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Faecal Calgranulin C Versus Faecal Calprotectin as Non Invasive Markers Distinguishing Functional From Organic Causes of Chronic Diarrhea

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The major challenge in inflammatory bowel disease is to achieve a sensitive and specific non invasive diagnostic marker. Recently, S100A12 (Calgranulin C) have been established to be elevated in the feces of patients with IBD. The objective was to investigate the utility of fecal S100A12, in comparison to fecal Calprotectin and standard inflammatory markers, as a screening and distinguishing marker for IBD and Irritable Bowel Syndrome (IBS) in patients with chronic diarrhea. Stool samples were obtained from 173 individuals presenting with gastrointestinal symptoms requiring endoscopy. Fecal S100A12, fecal Calprotectin and serum S100A12 levels were measured and correlated to final diagnosis and standard tests (ESR, CRP, platelet count, albumin, perinuclear anti-neutrophil and antineutrophil cytoplasmic antibodies. Full colonoscopy with histopathological examination was performed. Patients diagnosed with IBD had elevated fecal S100A12 (median 49.7 mg kg⁻¹) and Calprotectin (median 385 mg kg⁻¹) levels compared with the patients without IBD (n = 35, S100A12: Median 4.6 mg kg⁻¹, p<0.0001, Calprotectin: Median 30.5 mg kg⁻¹; p<0.0001). Both the sensitivity and specificity of fecal S100A12 (cutoff 8 mg kg⁻¹) for the detection of IBD were 93.91 and 97%, respectively whereas fecal Calprotectin (cutoff 35 mg kg⁻¹) gave a sensitivity of 93.96% and a specificity of 84.2%. Both fecal markers were superior to the sensitivities and specificities of any standard inflammatory test. Both fecal S100A12 and Calprotectin are sensitive markers of gastrointestinal inflammation but fecal S100A12 provided exceptional specificity in distinguishing patients with IBD from patients without IBD.

Key words: Inflammatory bowel disease, S100A12, Calprotectin

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INTRODUCTION

Inflammatory Bowel Disease (IBD) consisting mainly of Ulcerative Colitis (UC) and Crohn's Disease (CD) is chronic idiopathic inflammatory condition of the gut with a typically remitting and relapsing course characterized by symptoms of abdominal pain, diarrhea, urgency of defecation and occasionally rectal bleeding. Treatment goal is to induce and maintain disease remission (Hanauer, 2006). The diagnosis and differentiating IBD patients from those with Irritable Bowel Syndrome (IBS) represents the major challenge to gastroenterologists. The IBD can be challenging to diagnose and fecal markers of disease that correlate with its severity could potentially be clinically useful. The etiology of Inflammatory Bowel Disease (IBD) involves complex interactions among susceptibility genes, the environment and the immune system. These interactions lead to a cascade of events that involve the activation of neutrophils, production of pro-inflammatory mediators and tissue damage (Hanauer, 2006).

Although, all studied faecal biomarkers provide reliable and simple non invasive means in the differentiation of IBD and IBS, Calprotectin appears to represent the most accurate marker to discriminate these two common causes of chronic diarrhea (Schroder *et al.*, 2007). Endoscopic examination and histological analysis of biopsy specimens remain the "gold standard" methods for detecting and quantifying bowel inflammation.

As intestinal symptoms are a frequent cause of referrals to gastroenterologists, it is mandatory to differentiate between IBD and non-inflammatory Irritable Bowel Syndrome (IBS). The natural history of IBD is characterized by an unexpected variation in the degree of gut inflammation. The gold standard methods for detecting and quantifying bowel inflammation still remain the invasive endoscopic manoeuvres and histological analysis of biopsy specimens, however, these techniques are costly, invasive and repeated examinations are unpopular with patients. Disease activity questionnaires and the widely used laboratory inflammatory markers are unreliably correlated with endoscopy and histopathology. Anti-Saccharomyces Cerevisiae Antibody (ASCA) and atypical Perinuclear Antineutrophil Cytoplasmic Antibody (P-ANCA) are the most widely studied serologic markers and remain the best characterized markers in IBD. As these serum inflammatory markers are non specific, can be elevated in a variety of conditions,

new more specific fecal markers are needed for detecting and quantifying bowel inflammation (Pardi and Sandborn, 2005).

To date, there is a paucity of biological markers to determine intestinal inflammation (Vermeire *et al.*, 2006; Niederau *et al.*, 1997). Biological markers are needed to confirm disease remission, detect early relapses and to monitor reliably anti-inflammatory therapies. Serum markers of gut inflammation are still not very useful in determining intestinal inflammation (Brignola *et al.*, 1986; Nielsen *et al.*, 2000). While, assays that detect neutrophil-derived products in stool show great promise in this issue (Langhorst *et al.*, 2005).

The members of the S100 protein family are all calcium-binding proteins and exhibit a dimeric nature. To our knowledge, 25 tissue and cell-specific S100 proteins have been recognized to date in humans. It is thought that S100 proteins carry out a series of proposed biological tasks, such as regulation of protein phosphorylation, modulation of enzyme activity, promotion of cell growth, differentiation or apoptosis, participation in Ca⁺⁺ homeostasis, preservation of cell shape and motility, regulation of coagulation and induction of proinflammatory/inflammatory responses (Santamaria-Kisiel *et al.*, 2006).

Initiation and perturbation of inflammation in IBD is mainly through activation of innate immune mechanisms. Certain S100 proteins, S100A8/A9 heterodimer (Calprotectin), S100A12 (calgranulin C) have been identified to be released by infiltrating neutrophils during cell activation and lysis (Foell *et al.*, 2007). These proteins of the S100 family proved to have quite emerging trigger for intense research towards their involvement in many different inflammatory, malignant and degenerative diseases (Salama *et al.*, 2008). Also, these S100 proteins have been linked with both digestive and extra-digestive disorders, including IBD. There are multiple emerging evidences focusing on their role in the pathogenesis activity, diagnosis and therapeutic management of IBD (Foell *et al.*, 2003a; Donato, 2003). Apart from inflammation, certain S100 proteins have also been suggested to have a crucial role in alimentary tract-related tumorigenesis (Moskaluk *et al.*, 2002).

Calprotectin, a 36 kDa calcium and zinc binding protein, is detectable both in the serum and stool during intestinal inflammation and may probably be the most promising marker for IBD. It represents 60% of cytosolic proteins in granulocytes. The presence of Calprotectin in faeces can therefore be considered directly proportional to neutrophil migration to the gastrointestinal tract

(Vermeire *et al.*, 2006; Roth *et al.*, 2003). Recently fecal Calprotectin has been proposed as a faecal marker of gut inflammation reflecting the degree of phagocyte activation (Sherwood, 2012; Manolakis *et al.*, 2011).

However, variations in faecal Calprotectin assays still restrain its routine use as a sole parameter in clinical practice. The broad expression pattern of Calprotectin in granulocytes as well as monocytes, macrophages and epithelial cells is responsible for these observed variations (Foell *et al.*, 2004; Campeotto *et al.*, 2004).

In contrast with Calprotectin, S100A12 is more restricted and predominantly expressed in granulocytes. It is abundantly secreted by the activated infiltrating neutrophils in the intestinal mucosa of patients with IBD (Vogl *et al.*, 1999). Overexpression at the site of inflammation and correlation with disease activity in a variety of inflammatory disorders affirm the role of this granulocytic specific protein as a proinflammatory molecule (Kaiser *et al.*, 2007). The S100A12 promotes gut inflammation through long-term activation of Nuclear Factor kappa B (NF- κ B) via binding to the Receptor for Advanced Glycation End-products (RAGE) (Hofmann *et al.*, 1999). In experimental studies, (mouse models of colitis), blocking the interaction of S100A12 with RAGE proved to attenuate gut inflammation confirming the crucial role for S100A12 in pathogenesis of these disorders (De Jong *et al.*, 2006). The S100A12 is overexpressed during chronic active IBD and serves as a useful serum marker for disease activity in patients with IBD (Foell *et al.*, 2003b). De Jong *et al.* (2006) reported that S100A12 can be detected in the stool of children with Crohn's disease.

We investigated the utility of fecal S100A12, in comparison to fecal Calprotectin and standard inflammatory markers, as a screening and distinguishing marker for IBD and IBS patients with chronic diarrhea.

The correlation of Fecal S100A12 and Calprotectin levels with endoscopic and histological findings in participants were also studied.

MATERIALS AND METHODS

Subjects: A total of 173 adult consecutive subjects, older than 20 years, with gastrointestinal symptoms requiring endoscopy and 35 age and sex-matched healthy subjects were initially enrolled in this prospective study from January 2012 through 2014. This study, in agreement of the World Medical Association (WMA) of Helsinki declaration (WMA, 1964), was approved by the Ethical Commission and Institutional Review Board of Mansura University Hospital in Egypt. A written informed conscious consent was obtained from all patients before

their participation. Initially, all patients completed a detailed questionnaire regarding diet and habits, submitted to thorough history taking with special emphasis on abdominal pain, weight loss, rectal bleeding, diarrhea, constipation, malaise, lethargy, anorexia, nausea, tenesmus, abdominal distension, passage of mucous, vomiting and low-grade fever along with detailed physical examinations performed at fasting in the morning.

Exclusion criteria: Patients with incomplete ileocolonoscopy, past history of any malignant condition and major gastrointestinal surgical procedures, liver cell failure, coagulopathy, chronic renal failure, urinary incontinence (contaminate the stool samples), congestive heart failure, endocrinal diseases (diabetes, thyroid disease), cardiopulmonary diseases, immune-compromised patients or on chemotherapy, advanced chronic or psychiatric illness, pregnancy, smokers, drug or alcohol abuse, Non Steroidal Anti-Inflammatory Drugs (NSAIDs), aspirin intake and any special type of dieting or treatments for the previous 6 months were excluded from the study. Eight patients were excluded and the remaining 200 participants underwent routine laboratory and radiological investigations and gastrointestinal endoscopic evaluation at enrollment.

Endoscopy: Upper endoscopy (distal duodenum), total colonoscopy and ileoscopy (distal ileum) (a gastroscope/ colonoscope; XQ40, Pentax Fiberoptic, Tokyo) performed after overnight fast and/or cleansing enemas between 08:00 and 10:00 am. The macroscopic endoscopic features of mucosa were evaluated and multiple biopsies were taken from distal duodenum, colon or ileum (from both diseased and healthy areas) for histopathological examination which is the gold standard to detect and quantify IBD (asses the severity and extent of endoscopic findings). The macroscopic endoscopic signs of inflammation were recorded by the experienced gastroenterologist using the Simplified Endoscopic Activity Score for CD (SES-CD) (Daperno *et al.*, 2004) or Rachmilewitz endoscopic score for ulcerative colitis (Rachmilewitz, 1989). These macroscopic endoscopic features may be, normal endoscopic signs, active ulcerative colitis, active Crohn's disease, celiac disease, diverticula with peri-diverticular inflammation, ischemic colitis, colorectal cancer or polyps and miscellaneous diagnoses.

Histopathological determination of disease activity: The 135 patients confirmed to have IBD to define inflammatory activity in the gut, in addition to endoscopic features, multiple biopsies were taken from different sites,

embedded in Paraffin blocks, sectioned and stained with hematoxylin and eosin for conventional histopathological examination. Biopsy sections were encoded and analyzed semiquantitatively on a four-point scale (0, 1, 2, 3; non, mild, moderate and severe inflammation, respectively) by two independent histopathologists, who were blinded to the diagnosis and clinical data. The neutrophilic infiltration in the lamina propria and epithelium was graded as absent, mild, moderate, or severe according to the updated Sydney system (Dixon *et al.*, 1996). As a further inclusion criterion, the histology score had to be in accordance with endoscopic signs of inflammation. This histology inflammation score served as the reference standard to determine the absence of inflammation or active IBD in this study cohort. Celiac disease diagnosed according to Modified Marsh Classification (Oberhuber, 2000; Marsh, 1992).

In addition, clinical disease activity in CD was documented according to the Crohn's Disease Activity Index (CDAI) (Best *et al.*, 1976) and for ulcerative colitis by using the Colitis Activity Index (CAI) of Rachmilewitz (1989). Levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cell count, platelets, hemoglobin and hematocrit were determined in all patients. Inflammatory Bowel Disease (IBD) patients were considered inactive if there were no endoscopic and histological signs of inflammation (histology inflammation score < 1) and normal disease activity scores (CDAI < 150; CAI < 6). In addition, extra-intestinal disease and small bowel involvement were excluded in Crohn's disease patients by clinical examination and contrast enhanced Magnetic Resonance Imaging (MRI) using the Sellink technique (Ajaj *et al.*, 2005).

The fifty eight patients who have infectious gastroenteritis, in addition to clinical presentation, were confirmed by microbiological stool examination, including classic culture methods for enteropathogenic bacteria and the detection of enteropathogenic viruses according to standard procedures. In patients with acute gastroenteritis stool was collected in regular sterile sample containers and sent to the laboratory within 24 h.

Thirty five patients with IBS fulfilled the ROME III criteria (Tibble *et al.*, 2002). Symptoms suggesting gastrointestinal inflammation in IBS included straining during bowel movement, the passage of mucus, weight loss and fatigue. Endoscopic and histopathological evaluations of these patients negate IBD. The ROME III, criteria for IBS: Symptoms of recurrent abdominal pain or discomfort (uncomfortable sensation not described as pain) and a marked change in bowel habit for at least six months, with symptoms experienced on at least three months. Two or more of the following

must apply: Pain is relieved by a bowel movement, onset of pain is related to a change in stool frequency, onset of pain is related to a change in stool appearance.

Healthy controls: The control group comprised 35 age and sex-matched healthy subjects (volunteers, medical students and health workers) without any signs of inflammation or intestinal symptoms. They supplied a single stool sample after giving informed consent.

Laboratory investigations: Eight milliliters of whole blood were collected onto 3 tubes, 3 mL on EDTA for CBC and ESR, 2 mL of blood were collected onto citrate for immediate assay of P.T and P.T.T and lastly 3 mL blood was collected into plain tube, prompt separation of serum was done for assay of fasting blood glucose, kidney and liver function tests, electrolytes and CRP. Part of serum was stored at -200°C until assay of ASCA and P-ANCA. Another sample was withdrawn after two hours for assay of postprandial blood glucose.

Complete blood count was performed using coulter B66, Miami, Florida, USA, liver function tests including total protein, serum albumin, AST, ALT, total and direct serum bilirubin and kidney function tests including serum creatinine, blood urea nitrogen as well as electrolytes (Na and K) and glucose were all done on Synchro CX9 autoanalyzer (Brea, California, USA), P.T and P.T.T using coagulometer. Quantitative CRP was done using nephelometry, complete stool analysis and stool culture: To affirm the presence or absence of infectious gastroenteritis.

Calprotectin and S100A12: The whole stool specimens were collected by the patients 72 h before endoscopy (before starting bowel preparation). A single stool sample (about 5 g weight) placed in a suitable disposable plastic bucket-type device that avoids toilet water artifact and simplifies laboratory sampling were frozen to -70°C immediately after collection and sent to the laboratory. Calprotectin is stable in the feces at room temperature for at least 72 h and for more than 6 months when frozen. Avoid repeated freeze-thaw cycles. About 100 mg of the faecal sample were added to 4.9 mL of diluted (at 1:50 dilution) extraction buffer solution in a screw cap tube which was then shaken vigorously for 30 sec by means of a vortex mixer then homogenized 30 min on a shaker or roller as described previously (Langhorst *et al.*, 2005; De Jong *et al.*, 2006). One milliliter of the homogenate was transferred to an Eppendorf tube and centrifuged at 10000 rpm for 20 min. Then 0.5 mL of the clear extract supernatant was transferred to another eppendorf tube and was stored at -80°C until analysis for both

Calprotectin and S100A12. The analyses for these markers were performed using aliquots from identical samples. Samples were prepared and analyzed in accordance following manufacturer's instructions.

The concentrations of S100A12 (either fecal or serum) were determined by a double sandwich ELISA system as described previously (Foell *et al.*, 2003a). The inter-assay and intra-assay CV% were 12.1 and 4.8%, respectively.

Faecal Calprotectin was determined by a commercially available ELISA kit (Calpro AS, Oslo, Norway) following the manufacturer's instructions. The inter-assay and intra-assay CV% were 4.3-5.3 and 6.1-8.7%, respectively. The value of the normal range is 5-50 mg kg⁻¹. The readers of the laboratory assay were blinded to the clinical data and diagnosis.

IBD antibodies (ASCA and P-ANCA) were detected in the first group only. The ASCA (IgA and IgG) detection were done by the QUANTA-Lite ELISA assay (Inova Diagnostics, San Diego, CA) as described elsewhere (Seibold *et al.*, 2001) while atypical P-ANCA detection was done by indirect immunofluorescence (Inova Diagnostics, San Diego, CA/EuroImmun, Germany) as described elsewhere (Seibold *et al.*, 1992). The CD markers were defined as ASCA+ve/P-ANCA-ve or ASCA+ve/P-ANCA+ve and the UC markers as P-ANCA+ve/ASCA-ve (Papp *et al.*, 2007).

STATISTICAL ANALYSIS

Data was analyzed using SPSS software (Version 17.0). Quantitative data was expressed as (Mean±SD) while qualitative data was expressed as number and percentage. The qualitative or categorical variables were described as proportions. Proportions were compared using the chi-squared test or Fisher's exact test whenever applicable. Subgroups were compared by using the McNemar test. Continuous data are expressed as median (range) and were evaluated by appropriate statistical tests. Mann-Whitney U-test was used for the continuous ordinal data between two qualitative variables. Comparisons between the groups were performed using the Student's t-test whenever applicable. Kruskal-Wallis, one way analysis of variance (ANOVA) compared more than two groups. A value of p<0.05 was considered statistically significant. Correlations between fecal markers and other variables were evaluated using the Spearman rank correlation coefficient test. Variables that achieved statistical significance with the univariate analysis were included in multiple regression analysis to evaluate the independent factors associated with high fecal Calprotectin or Calgranulin C. Sensitivity, specificity and predictive values were calculated to study the overall predictability of other techniques according to the following equations:

Positive (+ve) predictive value = (N⁰ of true +ve cases/N⁰ of all +ve cases with screening test)×100

Negative (-ve) predictive value = (N⁰ of true -ve cases/N⁰ of all -ve cases with screening test)×100

Sensitivity = (N⁰ of true +ve cases/N⁰ of all +ve cases with reference test)×100

Specificity = (N⁰ of true -ve cases/N⁰ of all -ve cases with reference test)×100

Overall predictability (accuracy) = (N⁰ of true +ve and true -ve cases/total N⁰ of all +ve and all -ve cases) ×100

RESULTS

A total of 173 patients presented with gastrointestinal symptoms in outpatient clinic and 35 age and sex-matched healthy volunteers (Control group) underwent specific laboratory, radiological investigations and diagnostic upper and lower endoscopy. The Flow chart of the study and distribution of diseases in all studied populations were shown in Fig. 1. Eight patients were excluded; three with chronic liver disease, two with previous major gastrointestinal surgery and three with cardiopulmonary disease. From the remaining 165 studied patients, 135 patients were confirmed to have IBD, 38 with UC, 27 with CD, 3 with celiac disease, 4 with diverticulitis and 58 patients with infectious gastroenteritis (GE). The remaining 35 patients fulfilled the ROME III criteria and had IBS.

The characteristics of control and patients groups either organic (IBD; UC or CD) or non-organic (IBS) GIT diseases were mentioned in Table 1. Most of IBS patients were female gender (IBS more common in female). There was statistically significant difference between studied IBD, IBS and control groups as decreased hemoglobin and serum albumin levels, elevated white blood cells (although its value was within normal ranges), elevated ESR and CRP as markers of inflammation and occurrence of bleeding per rectum in IBD group (ANOVA = 0.0001). Also, there was a remarkably statistically significant difference between patients with IBD versus either patients with non organic diseases (IBS) or healthy control as regards fecal Calprotectin and fecal S100A12 levels being highly elevated in IBD group (ANOVA = 0.0001). On the other hand, no significant differences were demonstrated between IBS and Control groups (p = 0.0001) in any parameter.

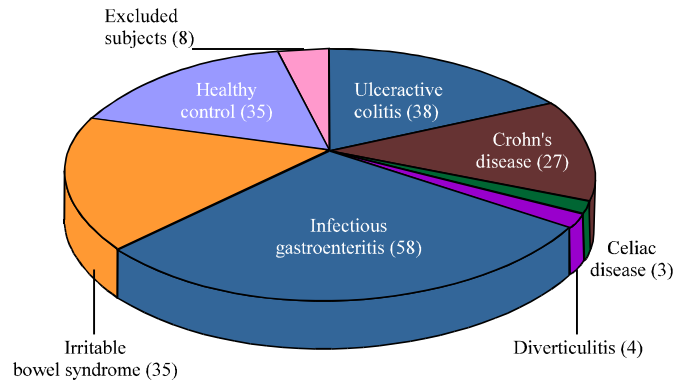


Fig. 1: Distribution of diseases in all studied populations (n = 208)

Table 1: Demographic data, clinical, serological and fecal characteristics of the studied groups

Parameters	IBD (n = 65)	IBS (n = 35)	HC (n = 35)	ANOVA (All groups)	P1 (IBD vs. IBS)	P2 (IBD vs. HC)	P3 (IBS vs. HC)
Age: Median (Range)	38 (24-60); 40.23±8.84	36 (24-60); 39.3±9.4	41 (24-60); 41.28±9.7	0.806	0.584	0.835	0.777
Sex: ♀/♂	36/29; 0.45±0.0	26/9; 0.46±0.5	20/15; 0.43±0.5	0.071	0.064	0.054	0.576
Temperature	37.2±0.29	37.3±0.28	37.3±0.4	0.812	0.572	0.835	0.554
Pulse	85.5±5.7	87.8±7.36	88.1±7.6	0.291	0.068	0.304	0.161
Diarrhea	2.42±0.77	1.34±0.48	-	0.0001	0.0001	0.0001	-
Bleeding/Rectum	0.78±0.4	0.029±0.17	-	0.0001	0.0001	0.0001	1.000
Hemoglobin	8.7±0.83	11.6±0.79	11.93±1.1	0.0001	0.0001	0.0001	0.487
Platelets	325.3±29.6	333.6±30.5	329.5±48.3	0.702	0.452	0.141	0.745
White blood cells	7.5±1.01	4.46±1.24	4.75±1.19	0.0001	0.0001	0.0001	0.904
Serum albumin	3.3±0.46	4.74±0.51	4.64±0.55	0.0001	0.0001	0.0001	0.166
Erythrocyte sedimentation rate	40.9±7.3	12.49±6.1	10.9±3.7	0.0001	0.0001	0.0001	0.113
C-reactive protein	30.4±5.1	2.86±1.3	2.85±1.2	0.0001	0.0001	0.0001	0.781
Fecal Calprotectin (µg g ⁻¹)	249.9±137.7	48.31±23.9	42.2±18.4	0.0001	0.0001	0.0001	0.183
Fecal S100A12 (µg g ⁻¹)	35.4±17.1	9.8±5.9	9.69±5.42	0.0001	0.0001	0.0001	0.671

IBD: Inflammatory bowel disease, IBS: Irritable bowel syndrome, HC: Healthy control, UC: Ulcerative colitis, CD: Crohn's disease. ANOVA compared IBD: IBS and control groups, P1-value: Compared IBD and IBS subgroups, P2-value: Compared IBD and control subgroups, P3-value: Compared IBS and control subgroups, Significance at the value ≤ 0.05

The patient's characteristics of ulcerative colitis and Crohn's disease were demonstrated in Table 2. No significant differences were observed between the two sets of patients (UC or CD) regarding demographic, clinical, serological and histopathological characteristics ($p = 0.05$). The majority of patients (19/21) with ASCA-/pANCA+ were confirmedly diagnosed as UC compared with only two who diagnosed as CD. On the other hand, the majority of patients (17/19) with ASCA+/pANCA+ or ASCA+/pANCA- were confirmedly diagnosed as CD compared with only two who diagnosed as UC. Twenty five patients were ASCA-/pANCA- (UC/CD; 17/8).

The correlations between fecal markers (fecal S100A12 and fecal Calprotectin) and other parameters in patients with IBD were demonstrated in Table 3. Both fecal S100A12 and fecal Calprotectin were significantly positively correlated with duration, chronicity and severity of the IBD, increased frequency of diarrhea, occurrence of bleeding per-rectum, increased

inflammatory markers as ESR and CRP, elevated WBC count and increased platelets and increased pulse rate ($p \leq 0.05$). Moreover, both fecal markers were significantly positively correlated with clinical disease activity indices (CAI and CDAI), endoscopic scores (Rachmilewitz and SES-CD) and histology inflammation score ($p \leq 0.05$). Also, fecal S100A12 was significantly positively correlated with fecal Calprotectin ($p \leq 0.05$). Contrary, fecal S100A12 and fecal Calprotectin were significantly negatively correlated with hemoglobin level ($p < 0.001$) and serum albumin ($p \leq 0.05$). Such correlations were not observed regarding age, sex, ASCA, P-ANCA and CMV ($p < 0.05$).

The effect of disease location on fecal S100A12, fecal Calprotectin and other inflammatory markers in patients with inflammatory bowel disease were shown in Table 4 and Fig. 2. All test markers performed equally well regardless the disease location. However, the test for fecal Calprotectin was significantly higher in case of colonic involvement.

Table 2: Demographic data, clinical, serological and fecal characteristics features of patients with inflammatory bowel diseases

Parameters	Ulcerative Colitis (n=38)	Crohn's disease (n=27)	p-value
	Mean± SD	Mean± SD	
Age: Median (Range)	38 (24-58); 39.9±9.17	41 (29-60); 40.7±8.49	0.954
Sex: ♀/♂	21/17; 0.45±0.5	15/12; 0.56±0.51	0.427
Temperature	37.3±0.29	37.2±0.27	0.949
Pulse (min)	85.6±5.76	86.7±5.36	0.073
Diarrhea	2.3±0.77	2.56±0.75	0.883
Bleeding/Rectum	0.71±0.46	0.6±0.498	0.339
Hemoglobin (g dL ⁻¹)	8.66±0.86	9.13±0.39	0.628
Platelets	334.4±30.4	331.3±27.6	0.240
White blood cells	7.89±1.1	7.25±0.84	0.101
Serum albumin	3.01±0.47	3.41±0.45	0.816
Erythrocyte sedimentation rate	40.05±7.2	42.2±7.4	0.581
C-reactive protein	29.68±5.1	31.4±7.01	0.486
Fecal Calprotectin (µg g ⁻¹)	204.9±142.8	252±144.7	0.786
Fecal S100A12 (µg g ⁻¹)	29.66±14.8	31.4±15.39	0.948
Inflammation score	2.37±0.99	2.44±1.1	0.148
Disease location	13 Right colic, 16 left colic, 5 pan-colic, 4 others (proctitis or sigmoiditis)	11 Ileum, 5 Caecum, 6 colonic, 5 others (proctitis or sigmoiditis)	-
Clinical disease activity index	CAI: 8.76±4.55	CDAI: 188.4±29.4	-
Endoscopic score	Rachmilewitz: 9.08±3.3	SES-CD: 15.07±9.7	-
ASCA-/pANCA+ (UC marker; n)	19	2	-
ASCA+/pANCA- (CD marker; n)	1	9	-
ASCA+/pANCA+ (CD marker; n)	1	8	-
ASCA-/pANCA- (n)	17	8	-
Cytomegalovirus (+/-)	23/15	17/10	-

CAI: Colitis activity index, CDAI: Crohn's disease activity index, ASCA: Anti-*Saccharomyces cerevisiae* antibody, SES-CD: Simple endoscopic score for Crohn's disease, P-ANCA: Peri-nuclear anti-neutrophil cytoplasmic antibody

Table 3: Spearman's rho correlations between fecal S100A12 and fecal Calprotectin and other parameters in patients with inflammatory bowel disease

Parameters	Fecal Calprotectin (µg g ⁻¹)		Fecal S100A12 (µg g ⁻¹)	
	r	p-value	r	p-value
Age	-0.036	0.773	0.072	0.5700
Sex	-0.012	0.922	-0.086	0.4970
Temperature	0.525*	0.0001	0.425*	0.0001
Pulse	0.449*	0.0001	0.539*	0.0001
Diarrhea	0.675*	0.0001	0.335*	0.0005
Disease duration (Chronicity)	0.541*	0.0001	0.531*	0.0001
Bleeding/Rectum	0.413*	0.0001	0.431*	0.0001
Hemoglobin	-0.320*	0.009	-0.390*	0.0001
Platelets	0.643*	0.0001	0.643*	0.0001
White blood cells	0.450*	0.0001	0.450*	0.0001
Serum albumin	-0.823*	0.0001	-0.823*	0.0001
C-reactive protein	0.721*	0.0001	0.614*	0.0001
Erythrocyte sedimentation rate	0.770*	0.0001	0.874*	0.0001
Fecal Calprotectin (µg g ⁻¹)	1.000	-	0.346*	0.005
Fecal S100A12 (µg g ⁻¹)	0.346*	0.005	1.000	-
Histology inflammation score	0.656*	0.0001	0.328*	0.0080
Crohn's disease activity index	0.547*	0.0001	0.470*	0.0001
Colitis activity index (Rachmilewitz)	0.594*	0.0001	0.423*	0.0001
Simple endoscopic score for CD	0.412*	0.01	0.439*	0.005
Rachmilewitz endoscopic score for UC	0.537*	0.0001	0.324*	0.009
Anti-saccharomyces cerevisiae antibody	0.236	0.059	0.223	0.074
Perinuclear anti-neutrophil cytoplasmic antibody	-0.079	0.531	0.075	0.551
Cytomegalovirus	-0.106	0.525	-0.011	0.948

*Correlation is significant if p<0.05 (2-tailed)

The linear regression analysis of fecal S100A12 and fecal Calprotectin to other parameters was demonstrated in Table 5. High level of fecal S100A12 as well as fecal Calprotectin could predict presence of high pulse rate, increased frequency of diarrhea, occurrence of bleeding

per-rectum, high WBC and platelets count, low level of hemoglobin, low serum albumin, high CRP and ESR and more colonic involvement. Moreover, high level of both fecal markers could significantly predict higher clinical disease activity indices (CAI and CDAI), endoscopic

Table 4: Fecal (S100A12 and Calprotectin) and other inflammatory markers in patients with inflammatory bowel disease according to disease location

Parameters	Disease location						Total	ANOVA
	Right colic	left colic	Pan-colic	Ileum	Caecum	Others, proctitis, sigmoiditis		
No. of patients	13	16	11	11	5	9	65	
Fecal S100A12 ($\mu\text{g g}^{-1}$)								
Mean	47.8	45.4	38.8	34.9	37.8	39.1	35.4	0.156
SD	15.6	13.8	13.8	12.3	8.58	4.3	17.1	
Fecal Calprotectin ($\mu\text{g g}^{-1}$)								
Mean	250	315	243.9	221.6	82.8	268.9	249.9	0.034
SD	120.8	122.8	156.9	150.7	32.7	119.6	137.7	
Erythrocyte sedimentation rate								
Mean	39	41.88	42.36	40.45	37.8	42.78	40.95	0.659
SD	6.3	8.02	7.839	7.047	8.76	6.99	7.3	
C-reactive protein								
Mean	29.1	30	31.81	30.09	29.4	32.1	30.4	0.707
SD	4.35	6.25	4.534	4.805	5.2	5.42	5.1	
Platelets								
Mean	319.2	327.7	328.3	326	319.4	328.7	325.3	0.959
SD	24.05	34.2	31.54	26.6	33.34	33.9	29.6	
White blood cells								
Mean	7.02	7.49	7.600	7.48	7.4	7.9	7.46	0.508
SD	1.49	0.78	0.67	1.126	0.92	0.748	1.01	
Serum albumin								
Mean	3.4	3.2	3.3	3.29	3.3	3.18	3.27	0.882
SD	0.43	0.46	0.53	0.473	0.49	0.49	0.46	

Table 5: Linear regression analysis of fecal S100A12 and fecal Calprotectin to other parameters in patients with inflammatory bowel disease

Parameters	Fecal S100A12		Fecal Calprotectin	
	B	p-value	B	p-value
Pulse rate (min)	0.439	0.0001	0.058	0.049
Diarrhea frequency	0.353	0.0005	0.476	0.008
Bleeding/Rectum	0.454	0.0001	0.313	0.007
Hemoglobin (g dL^{-1})	-0.395	0.0001	-0.220	0.031
Platelets (mm^3)	0.743	0.0001	0.343	0.015
White blood cells (mm^3)	0.350	0.001	0.250	0.037
Serum albumin (g dL^{-1})	-0.723	0.0001	-0.723	0.004
C-reactive protein	0.634	0.0001	0.621	0.0004
Erythrocyte sedimentation rate	0.864	0.0001	0.780	0.0001
Disease location (colonic)	0.531	0.0001	0.281	0.021
Fecal Calprotectin ($\mu\text{g g}^{-1}$)	0.326	0.005	-	-
Fecal S100A12 ($\mu\text{g g}^{-1}$)	-	-	0.346	0.0001
Histology inflammation score	0.347	0.008	0.457	0.0003
Crohn's disease activity index	0.573	0.0001	0.248	0.0019
Colitis activity index (Rachmilewitz)	0.634	0.0001	0.504	0.0013
Simple endoscopic score for CD	0.549	0.005	0.342	0.006
Rachmilewitz endoscopic score for UC	0.324	0.009	0.418	0.005

Significant if $p \leq 0.05$

Table 6: Diagnostic accuracy of fecal and other markers in distinguishing active inflammatory bowel disease from irritable bowel syndrome and healthy control

Parameters	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPP (%)	Accuracy (%)	AUC	95% CI
Fecal S100A12 ($\mu\text{g g}^{-1}$)	8	93.91	97.0	99.1	73.0	96.1	0.983	0.897-0.998
Fecal Calprotectin ($\mu\text{g g}^{-1}$)	35	93.96	84.2	97.3	69.6	92.6	0.797	0.669-0.887
C-reactive protein (mg mL^{-1})	5	63.20	60.0	78.9	40.6	62.2	0.673	0.540-0.809
Erythrocyte sedimentation rate (mm h^{-1})		60.00	59.0	78.3	37.0	60.0	0.654	0.512-0.776
Colitis activity index	6	68.80	100	100	37.5	73.77	0.675	0.635-0.788
Crohn's disease activity index	150	52.90	100	100	23.8	60.5	0.621	0.532-0.779

PPV: Positive predictive value, NPP: Negative predictive value, AUC: Area under the curve, 95% CI: 95% confidence interval

scores (Rachmilewitz and SES-CD) and histology inflammation score ($p = 0.05$). Also, high level of fecal S100A12, significantly predicted high level of fecal Calprotectin ($p \leq 0.05$).

The diagnostic accuracy of fecal S100A12 and fecal Calprotectin and other markers in distinguishing IBD from IBS and healthy control were shown in Table 6. Cutoff points of $8 \mu\text{g g}^{-1}$ and $35 \mu\text{g g}^{-1}$ for fecal S100A12 and

fecal Calprotectin, respectively were used to distinguish active IBD from IBS and healthy controls. Both fecal biomarkers (S100A12 and Calprotectin) were found to distinguish between IBD and IBS with almost equal sensitivity (~94%) but with higher specificity for fecal S100A12 (97% vs. 84.2% for fecal Calprotectin) and higher diagnostic accuracy (96.1% vs. 92.6%). The test accuracy indicated by sensitivity, specificity, PPV, NPP and odd

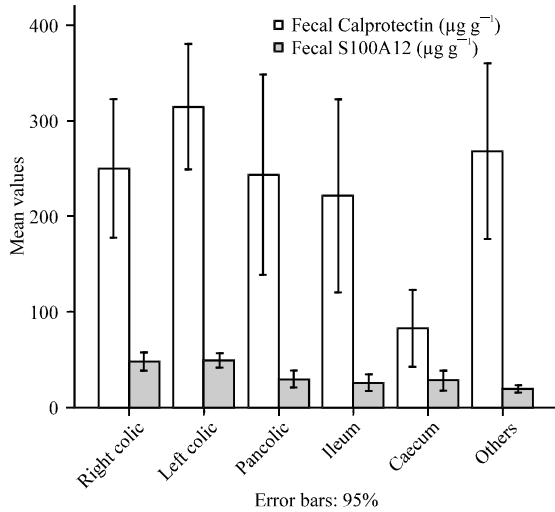


Fig. 2: Fecal markers in patients with inflammatory bowel disease in relation to disease location

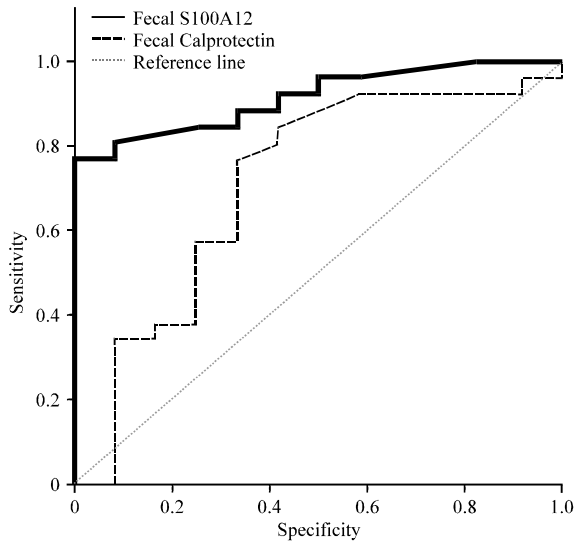


Fig. 3: Receiver Operating Characteristics (ROC) curve for fecal Calprotectin and fecal S100A12 in differentiating patients with inflammatory bowel disease from those with irritable bowel syndrome and healthy control

ratio was superior for fecal S100A12 compared to any other marker. Other markers including ESR, CRP, CDAI and CAI had much less diagnostic accuracy than fecal markers.

The receiver operating curve analyses was performed and demonstrated in Fig. 3 to determine cut off points for fecal markers measurements. Both fecal biomarkers (S100A12 and Calprotectin) were found to distinguish

IBD from IBS and healthy control with a better result for faecal S100A12 (AUC: 0.983, 95% CI: 0.897-0.998) than for fecal Calprotectin (AUC: 0.797, 95% CI: 0.669-0.885).

DISCUSSION

Various types of organic (IBD) and non organic (IBS) GIT disorders may have similar symptoms and clinicians suffer a lot in differentiating between the two categories especially the management is completely different and the clinical examination alone may not be sufficient to give a specific diagnosis. Since the proposed methods assessing bowel inflammation are nonspecific, complex and expensive or expose the patient to pain, ionizing radiation or other risks, there is a need for a simple, inexpensive, non invasive and objective method helping in screening and selecting patients for further additional investigation, for instance endoscopy and biopsy.

Damage to gastrointestinal mucosal layer occurred in most of organic GIT disorders like IBD vary from increased permeability to ulceration. Such injury provokes polymorphonuclear neutrophilic (PMN), granulocyte infiltration and migration to gut lumen where their contents either leaked or are released including Calprotectin and S100A12 proteins (Foell *et al.*, 2007).

Faecal estimation of these protein cytokines and markers of neutrophil activation in intestinal inflammation is attractive and promising in the clinical routine as they could provide a non-invasive examination that can help screening patients with mild GIT symptoms and may have roles in discriminating IBD from IBS. Moreover, their measurement could be helpful in follow up inflammation and early detection of relapse or subclinical activity (Pardi and Sandborn, 2005).

Lactoferrin, myeloperoxidase (MPO) and polymorphonuclear (PMN) elastase are faecal markers of neutrophil degranulation but are not expressed in neutrophils only and are of limited stability in stool samples. Other markers of neutrophil-derived proteins, lysozyme or human neutrophil lipocalin are less promising (Peterson *et al.*, 2002).

Recently, neutrophil-derived S100 proteins have been identified and extensively investigated as faecal markers distinguishing IBD from IBS. S100 proteins [S100A8/A9 (Calprotectin), S100A12 (Calgranulin C)] are cytosolic molecules released by activated or damaged cells during cell stress and have a remarkable stability in stool samples in room temperature (resist degradation by faecal bacteria) making them suitable markers for gut inflammation (Foell *et al.*, 2009).

As expected and as these proteins are expressed by infiltrating granulocytes, their fecal levels are significantly higher in IBD than in IBS. However, some conflicts about their levels in active disease compared with inactive IBD had been observed (Sugi *et al.*, 1996). The most extensively investigated faecal marker is Calprotectin (S100A8/S100A9 complex). In many previous studies, fecal Calprotectin has been claimed to be a specific, sensitive, non-invasive, cheap and accessible biomarker for gut inflammation that correlates well with disease activity in IBD and can distinguish IBD from IBS with a reported sensitivity varies between 65% and 95% and specificity between 75% and 91%, (Langhorst *et al.*, 2005; Foell and Roth, 2005; Judd *et al.*, 2011).

In the IBD, many symptoms are similar to the functional disorder IBS. Thus the major challenge is to distinguish symptoms of IBD from IBS. In this study, fecal Calprotectin can also significantly differentiate organic gastrointestinal diseases including IBD from non organic disorders including IBS patients with an overall diagnostic accuracy of 92.6%. The results obtained in this study for fecal Calprotectin are therefore within the range found in previous studies. In a previous study, Calprotectin levels were elevated in subgroups of IBD patients that are in remission and have coexisting IBS-like symptoms and can still differentiate IBD from IBS (Jelsness-Jorgensen *et al.*, 2013).

The observed variations of Calprotectin results could be explained by variable presentation of epitopes on the Calprotectin complex under different conditions and the broad expression pattern of Calprotectin induced in epithelial cells under certain circumstances influencing the results (Foell and Roth, 2005).

Also, faecal Calprotectin have been elevated in bacterial enteritis and rotavirus infections (Judd *et al.*, 2011). In contrast, faecal S100A12 was not elevated in viral gastroenteritis as was faecal Calprotectin and lactoferrin. Collectively, fecal S100A12 biomarker is more specific for neutrophils than Calprotectin and neutrophil activation is less involved in viral gastroenteritis than in bacterial infections.

Patients with IBD were significantly presented by high pulse rates although within normal value, high ESR and CRP, high WBC count, bleeding per rectum and high fecal Calprotectin level. Similarly, Xiang *et al.* (2008) found that patients with active UC had higher levels of CRP and ESR than patients with inactive UC and control group. This could be explained by the fact that these parameters are elevated in many systemic inflammatory conditions as acute phase reactant. The CRP is one of the many acute phase proteins that increase in the serum of patients with active IBD. It is more sensitive in cases of CD than UC but not specific for gut inflammation. So, the

use of CRP and ESR only to identify patients with IBD that need further evaluation would retard the diagnosis of many cases.

S100A12 is almost exclusively expressed in large amounts by granulocytes which play a crucial role in pathogenesis of IBD and does not form heterodimers with S100A8 or S100A9. It is secreted exclusively by activated neutrophils and promotes inflammation through Receptor for Advanced Glycation Endproducts (RAGE) activation (Schmidt *et al.*, 2001; Dabritz *et al.*, 2014). The neutrophil influx into the intestinal mucosa is closely related to IBD activity and inflammation. Therefore, neutrophil-derived S100A12 in tissues and exudates strongly correlates with inflammatory activity. Moreover, the excretion of this protein into the gut lumen could reflect the number and activation status of infiltrating neutrophils (Foell *et al.*, 2003b). Fecal S100A12 measurement is specific for neutrophil activity during bowel inflammation but is not strictly disease specific. Recently, it was reported that S100A12 is detectable in stool samples, where it is evenly distributed and is as stable as Calprotectin for up to 7 days (De Jong *et al.*, 2006).

In the current study, a strong significant correlation between fecal biomarkers (Calprotectin and S100A12) and intestinal inflammation confirmed either endoscopically (SES-CD or Rachmilewitz Endoscopic score for UC) or histologically. In contrast, a number of studies have found clinical and endoscopic scores to be of little value in monitoring of disease activity.

In this study, faecal S100A12 was demonstrated to discriminate between IBD and IBS with high diagnostic accuracy and significantly distinguish active from inactive disease and even could differentiate UC from CD. Moreover, the test for faecal S100A12 performs equally well regardless of disease location. These findings are analogous to Calprotectin which is also more accurate in ulcerative colitis (Costa *et al.*, 2005). De Jong *et al.* (2006) reported similar results. The present study also confirm the fact that neither faecal marker of intestinal inflammation is completely specific for IBD but rather for intestinal inflammation in general, because the levels of these phagocyte-derived proteins are also elevated during infectious gastroenteritis. In this regard, S100A12 as a specific marker of neutrophil activation may be useful in monitoring disease activity.

In the present study, the histology inflammation score was the gold standard for diagnosis. Faecal S100A12 strongly correlates with intestinal tissue inflammation suggesting that the infiltrating neutrophils are the main source of faecal S100A12. Kaiser *et al.* (2007) found that immunohistochemical staining of tissue sections confirmed S100A12 expression in the gut of patient with active IBD more than inactive disease and IBS

confirming that infiltrating neutrophils are the main source of faecal S100A12.

The clinical disease activity indices and endoscopic scores, hindered by inaccuracies due to subjective components, are too indistinct to reflect inflammatory activity in chronic IBD and cannot provide precise information about the patient status. Endoscopic and radiological procedures are too invasive, time-consuming or expensive for routine use, or carry risk of radiation exposure. Serological markers (e.g., CRP, ESR) reflect the presence and intensity of a systemic inflammation and are not specific for intestinal inflammatory disease. Whereas most serological biomarkers are of limited use, faecal markers of neutrophil activity can indicate intestinal inflammation. Once bacterial enteritis is ruled out, faecal S100A12 may be an excellent non-invasive marker of IBD activity superior to other biomarkers including faecal Calprotectin. Faecal Calprotectin being derived from monocytes and potentially from epithelial cells is less specific for infiltrating neutrophils.

CONCLUSION

Faecal S100A12 (Calgranulin C) is a novel non-invasive reliable reproducible surrogate biomarker which strongly correlated with inflammation score with the potential to discriminate chronic IBD with or without active mucosal lesions from those with IBS or healthy control, with superior sensitivity, specificity and diagnostic accuracy to other inflammatory biomarkers including fecal Calprotectin. It could be an adjunctive tool in overall evaluation of patients with nonspecific symptoms and as a practicable objective diagnostic tool in those with IBD. This neutrophil-derived S100A12 protein can significantly improve arsenal of non-invasive biomarkers of intestinal inflammation.

RECOMMENDATIONS

Further longitudinal studies are required in the future to consolidate the promising initial data, improve our knowledge about the biology of S100 proteins and determine the value of faecal S100A12 in diagnosis, monitoring of inflammation and prediction of the disease course in other GI disorders.

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