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Performance of Quantitative Fecal Hemoglobin Versus Fecal Calprotectin as Markers of Crohn's Disease Activity and Severity

KEYWORDS: Crohn's disease, fecal calprotectin, fecal hemoglobin, endoscopic score

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ABSTRACT *Background: Crohn's disease (CD) is characterized by recurring flares and remissions. Fecal biomarkers especially fecal calprotectin (f-Cal) have promising results as non invasive tools to assess disease activity and severity. However, these markers are often difficult to process and expensive. The aim of this study was to evaluate the performance of quantitative fecal hemoglobin (f-Hb) measurement in comparison to f-Cal measurement, in the assessment of CD activity and severity.*

Methods: This study was performed on 52 patients of previously diagnosed Crohn's disease, undergoing colonoscopy, and 26 healthy control subjects. Stool samples were collected for measurements of f-Hb and f-Cal, and blood samples for measurements of C-reactive protein (CRP) and routine blood tests. Endoscopic disease activity and severity were assessed and scored according to the Simple Endoscopic Score for CD (SES-CD).

Results: There were significant positive correlations between SES-CD and all tested markers; serum CRP, f-Cal and f-Hb (r=0.395, r=0.670 and r=0.663, respectively). Using the statistically determined cutoff levels of f-Cal and f-Hb (177 µg/g and 1.6 µg/g, respectively), the sensitivity, specificity and overall diagnostic accuracies of both f-Cal and f-Hb in determining disease activity were very close (87.5% vs 83.3, 85.7% vs 85.7%, and 86.5 vs 84.6%, respectively) and much higher than that of CRP. Likewise, the sensitivity, specificity and overall diagnostic accuracies of both f-Cal and f-Hb in determining disease severity were also close (83.3% vs 83.3, 75.0% vs 66.7%, and 79.2 vs 75.0%, respectively).

Conclusion: F-Hb can be used as a reliable simple and inexpensive marker of CD activity and severity with a diagnostic accuracy similar to f-Cal.

INTRODUCTION:

Crohn's disease (CD) is a devastating, lifelong disease characterized by inflammation of the gastrointestinal mucosa with recurrent flares of activity and remissions [1]. Physicians used to apply a combination of clinical, laboratory, and endoscopic criteria to detect activity, assess severity, predict relapse and monitor the effect of therapy in CD [2].

Many noninvasive systemic and fecal markers had been investigated in CD patients for objective measurement of disease activity and severity. Among the serum markers are C-reactive protein (CRP), erythrocyte sedimentation rate, platelets count, α1 acid glycoprotein, anti-Saccharomyces cerevisiae, perinuclear antineutrophil cytoplasmic antibody and bacterial flagellin [3]. Unfortunately, these systemic markers have insufficient specificity and sensitivity, and despite the advantages of CRP over other markers, it is still far from ideal [2,4].

Many fecal biomarkers e.g. fecal calprotectin (f-Cal), Alpha1 antitrypsin, alpha2-macroglobulin and lactoferrin, have emerged and most of them especially f-Cal showed better specificity than systemic markers [3]. There is accumulation of evidence for the promising role of f-Cal in the assessment of CD activity and severity [5-8]. F-Cal represents 60% of cytosolic proteins in granulocytes. Increased fecal concentrations of this protein are proportional to the neutrophils migration to the gastrointestinal mucosa [9]. Assessment of f-Cal level allows distinguishing inflammatory bowel disease (IBD) from non inflammatory diseases, such as irritable bowel syndrome [10]. However, the measurement of f-Cal is not simple, not available in all institutions and relatively costly [11]. Therefore an additional noninvasive, simple, easily available and inexpensive test that is sensitive and specific is needed for follow-up of CD patients.

Quantitative measurement of fecal hemoglobin (f-Hb) concentration has successfully replaced guaiac-based fecal occult blood tests for screening, assessment of severity and follow up of colorectal carcinoma in Japan and many Western countries [12-14]. Hence, f-Hb measurement was presumed to correlate with endoscopic assessment of CD activity and severity. The aim of this study was to evaluate the performance of quantitative f-Hb measurement in comparison to f-Cal measurement, in the assessment of CD activity and severity.

PATIENTS AND METHODS

A total of 52 patients with previously diagnosed CD were prospectively enrolled in this study from August 2012 to August 2014. The enrolled patients were those scheduled for follow up colonoscopy at gastroenterology unit in Royal Commission Medical Center (RCMC), Yanbu, Saudi Arabia, for whatever indication e.g. flare of the disease and assessment of endoscopic activity after medical treatment. Apparently healthy 26 control subjects (from patients relatives), not on any regular medication, were also enrolled in the study, but did not undergo colonoscopy. The study was approved from the ethical committee of RCMC, and an informed written consent was obtained from each participant. All patients were subjected to thorough clinical evaluation including clinical disease activity assessment.

Fresh stool samples were collected from control subjects as well as from all patients three days prior to colonoscopy (just before starting colonic preparation). A blood sample was also collected from all participants at the same day

Volume : 5 | Issue : 1 | Jan 2015 | ISSN - 2249-555X

of stool sample collection for assessment of CRP and routine laboratory parameters including complete blood cell counts (CBC).

All patients underwent complete colonoscopy including intubation of the terminal ileum, performed by the same expert gastroenterologist, under deep sedation. Endoscopic features of activity and severity of CD were recorded according to Simple Endoscopic Score for Crohn's Disease (SES-CD); each examined segment of the intestine (rectum, left colon, transverse colon, right colon, and ileum) was graded from 0 to 3 points according to the following endoscopic variables: presence and size of ulcers, extension of ulcerated surface and of affected surface, and presence and type of narrowings. An endoscopically active disease was defined as an SES-CD score > two points [15].

Exclusion criteria included incomplete colonoscopy (the terminal ileum not reached), poor colonic preparation incompatible with adequate endoscopic assessment, previous history or endoscopic evidence of another ulcerative or neoplastic gastrointestinal disease, history of infectious enterocolitis within one month before colonoscopy and regular intake of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs). Patients with coexisting and serious cardiopulmonary, hepatic, renal, neurologic, psychiatric, or rheumatologic disease, and those having had a colostomy or ileostomy were also excluded from the study.

F-Cal measurement:
Quantitative determination of f-Cal was done by lateral flow assay using the BÜHLMANN Quantum Blue® reader.

- Principle of the assay: Selective measurement of calprotectin antigen by sandwich immunoassay. A monoclonal capture antibody highly specific for calprotectin is coated onto the test membrane. A second monoclonal detection antibody conjugated to gold colloids is deposited onto the conjugate release pad and released into the reaction system after addition of the extracted and diluted stool sample. The calprotectin/anti-calprotectin gold conjugate binds to the anti-calprotectin antibody coated on the test membrane (test line) and the remaining free anti-calprotectin gold conjugate binds to the goat anti-mouse antibody coated on the test membrane (control line). The signal intensities of the test line and control line are measured quantitatively by the Quantum Blue® reader.
- Assay procedure: Extraction of stool sample (about 100 mg) was done using special extraction device, then put in clean tubes which can be kept at 2-8 C° for 6 days. The sample was diluted 1 : 16 with extraction buffer, then after dilution, the sample can be kept at – 20 C° up to 4 months before assay. Diluted extract was centrifuged for 5 min. at 3000 r.p.m. The test Cassette was loaded onto the Cassette holder of the reader, and 60 ul of diluted stool sample was added onto the sample loading port of test Cassette. Scanning was done automatically after 12 min., and f-Cal concentration was calculated by the reader using a lot-specific standard curve.

F-Hb measurement:
It was quantitatively estimated by latex agglutination immuno-turbidimetry using the OC-Sensor DIANA Autoanalyzer (Eiken Chemical Co.).

- Principle of the assay: Polyclonal antibodies directed against human hemoglobin A are adsorbed on latex particles. In the presence of blood in stool, they provoke the antigen-antibody reaction and agglutination. Changes in sample turbidity by latex agglutination are measured optically at 570 nm wave length.
- Assay procedure: 10 mg fecal sample was collected into OC auto sampling tubes containing 2 ml buffer using special sample probe, then the tubes were loaded into the Analyzer racks to measure the absorbance of light through these tubes.

CRP measurement:
It was quantitatively determined in serum on Cobas Integra 400 auto analyzer by particle - enhanced immune-turbidimetric assay (Roche diagnostics). Normal range is up to 5 mg /L.

Statistical analysis
Statistical analysis was performed using Statistical Package for Social Science (SPSS), version 19. Results of numerical data were presented as means, standard deviation (SD) and ranges or as numbers and percentages. One way ANOVA test and independent t test were used as appropriate. P value <0.05 was considered statistically significant. Spearman's correlation test using the Spearman's r correlation coefficient was applied. Receiver operating characteristic (ROC) analysis was helpful for the determination of the optimal cutoff levels of CRP, f-Cal, and f-Hb for discriminating CD endoscopic activity and severity. Determination of the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy were used for the comparison of the respective accuracies of biomarkers in predicting endoscopic activity and severity.

RESULTS
A total of 52 CD patients (mean age: 21.2±4.7 years, females: 59.9%) and 26 control subjects (mean age 23.2±6.2 years, females: 53.8%) were prospectively enrolled in the study. The location of the disease and medication used during study period were described in table (1). Table (2) shows various endoscopic activity groups and severity index subgroups with their corresponding concentrations of serum CRP, f-Cal and f-Hb. Based on the SES-CD, 46.2% (24/52) of patients had active disease; half of them had mild disease (12/52), one third of them had moderate disease (8/52), whereas, only four patients had severe disease. Overall, there were statistically significant trends toward increase of the concentrations of all tested markers with either the presence of CD, the active disease state or the increasing severity of the disease. In addition, using Spearman's correlation test, there were significant correlations between SES-CD score and all tested markers; serum CRP ($r=0.395$, $p=0.004$), f-Cal ($r=0.670$, $p<0.001$), and f-Hb ($r=0.663$, $p<0.001$). Table (3) shows the results of ROC statistics determining the respective accuracies of tested markers in assessment of CD activity and severity, as compared to SES-CD score. Using the statistically determined cut-off levels of serum CRP, f-Cal and f-Hb (5 mg/L, 177 µg/g and 1.6 µg/g, respectively), the sensitivity, specificity and overall diagnostic accuracies of both f-Cal and f-Hb in determining disease activity were very close (87.5% vs 83.3, 85.7% vs 85.7%, and 86.5 vs 84.6%, respectively) and much higher than that of CRP (figure 1). Likewise, the sensitivity, specificity and overall diagnostic accuracies of both f-Cal and f-Hb in determining disease severity, however being not satisfactory high, were close (83.3% vs 83.3, 75.0% vs 66.7%, and 79.2 vs 75.0%, respectively) and higher than that of CRP.

Volume : 5 | Issue : 1 | Jan 2015 | ISSN - 2249-555X

Table 1: Characteristics of all participants enrolled in the study.

	CD patients (n=52) No. (%), or mean±SD (range)	Control subjects (n=26) No. (%), or mean±SD (range)	P value
Age (years)	21.2±4.7 (16-40)	23.2±6.2 (16-39)	0.108
Female gender	31 (59.6)	14 (53.8)	0.627
Active cigarette smoking	4 (7.7)	3 (11.5)	0.575
Location of the disease			
– Ileocolonic	32 (61.5)	-	
– Ileal	18 (34.6)	-	
– Colonic	2 (3.8)	-	
Current Medication			
– 5-ASA	7 (13.5)	-	
– Steroids	10 (19.2)	-	
– Azathioprine	26 (50.0)	-	
– Biologic treatment	12 (23.1)	-	

Table 2: Variations of CRP, f-Cal and f-Hb concentrations according to SES-CD score.

Parameter	**Controls** (n= 26) Mean ± SD (range)	**CD patients** (n=52) Mean ± SD, (range)				
		Inactive CD SES-CD:0-2 (n=28)	**Active CD SES-CD: > 2** (n=28)			Overall CD SES-CD:0-32 (n=52)
			Mild CD SES-CD:3-6 (n=12)	**Moderate CD SES-CD:7-15** (n=8)	**Severe CD SES-CD:≥16** (n=4)	**All active CD SES-CD:3-32** (n=24)	
CRP (mg/L)	2.95±1.4[a]	5.8±5.3[b]	12.3±12.7[c]	40.6±51.1	120.5±91.1	39.8±59.0	21.5±43.3 (1.0-206.0)
f-Cal (µg/g)	35.8±11.6[a*]	157.8±142.3[b*]	695.5±671.9[c]	1210±588.8	1788.0±517.7	1049.1±725.0	569.2±670.1 (49.0-2228.0)
f-Hb (µg/g)	0.47±0.4[a*]	1.04±0.79[b*]	6.0±6.1[c]	10.3±6.4	18.4±1.8	9.5±7.1	4.95±6.4 (0.19-20.5)

[a]P< 0.05 CD patients versus controls. *[a*]P < 0.001 CD patients versus controls.*

[b]P< 0.05 active versus inactive disease. *[b*]P < 0.001 active versus inactive disease.*

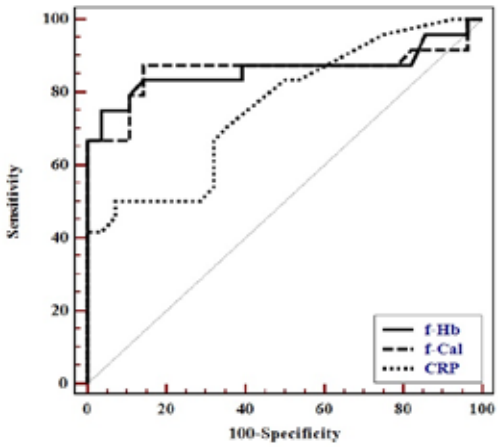
[c]P< 0.05 among the three severity index subgroups.

Table 3: ROC statistics determining the accuracies of CRP, f-Cal and f-Hb in assessment of CD activity and severity, as

	Accuracy in activity assessment					Accuracy in severity assessment				
	Cut-off value	**Sen (%)**	**Spe (%)**	**AUC (95% CI)**	**Overall Accuracy (%)**	**Cut-off value**	**Sen (%)**	**Spe (%)**	**AUC (95% CI)**	**Overall Accuracy (%)**
CRP (mg/L)	5.0	66.7	67.9	0.759 (0.63-0.89)	69.2	18.0	66.7	75.5	0.694 (0.47-0.92)	70.8
f-Cal (µg/g)	177.0	87.5	85.7	0.861 (0.74-0.98)	86.5	1028.0	83.3	75.0	0.785 (0.59-0.98)	79.2
f-Hb (µg/g)	1.6	83.3	85.7	0.861 (0.74-0.98)	84.6	7.7	83.3	66.7	0.788 (0.60-0.98)	75.0

Sen: sensitivity, Spe: specificity, CI: confidence interval

Volume : 5 | Issue : 1 | Jan 2015 | ISSN - 2249-555X



compared to SES-CD score.

Figure 1: ROC curve determining accuracy in assessment of CD activity, using f-Hb, f-Cal and CRP.

DISCUSSION

The evaluation of activity and severity is still one of the challenging aspects of CD management, as there is no single "gold standard" test or examination to rely on. Instead, physicians apply in this aspect a combination of, clinical features, laboratory indices, radiologic findings, and endoscopy with histology [16-17]. The correlation between patient symptoms and endoscopic activity and severity of CD is often limited. Colonoscopy has the drawbacks of being invasive, time-consuming, and expensive [18-19]. Moreover, most of serum and fecal biomarkers have several limitations, either due to poor reliability, high cost or unavailability in most centers [2,4,11,17].

In this study, according to the correlation with endoscopic scoring index, we found an overall nearly equal ability of f-Hb and f-Cal to correlate with the activity and severity of CD (r = 0.663 and r=0.670, respectively), and a superiority of both fecal markers to serum CRP (r=0.395). This was in agreement with other studies which concluded that, fecal biomarkers have better specificity and correlate better with endoscopic scoring, in IBD patients, than CRP and other serum markers, as the later could be affected by many systemic factors [3,17,18,19]. However, CRP has been recently demonstrated to be particularly helpful in predicting mucosal healing in response to treatment with antitumor necrosis factor agents [18].

Several previous studies in pediatric and adult patients, despite being usually concerned with ulcerative colitis (UC) rather than with CD, have demonstrated that f-Cal level is reflective of the activity and severity of mucosal inflammation in IBD [5-8,17]. The correlation coefficients between f-Cal and the severity of mucosal disease activity described previously, based on colonoscopic examination, were in accordance with those obtained in this study for f-Cal testing, because they ranged in previous studies between 0.48 and 0.83 in CD [19-21], and between 0.51 and 0.83 in UC [22-24]. Moreover, in the literature, when IBD activity was assessed by endoscopy, the sensitivities and specificities of f-Cal to identify active mucosal disease, ranged from 70% to 100% and from 44% to 100%, respectively, what were in accordance with those obtained in this study [19-20,22,24].

In the current study, we evaluated the accuracy of f-Cal testing in reflecting endoscopic activity and severity of CD, and used a cutoff level of f-Cal ≥177 µg/g derived from ROC statistics, which was near to that mentioned by other authors. The sensitivity, specificity and overall diagnostic accuracy in detecting active mucosal inflammation (SES-CD >2) in our CD patients were, 83.3%, 87.5% and 86.5% respectively. Whereas, using a much higher (statistically calculated) cutoff level of ≥1028 µg/g, the sensitivity, specificity and overall diagnostic accuracy of f-Cal in detecting moderate to severe mucosal inflammation (SES-CD >6) were, 83.3%, 66.7% and 75.0%, respectively.

Quantitative fecal immunochemical tests, which can rapidly quantify fecal blood with automated equipment, have been used for screening, assessment of severity and follow up of colorectal neoplasia [12-14]. Based on this fact, Nakarai et al., [25] have performed quantitative f-Hb assessment in 152 UC patients, and results were compared with colonoscopic findings, a positive f-Hb test (≥1 µg/g) predicted active mucosal inflammation with a sensitivity of 87% and a specificity of 60%. They recommended quantitative f-Hb assessment as an easy, rapid and effective method that can noninvasively help evaluate disease activity of UC.

Similarly, in this study we evaluated the accuracy of quantitative f-Hb measurement in reflecting endoscopic activity and severity of CD. Using a cutoff level of quantitative f-Hb ≥1.6 µg/g, the sensitivity, specificity and overall diagnostic accuracy in detecting CD activity in our patients were, 83.3%, 85.7% and 84.6%, respectively. Whereas, using a cutoff level of quantitative f-Hb ≥7.7 µg/g, its sensitivity, specificity and overall diagnostic accuracy in distinguishing moderate to severe mucosal inflammation from mild inflammation were, 83.3%, 66.7% and 75.0%, respectively.

In this study, according to the correlation with endoscopic scoring and the ROC derived diagnostic accuracy of each fecal marker, we found an overall strong and equal ability of f-Hb and f-Cal to correlate with the severity of mucosal damage and to distinguish between endoscopically active and inactive CD.

One of the main limitations to f-Cal use is that it is nonspecific and may be found elevated due to infectious enterocolitis, colorectal cancer or NSAIDs therapy [25]. Although, these limitations also exists with f-Hb, yet, it is much cheaper, more available and easily performed than f-Cal.

In conclusion, quantitative f-Hb measurement represents a novel and cheap biomarker, useful to easily detect and monitor CD activity and severity, that is as reliable as f-Cal.

Volume : 5 | Issue : 1 | Jan 2015 | ISSN - 2249-555X

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