Abstract. – Background and Objectives: Calprotectin is a protein especially expressed in neutrophil cytosol. In the last few years, Fecal calprotectin (FC) turned out to be a direct marker of gastrointestinal inflammation. Because of the simplicity of the method, it has been studied in several gastroenterologic diseases but no data are available about its concentration in children with Small Intestinal Bacterial Overgrowth (SIBO), a complex and not well known condition defined by an excessive germs proliferation, especially anaerobic, in the small bowel, and characterized by dyspeptic and malabsorption symptoms. The aim of this study was to evaluate FC values in children with SIBO, comparing to healthy subjects, in order to clarify if an inflammatory process coexists with SIBO.

Materials and Methods: We enrolled fifty-eight children affected by SIBO, as diagnosed by Lactulose Breath Test (LBT). They were assessed for FC values on stool samples. We compared them with a control population of 60 healthy children.

Results: In SIBO patients, a median value of 36.0 mg/kg and a mean value ± SD of 43.0 ± 31.6 mg/kg were calculated, while in healthy controls the median value was 29.5 mg/kg and the mean value ± SD was 35.7 ± 20.7 mg/kg, showing no statistically significant differences between the two groups (p = 0.07).

Conclusions: FC values are negative in children affected by SIBO, not differing from those obtained in healthy children, suggesting that no subclinical intestinal inflammation involving neutrophils occurs in patients with higher proliferation of bacteria in the small bowel. The presence of high FC levels in children affected by SIBO might not be caused by bacterial overgrowth itself and, in this case, another cause should be investigated.

Key Words:
Fecal calprotectin, Small intestinal bacterial overgrowth, Children, Lactulose breath test.
Intestinal diseases became possible. Because of the simplicity of the method, in the last few years fecal Calprotectin (FC) has been evaluated in various gastrointestinal disorders and has emerged as a sensible and useful marker of gastrointestinal inflammation, becoming an important aid in clinical practice.

Small Intestinal Bacterial Overgrowth (SIBO) is a qualitative and quantitative variation of intestinal flora characterized by an excessive germs proliferation, especially anaerobic, in the small bowel, exceeding 10^5 Colony Forming Unit (CFU) of organisms per ml of intestinal juice. This disorder is not actually well known, and for explaining its pathogenesis several factors have been thought to be involved. A number of conditions which can compromise the delicate equilibrium of the gastrointestinal tract have been supposed to play a role, such as intestinal dismotility (diabetic neuropathy, scleroderma, accelerated gastric emptying, chronic renal failure), gastrointestinal anatomy changes (gastric atrophy, small bowel diverticulosis, intestinal stenosis, gut surgery, resection of the ileocecal valve), hypo or achlorhydria, ageing, immunodeficiency and malnutrition. With regard to clinical aspects, patients affected by SIBO can suffer from dyspeptic and malabsorption symptoms, such as bloating, meteorism, abdominal discomfort or pain, flatulence, diarrhea, steatorrhea, weight loss and anaemia.

The diagnosis of SIBO can be assessed with different methods. The gold standard is the culture of upper intestinal aspirate but it is an invasive and difficult to perform technique, which requires an expert staff. Today, one of the most used is the Lactulose Breath Test (LBT), which is a more simple and less invasive and expensive methodic. LBT is characterized by high sensitivity and specificity.

At present, no data are available about FC concentrations in children with SIBO. This prospective study was designed to evaluate FC concentrations in children affected by SIBO, comparing them to a group of healthy controls, in order to clarify if an inflammatory process coexists with SIBO.

**Materials and Methods**

We evaluated fifty-eight consecutive children with SIBO as assessed by LBT. They were referred to the Pediatric Gastroenterology Outpatients Unit of Catholic University of the Sacred Heart, Gemelli Hospital of Rome between April 1st 2008 and September 1st 2009.

Children who took Non Steroidal Anti-Inflammatory Drugs (NSAIDs), antibiotics, gastric acidity inhibitors or drugs influencing gut motility within the previous 2 months were excluded. Children who were affected by other gastrointestinal disorders, respiratory or urinary infections, or chronic diseases such as rheumatoid arthritis, diabetes, thyroid diseases, connective tissue diseases, or had a history of intestinal surgery were excluded. Children who had nasal or menstrual bleeding in the last three weeks were excluded too.

The control population included sixty healthy children, without SIBO (as assessed by negative LBT). They were referred to our General Pediatrics Outpatients Unit for routine medical care.

All patients affected by SIBO and all healthy controls were assessed for FC values after stool sample measurements.

All children were clinically evaluated at three and six months of follow-up.

All patients and control subjects were enrolled with parents informed consent, according to the Ethics Committee of our University.

**Hydrogen/Methane Lactulose Breath Test**

Hydrogen (H2)/methane (CH4) Lactulose Breath Test (LBT) was performed under standard conditions. No patients had received laxatives in the 30 days preceding the test. Subjects were asked to have a carbohydrate-restricted dinner on the day before the test and to fast for at least 12 hours to minimize basal H2 excretion. On the day of testing, patients received a mouthwash with 20 mL of chlorhexidine 0.05%. Physical exercise was not allowed for 30 minutes before and during the test. End-alveolar breath samples were collected immediately before lactulose ingestion (lactulose 10 g in solution 20 mL). Samples were taken every 15 minutes for 4 hours with a 2 bag system, consisting of a mouthpiece, a T-valve, and 2 collapsible bags; the first one collects dead space air, the second one collects alveolar air. The breath sample was aspirated from this bag into a 20 mL plastic syringe. Samples were analyzed immediately for H2 and CH4 with a model DP Quintron gas chromatograph (Quintron Instrument Company, Milwaukee, WI, USA). The results were expressed as parts per million. A normal LBT was defined as the absence of an early rise in H2 or CH4 excretion of more than 20 parts per million within the first 90 minutes.
FC Measurement and Ranges

One hundred-eighteen stool samples were collected, using a disposable plastic test tube. Specimens were returned to the laboratory within 48 hours of defecation. The weight of the samples necessary for the test was 40-120 mg. This little amount was collected with a specific device and then diluted with a buffer solution containing citrate and urea in a weight per volume ratio 1:50 (20 µl of stool sample in 980 µl of buffer solution). If necessary, a second dilution 1:250 (200 µl of the first diluted solution in 800 µl of buffer solution) could be performed for very concentrated stool samples. After this procedure, the sample was mixed for 30 seconds by a vortex method, homogenized for 25 minutes and then one milliliter of the homogenate was centrifuged for 20 minutes. The supernatant was collected and kept refrigerated at –20°C. Within seven days, the samples were thawed at room temperature and then Calprotectin concentration was actually measured by the quantitative ELISA test Calprest® (Eurospital Spa, Trieste, Italy).

Laboratory ranges were expressed as mg of Calprotectin/kg of feces. The linearity of the method was 15-500 mg/kg. On the basis of data available in literature concerning the FC cut-off value in the pediatric age, a negative FC concentration was defined by a FC value lower than 100 mg/kg, while a positive FC concentration was defined by a FC value equal or higher than 100 mg/kg16,23.

Statistical Analysis

The statistical analysis was performed with ANOVA test. Student’s t-test was used for data analysis. A p value <0,05 has been considered statistically significant. All results have been presented as median and mean ± standard deviation (SD), or as absolute count numbers when appropriate.

Results

The results were reported on Tables I, II and Figures 1, 2.

Fifty-eight children affected by SIBO and sixty healthy subjects were evaluated.

The number of males/females was 39/19 in the group of patients affected by SIBO and 36/24 in the group of healthy controls. The age range of the children in the two groups was respectively 52-202 months and 52-211 months, with a mean age of 121.8 ± 38.9 months and 126.8 ± 46.9 months. Concerning demographic data, a p value of 0.26 was calculated, demonstrating that no statistically significant differences for sex and age were observed between the two groups.

Fifty-six (96.6%) patients affected by SIBO and sixty (100%) healthy children had a negative FC value. In particular, the range of FC values obtained in the two groups was <15-174 mg/kg and <15-89 mg/kg respectively. In the group of patients affected by SIBO, a median value of 36.0 mg/kg and a mean value ± SD of 43.0 ± 31.6 mg/kg were calculated, while in the group of healthy controls the median value was 29.5 mg/kg and the mean value ± SD was 35.7 ± 20.7 mg/kg. Evaluating these results obtained in the two groups, a p value of 0.07 was calculated, suggesting that no statistically significant differences came out between FC concentrations in patients affected by SIBO in comparison with healthy children.

Discussion

For the first time, our case control study shows that FC levels in children affected by SIBO are not statistically different from those obtained in healthy controls. Our findings are similar to those pointed out by Montalto et al24, who per-
formed the only study available in literature about the correlation between SIBO and FC concentrations. Their study was carried out on an adult population: they evaluated 40 patients affected by SIBO and 40 controls, demonstrating no statistically significant differences in FC concentrations between the two groups.

In the last few years, the importance of FC measurement in the management of gastrointestinal disorders is becoming more and more evident, and it is settling as an useful marker of gastrointestinal inflammation which can support the clinical practice\textsuperscript{8}.

In fact, FC concentration increases in a number of organic gastroenterologic conditions such as colorectal cancer, NSAIDs enteropathy, alcoholic enteropathy, active inflammatory bowel diseases (IBD), acute gastroenteritis, allergic colitis and gastro-esophageal reflux disease\textsuperscript{8, 25-28}. This happens because it is released from neutrophils in gut lumen during gastrointestinal inflammation, then it binds Ca\textsuperscript{2+}, becoming resistant against heat and proteolysis. Consequently, it is eliminated intact in feces and there it can remain stable at room temperature for about 7 days\textsuperscript{10, 11}. This allows to measure it by means of a simple and non invasive laboratory test, which requires a little amount of feces. These characteristics make FC measurement a convenient laboratory test, easy to be performed by patients, especially in the pediatric age.

Furthermore, supporting data that FC can constitute a direct marker of those gastrointestinal inflammatory processes in which neutrophils are involved, some studies which compared FC measurement with invasive techniques have shown interesting results.

Røseth et al\textsuperscript{29} investigated the correlation between the faecal excretion of the granulocyte marker protein and that of 111-Indium-labelled granulocytes in patients with IBD. In fact, faecal excretion of 111-Indium-labelled neutrophilic granulocytes has been suggested as the gold standard of disease activity, but it is a complex and expensive method which expose patients to ionizing irradiation. The results obtained in this study suggested that FC reflects the granulocyte migration through the gut wall in patients with IBD and hence could be used as a simple, inexpensive alternative to the 111-indium technique.

Limburg et al\textsuperscript{30} evaluated 110 subjects with chronic diarrhea who were referred for colonoscopy and observed that increased FC levels were significantly associated with the colonoscopic and histological findings of colorectal inflammation.

A recent metaanalysis has analyzed 30 prospective studies which compared FC levels against the histological diagnosis in patients with diagnosis of IBD. It evaluated FC concentrations of 5983 adults and children and demonstrated that FC has a sensitivity of 95\% and a specificity of 91\% in IBD diagnosis. The same metaanalysis shows that the diagnostic precision in childhood
population is higher than in the adult population. Furthermore, FC values of children with IBDs in remission turn into normal ranges becoming non statistically different from those of healthy children, while they increase again in relapses, preceding clinical symptoms.

Moreover, FC values in functional symptoms have been demonstrated to be not statistically different from controls, and this is true in children affected by IBD too. So, FC can help in distinguishing functional pains from relapses in a child affected by IBD, and this is very important for these subjects because they present with an increased frequency bowel movements, urgency and abdominal cramping, and these symptoms can be mistakenly interpreted as a flare-up.

Concerning literature which has examined FC levels specifically in the pediatric age, a remarkable study is that of Berni Canani et al., who enrolled 281 children assessed for gastrointestinal symptoms. Among these subjects, those of them affected by a disease characterized by gastrointestinal mucosal inflammation, such as Crohn’s disease (38 children), ulcerative colitis (45 children) had increased FC concentrations, while 44 children suffering from functional gastrointestinal disorders (FGIDs) showed normal values. Therefore, they pointed out that FC is a sensitive but not disease specific marker to easily detect inflammation throughout the whole gastrointestinal tract and may help in identifying an organic disease and in the differential diagnosis of functional bowel disorders.

All these results impact on clinical practice because suggest that several invasive diagnostic techniques can be avoided, and this is even more important in Pediatrics.

SIBO is a condition characterized by an excessive germs proliferation, especially anaerobic, in the small bowel (more than 10⁵ CFU/ml of intestinal juice), liable to antibiotic treatment, which improves gastrointestinal symptoms.

Generally, in the intestinal tract there are 10³-10⁹ CFU/ml of bacteria such as Enterococcus and Lactobacillus, and there are a number of factors which permit to restrain bacterial overgrowth. Among these, there are anatomical and functional factors (such as gastric acidity, ileocecal valve continence, gall and pancreatic secretions and their antibacterial activity), mechanical factors (the peristalsis) and factors which inhibit bacterial adhesion to the epithelium (gastric mucus, secretory IgA and epithelial desquamation).

Moreover, gut microflora plays a crucial role in the development of intestinal defences: the colonization with diverse intestinal microbes, in fact, is necessary for the synthesis and the secretion of polymeric immunoglobulin A and the generation of a balanced T Helper cell response. By studying germ-free animals, it results that neither function exists in the germ-free state, but rapidly develops after germ colonization. Intestinal bacteria maintain “a physiological inflammation” in the human gut which is efficiently protective and necessary to have an appropriate local immune response, while a dis regulates of the mucosal immune response can switch a “controlled” toward an “uncontrolled” intestinal inflammation, paving the way to pathology. Therefore, when intestinal bacteria exceed, this label equilibrium can be broken. The presence of a higher bacterial number in the small bowel causes a premature and abnormal deconjugation of the bile acids, determining a larger jejunal reabsorption and secondary lipid malabsorption. Moreover, contaminant bacteria can cause a direct damage on entherocytes because of their adesivity on epithelial surface and because of their competition with entherocytes for the link of the complex vitamin B12 – intrinsic factor. This results in a reduction of the vitamin B12 absorption. Even if some type of bacteria can produce the vitamin theirselves, finally the subject has reduced levels of bio-availability of vitamin B12 and can have malabsorption symptoms.

Otherwise, patients affected by SIBO often suffer from a nebulous symptomatology characterized by diarrhea, flatulence, abdominal pain or discomfort. Underlying these symptoms, there is the glucidic malabsorption, which causes an accentuated fermentation and then higher production of water, short chain fatty acids and gas such as carbon dioxide, hydrogen and methane.

Whether the presence of SIBO leads to small intestinal mucosal changes is not well known. There are some investigations about the histological changes caused by SIBO in animal models, where changes of villus and crypt architecture and an increase in chronic inflammatory cells number – mostly lymphocytes of the lamina propria – have been shown.

Recently, a retrospective study has been performed on 122 subjects who underwent upper gastrointestinal endoscopic examination because of gastrointestinal symptoms. Among these patients, 67 was affected by SIBO (as assessed by duodenal aspirate culture >10⁵ CFU/ml), while
55 had a negative culture (<10^3 CFU/ml) and they were considered controls. From these duo- 
dental biopsy has emerged one feature significant- 
ly more frequent in SIBO than in controls, which 
was villous blunting to crypt ratio (<3:1)48. 
SIBO seems, also, to determine a higher level of 
IgA in the proximal small intestine particular- 
ly when the overgrowth is caused by colonic type 
bacteria49. Nevertheless, no study performed on 
patients with SIBO about direct parameters that 
indicates the number of leucocytes neutrophils in 
the gut wall are available. Montalto et al24 have 
published about FC concentrations in adults af-
fected by SIBO considering it as an indirect para-
meter of intestinal inflammation. Their results 
suggested that no inflammatory changes involv-
ing neutrophils occurs in patients with high-
er proliferation of bacteria in the small bowel. 
The presence of high FC levels in children affect-
ed by SIBO might not be caused by bacterial overgrowth itself and, in this case, another cause should be investigated.

In conclusion, our study demonstrates for the 
first time that Fecal Calprotectin values do not 
increase in children affected by SIBO. Our re-
results are similar to the findings obtained in 
adults, supporting the hypothesis that no subclini-
cal intestinal inflammation involving neutrophils 
occur in SIBO.

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