

Original Article

Fecal Calprotectin, CRP and Leucocytes in IBD Patients: Comparison of Biomarkers With Biopsy Results

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Abstract

Background: This study aimed to compare fecal calprotectin (FC) levels with other commonly used parameters as part of patient care during evaluation for inflammatory bowel disease (IBD).

Methods: We recruited adult IBD patients with ulcerative colitis (UC) and Crohn's disease (CD) and compared the results of the patient's biopsy results (i.e., inflamed versus noninflamed) for six sites (i.e., ileum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum) with concentrations of C-reactive protein (CRP), total leucocytes and fecal calprotectin (FC).

Results: We found that FC was significantly elevated in a concentration-dependent manner that correlated with the number of active inflammation sites reported in biopsy. Although CRP and leucocyte measurements trended upwards in line with inflammation reported from biopsy, the results were highly variable and highlighted poor reliability of these biomarkers for indicating IBD inflammation.

Conclusions: These results strongly suggest that FC correlates best with biopsy reports and is a superior marker than CRP and leucocytes.

Keywords: *Fecal calprotectin; Inflammatory bowel disease; c-reactive protein; Bowel biopsy*

Introduction

The detection and monitoring of intestinal inflammation are the basis of diagnosis and follow-up of the two major forms of inflammatory bowel disease (IBD): Crohn's disease (CD) and ulcerative colitis (UC) (1). Endoscopy is the current gold standard for characterizing IBD and is employed to directly assess ongoing mucosal inflammation (2). This procedure is expensive, invasive and associated with significant patient preparation, waiting times and discomfort while carrying a small but significant risk of complications (3,4). Currently used

blood and serological laboratory tests such as total leucocyte count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) offer indirect, objective but nonspecific markers for IBD in most patients (5,6). Several studies have demonstrated relatively poor sensitivity and specificity for utility of these biomarkers in IBD diagnosis and monitoring treatment (4,5,7). CRP, for example, has been shown in ~50% of the patients with UC to be in the normal range (7,8). Additionally, CRP can be elevated due to many factors unrelated to IBD including infection, rheumatoid and autoimmune diseases (7). Recent

literature indicates that fecal biomarkers show a much stronger correlation with mucosal inflammation in IBD (8,9).

Calprotectin, a calcium- and zinc-binding protein produced in abundance by activated neutrophils (accounting for ~40% of cytosolic proteins of neutrophils (10,11)), represents a more sensitive (93%) and specific (96%) biomarker of IBD when compared to traditional blood and serological markers (12). The relatively high amount of neutrophil migration and turnover in the gut lumen provides an opportunity to utilize calprotectin in stool (fecal calprotectin [FC]) as a biomarker of IBD. Other inflammatory and infectious conditions are also known to elevate FC (e.g., autoimmune enteropathy, diverticulitis, bacterial dysentery, intestinal polyps and colon cancer) (8,13,14). The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is also known to lead to inflammatory flares and elevate FC levels (15). FC is highly resistant to pancreatic and intestinal enzyme degradation, and thus, FC protein properties are ideally suited for home collections in stool samples, exhibiting impressive stability performance in most assays (approximately 7 days at room temperature), offering ample time for patients to transport specimens to the clinical laboratory for analysis. Many studies are currently evaluating the utility of FC for both identification of IBD as well as its quantitative relationship with severity of IBD (16–18). However, relatively little information is available characterizing the relationship between each parameter (i.e., CRP, leucocytes, FC and active inflammation) in patients being monitored. In this study, we recruited adult patients diagnosed with either CD or UC undergoing care at the Division of Gastroenterology, McMaster University, and compared the reported concentration of blood and serological and stool analytes (CRP, leucocytes, calprotectin) to biopsy reports.

METHODS

Twenty-six IBD patients (comprising 5 CD and 21 UC patients, 6 male and 20 female, average age 41.3 ± 13.8 years) were recruited following approval from the Hamilton Integrated Research Ethics Board (HIREB) by informed consent. The exclusion criteria for IBD patients included (1) any positive results from stool culture, ova, and parasites or *Clostridium difficile* tests (via: loop-mediated isothermal amplification test), (2) inability to provide informed consent, (3) presence of serious life-threatening comorbidities, (4) colectomy, (5) toxic megacolon and (6) acute gastrointestinal bleeding. The endoscopic evaluation of disease activity was noted as normal or as inflamed with disease location and histological assessment by the pathologist based on standard criteria. For each of the 26 IBD patients included in the study, biopsy result for six sites (i.e., ileum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum), were assigned as either inflamed or noninflamed, and for statistical analysis patients

were grouped into four inflammation site counts (i.e., 0, 1 to 2, 3 to 5, >5 sites). FC extractions were performed within 48 hours of sample collection using the Smart Prep Extraction device from Roche Diagnostics for liquid stool and Stool Sample Application System (SAS) from Immunodiagnostik for sift/firm stool according to the respective manufacturer's instructions. Following homogenization, the extracts were centrifuged at $3000 \times g$ for 5 minutes; thereafter, supernatants were collected and stored at -20°C until assay. FC was measured using the two-site ELISA sandwich technique, whereby monoclonal capture antibodies highly specific to the calprotectin heterodimeric and polymeric complex are coated on the microtiter plate (PhiCal Calprotectin-EIA, Immunodiagnostik, Cat# K6927) kits were used according to the manufacturer's instructions on an automated DSX platform.

Lithium heparin and potassium EDTA tubes were utilized for CRP and leucocyte blood collections, respectively. All biomarkers were measured within 3 weeks of biopsy specimen collection from the following six bowel sites: ileum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. Patients were instructed to avoid NSAID use and there was no change in care during the period between blood work/stool sample collection, and biopsies (median interval 10 days, IQR 5.5 days.)

Data were plotted and analyzed using Microsoft Excel (2016) and Graphpad Prism 8 (2019) software (one-way analysis of variance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). ROC curves, sensitivity, specificity, negative and positive predictive values analyzed and generated using MedCalc version 18.11.6 (2019) software.

RESULTS

Results of Biomarkers in IBD Patients with Known Biopsy Reports

To determine the relationship between the various biomarkers and GI tract inflammation within our limited sample group, we grouped known IBD patients according to the explicit biopsy results reporting either inflammation or no inflammation into groups of zero inflammation sites ($n = 7$), one to two inflammation sites ($n = 8$), three to five inflammation sites ($n = 5$) or more than five inflammation sites ($n = 6$). Figure 1A demonstrates a concentration-dependent rise in FC as a function of number of inflammatory sites. In patients with no inflammatory sites identified (i.e., 0 sites), the average FC concentration was 87.9 ± 18.7 (42 to 134 confidence interval [CI]; expressed as mean mg/kg \pm SEM, 95% lower and upper CIs). The average FC concentration in patients with one to two inflammatory detected increased to 606.5 ± 207 mg/kg (117 to 1096 CIs). Although substantial, this elevation was not statistically significant compared to 0 site group in our sample group comparison (P -value = 0.3). In patients with three to five inflammation sites,

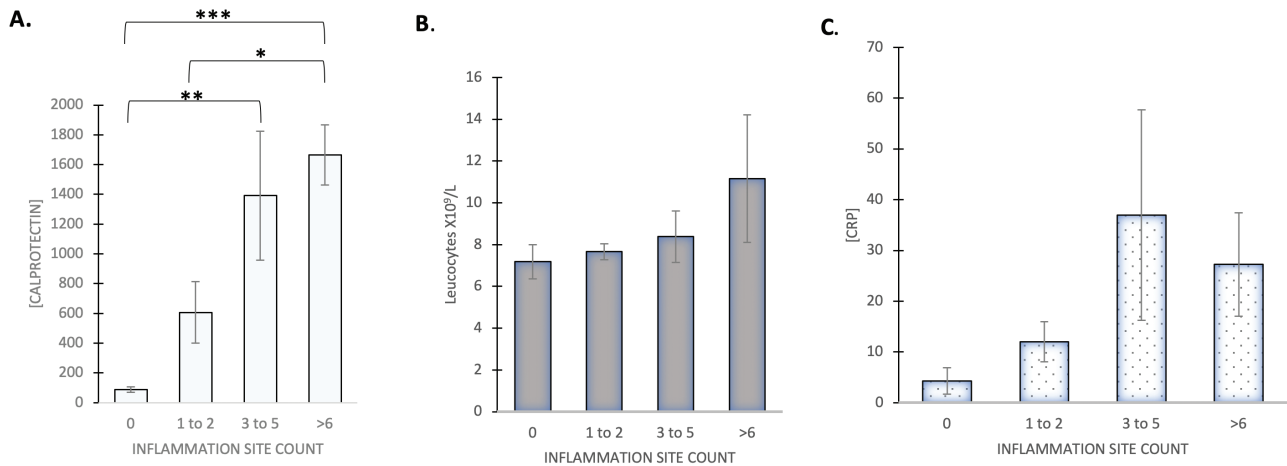


Figure 1. The magnitude of FC concentrations are higher in IBD patients ($n = 26$) with more active inflammation sites throughout the gastrointestinal tract. (A) Statistically significant relationship between the active inflammation sites and the concentration of FC. (B) Leucocyte relationships in the same patients demonstrated a modest rise as a function of inflammation sites. (C) CRP concentrations were generally higher but more variable with active sites. One-way analysis of variance analysis, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. CRP, C-reactive protein; FC, Fecal calprotectin; IBD, Inflammatory bowel disease.

the average FC concentration was 1391 ± 433 (118 to 2594 CIs). This rise was significantly elevated when compared to the 0 site group (P -value < 0.01). In patients with greater than five sites noted (i.e., >5 group), the average calprotectin concentration was 1664 ± 203 (1144 to 2185 CIs). This 19-fold rise was statistically significant when compared to zero sites (P -value < 0.001) and was also significantly different when compared to one to two inflammation sites ($P < 0.01$). These results demonstrate that FC levels correlate with the number of inflammation sites in our patient population.

Next, we compared the total leucocyte count to the inflammation site count groups (Figure 1B). The average total leucocyte count in patients with zero sites was 7.2 ± 0.82 (5.2 to 9.2; results expressed as mean $\times 10^9/L \pm SEM$ [95% lower and upper CIs]). A modest rise in leucocytes numbers was observed in patients with one to two inflammation sites (7.7 ± 0.38 [6.8 to 8.6]) as well as in patients with three to five inflammation sites (8.4 ± 1.2 [5 to 11.3]). Patients with more than five sites exhibited a mean leucocyte count of 11.2 ± 1.2 (7.3 to 19), which was not significantly different to any other group (e.g., P -value = 0.3 compared to 0 sites).

We then evaluated the serological inflammatory marker CRP and compared its levels to the inflammation site count groups (Figure 1C). The average CRP concentration in patients with zero sites was 4.3 ± 2.62 (-2.1 to 10.7 CI; units expressed as mg/L $\pm SEM$ [95% lower and upper CIs]). We observed an increase in CRP concentrations, albeit not statistically significant in patients with one to two inflammation sites (12 ± 3.92 [2.7 to 21.3]). A more substantial but highly variable insignificant rise (P -value = 0.13 compared to 0 sites group) in CRP concentration was seen in patients with three to five inflammation

sites (36.9 ± 20.73 [-20.6 to 94.5 CIs]). Patients with more than five sites exhibited an average CRP of 27.2 ± 10.22 (1 to 53.5 CIs), which was again not significantly different to any other group (e.g., $P = 0.34$ compared to 0 sites group.)

Correlation of Biomarkers to Inflammation

We next examined the relationship between each biomarker and inflammation site count identified by biopsy reports. As Figure 2A illustrates, there was a linear relationship between FC concentration with the inflammation site count as FC concentrations generally increased as a function of the inflammation site count ($R^2: 0.48$). This relationship significantly exceeded the correlation performance of CRP ($R^2: 0.12$) and leucocyte ($R^2: 0.11$) to inflammation sites count. When compared between themselves (Figure 3), blood biomarkers exhibited a very poor performance: CRP and FC R^2 value was 0.07, leucocyte and FC R^2 value was 0.08, and CRP and leucocyte R^2 value was 0.02.

Finally, we generated receiver operator characteristic (ROC) curves of our patient group graphically depicting the test performance (Figure 4). As anticipated, the cut-off value of 100 mg/kg exhibited impressive sensitivity (89.5%); however, the suboptimal specificity (58.9%) remains a distinct limitation of Utilizing this cut-off. The positive predictive value (PPV, 100%) and negative predictive value (NPV, 77.8, [CI: 48.5 to 92.9]) indicated impressive clinical utility. The 250 mg/kg had a slightly lower sensitivity (77%), but the specificity was ideal at 100%. The PPV (100%) and NPV (63.6% CI: 42.3 to 80.7) indicate that the test is more useful for PPV interpretation. CRP test performance at the cut-off (>5.1) demonstrated sensitivity and specificity of 78.9% and 85.7%, respectively, and PPV and

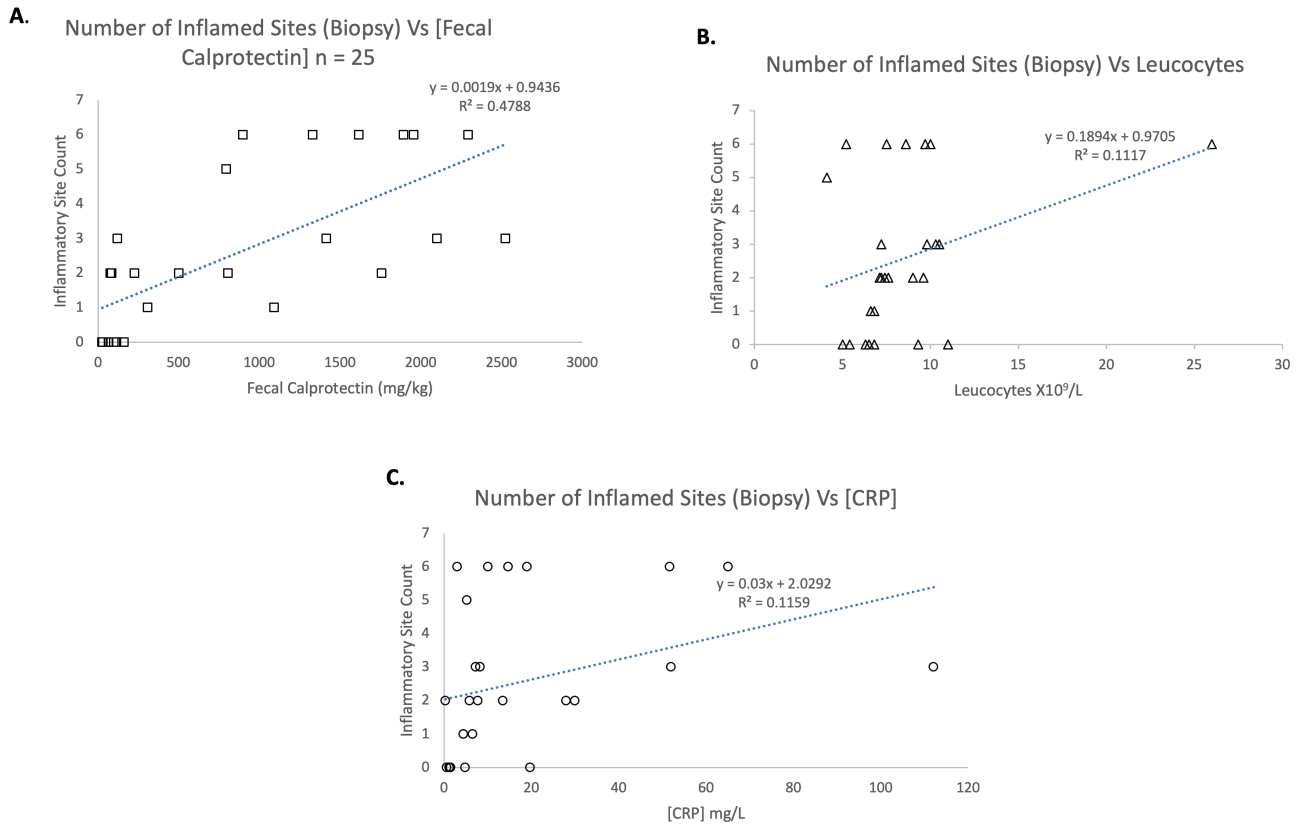


Figure 2. Comparison of inflammatory sites to FC, leucocytes and CRP concentration. CRP, C-reactive protein; FC, Fecal calprotectin; IBD, Inflammatory bowel disease.

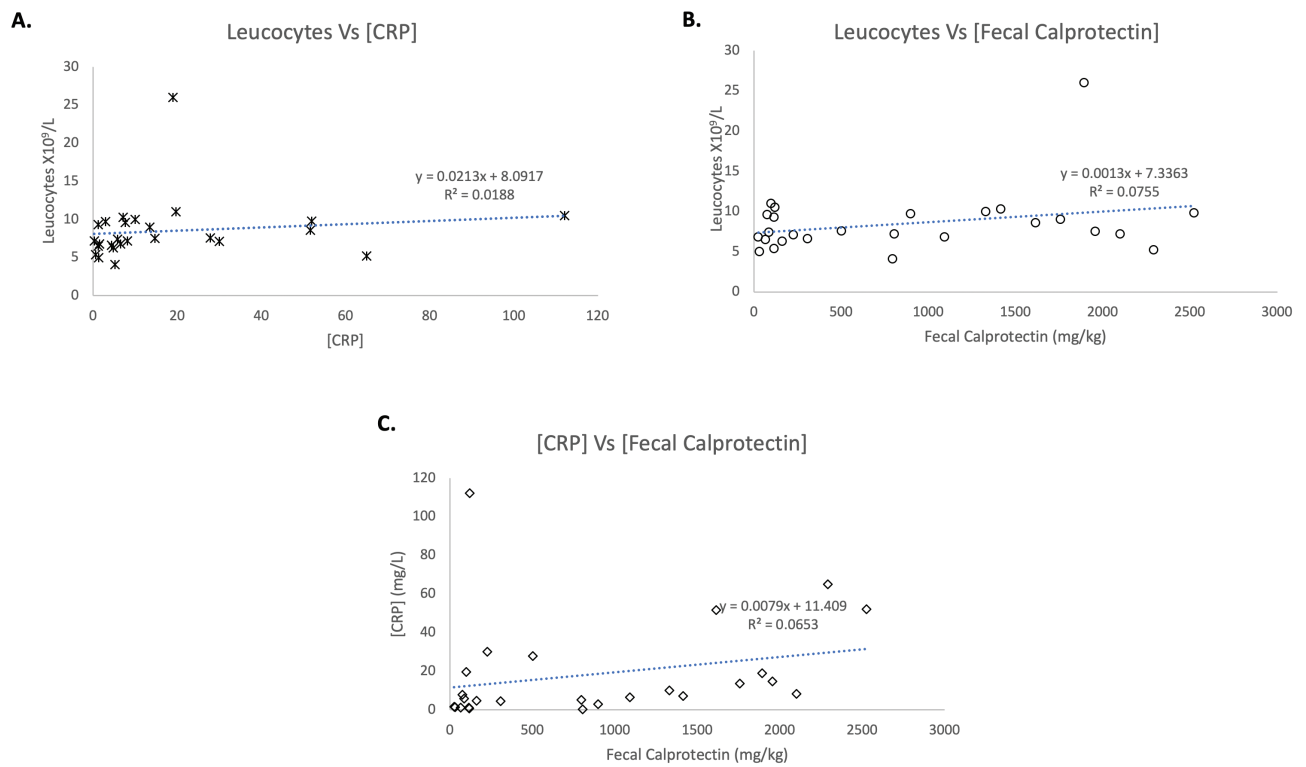


Figure 3. Comparison of inflammatory sites to FC, leucocytes and CRP concentrations. CRP, C-reactive protein; FC, Fecal calprotectin; IBD, Inflammatory bowel disease.

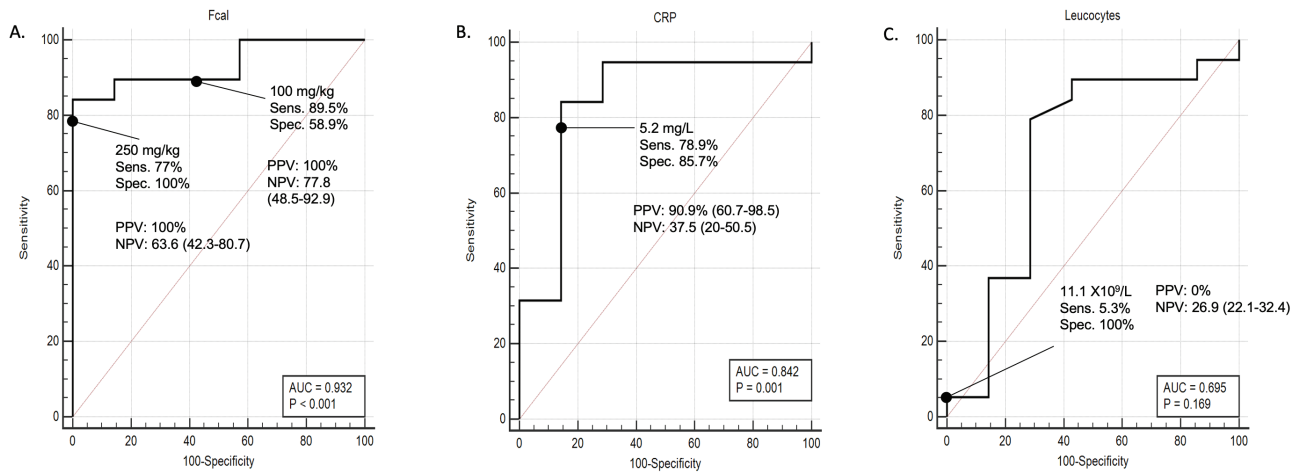


Figure 4. ROC curve graphical illustration for FC, CRP and leucocytes for the 26 patients. Patients with histological inflammatory sites >0 positive were assigned positive status (i.e., ‘gold-standard’ positive). Confidence intervals (95%) are shown in brackets. **(A)** FC ROC curve analysis demonstrated the best test performance (AUC: 0.93) throughout concentration range. **(B)** CRP curve analysis demonstrated the next best performance (AUC 0.84). **(C)** Leucocyte ROC curves illustrated the poor utility of this test biomarker (AUC 0.7). AUC, Area under the curve; CRP, C-reactive protein; FC, Fecal calprotectin; IBD, Inflammatory bowel disease, ROC, Receiver operator characteristic.

NPV of 90.9% (CI: 60.7 to 98.5) and 37.5% (CI: 20 to 50.5). Leucocytes had particularly poor sensitivity (5.3%) although the specificity at the recommended cut-off was 100%. This translated to a PPV of 0% and NPV of 26.9% (22.1 to 32.4).

Discussion

In the current study, we evaluated the commonly used blood biomarkers (namely CRP, leucocyte count) and FC to monitor IBD patients and how they correlate with the severity of inflammation. This study directly demonstrates that FC has substantially better association with biopsy results for IBD when compared to traditional biochemical and cellular markers of IBD in blood. While many recent studies explore the relationship between FC and biopsy reports (12,13,18,19), and FC and blood biomarkers (6,8,20), few have collectively explored all of the parameter relationships in the same patients.

Biomarker Results in Patients with no Active Inflammation Sites

With none of the six sites biopsied exhibiting active inflammation, all biomarkers generally showed a low, sub-cut-off trend. Of the six patients with negative biopsy results, four would have fallen under the 100 mg/kg FC cut-off value many clinicians use (18,21–23) (mean: 87.9 mg/kg). Three patients exceeded the 100 mg/kg value into the ‘grey zone’ (i.e., 100 to 250 mg/kg area). None breached the commonly used upper cut-off (18) of 250 mg/kg for likely inflammation. All but one of the CRP levels in the same patients were below the CRP cut-off (<5.1 mg/L, mean: 4.3 mg/L). All patients had leucocyte levels below the cut-off range (i.e., 4.00 to 11.0 × 10⁹/L, mean: 7.2). These findings indicate that reliance on the CRP level in one patient in our study

(19.7 mg/L) would have resulted in inappropriate endoscope procedure despite the absence of active inflammation on biopsy. FC concentration (98.4 mg/kg) would have had utility in ruling out the likelihood of inflammation in this particular patient.

Biomarker Results in Patients with One to Two Active Inflammation Sites

In eight patients with one to two active sites identified, six patients had FC concentrations exceeding the 100 mg/kg cut-off value, five of which also exceeded the 250 mg/kg (mean: 606.5 mg/kg). CRP values were highly variable, with two patients below the cut-off (mean 12 mg/L). Leucocytes showed a modest elevation (mean 7.7 × 10⁹/L), none of the patients exceeded the cut-off. These findings demonstrate that using either FC or CRP values would have resulted in two patients falling below cut-off with active inflammation.

Biomarker Results in Patients with three to five Active Inflammation Sites

In five patients with three to five active sites identified, all patients had FC concentrations exceeding the 100 mg/kg and 250 mg/kg cut-off values (mean: 1391 mg/kg). Again, CRP values were highly variable (ranging 5.2 to 112.1 mg/L, mean 36.9 mg/L), with three patients in the normal range. As highlighted in the result section of this study, leucocytes showed a modest elevation (mean 8.4 × 10⁹/L), and none of the patients exceeded the cut-off. These findings demonstrate that FC concentrations (i.e., a measure of neutrophil activity (24, 25)) strongly correlate with the degree of inflammation (i.e., good sensitivity), while CRP was within the reference interval in three out of five patients. Such a discrepancy would have resulted in a failure to identify inflammation in these patients. Leucocytes were of particularly low utility in all patients.

Biomarker Results in Patients with Three to Five Active Inflammation Sites

The most impressive demonstration of the utility of the FC test occurred in patients with more than five sites of inflammation identified by biopsy. FC exceeded the commonly used cut-offs (i.e., 100 and 250 mg/kg) by an order of magnitude, correlating well with the biopsy data. These findings support the observation that the magnitude of FC elevation correlates with the degree of inflammation, an ideal property for a reliable biomarker. Surprisingly, in one patient with more than five site of inflammation, and FC of 899 mg/kg, the CRP concentration (3 mg/L) was still below the cut-off, raising concern that in some patients, even during extensive inflammation in process, CRP clinical sensitivity remains inadequate to identify such processes. The CRP level is also unreliable in interpreting the magnitude of inflammation, given that it ranged from 3 to 65 in this group of patients (i.e., >5 inflammation sites). As before, leucocytes were generally mildly elevated relative to 0 site group, but only one patient ($26 \times 10^9/L$) exceeded the cut-off.

Correlation of Biomarkers and Inflammation

Consistent with the literature, FC far out-performed the CRP and leucocyte biomarkers of inflammation, steadily trending upwards in concentration as a function of the inflammation site count. These data further support the previous data illustrating the significant concentration-dependent relationship of FC to inflammation site count, and the poor reliability and utility of CRP and leucocytes for determining inflammation related to IBD.

The superior test performance of FC was evident when we examined the parameters of sensitivity, specificity, PPV and NPV at the relevant cut-off concentrations. FC, examined at 100 mg/kg and 250 mg/kg was generally superior to CRP for each parameter with the exception of sensitivity at 250 mg/kg for FC (77%) compared to CRP (78.9%). This was further supported by a superior area under the curve value for FC when analyzed via ROC curve analysis when compared to both CRP and leucocytes. Additionally, this analysis starkly highlights the lack of clinical utility for leucocytes as a meaningful marker of inflammation for the IBD patient group (5.3% sensitivity).

Conclusions

Our study highlights the impressive clinical sensitivity and specificity utilities of FC in identifying patients with ongoing inflammation. This study is based on a small cohort of participants, which is one of the limitations that must be considered. However, our findings revealed notable information on the utility of biomarkers in diagnosing and monitoring IBD and do emphasize the need for future studies with larger populations.

FC concentrations were significantly elevated in a concentration-dependent manner in line with biopsy reports.

The poor utility of CRP and leucocytes were evident, lacking in a meaningful significant relationship (i.e., linear) with the biopsy reports. Reliance on these biomarkers should be used with caution as we demonstrate that several patients would have fallen in the normal range, which was in contrast to the biopsy reports indicating ongoing active inflammation. These results strongly suggest that FC demonstrates a better correlation with biopsy reports and is a superior marker than CRP and leucocytes count.

Author Contributions: B.D.K., T.A.A., S.H. and W.I.K. designed the study. B.D.K., T.A.A., M.S.S., J.M., U.C. and S.H. recruited the patients. B.D.K. analyzed the data and generated figures. B.D.K., S.I. and W.I.K. wrote the manuscript.

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