel transit (3). Although it is not a life threatening condition, IBS is a chronic debilitating disease that impairs the quality of life (4,5).

According to Rome criteria, IBS patients are divided into subtypes: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (3). IBS can be triggered by a precedent gastrointestinal infection, post-infectious IBS (PI-IBS) or by other causes (non-PI-IBS) (6,7).

Diagnosis still relies on symptom-based criteria (1-3). Therefore, the need for a reliable test or marker that could help improve diagnosis strategy for IBS is reflected in a high number of studies addressing IBS.

Several studies have stressed the importance of a reliable test or marker to improve the knowledge and the management of IBS. Intestinal inflammation has been proposed as a potential mechanism since 1960s (8), later microscopic inflammation was considered a strong candidate in the pathogenesis (9). Studies have shown the role of inflammation (8-10),

confirming a persistent state of inflammation especially in PI-IBS patients. To date, the multifactorial pathogenesis of IBS includes altered gastrointestinal motility, brain-gut interactions, visceral hypersensitivity, bacterial overgrowth, perturbation of microbiota, and food sensitivity (11-16).

A chemotactic cytokine, named in 1989 as monocyte chemoattractant protein-1 (MCP-1) (17,18) is one of the members of the CC chemokine subfamily that regulates the migration and recruitment of leukocytes to inflammatory regions (19,20). It has been shown that MCP-1 recruits leukocytes (monocytes or macrophages) to inflammatory sites in several conditions such as: interstitial lung disease (21), and atherosclerosis (22). Its potential role in IBS pathogenesis has been hypothesized (20) but its value, as a serological marker it is not established.

An inflammatory cascade that begins with an infiltration of inflammatory cells in the mucosa and the release of pro-inflammatory mediators such as reactive oxygen metabolites, which provides support for the relation between oxidative stress and inflammation has been cited (23). The role of oxidative stress in IBS etiopathogenesis is suggested also by another study (24).

Free and protein-bound tyrosine residues react with nitrating/nitrosating agents leading to nitrotyrosine (NT), which was proposed as a marker for nitrosation and nitration (22). Detection of NT provides evidence for generation of nitrogen species (23).

The aim of the present study was to evaluate two biomarkers, of two different putative pathways of the pathogenesis of IBS as potential serologic biomarkers for IBS.

Patients and methods

Subject selection. A total number of 42 consecutive patients that fulfilled Rome III criteria were prospectively included. IBS patients were recruited from a tertiary care center. Any other confounding condition (gastrointestinal disorders, inflammatory processes) was ruled out. Standard laboratory workout, including inflammation markers: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and fibrinogen were assessed in order to exclude active inflammation.

Patients with IBS were also subdivided into PI-IBS and non-PI-IBS, according to previous methodology (8,25) by asking patients about their medical history over the year before the onset of IBS. If patients recognized or described that IBS symptoms occurred after a triggering event consisting of an acute episode of gastroenteritis (nausea, vomiting and diarrhea), they were assigned to PI-IBS group analysis. Thirty-five consecutive controls were recruited by similar approach, including local advertising. For both groups, body mass index (BMI) was calculated using the formula: weight (kg) / [height (m)]^2. BMI below 18.5 kg/m^2 is considered underweight, a BMI ranging between 18.5 and 24.9 kg/m^2 represents a normoponderal status while a BMI higher than 25 kg/m^2 is referred as overweight. Exclusion criteria for all subjects were alcohol and substance abuse or dependence, presence of severe organic disorders, use of antioxidants and antibiotics in the previous month or anti-inflammatories in the week prior to inclusion.

Ethical considerations. The study was approved by the Ethics Committee of the 'Iuliu Haţieganu' University of Medicine and Pharmacy and was carried out in accordance with the Declaration of Helsinki. All participants were informed about the study protocol and all subjects signed an informed consent prior to inclusion in the study.

Assessment of biomarkers. Subsequent to overnight fasting, a whole venous blood sample of 5 ml volume was collected. Samples were centrifuged immediately 10 min at 2,000 x g at room temperature (21˚C) and separated serum frozen at -80˚C until use. Serum levels of the biomarkers were measured using solid-phase sandwich enzyme-linked immunosorbent assays (ELISA). ELISA is a widely used approach that allows quantitative measurement of proteins in biological specimens, including serum (25).

For MCP-1 human MCP-1 ELISA kit (OmniKine™) was used with 50 μl of serum diluted 1:5, based on the recommended protocol. For NT the OxiSelect™ Nitrotyrosine ELISA kit (Cell Biolabs Inc.) was used, according to product specifications, using 100 μl of serum. The absorbance values from each serum were plotted against the standard curve obtained for each kit and the results were extrapolated by representative extrapolation model using GraphPad Prism software.

Statistical analysis. We used descriptive statistics to characterize the groups. Comparison of parametric data was performed with the Mann-Whitney test and of the non-parametric data with Spearman's rank correlation. In order to evaluate the association between the expression of the serum biomarkers as quantitative variables, a bivariate correlation was performed, with Spearman's correlation test by using GraphPad Prism 6 (GraphPad Software, Inc.). Simple and multiple linear regression analysis were performed using SPSS version 15.0 (SPSS Inc.). For all tests, $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

IBS group consisted of 42 patients, 30 females, 12 males: mean age, 55±14 years with a sex ratio of 1.4. Regarding IBS types there were 21 patients (50%) IBS-C, 14 patients (33.33%) IBS-D and 7 patients (16.66%) IBS-M. Control group included 35 individuals, 18 females, 17 males; mean age, 50±16 years. Mean serum levels for the two biomarkers are displayed in Table I.

All the samples evaluated had values within the detection limits of the MCP-1 kit. The concentration interval for the determination of MCP-1 in samples was between 15.6-1,000 pg/ml. The serum levels of MCP-1 were higher in the IBS group, but not statistically significant (204±130 vs. 174±73 pg/ml; P=0.311).

NT, a marker of RNS, has a detection limit between 20 and 8,000 nM accordingly to producer's specifications. In our study NT showed statistically significant lower levels (P=0.050) for the IBS group (average, 30±12 nM) than for the control group (average, 35±15 nM).

Bioinformatics analysis did not show a statistically significant difference of the parameters analyzed in relation to sex in the control group versus IBS patients: age, MCP-1, and NT (Table II). Data regarding BMI in the IBS group and in controls is listed in Table I.

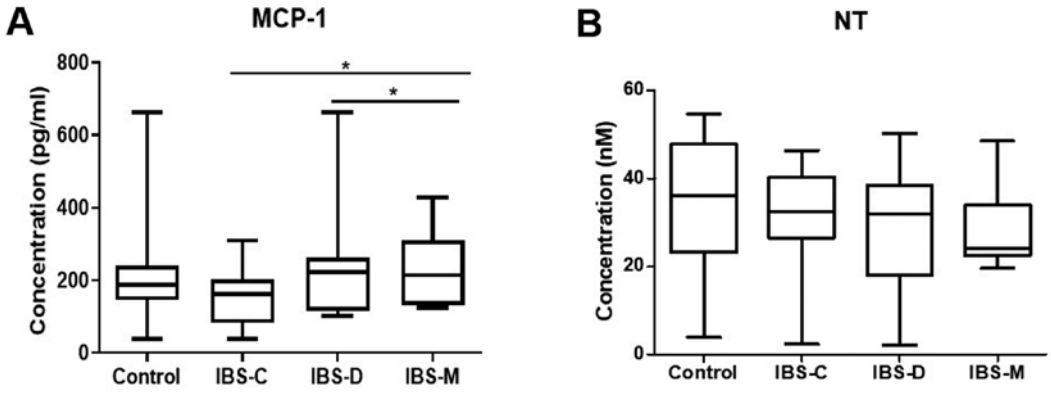

Figure 1. Serum biomarker levels of MCP-1 (A) and of NT (B) in IBS subtypes and controls. *P≤0.05. MCP-1, monocyte chemoattractant protein-1; NT, nitrotyrosine; IBS, irritable bowel syndrome.

Table I. Mean values in IBS and controls.

Baseline data	Control (n=35)	IBS (n=42)	P-value[a]
Age			
Mean ± SD	49.73±16.31	55.39±14.15	
Females	49. 88±15.63	55.17±14.57	
Males	48.05±16.94	55.92±13.07	
P-value[b]	0.749	0.882	0.072
BMI			
<18.5 kg/m^2	5	8	
18.5-24.9 kg/m^2	14	17	
>25 kg/m^2	16	17	
MCP-1 (pg/ml)			
Mean ± SD	174.26±13.39	203.65±129.99	
Females	164.58±78.18	201.94± 141.79	
Males	184.51±73.07	211.23±92.08	
P-value[b]	0.455	0.895	0.311
NT (nM)			
Mean ± SD	34.93±14.39	30.36±11.65	
Females	34.98±15.32	31.87±10.64	
Males	34.86±12.86	26.58±13.11	
P-value[b]	0.980	0.192	0.050

BMI, body mass index; IBS, irritable bowel syndrome; MCP-1, monocyte chemoattractant protein-1; NT, nitrotyrosine; SD, standard deviation; [a]P-value: IBS versus control; [b]P-value: male versus female.

Table II. Statistical analysis of IBS-D (PI-IBS, non-PI-IBS) patients and controls.

Mean and P-value	MCP-1	NT
Mean values ± SD		
PI-IBS	343.89±215.00	27.48±15.66
Non PI-IBS	167.94±76.75	28.03±17.79
Control	174.26±73.39	34.93±14.39
P-value		
PI-IBS vs. non-PI-IBS	0.064	0.952
Control vs. non-PI-IBS	0.376	0.271
Control vs. PI-IBS	0.041	0.224

IBS-D, irritable bowel syndrome with diarrhea; MCP-1, monocyte chemoattractant protein-1; NT, nitrotyrosine; SD, standard deviation; PI, post-infectious.

Biomarker levels in relation to IBS subtypes. Fig. 1 presents the serum levels for MCP-1 and NT in IBS subtypes and controls. For MCP-1 serum levels were statistically significantly higher in IBS-D patients (167±165 pg/ml; P=0.032) and IBS-M patients (236±92 pg/ml; P=0.040) when compared with IBS-C (168±80 pg/ml).

Serum concentrations for NT had similar values in the IBS subtypes (IBS-C, 33±33 nM; IBS-D, 29±15 nM; IBS-M, 30±11 nM). Related to PI-IBS status, of the 42 patients with IBS, 8 patients (19%) were included as PI-IBS, of whom five were females. Seven patients of the PI-IBS group were IBS-D (87.5%) and one (12.5%) IBS-C. In the group of non-PI-IBS of 34 patients, there were 7 patients with IBS-D, 20 patients with IBS-C and 7 patients with IBS-M. MCP-1 had statistically significantly higher values (P=0.004) in PI-IBS versus non-PI-IBS (310±211 vs. 163±89 pg/ml) (Fig. 2).

Biomarkers, age and metabolic syndrome. Fig. 3 presents correlation of the biomarkers analyzed in relation with age in IBS and control group, and statistical data are displayed in Table S1. Only MCP-1 concentrations were statistically significantly correlated with age (P=0.019, R^2=0.128) in the IBS group. Spearman's correlation for serum biomarker expression and P-values are listed in Table S2.

MCP-1 levels were higher in IBS patients with metabolic syndrome vs. IBS patients without metabolic syndrome (239±153 vs. 168±120 pg/ml; P=0.948), controls with metabolic syndrome (174±56 pg/ml) or controls without metabolic syndrome (157±89 pg/ml). MCP-1 serum levels were statistically significantly higher in IBS patients with metabolic syndrome than in controls (239±153 vs. 157±89 pg/ml; P=0.037). NT levels were statistically significantly lower in

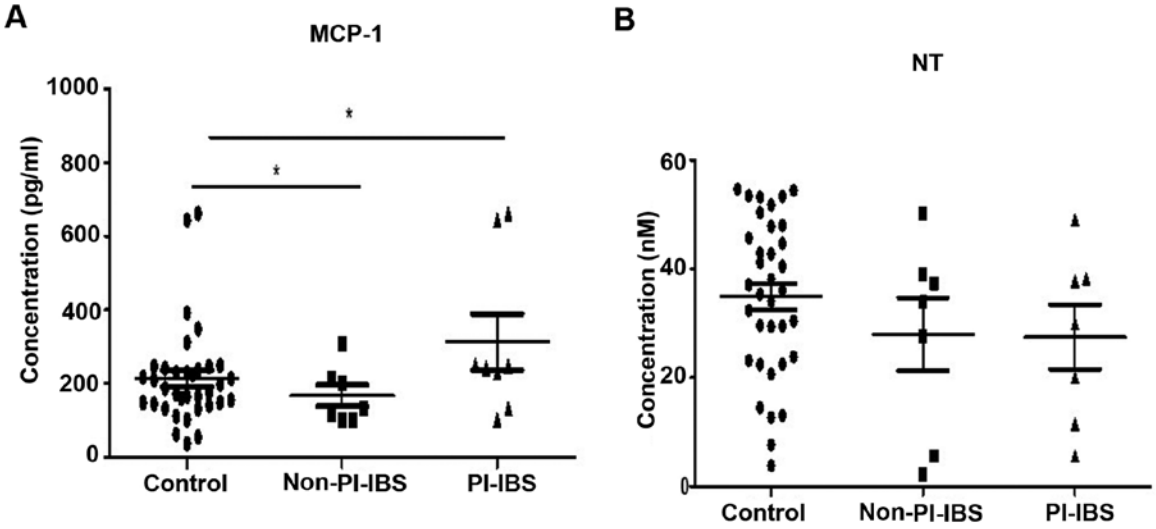

Figure 2. Serum biomarker levels of MCP-1 (A) and NT (B) in PI-IBS, non-PI-IBS and controls. *P≤0.05. MCP-1, monocyte chemoattractant protein-1; NT, nitrotyrosine; IBS, irritable bowel syndrome; PI, post-infectious.

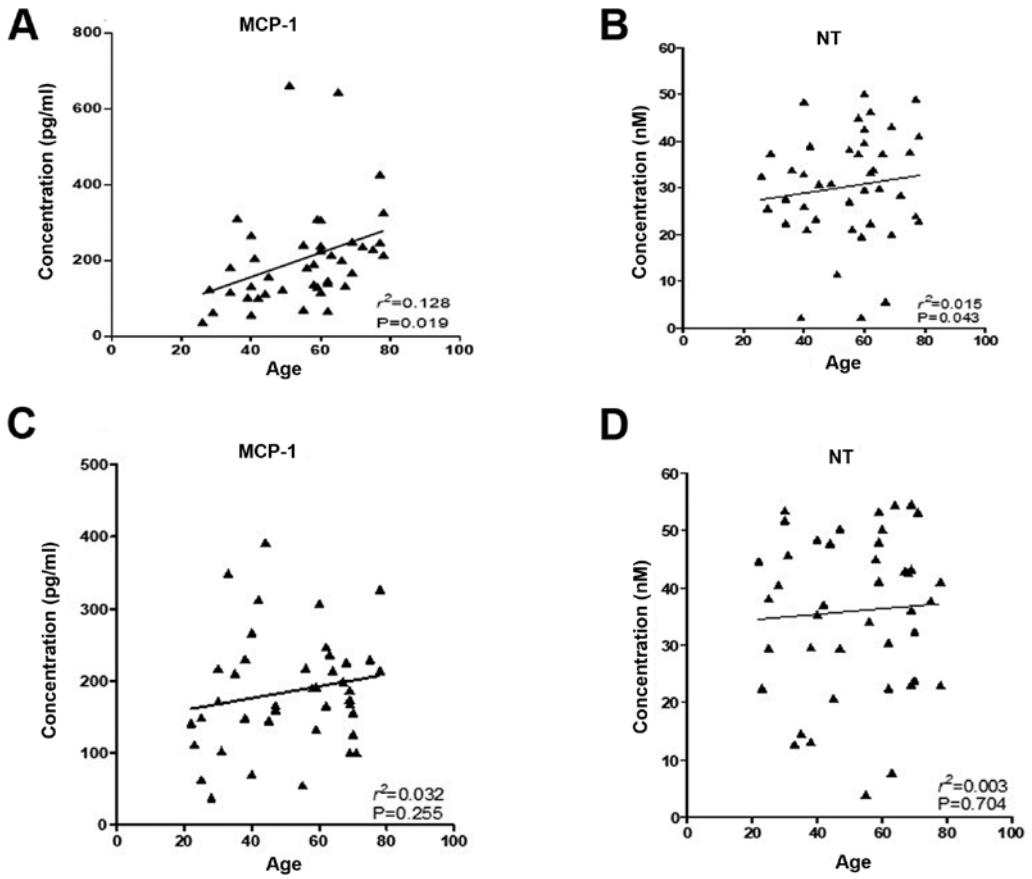

Figure 3. Correlation of the biomarker levels and age: MCP-1 (A) and NT (B) in IBS group and MCP-1 (C) and NT (D) in control group. MCP-1, monocyte chemoattractant protein-1; NT, nitrotyrosine; IBS, irritable bowel syndrome.

IBS patients without metabolic syndrome than in controls without metabolic syndrome (30 ±11 vs. 38±11 nM; P=0.004).

Discussion

IBS is a complex functional gastrointestinal disorder, highly prevalent worldwide, with most of the studies indicating a female predominance (3). Although there are many studies addressed to its pathogenesis, it still remains incompletely unraveled, and there is a need for a reliable diagnostic biomarker.

The available biomarkers or the ones investigated in IBS have recently been reviewed (26,27). In our study, we looked at two biomarkers: MCP-1 and NT. They were chosen to define

as possible biomarkers, due to paucity of data involving them in IBS. There are scarce data in literature concerning MCP-1 in IBS and also concerning oxidative stress and its relation to IBS, indicating that patients with IBS have higher levels of oxidative stress. Regarding NT, to our knowledge, there are no studies assessing NT, as a marker of reactive oxygen or nitrogen species in IBS patients.

The first study that investigated MCP-1 serum levels found that the levels were elevated in IBS patients (20). However, MCP-1 levels were not significantly different between idiopathic IBS and PI-IBS (20). Data suggest that even if the etiopathogenesis is different, both forms of IBS, PI-IBS and idiopathic IBS respectively, present similar phenotypes (20). In a previous study, Tülübaş *et al* (28) found that MCP-1 levels were significantly higher (P=0.000) in the IBS group compared with the control group, concordant with the study of Darkoh *et al* (20). Our data are consistent with these studies, with MCP-1 serum higher in the IBS group, but we found lower levels than the two previous studies (20,28). A previous study also reported that plasmatic concentrations of MCP-1 did not exhibit differences in IBS patients with vestibulodinia versus healthy controls (29). Lower levels in healthy controls than those reported by Darkoh *et al* (20) and Tülübaş *et al* (28) were found also by other studies (29,30).

There are no data available regarding MCP-1 levels in IBS subgroups (D, C and M) our data indicate statistically significant higher serum levels in IBS-D patients (P=0.032) and IBS-M patients (P=0.040) when compared with IBS-C. Regarding age and MCP-1 levels, a previous study found that MCP-1 levels increase with age in healthy individuals (31) supporting the hypothesis that MCP-1 concentration could attest existence of atherosclerosis as suggested by other literature data (31). Same authors also observed a sex difference, higher levels being found in males (31). In another study, MCP-1 levels were not related to age or sex (30). In our study, MCP-1 levels increased with age (P=0.001), which might explain similar serum levels in controls and IBS, higher MCP-1 serum levels being observed in elderly (Fig. 3).

A possible explanation for the difference encountered in various studies might be variations in methodology (including type of kit) used or mainly genetic polymorphisms, hypothesis supported by literature data regarding MCP-1 polymorphism that has already been investigated in several conditions (32,33). A genetic polymorphism of MCP-1 and the risk of inflammatory bowel disease development have been reported. This opens the perspectives to investigate this polymorphism also in IBS.

Since some of the patients exclude certain food groups (such as fermentable oligo-, di-, monosaccharides and polyols) they replace them with other groups such as lipids, which may be related to their BMI. Other studies also found an important percentage of the study group to be overweight (29% of healthy controls and 27% of IBS patients) (34). It is possible that overweight status might influence some symptoms or their persistence in IBS (34). Also, higher BMI was in some studies associated with reduced psychological well-being (35).

PI-IBS, accounted for 19% of IBS subjects in this study, our data indicate statistically significant higher MCP-1 values in PI-IBS versus non-PI-IBS (P=0.004). Also for the subgroup of PI-IBS in IBS-D we found a statistically significant difference when IBS-D were compared to control group (P=0.001), supporting infection-inflammation pathway in IBS etiopathogenesis and confirming their value as biomarkers.

A role for inflammatory, oxidative and nitrosative stress in inducing psychosomatic symptoms have been found in chronic fatigue, somatization disorders (36), ROS being involved in many inflammatory conditions, including those of the gastrointestinal tract (37).

The first study that investigated oxidative stress species in IBS found higher levels of malondialdehyde and nitric oxide in IBS patients versus control in plasma (P<0.01 respectively P<0.05) (24). The explanation for our results, with lower values of NT in IBS and also the NT levels statistically significantly lower in IBS without metabolic syndrome than in controls without metabolic syndrome (P=0.004) group might be the results of diet and lifestyle adopted by patients with IBS, either self-imposed, or as a medical recommendation, which may lead subsequently to lower levels of reactive oxygen species.

Biomarkers have been studied in serum. The study of Yu *et al* (38) showed that even if serum and plasma concentrations of biomarkers are in general similar, they found higher metabolite concentrations in serum, suggesting that serum would provide more sensitive results in biomarker detection.

Data regarding inflammatory status were not available for the previous study (24). In our group of patients, active inflammation was a rule out criteria, based on current inflammatory markers. However, both our and Mete *et al* (24) studies were conducted on a small number of cases, therefore it is possible that in a relatively small IBS patient group significant differences might not be visible.

Literature data regarding MCP-1 in IBS patients are limited (28), and there is no data regarding NT in IBS patients, most of these studies being conducted on small number of patients.

Analyzing the levels of the two serum biomarkers, we found that lipid profile does not correlate with MCP-1 or NT. Also, other study did not find a correlation between NT plasma levels and oxidized low-density lipoprotein in the patient group (Alzheimer's disease) (39). Though it was not the primary aim of our study we found a statistically significant difference with higher MCP-1 levels in IBS patients with metabolic syndrome versus controls without metabolic syndrome (P=0.037), which supports previous data that showed that elevated MCP-1 levels contribute to the development of certain pathologies associated with hyperinsulinemia and obesity (40), such as in our case the metabolic syndrome.

In conclusion, MCP-1 levels were significantly higher in IBS patients with metabolic syndrome than in controls, while nitrotyrosine levels were significantly lower in the IBS patients, suggesting multiple factors being involved, particularly the diet and its relation with the metabolic syndrome. MCP-1 levels increase with age, suggesting that MCP-1 could represent a marker for subclinical atherosclerosis. Low-grade inflammation that might be related to lipid peroxidation or oxidative stress could play an underestimated role in the pathogenesis of IBS. We consider that nutritional status and diet should be more frequently assessed in studies that investigate FGID and biomarkers in order to detect other potential liaisons.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available in part also from the Supplementary files and from the corresponding author on reasonable request.

Authors' contributions

AC and RIC drafted the manuscript. AC and DLD designed the study. CB and LB performed the biomarker assessment and part of the analysis and interpretation of data under the supervision of IBN. AC and RIC acquired part of data and performed part of data analysis and interpretation. RIC, IBN and DLD critically revised the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the 'Iuliu Haţieganu' University of Medicine and Pharmacy, (Cluj-Napoca, Romania). Signed informed consent was obtained from the patients for the inclusion in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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