Prospective evaluation of faecal neutrophil-derived proteins in identifying intestinal inflammation: combination of parameters does not improve diagnostic accuracy of calprotectin

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SUMMARY

Background
Differentiating symptoms of irritable bowel syndrome from those of organic intestinal disease is a common clinical problem. Several neutrophil-derived proteins have been proposed as a marker of inflammatory bowel disease.

Aim
To compare the diagnostic value of faecal calprotectin, lactoferrin and polymorphonuclear neutrophil-elastase in distinguishing inflammatory bowel disease from irritable bowel syndrome.

Methods
Eighty-eight adult patients with a history of chronic diarrhoea of unknown origin were screened. All patients underwent a complete work-up to identify the underlying cause. In addition, a single stool sample was assayed for faecal calprotectin, lactoferrin and polymorphonuclear neutrophil-elastase by enzyme-linked immunosorbent assay.

Results
Within the study cohort inflammatory bowel disease was diagnosed in 45 patients and irritable bowel syndrome in 31 patients. The sensitivity and specificity of calprotectin for inflammatory bowel disease were 93% and 100%, respectively. In contrast, the respective diagnostic values for lactoferrin and polymorphonuclear neutrophil-elastase were 82% and 100% and 84% and 87%, respectively. Neither combination of markers did improve the diagnostic power compared with calprotectin alone.

Conclusions
Although all faecal biomarkers studied provide a reliable and simple non-invasive means in the differentiation of inflammatory bowel disease and irritable bowel syndrome, calprotectin appears to represent the most accurate marker to discriminate between these two common causes of chronic diarrhoea.

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INTRODUCTION

Irritable bowel syndrome (IBS) is probably one of the most frequent causes of chronic diarrhoea in adults affecting 6–22% of the general population in industrialized countries with an annual incidence ranging from 6% to 9% (for review see Ref.1). In routine clinical practice, gastroenterologists are often faced with the diagnostic difficulty of differentiating between patients with IBS and those with intestinal pathology, in particular, inflammatory bowel disease (IBD). Many symptoms are common in both conditions such as diarrhoea, abdominal pain, bloating and excessive flatus, whereas rectal bleeding and systemic illness will increase the likelihood of inflammatory disease. Because the clinical differentiation remains problematic, many patients within the IBS category are investigated extensively with invasive endoscopic and radiographic imaging to make a diagnosis of exclusion. This has a significant impact for both health costs and exposing patients to the inherent risks associated with such diagnostic procedures.

During the last decade, several leucocyte products excreted in faeces have emerged as candidate biomarkers of intestinal inflammation (for review see Ref.2). Among those neutrophil-derived proteins calprotectin and lactoferrin appear to be the most promising surrogate parameters. Lactoferrin is a 76 kDa iron-binding glycoprotein that is the major component of the secondary granules of polymorphonuclear neutrophils (PMN) and is secreted by most mucosal membranes. The secondary granules are released by PMNs when they degranulate during inflammatory processes as lactoferrin has an important role in the innate immunity as a bactericidal.3, 4 During intestinal inflammation, PMN infiltrate the mucosa, resulting in an increase in the concentration of lactoferrin in the faeces and its presence is proportional to neutrophil translocation to the gastrointestinal tract.5 Calprotectin, also known as L1 protein, MRP-8/14, calgranulin and cystic fibrosis antigen, is a 36 kDa calcium- and zinc-binding protein found in neutrophils and comprises up to 60% of their total cytosolic protein content. The protein is a heterocomplex protein consisting of two heavy chains and one light chain, which are non-covalently linked.6 Calprotectin appears to play a regulatory role in the inflammatory process and comprises both antimicrobial and antiproliferative capacities.7 Faecal calprotectin correlates well with the faecal excretion of Indium-111-labeled leucocytes which is considered to be the gold standard stool marker of inflammation.8 This indicates an increased translocation of granulocytes into the intestinal mucosa in an inflammatory setting resulting in higher levels of proteins from such cells in faeces. Furthermore, faecal concentrations of lysozyme,9 myeloperoxidase,10 PMN-elastase,11 tumour necrosis factor-alpha12 and S100A1213 were all shown to correlate with intestinal inflammation.

To date, information regarding the comparative performance of several faecal proteins in the identification of intestinal inflammation is sparse. Thus, in an effort to overcome this lack of data we prospectively evaluated the clinical utility of calprotectin, lactoferrin and PMN-elastase in faeces alone or in combination to detect active gastrointestinal inflammation in patients suggestive of either having IBD or the non-inflammatory condition, IBS.

MATERIAL AND METHODS

Subjects

This prospective study was performed at the 1st Department of Internal Medicine, Division of Gastroenterology, J.W. Goethe-University, Frankfurt am Main, Germany. Consecutive patients referred for diagnostic clarification of chronic diarrhoea were recruited from tertiary referral practices in the Rhein-Main-area.

Protocol

The inclusion criteria were a history of chronic diarrhoea of unknown origin, lasting for more than 4 weeks, with or without abdominal pain. Exclusion criteria included previous evaluation for chronic diarrhoea, overt gastrointestinal bleeding, sigmoidoscopy or colonoscopy during the previous 2 months performed for any cause, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer syndrome and pregnancy.

A full medical history including the use of non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin was obtained from all adults enrolled in the study, and a physical examination was performed. In addition, all patients underwent first-step haematology and chemistry tests (including erythrocyte sedimentation rate, serum C-reactive protein, blood cell counts, electrolytes, thyroid, liver and renal function, IgA); serological assays for suspected coeliac disease (antigliadin
IgA and IgG and antitransglutaminase IgA and IgG; stool examination for pathogenic bacteria, ova and parasites; and a lactose-H\textsubscript{2} breath test.

Patients supplied fresh stool samples for determination of the concentration of calprotectin, lactoferrin and PMN-elastase within 1 week prior to colonoscopy. Laboratory personnel unaware of the clinical diagnoses or details of the patients’ clinical histories retrieved the faecal specimens within 24 h of defection. Upon receipt, stool was aliquoted for immediate assay or stored at \(-20^\circ\text{C}\) until assay performance. After thawing two 100 mg faecal aliquots from a single stool sample from each participant were assayed and the mean of the two measurements was recorded.

Calprotectin concentrations were determined using a commercially available monoclonal antibody-based enzyme-linked immunosorbent assay (Immundiagnostik AG, Bensheim, Germany); lactoferrin and PMN-elastase were quantified using commercial ELISA test systems (IBD-Scan, TechLab, the Netherlands and Immundiagnostik AG, respectively). The cut-off values for a positive result for calprotectin, lactoferrin and PMN-elastase according to the manufacturer’s instructions were 15 \(\mu\text{g/g}\), 7.3 \(\mu\text{g/g}\) and 62 ng/g, respectively. Coefficients of variation in intraday assays for the three proteins were <15%, respectively.

Colonoscopies were performed by experienced staff gastroenterologists, who were unaware of the faecal results. Mucosal abnormalities were recorded by anatomic location and biopsies were obtained routinely from each segment of the colon. Inflammation was defined and graded by standard histological criteria and subtyped by endoscopic and histological features as Crohn’s disease (CD), ulcerative colitis (UC) and other.

Patients with negative results for all of the examinations described above and with the clinical history indicative of IBS according to Rome II criteria were then considered to have a diagnosis of IBS.\textsuperscript{14} If IBD was diagnosed, disease activity was evaluated clinically by either using the Crohn’s Disease Activity Index (CDAI) for CD\textsuperscript{15} or the Colitis Activity Index (CAI) for UC,\textsuperscript{16} respectively.

The protocol was approved by the local ethics committee, and informed consent was obtained from all patients included in the study.

### Statistical analysis

All statistical analyses were performed using Bias. For Windows Version 8.1 (Frankfurt, Germany) except for receiver-operated curve (ROC) analysis, which was performed by applying the MEDCALC Software (Mariakerke, Belgium). ROCs were used to determine the optimal cut-off for identifying IBD. Descriptive statistics are given as median and range. Sensitivity, specificity, positive predictive value, negative predictive value and the likelihood ratios (LR) were calculated for each test. Statistical comparison between study groups was performed using the Mann–Whitney non-parametric test and Spearman’s correlation coefficient was used to assess correlations. A \(P\)-value of <0.05 was considered to be statistically significant.

### RESULTS

A total of 88 consecutive patients were screened between August 2002 and January 2004. From that cohort 12 patients had to be excluded from further study participation because of verification of lactose malabsorption by a pathological lactose-H\textsubscript{2} breath test \((n = 9)\) or history of chronic use of NSAIDs \((n = 3)\).

Among the 76 individuals included in the study, IBD was diagnosed in 45 patients, and 31 patients were diagnosed as suffering from IBS. In the population with IBD final diagnosis was CD in 25 patients and UC in 20 patients. Demographic details are shown in Table 1.

| Table 1. Demographics of study population as well as median and range of faecal biomarker concentrations among the study groups |
|-----------------|--------|--------|--------|
|                 | CD     | UC     | IBS    |
| \(N\)           | 25     | 20     | 31     |
| Age (years)     | 40 (25–59) | 38 (24–75) | 43 (20–72) |
| Sex (m/f)       | 7/18   | 15/5   | 11/20  |
| Disease activity score\textsuperscript{*} | 266 (183–446) | 6 (4–11) | – |
| Faecal biomarker |        |        |        |
| Calprotectin \((\mu\text{g/g})\) | 143 (15–1535) | 137 (16–2553) | 6 (0–24) |
| Lactoferrin \((\mu\text{g/g})\) | 45 (0–499) | 63 (0–306) | 0.2 (0–9) |
| PMN-elastase \((\text{ng/g})\) | 55 (4–640) | 40 (4–500) | 6 (0–35) |

\* CDAI for CD and CAI for UC.

CAI, colitis activity index; CD, Crohn’s disease; CDAI, Crohn’s Disease Activity Index; IBS, irritable bowel syndrome; PMN, polymorphonuclear neutrophil; UC, ulcerative colitis.
The median faecal calprotectin concentration was 143 µg/g in patients with CD, 137 µg/g in patients with UC and 6 µg/g in patients with IBS, respectively. The median faecal lactoferrin concentration was 45 µg/g in patients with CD, 63 µg/g in patients with UC and 0.2 µg/g in patients with IBS, respectively. Finally, the median faecal PMN-elastase concentration was 55 ng/g in patients with CD, 40 ng/g in patients with UC and 6 ng/g in patients with IBS, respectively (Table 1).

Concentrations of each faecal marker for patients with either form of IBD were significantly elevated compared with those of IBS patients (Figures 1 and 2). The respective P-values for calprotectin, lactoferrin and PMN-elastase were each <0.0001.

Table 2 demonstrates the sensitivity, specificity and predictive values of the individual faecal biomarker assays in identifying patients with IBD as determined by ROC analysis. ROCs for each test system evaluated are illustrated in Figure 3. The maximal sum of sensitivity and specificity for faecal calprotectin was achieved at a value of 24.3 µg/g, respectively; accordingly, maximal sum of sensitivity and specificity for faecal lactoferrin and PMN-elastase were 8.9 µg/g and 19 ng/g, respectively. Although there was a clear trend towards superiority of the diagnostic accuracy of calprotectin in favour of lactoferrin and PMN-elastase analysis did not reach statistical significance (calprotectin vs. lactoferrin: \(P = 0.44\); calprotectin vs. PMN-elastase: \(P = 0.12\); lactoferrin vs. PMN-elastase: \(P = 0.41\)).

Odds ratio (OR) and positive and negative likelihood ratios (LRs) for each individual stool marker for the detection of intestinal inflammation are listed in Table 3. The OR for having intestinal inflammation with an elevated faecal calprotectin level was 765 compared with increased faecal concentrations of lactoferrin and PMN-elastase in which the OR for organic disease was 278 and 37, respectively. In addition, the negative LRs of all biomarkers indicated that all test systems are very good with calprotectin again achieving the best test result although not being statistically significant.

In addition to the performance of each individual test system, diagnostic values of the various combinations of biomarkers in differentiating organic vs. non-organic patient groups were determined. As shown in Table 2, no statistical improvement could be obtained by assessing combinations of biomarkers when compared with each individual marker.

No statistical difference in faecal neutrophil-derived protein concentrations between clinically active CD and UC was observed. The respective P-values for calprotectin, lactoferrin and PMN-elastase were 0.65, 0.36 and 0.50, respectively. Analysis of IBD patients based...
on gender and age also failed to reveal any statistical difference between groups ($P > 0.4$ for all faecal biomarkers).

Faecal biomarker concentrations were also analysed with respect to disease location in CD patients according to the Vienna-classification.\textsuperscript{17} Of the 25 study patients with CD, the terminal ileum (L1) was involved in six patients, five patients had isolated colonic disease (L2), whereas 14 patients showed affection of the ileocolon (L3). None of the CD patients in the study cohort presented with isolated disease of the upper gastrointestinal tract (L4). The median calprotectin concentrations according to disease locations L1, L2 and L3 were 100 µg/g, 161 µg/g and 127 µg/g, respectively. For lactoferrin, the location-based median levels were 28 µg/g, 179 µg/g and 25 µg/g, respectively. Finally, the median concentration of PMN-elastase in patients with affection of the terminal ileum, the colon or the ileocolon were of 40 ng/g, 215 ng/g and 50 ng/g, respectively. No statistical difference was found between the subgroups, although there was a clear trend towards higher levels of faecal neutrophil-derived proteins, at least lactoferrin and PMN-elastase, in isolated colonic disease when compared with involvement of the ileum alone or the ileocolon. However, it has to be noted that the numbers in the three subgroups were very small.

Finally, no significant correlation between each stool parameter and the clinical disease activity of either IBD form, CD and UC, could be revealed as

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
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<tbody>
<tr>
<td>Cal</td>
<td>93</td>
<td>100</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>Lac</td>
<td>82</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>PMN-e</td>
<td>84</td>
<td>87</td>
<td>91</td>
<td>79</td>
</tr>
<tr>
<td>Cal + Lac</td>
<td>93</td>
<td>100</td>
<td>100</td>
<td>91</td>
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<tr>
<td>Cal + PMN-e</td>
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<tr>
<td>Cal + Lac + PMN-e</td>
<td>96</td>
<td>100</td>
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NPV, negative predictive value; PPV, positive predictive value; Cal, calprotectin; Lac, lactoferrin; PMN-e, polymorphonuclear neutrophil-elastase; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.

Figure 2. Head-to-head comparison of the concentrations of faecal neutrophil-derived proteins in the two diagnostic groups [inflammatory bowel disease (IBD) = closed symbols, irritable bowel syndrome (IBS) = open symbols]. The respective cut-off values as determined by receiver-operated curves are shown by the dashed horizontal and vertical lines. (a) Calprotectin plotted against lactoferrin with \( r = 0.31, P = 0.09 \) for IBS patients and \( r = 0.75, P < 0.0001 \) for IBD patients, (b) calprotectin plotted against polymorphonuclear neutrophil (PMN)-elastase with \( r = 0.22, P = 0.23 \) for IBS patients and \( r = 0.81, P < 0.0001 \) for IBD patients and (c) lactoferrin plotted against PMN-elastase with \( r = 0.03, P = 0.87 \) for IBS patients and \( r = 0.75, P < 0.0001 \) for IBD patients.

Table 2. Sensitivity, specificity and predictive values of individual as well as combinations of different fecal biomarkers in distinguishing between IBD and IBS.
DISCUSSION

Although the general usefulness of investigating the faecal concentration of any of these biomarkers has been demonstrated, the studies to date on the use of faecal neutrophil-derived proteins in intestinal inflammation have been very heterogeneous, making a direct comparison between different proteins and their clinical application difficult. Silberer et al.\textsuperscript{18} were the first to compare the clinical use of several neutrophil-derived proteins in prediagnosed patients. The authors concluded that calprotectin and PMN-elastase were superior to lactoferrin, lysozyme and myeloperoxidase in differentiating active IBD from IBS. In another study, calprotectin, lactoferrin and PMN-elastase but not lysozyme were found to represent suitable markers to monitor disease activity in patients with UC.\textsuperscript{19} To our best knowledge, the study at hand addressed for the first time the issue of comparing the performance of several of these surrogate parameters in identifying intestinal inflammation in a prospective manner.

We report high specificities to exclude intestinal inflammation for all three assays tested ranging from 87\% to 100\% with calprotectin and lactoferrin superior to PMN-elastase though not being statistically significant. In contrast, direct comparison of the sensitivity of each individual neutrophil-derived protein in identifying inflammation demonstrated that calprotectin appeared to be more accurate than lactoferrin and PMN-elastase (93\% vs. 82\% and 84\%, respectively), although again a statistically significant level was not achieved. In addition, a combination of two or even all three markers did not improve the diagnostic power compared with each individual marker alone.

A recent meta-analysis by von Roon et al.\textsuperscript{20} reported pooled sensitivities and specificities of faecal calprotectin in differentiating IBD from non-IBD diagnoses (in particular IBS) of 86\% (CI: 83–89\%) and 81\% (CI: 78–84\%), respectively, suggesting that faecal calprotectin has a good diagnostic precision for

\begin{table}
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\begin{tabular}{|l|l|l|l|}
\hline
Parameter & OR (95\% CI) & LR+ & LR− \\
\hline
Calprotectin & 765 (150–3901) & n.a. & 0.07 \\
Lactoferrin & 278 (58–1343) & n.a. & 0.18\textsuperscript{N.S.} \\
PMN-elastase & 37 (12–116) & 6.2 & 0.18\textsuperscript{N.S.} \\
\hline
\end{tabular}
\caption{Odds ratio and likelihood ratios for the different neutrophil-derived proteins in the detection of IBD}
\end{table}

n.a., not assignable due to a specificity of 100\% of each test system; PMN, polymorphonuclear neutrophil; IBD, inflammatory bowel disease.
separating IBD from IBS overall. The distinct higher diagnostic values found in our cohort here may be due to both differences in the disease severity of the underlying IBD and dissimilarities in the pretest probability of IBD based on clinical assessment. As disease activity was not consistently classified or even not performed in previous studies, it remains obscure if the extent of disease activity may have influenced the diagnostic accuracy in our IBD population. In contrast, a selection bias is existent in our study cohort as our hospital represents a referral centre for IBD. Selection of patients with a strong suspicion of IBD, as also indicated by the exceptionally high number of patients suffering from IBD when compared with IBS in our overall patient cohort equivalent to a pretest odds of 1.5 (or 51% of all screened patients), may have presumably positively influenced the diagnostic precision of the tests applied. In addition, patients suffering from other organic intestinal diseases or consuming NSAIDs, both conditions, which have been recognized to be associated with elevated faecal biomarkers,21 were excluded from the study.

Besides calprotectin, faecal lactoferrin appears to be the most used and also the most useful biomarker of intestinal inflammation. In 2003, Kane et al.22 determined the value of faecal lactoferrin in the identification of intestinal inflammation in a population of more than 200 prediagnosed IBD and IBS patients pointing out that faecal lactoferrin is a reliable marker of bowel inflammation with a sensitivity of 86% and specificity of 100%. A smaller second study reported a comparable sensitivity and specificity of 90% and 98%, respectively.23 After adjusting the recommended cut-off values by ROC analysis, our findings are well in accordance with the reported diagnostic accuracy for lactoferrin in the differential diagnosis of IBD. Nevertheless, the data obtained indicate an advantage of calprotectin over lactoferrin in the detection of intestinal organic disease.

In contrast to calprotectin and lactoferrin, data regarding the diagnostic value of PMN-elastase in distinguishing functional vs. inflammatory intestinal disease are lacking. Adeyemi et al.11 reported a significant correlation between faecal PMN-elastase with the CDAI in CD and a numeric index in UC, suggesting that PMN-elastase is helpful in the assessment of disease activity of IBD. However, direct comparison of accuracy and cost-effectiveness revealed that lactoferrin was a better neutrophil-derived faecal marker of inflammation than elastase, lysozyme and myeloperoxidase.24 Thus, our findings establish for the first time PMN-elastase as a tool for the differential diagnosis of IBD; however, supposedly with an overall lower diagnostic accuracy when compared with calprotectin and lactoferrin.

Apart from screening, faecal biomarkers may also be used for the clinical management of IBD. In this study, only a very poor correlation between the concentration of each individual faecal biomarker studied and the respective clinical index score (CDAI or CAI) in the IBD patient population was observed. Thus, it was impossible to stratify the disease activity into mild, moderate and severe by the level of calprotectin, lactoferrin or PMN-elastase. In contrast, in previous reports faecal calprotectin concentrations were shown to correlate with endoscopic and histological assessment of disease activity in adults and children with IBD.25–27 Moreover, stratification of disease activity in active vs. inactive as determined by clinical activity scores demonstrated significant differences in faecal lactoferrin concentrations.22, 28 These apparently conflicting results may be presumably explained by the fact that disease evaluation by using clinical activity indices is largely based on subjective symptoms, which do not necessarily correspond to the endoscopic and histological assessment. Moreover, the different stratification procedures (active vs. inactive on one hand and mild, moderate, severe on the other hand) cannot directly be compared.

In conclusion, although possibly limited with respect to study size and selection bias, the results of our study implicate an advantage of calprotectin over lactoferrin and PMN-elastase in the detection of intestinal inflammation provoked by IBD when compared with the non-inflammatory condition, IBS. Moreover, simultaneous determination of several biomarkers does not seem to improve the diagnostic power compared with each individual faecal neutrophil-derived protein. Based on our findings the diagnostic precision of faecal calprotectin should encourage its use in the clinical evaluation of patients with chronic diarrhoea.

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