Manuscript Doi : 10.1093/ecco-jcc/jjw044
Journal of Crohn's and Colitis Advance Access published February 13, 2016

FECAL LACTOFERRIN, CALPROTECTIN, PMN-ELASTASE, CRP AND WHITE BLOOD CELL COUNT AS AN INDICATOR FOR MUCOSAL HEALING AND CLINICAL COURSE OF DISEASE IN PATIENTS WITH MILD TO MODERATE ULCERATIVE COLITIS: POST HOC ANALYSIS OF A PROSPECTIVE CLINICAL TRIAL

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Short title: Non-invasive biomarkers in ulcerative colitis

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Manuscript Doi : 10.1093/ecco-jcc/jjw044

ABSTRACT

OBJECTIVES: We evaluated the performance of blood and fecal biomarkers for differentiating between endoscopic inflammation and mucosal healing, clinically active disease and sustained clinical remission and determined the predictive value for a flare in patients with ulcerative colitis (UC).

METHODS: Clinical activity Index (CAI), fecal lactoferrin (FLA), calprotectin (CAL), PMN-elastase (PMN-e), CRP, WBC, endoscopic index (EI) and UC-Disease activity Index (DAI) were determined repeatedly during 12-months and at acute flares.

RESULTS: Of 91 patients (45 female; mean age 48.1±13.4 years) entering in remission, 42 (46%) patients developed a clinical flare. A total of 529 CAI and 179 EI assessments were performed. Median levels for active disease confirmed by EI (n=35) vs clinical remission with endoscopic inflammation (n=37) vs mucosal healing (n=107) for FLA were 44/37/4µg/g, CAL 25/20/10µg/g (both p<0.0001), PMN-e 0.06/0.03/0.02µg/g, CRP 0.7/0.2/0.2mg/dl (both p<0.001) and WBC 7.0/6.5/6.4/nl (p=0.1). There was no difference for any of the markers for defining mucosal healing by EI=0 vs. EI=1 with the exception of PMN-e (p=0.03), where the difference was very small and with questionable clinical relevance. Using manufacturers' cut-offs, only FLA at baseline was associated with a significant higher Relative Risk (RR) of flaring (RR1.69; p=0.018). Using optimized cut-offs Cal, PMN-e and CRP were also predictive of a flare.

CONCLUSIONS: Fecal biomarkers FLA, CAL and PMN-e were able to distinguish between UC patients with mucosal healing from clinical remission and mild disease, showed significant correlations with endoscopy and were predictive of a flare.

Key words: Ulcerative colitis; relapse; mucosal healing; fecal lactoferrin, calprotectin, PMN-elastase, CRP, white blood cell count; monitoring

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Specific author contributions: Jost Langhorst: planning of the study, study design, data interpretation, writing of the article; James Boone: study design, writing of the article; Romy Lauche: data interpretation, writing of the article; Andreas Rueffer: laboratory analyses, interpretation of the data; Gustav Dobos: planning of the study, final review of the manuscript.

Special thanks to Mrs. Annette Tengelmann, Mrs. Anna Propson Mrs. Miriam Mohr and Mrs. Ines Varnhagen for their contribution to the study.

Financial support: This study was performed as an independent sub-study of a drug trial comparing the efficacy of a herbal preparation of myrrh, dry extract of chamomile flowers and coffee charcoal to mesalamine as treatment for maintaining remission in ulcerative colitis which was funded in full by REPHA GmbH Biologische Arzneimittel, Hannover, Germany. This study was supported in part by a research grant from TechLab.

Potential competing interests: J. Langhorst has received research and travel grants from TechLab, Inc, J. Boone is an employee of TechLab, Inc., Romy Lauche: none to declare, Andreas Rueffer: none to declare, Gustav Dobos: none to declare

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Introduction

Ulcerative colitis (UC) is a chronic relapsing disease. The determination of inflammatory activity is crucial for the assessment of clinical decision-making and for the tailoring of therapy[1]. To define remission in ulcerative colitis a standard based on clinical symptoms and/or endoscopy is proposed. A variety of disease activity indices are available for UC and several different symptom-based activity scores, composite scores and patient evaluation scoring systems have been used and published (1-4). Two widely used scores are the CAI by Rachmilewitz (5) and the Mayo UC Disease Activity Index by Sutherland (6).

The Colitis Activity Index (CAI) inaugurated by Rachmilewitz (5) uses a threshold of ≤ 4 to define clinical remission. It is based mainly on symptoms and clinical examinations which might be hampered by inaccuracy due to the subjective nature of symptom reports (2). In addition, a score of 4 points allows a level of symptoms which may include an increased stool frequency and/or rectal bleeding which is not consistent with remission (2). Consequently, in more recent discussions, the definition of clinical remission should include both the absence of rectal bleeding and a normal threshold for stool frequency as crucial components (2).

The UC Disease Activity Index (UC-DAI) is a composite score incorporating the four variables stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity (6). The index has been adopted in large clinical studies (3). Of particular note, a score <2.5 points correlates with patient-defined remission (7) although the index has not been formally validated.

When targeting intestinal inflammation, conventional colonoscopy in conjunction with histopathological biopsy is considered the gold standard for the detection and quantification of intestinal inflammation in UC (8-9). More recently, studies have shown the use of mucosal healing as a treatment endpoint as having a potential protective role in preventing future flares (10-14). The current definition of mucosal healing requires an endoscopy score of 0 or 1 of the Mayo Clinic score, the UC-DAI or the Endoscopy Index (EI) including normal appearance of the rectal mucosa or erythema only (2). However, there are several drawbacks related to the invasiveness, expense, procedure-related discomfort, risk of bowel perforation and relatively poor patient acceptance for colonoscopy (15-17).

Consequently, in an attempt to overcome these problems, a number of laboratory markers have been evaluated. Blood tests, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), are in common use but achieve only suboptimal sensitivity and specificity for intestinal inflammation (1). Fecal biomarkers are non-invasive and used to specifically measure intestinal inflammation and to assess disease activity in UC. In recent decades, a number of fecal biomarkers have been evaluated for their ability to differentiate and monitor IBD disease activity (1;2;18-22). In an earlier study of our work-group, fecal

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lactoferrin (FLA), calprotectin (CAL) and polymorphonuclear neutrophil elastase (PMN-e) were able to differentiate active from inactive IBD and IBS and showed higher diagnostic accuracy than CRP (1). But while relevance and acceptance of fecal biomarkers in clinical care has increased in recent years allowing repeated non-invasive testing and evaluation of intestinal inflammation, their full potential is still being investigated.

Therefore, the aim of this study was to evaluate the performance of fecal FLA, CAL, and PMN-e and the blood biomarkers serum CRP and white blood count (WBC) with a comparison to clinical course of disease using the CAI, the UC-DAI and the Rachmilewitz-endoscopy score in a patient cohort with UC over a 12 month period.

MATERIALS AND METHODS

Study design and patient population

The study was conducted from June 2008 to July 2010, as a single center trial in the department for Internal and Integrative Medicine, Kliniken Essen-Mitte, Germany as part of a study comparing the efficacy of a herbal preparation of myrrh, dry extract of chamomile flowers and coffee charcoal to mesalamine as treatment for maintaining remission in ulcerative colitis (UC) (23). The patients were randomly assigned to a treatment with an oral preparation of 100mg myrrh, 70mg chamomile extract and 50mg coffee charcoal four tablets three times daily or one tablet of mesalazine 500mg three times daily in a double-blind, double-dummy setting.

Inclusion criteria were: (a) A previous diagnosis of UC, verified by the defining symptoms (rectal bleeding, diarrhea), endoscopy and histopathology, (b) age ≥18 and ≤75 years, (c) patients with inactive UC as defined by a CAI score ≤4.

Exclusion criteria for all participating subjects were clinically active disease at baseline using CAI, a treatment within the last 3 months with biologic therapies or other immunosuppressive drugs (azathioprine, methotrexate), infectious or chronic active colitis, relevant somatic comorbidities or pregnancy.

Patients were followed for a 12 month period, or less if they relapsed. The Colitis Activity Index (CAI; Rachmilewitz) (5) which was used in the clinical study was calculated at the six predefined time-points (baseline, after 1, 3, 6, 9 and 12 months) during the 12-month interval and in addition when the patient reported symptoms of an acute flare. Patients were instructed to contact the research coordinator if they developed symptoms of an acute flare. Acute flare was defined as a CAI >4.

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Sigmoidoscopy with histology was performed at baseline, in the event of an acute flare (CAI >4) when possible or at the end of the 12 month period to determine the presence of mucosal inflammation. Endoscopy score (EI) was used to confirm mucosal healing.

Ethics

All patients gave informed consent for participation and the study protocol was approved by the Ethics Committee of the Medical Faculty, University of Duisburg-Essen.

Clinical Measures

Colitis Activity Index

The clinical disease activity was determined with the CAI by Rachmilewitz (5) which includes a combination of laboratory parameters and clinical symptoms, namely weekly calculation of bowel frequency, blood in stool, well-being, abdominal pain, fever, extraintestinal symptoms, erythrocyte sedimentation rate and hemoglobin, with a score of >4 indicating active disease as described before. A score ≤4 and >2 defines clinical remission and a score ≤2 with normal bowel frequency and no blood in stool indicates sustained clinical remission.

UC-DAI

Mayo Disease activity was calculated at the time points when an endoscopic procedure was performed including stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity (6).

Endoscopy

All procedures were performed by one experienced gastroenterologist who was blinded to the CAI at the time of endoscopy.

The endoscopic activity index score (EI; Rachmilewitz) (5) was calculated using the following subscales: 1. Granulation scattering reflected light (No 0; Yes 2 points); 2. Vascular pattern: (normal 0; faded/disturbed 1; completely absent 2 points); 3. Vulnerability of mucosa: (none 0; slightly increased (contact bleeding) 2; greatly increased (spontaneous bleeding) 4 points); 4. Mucosal damage: (mucus, fibrin, exudates, erosions, ulcer) (none 0; slight 2; pronounced 4). The Endoscopic Activity Index ranges from 0 to 12 points (16). Mucosal healing was defined by a score ≤ 1 with additional analysis for comparing EI=0 versus EI=1

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Combined Clinical and Endoscopic Disease Activity

Clinical and endoscopic disease activity was defined by the CAI and endoscopy as follows: Patients in an acute clinical flare with endoscopic intestinal inflammation, patients in clinical remission with endoscopic intestinal inflammation, patients in clinical remission with mucosal healing.

Laboratory measures

Fecal biomarkers, C-reactive Protein and white blood cell count

Blood and fecal samples were collected at baseline and at each of the scheduled time-points or in the event of an acute flare (CAI >4). It was recommended to harvest the fecal samples in the morning when possible. Blood and serum were evaluated for white blood cell count (WBC; cut-off: 8.5/nl) and C-reactive protein (CRP; cut-off: 0.5mg/dl) respectively, fecal samples were evaluated for lactoferrin (FLA; cut-off: ≥ 7.25µg/g), Calprotectin (CAL; cut-off: > 50µg/g) and PMN-elastase (PMN-e; cut-off: >0.06µg/g), respectively as described before.

Serum CRP was determined using a latex immunoturbidimetric test (CRPLX, Tina-quant, Roche/Hitachi). The whole stool specimens were collected by the patients using a disposable plastic bucket-type device which avoids toilet water artifact and simplifies laboratory sampling. The stool specimens were frozen at -30℃ immediately after collection, sent to the laboratory (Labor L+S AG, Bad Bocklet-Großenbrach, Germany) and analyzed. Each specimen was tested for concentrations of FLA, CAL, and PMN-e with ELISA (enzyme-linked immunosorbent assay) according to the manufacturer's instructions (*IDK*® Calprotectin ELISA and PMN-Elastase ELISA from Immundiagnostik, Bensheim, Germany for calprotectin and PMN-e; LACTOFERRIN SCAN® kit from TechLab, Blacksburg, USA for FLA). Briefly, fecal samples were suspended in diluent-buffer. After homogenization and if necessary centrifugation, the homogenates respectively supernatants were transferred to microplates, each coated with antibodies specific for the respective inflammatory markers. Anti-lactoferrin, anti-calprotectin or anti-PMN-elastase antibodies, each conjugated with peroxidase, were used for development. In addition to the fecal samples, for all parameters standards as well as positive and negative controls were tested. For each well, the optical density was measured at 450 nm with a microplate ELISA reader (Dynex, Germany). The results of the test samples were calculated from the standard curve, and were expressed as µg/g for FLA and CAL, and ng/ml for PMN-e.

Outcomes:

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1. Patients were compared based on fecal biomarkers, CRP and WBC according to a combined evaluation of the CAI and the EI (*acute clinical flare with endoscopic intestinal inflammation* CAI>4/EI≥2 vs. *clinical remission with endoscopic intestinal inflammation* CAI≤4/EI≥2 vs. *clinical remission with mucosal healing* CAI≤4/EI<2).

2. Diagnostic accuracy of fecal biomarkers, CRP and WBC (normal or elevated according to established cut-offs) were calculated compared to CAI (CAI≤2, normal bowel frequency, no blood in stool) and endoscopic index (EI<2) as gold standard. It includes sensitivity, specificity, positive and negative predictive values (PPV, NPV). In addition optimized cut-offs for fecal biomarkers, CRP and WBC were calculated using the EI as gold standard (EI<2).

3. Correlation between clinical activity indices and fecal biomarkers, CRP and WBC were calculated.

4. The ability of fecal biomarkers, CRP and WBC (baseline scores) to predict a flare during the 12 months period was calculated.

Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, U.S.A., version 20.0) software. For all analyses the level for statistical significance was set at 0.05, no correction for multiple testing was applied. Unless otherwise specified, results are presented as median + range. Before analyses, normal distribution of data was checked.

1. Correlational analyses were conducted using Spearman's rho correlational coefficient for non-parametric correlations. It also does not require normal distribution of data.
2. Group differences were calculated using Mann-Whitney U test for data with non-normal distribution.
3. The following indicators of diagnostic accuracy were calculated: sensitivity (proportion of correctly identified diseased relative to all diseased), specificity (proportion of correctly identified healthy people relative to all healthy), positive predictive values (proportion of correctly identified diseased relative to all who were identified as diseased) and negative predictive values (proportion of correctly identified healthy relative to all who were identified as healthy). For optimized cut-offs receiver operator characteristics (ROC) were used to illustrate the optimal cut-off between sensitivity and specificity of each parameter.
4. The predictive value for flare prediction was calculated using Chi^2 tests. Risk ratios were determined in case of significant effects.

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RESULTS

Patient characteristics

Of the 118 patients screened, 27 (23%) did not satisfy the eligibility criteria. Thus, 91 subjects (45 female – 50%) in clinical remission were included in the study. The mean age was 48.1 ± 13.4 (range 19 to 75 years). All patients were in clinical remission at baseline according to the CAI ≤4. There were 87 patients (96%) on oral mesalamine as maintenance medication and 14 patients on additional topical mesalamine. No patient was treated with immunosuppressive drugs. The mean±SD duration of disease was 11.7 ± 10.9 years. Medical histories showed 12 patients had a previous diagnosis of proctitis, 48 left-sided colitis and 31 pancolitis.

Clinical course of disease

Of the 91 patients, a total of 42 (46%) patients developed one or more clinical flares (CAI > 4) with a mean ± SD CAI of 7.2 ± 2.4 indicating a predominantly mild course of disease and a range of 5 to 14 during the 12 month interval. Of these, only 6 flares were over a CAI of 10. For the remaining 49 patients, clinical remission (CAI ≤) was maintained during the 12-month period.

CAI

A total of 529 CAI (Rachmilewitz) were retrieved from 91 patients relating to about 6 assessments for each patient at baseline, 1, 3, 6, 9 and 12 month time-points and at flare. Of those assessments, 52 (10%) indicated periods of acute flare (CAI > 4), 175 (33%) indicated clinical remission (CAI ≤4 and >2) and 302 (57%) fulfilled the criteria of sustained clinical remission (CAI ≤2, a normal bowel frequency and no blood in stool).

Endoscopy

A total of 179 endoscopic procedures in 91 patients were conducted and included in this study. A single routine sigmoidoscopy at baseline was performed in 11 patients (4 remained in clinical remission during the 12-month period and 7 developed a clinical flare and did not have an additional endoscopy), two routine sigmoidoscopies (baseline and 12 months) for 41, two sigmoidoscopies (baseline and during remission before 12months) in 4, two sigmoidoscopies (baseline and flare) in 27 patients, three (baseline, flare, 12-months) in 7 patients and three (baseline, flare, flare) in 1 patient.

At baseline, 67 of the 91 (74%) patients showed mucosal healing (39 EI score of 0 and 28 EI score of 1) and 24 (26%) showed signs of endoscopic inflammation (EI>1). Of the 24 with endoscopic disease activity, a total of 14 (58%) developed a flare in the following 12 months.

Endoscopy confirmed the presence of intestinal inflammation in 33 of the 35 (94%) flaring patients with a mean ± SD EI of 6.5±1.8 and a range of 2 to 10 indicating mild to moderate disease. The remaining 2 patients (6%) showing no endoscopic signs of acute intestinal inflammation.

There were 41 patients in clinical remission for the entire study period that underwent an endoscopy at 12 months. Of these, a total of 34 (83%) patients showed mucosal healing (9 EI score of 0 and 25 EI score of 1) and 7 (17%) had endoscopic inflammation with a mean±SD EI score of 3.1±0.9 and a range of 2 to 4.

Differences in fecal biomarkers, CRP and WBC according to the combined clinical disease activity index (CAI) and endoscopic index (EI)

The median and range for levels of fecal biomarkers, CRP and WBC for those during a flare with endoscopic inflammation, those in clinical remission but having endoscopic inflammation, and those with mucosal healing are shown in Table 1. Only CRP was significantly different between acute flare and clinical remission with endoscopic inflammation. All 3 fecal biomarkers were different between both clinical flare and clinical remission with endoscopic inflammation and patients with mucosal healing. CRP and WBC were both significantly different between flare and mucosal healing but neither showed a difference between clinical remission with endoscopic inflammation and mucosal healing.

Median scores of fecal markers for patients with an endoscopic index of 0 vs. patients with an endoscopic index 1 were as follows: FLA 2.9µg/g (range 0-126.9) / 5.5 µg/g (0.1-93.3) (p=0.190); Cal 8.9 µg/g (0-62.1) / 12.2 µg/g (0-47.8) (P=0.283); PMN-e 0.02 µg/g (0-0.7) / 0.02 µg/g (0-0.4) (p=0.03); CRP 0.2mg/dl (0.1-2.8) / 0.2mg/dl (0.1-1.4) (P=0.441) and WBC 6.1/nl (3.8-12.8) / 6.8/nl (4.1-13) (p=0.088).

Diagnostic accuracy of fecal biomarkers, CRP and WBC for detecting sustained clinical remission and mucosal healing

Sensitivity, specificity, positive and negative predictive values are shown for detecting mucosal healing using endoscopy as the reference in 179 assessments in Table 2, boxplots are shown in figure 1.

The results of the ROC curves for detecting mucosal healing using endoscopy as the reference are shown in Table 3. FLA had the highest AUC of 0.73 followed by CAL with 0.70, PMN-e with 0.70, CRP with 0.65 and WBC with 0.57. Diagnostic cut-offs were optimized for each biomarker and changed from 7.25 to 11.9 µg/g for FLA, 50 to 13.9 µg/g for CAL, 0.062 to 0.035 µg/g for PMN-e, and 0.5 mg/dl to 0.25 mg/dl for CRP. Resulting diagnostic accuracies were 70% for FLA, 64% for CAL and PMN-e, and 63% for CRP. Optimized cut-

offs, area under the curve, p-value for ROC analyses, diagnostic accuracy and Risk Ratio to develop a flare using the optimized cut-offs at baseline are listed in Table 3.

Correlation between clinical activity indices and fecal biomarkers, CRP and WBC

The CAI significantly correlated with FLA (ρ=0.30), CAL (ρ=0.30), PMN-e (ρ=0.30), CRP(ρ=0.30) and with WBC (ρ=0.20) (all p < .001).

The endoscopic activity index by Rachmilewitz significantly correlated with FLA (ρ=0.4), CAL (ρ=0.35), PMN-e (ρ=0.38), CRP (ρ=0.29) (all p < .000), and with WBC (ρ=0.19; p=0.013).

The UC-DAI significantly correlated with FLA (ρ=0.37), CAL (ρ=0.32), PMN-e (0.29), CRP (ρ=0.34) (all p < .000), but not with white blood count (ρ=0.11; p < .15).

Prediction of flare during the study period

Using manufactures' cut-offs, the results of the Chi² tests indicated a significant risk ratio for FLA only. Patients with elevated FLA levels at baseline were 1.69 times more likely to experience a flare during the 12 month study period (95% CI 1.104 to 2.575, p=0.018). For all other parameters no such effect could be found. However, when optimized cut-offs were applied FLA, Cal, PMN-e and CRP were predictive of a flare. These results are presented in Table 3.

Discussion

This paper provides three findings we believe to be important. First, the fecal biomarkers lactoferrin, calprotectin and PMN-elastase are able to differentiate patients with mucosal healing from endoscopic inflammation in patients with UC. Second, levels of fecal biomarkers at baseline had predictive value for a flare when using the manufactures' cut-off for FLA and optimized cut-offs for CAL and PMN-e in this prospective 12-months follow-up study. Third, these findings were determined in UC-patients with predominantly mild disease not on immunosuppressive medication.

The clinical course of UC is quite variable and characterized by episodes of relapse and remission. In clinical trials, relapse rates among patients receiving placebo range up to 76% during a 12-month interval (24). The best outcomes include only a 65% rate of sustained remission – in other words, at least 35% of patients receiving standardized care will experience at least one relapse over the course of a year (24). Assessing the activity of UC is important for our daily practice with treating patients. The assessment of disease activity impacts our approach to therapeutic decisions.

While endoscopy with histology remains the gold standard for assessing mucosal inflammation, there are several drawbacks related to the invasiveness, procedure-related discomfort, risk of bowel perforation and relatively poor patient acceptance for colonoscopy

(15-17). Hence, reliable non-invasive markers are highly required for daily routine. A variety of disease activity indices are used for UC including different symptom-based activity scores, composite scores and patient evaluation scoring systems (2). The Rachmilewitz index (clinical activity index, CAI) has been used in multiple studies in UC (5). While it comes relatively close to the clinical reality of all-day patient care its main weakness is that clinical remission has come to be defined as any score less than that used to define disease activity (CAI score >4) (14). A score of ≤4 points allows a level of symptoms (which may include a stool frequency of 36–60 per week) that cannot conceivably define remission. It fails to recognize that there is a 'gray zone' in scoring systems between the threshold for defining disease activity and that used to define remission. To overcome this problem, we included sustained clinical remission defined by a CAI score ≤2 with additional normal bowel frequency and no blood in stool as a reference group.

Fecal biomarkers are becoming more accepted as routine diagnostics for assessing disease activity in patients with IBD. Fecal calprotectin and lactoferrin are the most validated biomarkers of intestinal inflammation and are widely available by larger reference labs. These tests are FDA-cleared and available in many European countries and may be performed in the hospital clinical lab, increasing turn-around time for receiving results for managing patient care. Costs for these assays vary through-out Europe and the U.S. but in general are a small fraction of the cost for endoscopy with histology even when performed repeatedly for monitoring treatment.

In the recent years, the potential protective role of mucosal healing against relapse has been described (10-14). Definition of mucosal healing requires an endoscopy score of 0 or 1 of the Mayo Clinic score or Ulcerative Colitis Disease Activity Index including normal appearance of the rectal mucosa or erythema only (2). The importance of mucosal healing was first discussed in 2001 when basal plasmocytosis on rectal biopsy (12) was recognized as predictor for clinical relapse. Since this study, monitoring of inflammatory activity on the mucosal level is gaining more attention and mucosal healing is discussed as a relevant endpoint in clinical trials, especially in trials evaluating biologics in patients with moderate to high disease activity (25-26). While a predictive impact of mucosal healing in prevention of relapse could not be shown in a UC cohort in remission and not on immunosuppressive drugs in our work group (27) in a 12 month approach, achievement of long-term mucosal healing has been associated with a decreased risk of colectomy and colorectal cancer in ulcerative colitis patients (14). In a study by Ardizzone et al. (28), 157 newly diagnosed ulcerative colitis patients were treated with corticosteroid therapy and then followed for 5 years using endoscopy. Results from this study showed that patients without mucosal healing following the initial treatment suffered a higher rate of negative clinical outcomes including hospitalization, use of immunosuppressive drugs and colectomy compared to early

responders (49% vs 27%). The authors concluded that determining endoscopic response in addition to clinical assessments may prove to be a more accurate prognostic tool for following response to treatment. However, a definite proof that therapy escalation for patients in clinical remission not achieving mucosal healing will be beneficial is still lacking.

In the light of clinical practice, looking beyond clinical activity and using a biomarker for mucosal healing is needed. Considering the quantitative results, FLA, CAL and PMN-e were able to differentiate UC patients with mucosal healing from those with endoscopic inflammation. Of notice, median scores of fecal markers for patients with an endoscopic index of 0 vs. 1 did not show a significant difference, with the exception of PMN-e, where the difference was very small and with questionable clinical relevance. CRP and WBC failed to discriminate between patients in clinical remission showing endoscopic inflammation from patients with mucosal healing. In addition, CAL and PMN-e, showed median levels within the predefined normal range for all three groups, independent if mucosal healing or active inflammation was proven. Only FLA showed median level above the manufacturers' cut-off (7.25µg/g) for active inflammation in this group of patients showing mild to moderate inflammation activity proven by endoscopy which might be of special interest in daily routine due to the impact on long-term course of disease. The negative predictive value using the manufacturers' cut-offs, which is of special importance for the used biomarkers was highest in FLA with 80%.

Multiple studies investigating fecal biomarkers have shown sufficient diagnostic accuracy for active disease when compared to endoscopy (1;22;32-47) in patients with UC. Nevertheless, meta-analyses are available only for FLA and CAL. In a recent meta-analysis including 1012 patients, FLA was 78% sensitive and 94% specific for differentiating IBD from IBS (30). Another meta-analysis showed that fecal CAL concentrations correlated well with endoscopic grading of disease activity at a suggested cut-off value of 50 µg/g for adults and 100 µg/g for children based on a meta-analysis of 30 studies and 5983 patients (30). However, the variation in cut-offs for defining active IBD remains a limitation for fecal biomarkers, especially for calprotectin. In our study, the maximum calprotectin values in the group with an acute UC flare and endoscopic inflammation was 105 µg/g feces which is surprisingly low when compared to almost all other studies even with patients having mild to moderate disease (30). A possible reason for this discrepancy is that the calprotectin ELISA test used for our sample analysis is monoclonal-based system compared to other commercial assays that utilize polyclonal antibodies. The restrictive nature of binding to fewer calprotectin epitopes by the monoclonal antibodies could have led to the lower sensitivity observed in this study. The commercially available cut-off providing a sensitivity of 10% in our study is obviously not ideal and comparative studies evaluating the different commercially available

calprotectin kits and optimization of cut-offs in the same patient cohort are necessary to examine the inherent differences.

In addition, a combination of markers may prove to be more useful in clinical practice and should be addressed in future studies. Our study can add to the available data especially in the way that the inclusion of patients with predominantly mild UC not on immunosuppressive medication further challenge the diagnostic utility of fecal and blood biomarkers.

In regard to the predictive value for flaring UC, FLA and CAL showed the highest potential for predicting a flare following optimization of the cut-off. The diagnostic accuracy for FLA was 70% with a Risk Ratio of 1.99 (95% CI: 1.47-2.71). The cut-off for FLA was optimized from 7.25 to 11.9µg/g. In the literature, a study by Gisbert et al. (35) including 74 UC patients showed sensitivity and specificity to predict relapses of 46% and 61%. The diagnostic accuracy for CAL in our study reached 64% with a Risk Ratio of 1.58 (95% CI: 1.20-2.09). The cut-off for CAL was optimized from 50 to 13.9µg/g. In CAL, Mao et al. (44) performed a meta-analysis in 2012 investigating the predictive value of CAL concentrations at remission in predicting relapse of CD and UC. A total of 672 IBD patients (318 UC and 354 CD) from six different studies were analyzed. The pooled sensitivity and specificity of CAL to predict relapse of quiescent IBD was 78% (95% CI: 72–83) and 73% (95% CI: 68–77), respectively. It is plausible but has to be shown yet that a modified treatment after detecting mucosal inflammation prior to symptoms could change the course of disease and improve patient outcome.

The major strength of this study was the longitudinal design with multiple follow-up time points in the same patient group. Furthermore, while mucosal healing was as yet of mayor interest especially in UC patients treated with biologics, the UC patient cohort in our study was rather homogenous regarding the absence of immunosuppressive therapy and a course of predominantly mild disease.

The major limitation of the study is that patients underwent mainly sigmoidoscopy at baseline. Though the rectum is involved in active disease in at least 95% (48) of patients in ulcerative colitis, it cannot be ruled out that active inflammation in the proximal colon was present. Our findings are restricted to UC-patients in remission achieved without immunosuppressive agents and/or biologics.

Manuscript Doi : 10.1093/ecco-jcc/jjw044

In conclusion, assessment of activity in UC can be performed on different levels such as clinical activity, biochemical activity by measuring blood or fecal biomarkers, endoscopy, and histology. Clinical remission in UC does not necessarily imply biochemical, endoscopic, or histologic remission. Non-invasive fecal biomarkers like FLA, CAL and PMN-e are highly sensitive to a mucosal level and have the potential to significantly add to our understanding of active inflammation in every day patient care.

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Manuscript Doi : 10.1093/ecco-jcc/jjw044

Table 1: Median levels and p-values of the five diagnostic tools according to the three groups (patients in clinical remission and mucosal healing; patients in clinical remission and endoscopic intestinal inflammation; patients in an acute clinical flare and endoscopic intestinal inflammation) **as defined by the CAI and endoscopy**

Diagnostic tool median (range)	Acute flare with endoscopic inflammation N = 35	clinical remission with endoscopic inflammation N = 37	Mucosal healing N = 107	p-value from Mann Whitney U test
Lactoferrin	43.7 (0.1 – 145.0)	36.7 (0.2 – 160.7)		0.687
µg/g	43.7 (0.1 – 145.0)		4.4 (0.0 – 126.9)	***< 0.000***
		36.7 (0.2 – 160.7)	4.4 (0.0 – 126.9)	***< 0.000***
Calprotectin	25.0 (1.7 – 105.6)	19.8 (1.4 – 98.5)		0.292
µg/g	25.0 (1.7 – 105.6)		10.4 (0.01 – 62.1)	***< 0.000***
		19.8 (1.4 – 98.5)	10.4 (0.01 – 62.1)	***0.003***
PMN-elastase	0.06 (0.0 – 0.4)	0.03 (0.0 – 0.4)		0.052
µg/g	0.06 (0.0 – 0.4)		0.02 (0.0 – 0.7)	***< 0.000***
		0.03 (0.0 – 0.4)	0.02 (0.0 – 0.7)	***< 0.011***
CRP	0.7 (0.1 – 10.6)	0.2 (0.2 – 9.9)		***0.011***
mg/dl	0.7 (0.1 – 10.6)		0.2 (0.0 – 2.8)	***< 0.000***
		0.2 (0.2 – 9.9)	0.2 (0.0 – 2.8)	0.243
WBC	7.0 (3.0 – 14.7)	6.5 (3.7 – 13.0)		0.098
/nl	7.0 (3.0 – 14.7)		6.4 (3.8 – 13.0)	***0.036***
		6.5 (3.7 – 13.0)	6.4 (3.8 – 13.0)	0.793

CRP – C-reactive protein; WBC – white blood count

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Table2: Sensitivity and specificity, PPV and NPV for the five diagnostic tools **compared to presence of mucosal healing by endoscopy**

Diagnostic tool		sensitivity in %	specificity in %	PPV in %	NPV in %
Lactoferrin	n = 174	75.0 (62.6; 85.0)	62.5 (52.0; 72.2)	57.1 (45.9; 67.9)	78.9 (68.1; 87.5)
Calprotectin	n = 174	10.9 (4.5; 21.3)	99.0 (94.3; 100.0)	87.5 (47.4; 99.7)	62.5 (54.3; 70.2)
PMN-elastase	n = 174	39.1 (27.1; 52.1)	86.5 (78.0; 92.6)	65.8 (48.7; 80.4)	68.0 (59.0; 76.2)
CRP	n = 176	45.5 (33.1; 58.2)	82.3 (73.2; 89.3)	63.8 (48.5; 77.3)	68.7 (59.4; 77.0)
WBC	n = 179	23.9 (14.3; 35.9)	88.8 (80.8; 94.3)	59.3 (38.8; 77.6)	63.0 (54.4; 71.1)

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Table 3: Optimized cut-off, area under the curve, p-value for ROC analyses and Risk Ratio **to develop a flare using the optimized cut-offs at baseline**

Diagnostic tool	Cut-off	AUC (95% CI)	p-value	Diagnostic Accuracy In %	RR (95% CI), p-value
Lactoferrin	**11.9** µg/g	0.734 (0.654 – 0.813)	***< 0.000***	***70.2***	***1.99 (1.47 – 2.71), p<0.001***
Calprotectin	**13.9** µg/g	0.700 0.619 – 0.782)	***< 0.000***	***64.0***	***1.58 (1.20 – 2.09), p=0.001***
PMN-elastase	**0.035** µg/g	0.697 (0.614 – 0.780)	***< 0.000***	***64.0***	***1.67 (1.21 – 2.29) p<0.001***
CRP	**0.25 mg/dl**	0.651 (0.562 – 0.740	***0.001***	***62.5***	***1.52 (1.15 – 2.0) p=0.002***
WBC	**n.s.**	0.569 (0.477 – 0.660)	0.133	na	na

Figure 1: Boxplots