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S100B as a new fecal biomarker of inflammatory bowel diseases

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Abstract. – OBJECTIVE: S100 proteins are demonstrated to exert a protective role in the gastrointestinal tract. In the present study, we investigated whether S100B protein, that is typically expressed by enteroglial cells, is detectable in feces and could be a useful noninvasive indicator of gut chronic inflammation.

PATIENTS AND METHODS: This clinical prospective study included n=48 patients suffering Crohn's disease (CD) or ulcerative colitis (UC) and non IBD-controls. The clinical disease activity was evaluated using Harvey-Bradshaw or Mayo Score Index while the diagnosis of IBD was defined based on standard endoscopic and histological criteria. S100B and calprotectin were extracted and analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits.

RESULTS: Unlike calprotectin, S100B was significantly decreased in both CD and UC compared to non IBD-patients. The strongest quantitative alterations of S100B were detected concomitantly with signs of active or quiescent disease, including high/normal expression of fecal calprotectin, mucosal damage/cryptitis, mucin depletion and inflammatory infiltrate, as defined by endoscopic evaluation and histological analysis. At the onset of disease and under no Infliximab-based therapy, the lowest was detected suggesting that S100B in feces could have a potential diagnostic value for IBD.

CONCLUSIONS: Testing for S100B and calprotectin could be a useful screening tool to better predict IBD activity.

Key Words:
S100B, Feces, Calprotectin, IBD biomarkers, Gut inflammation.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) represent the two major clinically defined forms of inflammatory bowel disease (IBD), and are associated with an increased risk of developing colon cancer, being more common in developed countries, with the highest incidence rates and prevalence in North America and Europe[1].

Although dysfunction of the mucosal immune system plays an undoubtable role in the pathogenesis of IBD[2], in recent years it has become clear that the mucosal immune system alone may not account for all the aspects of IBD; thus, a role for the enteric nervous system (ENS), has been recognized in intestinal inflammation, also involving interactions with the intestinal microbiota[3]. The ENS is located within the wall of the gastrointestinal tract and, independently from the central nervous system, coordinates many aspects of digestive functions such as motility, blood flow, and immune/inflammatory processes[4]. Among different ENS cell types, enteric glial cells (EGCs) appear to deserve special

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interest for IBD pathogenic processes. EGCs resemble astrocytes in the central nervous system, and form vast communication networks through a complex repertoire of calcium signals enabling EGCs to integrate information transmitted by neurons, glial cells, immune cells or other cells in the gut microenvironment; in disease states, inflammation can convert EGCs to a "reactive EGC phenotype", resembling the behavior of astrocytes in the central nervous system[5,6]. In addition, EGCs express typical astrocytic identification markers, such as, among others, the S100B protein[7].

S100B is a calcium-binding protein which, in the nervous system, is concentrated in astrocytes, although it is expressed also in definite extra-neural cell types, including adipocytes, which also constitute a site of concentration for the protein. When secreted, S100B is believed to have paracrine/autocrine trophic effects at physiological concentrations, but also toxic effects participating in inflammatory processes at higher concentrations, behaving as a damage/danger-associated molecular pattern molecule (DAMP). Elevated S100B levels in biological fluids (cerebrospinal fluid, blood, urine, saliva, amniotic fluid) are thus currently regarded as a reliable biomarker of pathological conditions in the nervous system[4,8]. Notably, enteric glial-derived S100B has been shown to be overexpressed, to correlate with the gut's inflammatory status and to stimulate NO production in human ulcerative colitis[9,10]. In contrast, the S100B inhibitor pentamidine ameliorates the severity of experimentally dextran sodium sulphate (DSS)-induced acute colitis in mice, as shown by macroscopic evaluation and histological/biochemical assays in colonic tissues and in plasma[11]. Interestingly, data have been reported suggesting a common pathway involving S100B upregulation and Toll-like receptor (TLR) stimulation in the colon inflammatory process[12,13]. Thus, enteroglial S100B protein, when over-expressed and released, is regarded as a pivotal participant in the cascade of events able to induce chronic inflammatory changes in gut mucosa[6,14].

Serum S100B levels have been tested as a biomarker for enteroglial activation in patients with ulcerative colitis, having been reported to be significantly lower than in healthy controls. It should also be noted, in this respect, that the authors point out the limitations of their study, including the restricted number of untreated patients[15]. Reasonably, levels of S100B in feces reflect more directly the behavior of S100B in the gastrointestinal tract, thus offering a reliable biomarker for IBD, since the protein appears to be actively involved in IBD pathogenic processes. The present study investigates the levels of S100B in feces of IBD patients, also offering a correlation with clinical parameters.

Patients and Methods

Patients

Thirty-three UC [mean age=49 years; range=20-72] fifteen CD [mean age=43 years; range=21-65 years] and twenty-one control donors [mean age=50 years; range: 33-63] (Table I) were enrolled between June 2016 and October 2016 in this prospective study. Standard clinical, endoscopic and histological criteria were used for the diagnosis of CD and UC. Clinical disease activity was defined by Harvey-Bradshaw index (HBI) or MAYO score, considering a score lower than 5 for CD or 2 for UC as indicative for remission. Subjects listed for routine preventive care and not affected by IBD organic diseases were enrolled as control donors. All procedures were approved by the Ethics Committee of the University Hospital of Padova (protocol No. 46093).

Samples

They were collected at the Department of Laboratory Medicine, University-Hospital of Padova, and kept frozen at −80°C until use. For the analysis of S100B and calprotectin, 25 mg of stool were dissolved in 2 mL of extraction saline buffer (Immundiagnostik AG, Bensheim, Germany) for 15 min at room temperature (RT). After centrifugation at 10,000 rpm for 10 min, the supernatant was collected and frozen at −20°C. The preparation of fecal protein extracts and the quantification of calprotectin were performed at the Department of Laboratory Medicine, University-Hospital of Padova, following a standard protocol.

S100B Assay

The enzyme-linked immunosorbent assay (ELISA) of S100B was performed using Human S100B (Protein S100-B) ELISA Kit (Immunological Sciences, Rome, Italy) according to the manufacturer's protocol. Briefly, after dilution (1:2-1:10) in the provided buffer, fecal protein extracts and standards were incubated in microtiter plates for 90 min at 37°C. In parallel,

Table I. Classification of patients and control donors.

Indicators	Crohn's disease	Ulcerative colitis	Controls
No.	15	33	22*
Gender (M/F)	11/4	21/12	15/7
Age/years (mean ± SD)	43.13 ± 11.42	48.78 ± 15,22	49.55 ± 7.38
Age at diagnosis/years (mean ± SD)	28.63 ± 13.36	36.02 ±13.23	–
Disease activity (Mayo > 1 or HBI > 4)			
Remission	14	21	–
Low	0	8	–
Moderate	1	3	–
Severe	0	1	–
Therapy (immunosuppressive agents)			
None	12	25	22
Steroids	2	5	–
Azathioprine	1	3	-
Therapy (biologics)			
None	6	27	22
Infliximab/Humira	9	6	–
Location of inflammation	L1 L2 L3 L4	E1 E2 E3	
(n=patients)	4 5 6 0	3 11 19	
CD phenotype			
B1-Non stricturing, non-penetrating	5		
B2-Stricturing	8		
B3-Penetranting	2		

*N=1 control sample presenting calprotectin = 324.28 mg/kg was excluded from the study; location of inflammation in Crohn's disease: (L1) terminal ileum; (L2) colon; (L3) ileo-colon; (L4) upper gastrointestinal tract; location of inflammation in Ulcerative Colitis: (E1) proctitis); (E2) left-sided colitis; (E3) pancolitis. SD: Standard Deviation.

control (zero) wells were prepared for detecting background noise. When the plate content was discarded, the treatment with biotin-detection antibody solution was carried out for 60 min at 37°C. Plates were washed three times and horseradish peroxidase (HRP)-Streptavidin conjugate was added for incubation for 30 min at 37°C. Finally, after five washings, the treatment with 3,3',5,5'-tetramethylbenzidine substrate solution was performed for 10-20 min at RT. The colorimetric reaction was blocked by addition of a stop solution and the optical density (OD) was measured at 450 nm (OD_{450}) using an EL311SK plate reader (Bio Tek Instruments Inc., Winooski, VT, USA). For calculation, the relative OD_{450} for each sample and standard solution was defined by subtracting the plate background. The concentration of S100B (µg/L) was interpolated from the standard curve plotted as the relative OD_{450} *vs*. the respective concentration of the standard solutions. In this study, calprotectin (mg/kg) of each sample was measured by standard procedure and used as reference. All results were expressed as box and whisker plots, wherein box extends from the 25th percentile to the 75th percentile of values and whiskers display values from the highest and the lowest value of the total data set. The median (50th percentile) was drawn inside the box.

Histological Analysis

Paraffin embedded biopsies from *n*=10 representative donors (*n*=5 UC active, *n*=1 UC inactive, *n*=2 CD active, *n*=2 CD inactive) were sectioned to 5-µm thickness and mounted on standard glass slides (Thermo Fisher Scientific, Waltham, MA, USA). After deparaffinization and rehydration, the specimens were stained with Hoechst 33342 (3.3 µg/mL) (Invitrogen Life Technology, Carlsbad, CA, USA) in Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences, Hatfield, PA, USA) for the histological study. Images were acquired using a Zeiss LSM800 Axio Observer Z1 inverted microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

To analyse data, the GraphPad Prism 5.0 software package (GraphPad Software, La Jolla, CA, USA) was used. When CD or UC patients were compared to controls, Student's *t*-test was applied. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Patients

The analysis of demographic and laboratory data of IBD patients and control subjects (Table I) evidenced no significant differences (p>0.05) in terms of age or sex or disease onset. UC patients exhibited pancolitis (E2, E3: 57.58%) or proctitis (E1: 9.09%) with a remission rate of ~63%. In contrast, CD patients showed a prevalent remission (93.33%), a disease location at ileum (L1: 26.67%), ileum-colon (L2: 33.33%), colon (L3: 40%), and a predominant stricturing phenotype (B2: 53.85%). Moreover, the patients enrolled in the study resulted being treated with Azathioprine (CD: n=1; UC: 3) or Corticosteroids (CD: n=2; UC: 5) or anti-TNFα agents (CD:60%; UC:18.16%).

S100B and Histological Analysis

As reported in Table II and Figure 1A, a decreased ($p \leq 0.01$) expression of S100B was detected in the stool of CD (43.61-484 μg/L) and UC (27.37-937.541 μg/L) groups compared to controls (65.22-2018 μg/L). The histological analysis by Hoechst staining (Figure 2) evidenced in the representative biopsy specimens a correlation among the decreased expression of S100B, calprotectin upregulation and signs of severe or moderate inflammation. Disease activity or remission was defined based on endoscopic and/or histological analysis performed at the University Hospital of Padova and data are reported as following: (Figure 2-A) UC patient showing severe activity at onset, pancolic distribution, mucin depletion, inflammatory infiltrate and signs of cryptisis; (Figure 2-B) UC patient under Infliximab-based therapy, presenting moderate activity at 20 years from diagnosis, pancolic distribution; (Figure 2-C) CD patient under remission following colectomy, characterized by ileocolic distribution at 7 years from the onset of disease; (Figure 2-D) CD patient under Infliximab-based therapy, showing severe activity after colectomy, ileocolic distribution of inflammation, at 23 years from IBD diagnosis; (Figure 2-E) UC patient revealing low/moderate activity, pancolic distribution, at 2 years from diagnosis; (Figure 2-F) UC patient presenting low/moderate activity, pancolic distribution, at 18 years from diagnosis (Figure 2G) UC patient showing severe activity, sigma-rectal distribution, at 16 years from diagnosis; (Figure 2-H) CD patient under Infliximab-based therapy, expressing low activity, sigma-rectal, at 32 years from diagnosis, stenosis, goblet cell hyperplasia; (Figure 2-I) CD patient under remission with Humira-based therapy, at 11 years from diagnosis; (Figure 2-J) UC patient showing remission, minimal inflammatory infiltrate inside lamina propria, at 7 years from diagnosis. High grade of crypt architectural distortions was detected in UC patient at disease onset (Figure 2-A).

In some cases (CD: n=5; UC: n=10), IBD patients evidenced a normalized (<100 mg/kg) expression of calprotectin (Table III, Figure 1B) and endoscopic/histological signs of remission (Figure 2I-J). In contrast, most of all CD and UC subjects showed an altered level of both S100B level and calprotectin (>100 mg/kg) (Figure 1B) concomitantly with mucosal damage/cryptitis, mucin depletion and inflammatory infiltrate (Figure 2I-J). Although no correlation among S100B expression and anti-TNFα-based therapy or patient age was detectable ($p > 0.05$), a case of severe inflammatory activity at onset (Figure 2A) evidenced a drastically reduced expression of S100B (79.37 μg/L) and high grade of mucosal damage (calprotectin: 851.15 mg/kg, cryptisis, mucin depletion, inflammatory infiltrate) suggesting that S100B could exert an active protective role of mucosal integrity.

Discussion

The present data show that the S100B protein is detectable in feces of normal subjects and that

Table II. Expression of faecal calprotectin and S100B levels in patients affected by Crohn's disease and ulcerative colitis.

Fecal biomarkers	Controls	CD	UC	*p*-value
Calprotectin (mg/kg) median (range)	40.07 (4.11-324)	146.00 (42.21-753.44)	265.81 (6.47-2100)	≤ 0.01
S100B (μg/L) median (range)	467.31 (65.22-2018)	202.00 (43.61-484)	225.00 (27.37-937.541)	≤ 0.01

The expression of both indicators in non IBD subjects was used as reference.

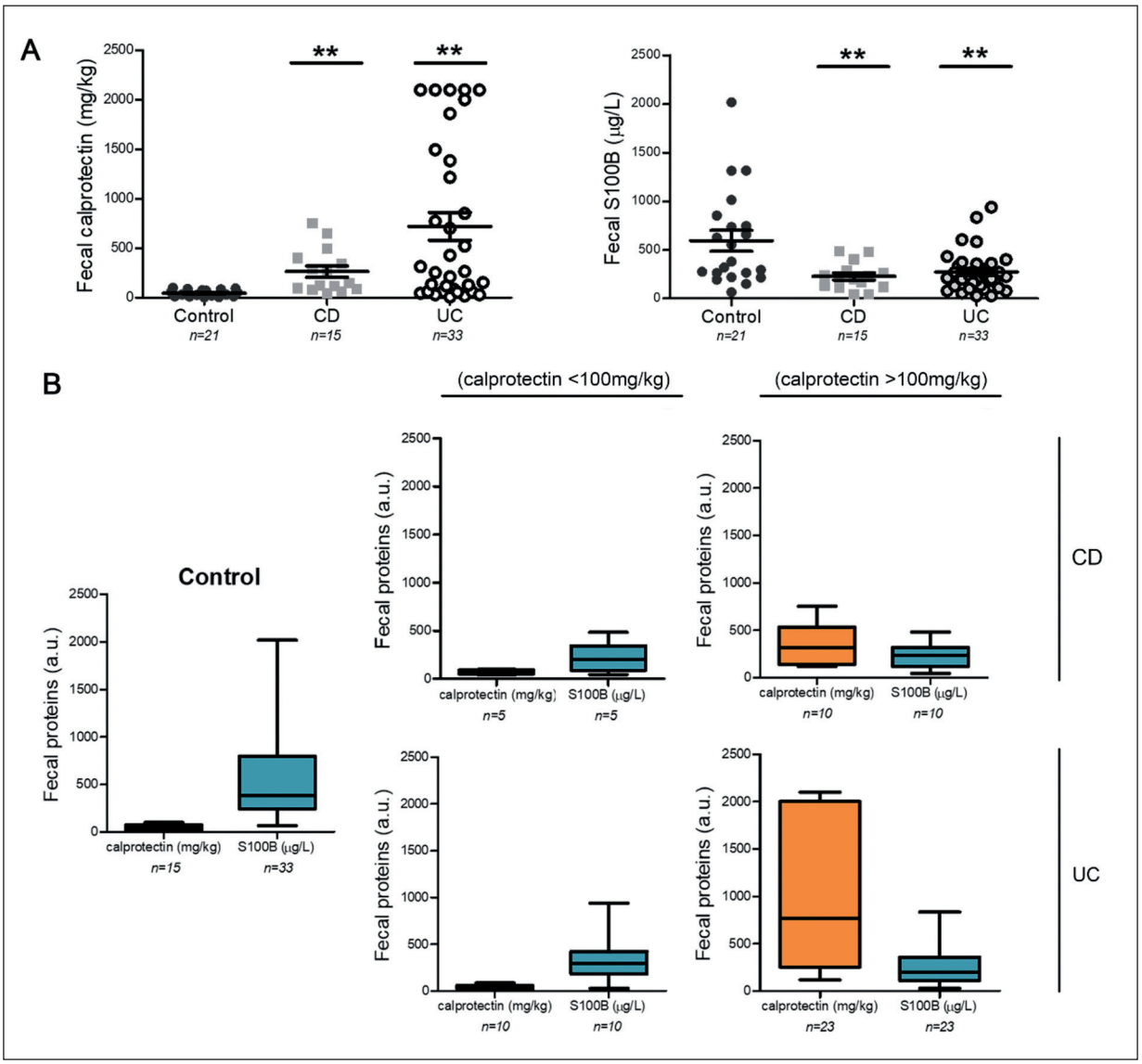

Figure 1. **A**, Detection of faecal calprotectin (mg/kg) and S100B (μg/L) in patients affected by Crohn's disease (CD) and Ulcerative Colitis (UC) by using enzyme-linked immunosorbent assay (ELISA) tests. In parallel, the analysis was performed on control samples obtained from donors listed for routine preventive care and not affected by IBD organic diseases. **B**, Discriminative analysis of S100B level compared to unchanged or increased calprotectin expression. ***p*-value < 0.01 *vs.* control.

its concentration is significantly reduced in feces of untreated patients affected by ulcerative colitis.

After the earlier finding of S100B in cerebrospinal fluid (CSF) of multiple sclerosis patients in the acute phase[16], research on S100B as a biomarker of neural injury has been extended to other biological fluids besides CSF. Peripheral blood[17], cord blood[18], amniotic fluid[19], urine[20], and saliva[21] have all been shown to contain detectable levels of S100B, which have been found to be altered in a variety of pathological conditions of the nervous system. These include acute brain injury (cardiovascular disorders and traumatic injury), neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis), congenital/perinatal disorders (Trisomy 21, pre-term and full term asphyxiated newborns, intrauterine growth retarded fetuses), psychiatric disorders (schizophrenia, mood disorders)[8].

Feces have never been used, before the present study, in order to evaluate the presence of S100B and its possible alteration as a biomarker in pathological conditions. The contiguity of feces with the

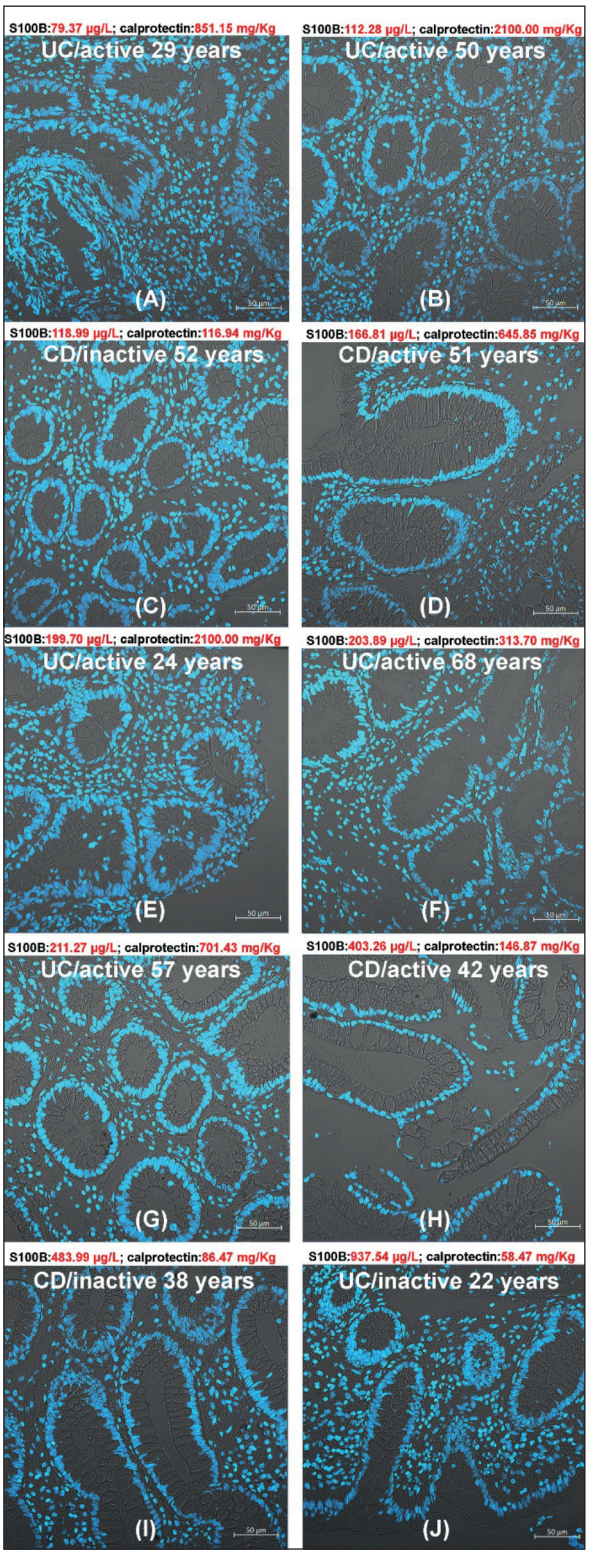

Figure 2. Confocal microscope images from some representative gut biopsies of patients included in the study. Paraffin sections were stained with Hoechst 33342 (nuclear stain) and images were acquired at 40× magnification. All pictures were optimized by brightness- and contrast-enhancement. The age and the disease activity of patients is reported in all pictures. UC: Ulcerative Colitis; CD: Crohn Disease; **A,** UC [severe activity at onset, pancolic distribution, signs of cryptisis]; **B**, UC [moderate activity, pancolic distribution, 20 years from diagnosis, Infliximab-based therapy]; **C**, CD [remission, ileocolic distribution, 7 years from diagnosis]; **D**, CD [severe activity, ileocolic distribution, 23 years from diagnosis, Infliximab-based therapy]; **E**, UC [low/moderate activity, pancolic distribution, 2 years from diagnosis]; **F**, UC [low/moderate activity, pancolic distribution, 18 years from diagnosis]; **G**, UC [severe activity, sigma-rectal distribution, 16 years from diagnosis]; **H**, CD [low activity, sigma-rectal, 32 years from diagnosis, stenosis, goblet cell hyperplasia, Infliximab-based therapy]; **I**, [remission, 11 years from onset, Humira-based therapy]; **J**, UC [remission, minimal inflammatory infiltrate in LP, 7 years from diagnosis]. Scale bar: 50 µm.

wall of the intestinal tract proposes this material as particularly suitable to evaluate the gastroenteric apparatus and, as in the case of calprotectin, is in fact usefully employed as a source of this accurate biomarker of disease activity in IBD[22]. Indeed, S100B is known to be overexpressed and to correlate with the gut's inflammatory status in human ulcerative colitis[9,10], so that the finding of

Table III. S100B level in IBD patients showing normal (<100 mg/kg) or altered (>100 mg/kg) expression of calprotectin.

Classification	Faecal indicators	Protein concentration		
		CD	UC	*p*-value
Subgroup 1	Calprotectin (mg/kg) median (range)	81.64 (42.21-96.67)	50.01 (6.47-85.53)	0.3370
	S100B (μg/L) median (range)	199.35 (43.61-483.99)	292.61 (55.44-937.54)	0.0371
Subgroup 2	Calprotectin (mg/kg) median (range)	315.47 (116.94-753.44)	771.13 (118.22-2100)	0.0001 0.0004
	S100B (μg/L) median (range)	234.78 (44.85-481.22)	190.70 (27.48-584.67)	

In parallel, the expression of both faecal proteins from control groups was reported. *p*-value *vs.* controls.

the significant lowering of its levels in feces of patients affected by ulcerative colitis, negatively correlating with the level of the established biomarker calprotectin, may be surprising. Moreover, other members of the S100 protein family, in addition to calprotectin, are overexpressed and detectable at high levels in feces of patients affected by IBD[23]. However, consistently with the present results, serum S100B levels, when tested as a biomarker for enteroglial activation in patients with ulcerative colitis, have been shown to be significantly reduced in myenteric ganglia of patients affected by UC[24]. Thus, the reduced S100B level observed in feces of IBD patients could be indicative of gut mucosal dysfunction, and the persistent inflammation driven by pathogenic bacteria (i.e., *Rhodococcus spp., Shigella spp.,* and *Escherichia spp*), that is frequently described in UC patients[25], could lead to a severe, widespread damage of the enteroglial architecture[26], thus promoting a reduced extracellular secretion of S100B protein in human colon. It has also been reported in mice, in this respect, that experimental ablation of the enteric glia, which is a component of the innate, nonspecific mucosal defense system, that maintains and regulates gut homeostasis[27], besides controlling the expression of immunomodulatory molecules and cytokines[9], it provokes fulminant intestinal inflammation, with an initial alteration of mucosal[28] and vascular integrity[29]. Interestingly, our histological evaluations evidenced a correlation among the decreased levels of S100B, high calprotectin levels and signs of severe or moderate intestinal inflammation accompanied by alteration of mucosal integrity.

Taken together, the present results appear to suggest that the reduced S100B fecal levels are an index of lack or decrease of ENS activity in IBD. However, on the other side, enteric glial-derived S100B has also been shown to be overexpressed, to correlate with the gut's inflammatory status and to stimulate NO production in human ulcerative colitis[9,10]. In addition, a "reactive human enteric glial cell phenotype", also exhibiting upregulated S100B mRNA, has been shown to characterize inflammatory gut disorders[30]. Intriguingly, these inflammatory enteric glial cells exhibit a reduced secretion of S100B, although their S100B mRNA is upregulated. This finding, which remains at present unexplained, may be consistent with the present findings indicating reduced fecal levels of S100B in IBD patients. An alternative tentative explanation for the apparently paradoxical finding of this protein which, during IBD, at the same time appears to be overexpressed in the gut tissue and reduced in the feces, may reside in an entrapment of the molecule, captured by its receptor, which accumulates in the tissue enhancing the cascade of events leading to the pathologic process. Extracellular S100B, together with other DAMPs, has been shown to interact with surrounding cell types through the receptor for advanced glycation end products (RAGE)[31,32]. RAGE is a ubiquitous, transmembrane immunoglobulin-like receptor that binds to a diverse range of extracellular ligands and intracellular effectors, initiating a complex intracellular signaling cascade, which may also be associated with a series of pathological conditions, and concomitantly resulting in an upregulation of RAGE itself[33]. In fact, RAGE has been shown to upregulate in gut tissue of IBD patients, crucially participating in the proinflammatory activity exerted by S100B

protein[10,34]. Interestingly, multimeric S100B has been shown to exhibit a higher binding affinity to RAGE than dimeric S100B, which is the physiologically more diffuse form of the protein[35,36], so that a stronger binding of the protein with its receptor may result at high concentration, possibly also involved in an inflammasome protein complex[37] in the tissue, where DAMPs and RAGE are actually regarded as active participants[38].

Finally, it should be noted that the fecal biomarkers of IBD currently regarded as the most reliable, such as calprotectin and lactoferrin, exhibit elevated levels during the active phase of the disease[39,40] Indeed, they are proteins released during neutrophil degranulation, while S100B is a constituent of enteroglial tissue cells.

In any case, the question remains about the different findings of S100B in biological fluids as a biomarker of neural injury in other pathological conditions: in neurodegenerative diseases, acute brain injury and psychiatric disorders, the protein is constantly increased4, while, in feces of IBD patients, it is reduced as reported in the present study. In this respect, the structural/functional peculiarities of the enteric nervous system and its interaction with the intestinal epithelial barrier (IEB)[41] may play a role in this discrepancy. Also the possible interaction of the protein with the intestinal microbiota, which also differs in healthy and pathological conditions[42] may interfere with S100B findings in feces. In this respect, intriguingly, S100B has been reported indeed to be overexpressed in cultured enteric glial cells after exposure to pathogen but not probiotic bacteria[13], but a degradation of the fecal protein operated by pathogen bacteria[43] might explain the discrepancy.

To summarize, the present results show for the first time the presence of S100B in feces, at significantly different levels in healthy subjects and in patients with IBD, thus proposing S100B as a biomarker for this disease. Whether the reduced levels of fecal S100B in IBD reflect lack of ENS activity or a peculiarity of S100B dynamics in the gut compartment remains to be investigated. Studies on patients affected by irritable bowel syndrome, an intestinal disorder sharing some characteristics with IBD (for review, Spiller and Major, 2016)[44], and/or on the interaction of S100B with microbiota, which constitutes an especially promising field of investigation, may be illuminating in this respect.

Conclusions

Compelling evidence indicates that intestinal barrier dysfunction exerts a pathogenic role in chronic inflammatory bowel diseases. Therefore, the identification of mediators associated with reinforcing or re-establishing IEB functions could be of great clinical interest. In this study, the glial mediator S100B is found at lower level in feces of IBD patients compared to healthy subjects. While further studies are required to clarify the processes underlying this phenomenon, S100B is proposed as a new biomarker for the clinical evaluation of IBD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Conceptualization: R.D.L., R.D.I. and F.M.; execution of experiments: M.P., S.S., C.D.M., C.C., A.P., N.C., M.S. and A.P.; theoretical calculations: R.D.L., R.D.I. and F.M., writing of the original draft: R.D.L., M.P. and F.M.; review and editing: R.D.L., R.S.V., M.T.C., P.P.P., F. M.

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