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Original Article

The Intra-Individual Variability of Faecal Calprotectin: A Prospective Study In Patients With Active Ulcerative Colitis

Anders Lasson[a],[*], Per-Ove Stotzer[b], Lena Öhman[b],[c], Stefan Isaksson[c],[b] Maria Sapnara[c] , Hans Strid[b]

[a]Department of Internal Medicine, Södra Älvsborgs Hospital, Borås, Sweden [b]Department of Internal Medicine, Sahlgrenska University Hospital, Gothenburg, Sweden [c]Department of Biomedicine, Sahlgrenska Academy at the University of Gothenburg, Sweden

*Corresponding author at: Department of Internal Medicine, Södra Älvsborgs Hospital, 401 82 Borås, Sweden. Tel.: +46 33 616 2249. email:anders.lasson@vgregion.se

Abstract

Background and aims: Leukocyte-derived proteins in faeces, especially calprotectin, are increasingly used to assess disease activity in ulcerative colitis. The objectives of the present study were to assess the importance of factors related to the stool sampling procedure.
Methods: For 2 days, patients with active ulcerative colitis collected two stool samples at each bowel movement. The time of defecation, consistency and presence of blood were self-recorded in a diary. The variability in the concentrations of calprotectin during the day and between two consecutive days was assessed, as was the stability of calprotectin concentrations in samples stored at room temperature.
Results: Altogether, 18 patients collected 287 stool samples. The intraclass correlation coefficient in pairs of samples from 132 bowel movements was 0.79 (95% CI 0.48–0.90). The median individual coefficient of variation in samples collected during the same day was 52% (4–178). There was a correlation between the level of calprotectin and the time between bowel movements (r = 0.5; p = 0.013). After 3 days at room temperature the calprotectin concentrations in stool samples were unchanged, but after 7 days a significant (p < 0.01) decrease was found (mean 28%; 95% CI 0.10–0.47).
Conclusion: The present data reveal a great variability in the concentrations of calprotectin in stool samples collected during a single day. Since the levels of calprotectin increased with longer time between the bowel movements, it seems most appropriate to analyse stool from the first bowel movement in the morning. Moreover, storage of stool samples at room temperature for more than 3 days is not advisable.

Keywords: Ulcerative colitis; Calprotectin; Disease activity

Abbreviations

CAI	Clinical Activity Index
SD	standard deviation
ICC	intraclass correlation coefficient
CV	coefficient of variation.

1. Introduction

To assess disease activity in ulcerative colitis, colonoscopy with endoscopic and histologic assessment is the gold standard, although the procedure is invasive, expensive and sometimes inconvenient for the patient.[1] In ulcerative colitis, there is often incongruence between

symptoms, clinical examination and serologic markers since abdominal and bowel symptoms are common even without inflammatory activity.[2] On the other hand, significant inflammation may be present without obvious symptoms or changes in serologic markers like the C-reactive protein (CRP). To overcome these problems, activity scores, such as the Mayo score and the Clinical Activity Index (CAI), are frequently used in clinical trials.[3,4] However, to what extent these scores actually reflect inflammatory activity has been questioned.[5,6]

In recent years, leukocyte-derived proteins, such as calprotectin and lactoferrin, quantified in faeces have emerged as sensitive markers of disease activity in inflammatory bowel diseases (IBD).[7,8] In clinical practice calprotectin has been the most widely used faecal marker for IBD. In several studies, faecal calprotectin has been shown to be a useful tool in the diagnosis of IBD, evaluation of response to therapy and prediction of the disease course.[7,9–15] Moreover, compared to CRP, the Mayo score and the CAI, the faecal concentration of calprotectin significantly better reflects the endoscopic and histologic disease activity.[5,6]

Although faecal markers are proved to be the best markers of disease activity in IBD, some concerns have been raised. When groups of patients with IBD and different levels of disease activity are compared, considerable overlaps in calprotectin concentrations between the groups are seen.[8,11] This makes it difficult to interpret the results for the individual patient. A significant day-to-day variation in faecal calprotectin concentrations in patients with IBD has also been described, and the representativeness of small random stool samples has not been extensively evaluated.[10,16–19]

Although faecal calprotectin has been evaluated in many studies and been in clinical use for years, further investigations regarding the importance of the stool sampling procedure itself have not been made. Therefore, the objectives of this study were to evaluate: 1) the differences in calprotectin concentrations in two random samples collected at each bowel movement, 2) the variations in calprotectin concentrations in faeces over the day, 3) the variations in calprotectin concentrations on a day-to-day basis, 4) the stability of calprotectin in faeces kept at room temperature for up to a week, and 5) the patients' opinions regarding the stool sampling procedure.

2. Patients and methods

Adult patients with left-sided or extensive ulcerative colitis and with a present flare of mild or moderate disease activity according to the modified Truelove–Witts criteria were eligible for the study.[20] Patients with proctitis were excluded from the study, as were patients with topical treatment for their colitis and patients who were using non-steroidal anti-inflammatory drugs. Patients with severe comorbid disease affecting their ability to comply with the study protocol (i.e., severe cardiovascular disease, neurological disease and mental disorder), respiratory tract infection, ongoing menstruation or pregnancy were also excluded.

All patients gave written informed consent according to the Declaration of Helsinki. The study was approved by the Regional Ethical Review Board at the University of Gothenburg.

2.1 Study procedures

At inclusion (Day 0), demographic data and disease characteristics were collected and the disease activity was evaluated according to the Mayo score. The Mayo score is an activity index that consists of four items: stool frequency, rectal bleeding, endoscopic findings, and a physician global assessment.[3] Each item was scored from 0 to

3 and the total score ranges from 0 to 12 points. Active disease was defined as a Mayo score ≥ 3.

Patients were instructed to record in a diary the time of defecation, faecal consistency and the occurrence of blood in the faeces for every bowel movement starting immediately after the inclusion visit and for the following two days. The Bristol stool form scale (range 1–7, i.e., separate hard lumps like nuts–entirely liquid stool) was used to assess faecal consistency and the rectal bleeding subscore in the Mayo score (range 0–3, i.e., no blood–only blood) was used to specify the occurrence of blood in the faeces.[3,21] Stool sampling was done over two days (Days 1–2) beginning the day after the enrolment.

After the two days of sampling, the patients delivered all the stool samples and the diary on Day 3. A flexible sigmoidoscopy was performed and the mucosa was assessed according to the endoscopic Mayo subscore. Biopsies were taken from the rectum and sigmoid colon for histological assessment. Thereafter, an individualized treatment for the inflammatory flare was prescribed. Thus, the treatment was unchanged during the study period and no therapy, except ongoing maintenance treatment, was given for the present disease flare until the study was finished.

2.2 Stool collection procedure

Instructions for the stool collection were carefully described to each patient. A faeces collection paper (Stuhlfänger; Süsse Labortechnik, Gudensberg, Germany) had to be used and 2–3 spoons (approximately 2–3 g) of faeces were collected from two different parts of the faeces and placed in two different plastic tubes (Faeces tube; Sarstedt, Nürnbrecht, Germany) at every bowel movement for two days. Specification of time, consistency and occurrence of blood were recorded in the diary. The patients were instructed to keep the filled faeces tubes in a covered plastic box in a refrigerator until delivery at the gastroenterology outpatient clinic the day after completion of sampling (Day 3). All these samples were immediately frozen and stored at – 70 °C until analysis.

When the first samples were collected on Day 2, an extra plastic tube with at least 4 spoons of faeces was filled. This sample was used to assess the stability of the calprotectin concentration in a sample kept at room temperature for up to a week. At delivery this sample was split into three different plastic tubes, one of them was frozen immediately (i.e., approximately 24 h after sampling) and the other two were kept at room temperature (19–23 °C) and frozen after another 2 and 6 days (i.e., 3 and 7 days after sampling), respectively.

2.3 Patient questionnaire

To evaluate the patient's experience of the sampling itself, a questionnaire was filled. The patients were asked to answer four questions using a 4-graded Likert scale. The questions and corresponding response options are shown in Table 3. The patients also had to indicate whether they had used the special spoon and the faeces collection paper before, and if they would prefer to collect samples with or without the collecting paper in the future.

2.4 Laboratory analyses

Faecal calprotectin was analysed by a sandwich enzyme-linked immunosorbent assay (Calprotectin ELISA; Bühlmann Laboratories AG, Basel, Switzerland) using a monoclonal capture antibody specific for calprotectin, according to the manufacturer's instructions. According to the manufacturer, the normal range for faecal calprotectin is < 50 μg/g. Samples were initially diluted 1/50, and if

calprotectin levels were above the standard curve the samples were diluted 1/400. If a noticeable variation between the two samples collected at the same bowel movement was recorded, both samples were reanalysed after performing new extractions from the original samples.

Routine histology was performed on the mucosal specimens stained with haematoxylin and eosin.

2.5 Statistical analyses

Continuous variables are shown as median and range values. In Table 2, the mean values and standard deviations (SD) for calprotectin are included. Categorical variables are presented as percentages. The results are presented with two significant figures in all tables. The correlation between the concentrations of calprotectin in the two samples collected at the same bowel movement was measured with the intraclass correlation coefficient (ICC). This would be equal to 1 if there was no within-stool variation. For the ICC, the 95% confidence interval (CI) was calculated. To assess the within-day and day-to-day variations, the coefficient of variation (CV) was calculated. The Spearman rho was used to calculate the correlation between either the individual concentrations of calprotectin or the variability of calprotectin during the day and the variables: time between bowel movements, stool consistency and the Mayo subscore for bleeding. The Wilcoxon Signed Rank Test was used to evaluate the stability of calprotectin in stool samples kept at room temperature for up to one week. Statistical significance was set at $p < 0.05$.

3. Results

In all, 18 patients were included at the Gastroenterology Unit, Södra Älvsborgs Hospital, Borås. The clinicopathologic characteristics of the patients are shown in Table 1. Disease location was grouped according to the Montreal Classification.[23] Seven patients were under treatment with oral steroids, three of them in a stable low dose as maintenance treatment and combined with an anti-TNF agent in two patients. Another four patients were tapering their steroids for a previous flare when they relapsed and entered this study.

Altogether, 18 patients delivered 287 stool samples, including one sample each for assessment of stability. One patient made the sampling for just one day, and 17 patients delivered samples from two consecutive days. There were five single samples

Table 1. Characteristics of the 18 patients in the study.

Characteristics	N (%)
Age, years, median (range)	43 (18–73)
Female sex	10 (56)
Smoking	
Current	2 (11)
Former	7 (39)
Never	9 (50)
Disease duration	
Years, median (range)	6 (1–43)
Extent of colitis	
Left-sided	11 (61)
Extensive	7 (39)
Mayo-score, median (range)	7 (3–9)
Therapy (ongoing)	
Mesalazine	15 (83)
Azathioprine	5 (28)
Anti-TNF agent	2 (11)
Steroids	7 (39)

and 132 paired samples from 137 bowel movements, 71 from day one and 66 from day two. In the diaries, another 45 bowel movements were registered, from which no samples were taken. The median number of bowel movements for all patients was 5 (range 2–13) and 4 (range 1–13) during day one and day two, respectively. In Fig. 1, the distribution of all faecal calprotectin results is shown.

Using sigmoidoscopy, endoscopic as well as histological active disease was confirmed in all the patients (Mayo endoscopic subscore one in 7 patients and subscore two in 11 patients).

3.1 Distribution of calprotectin in stool

To determine whether calprotectin was evenly distributed within the stool, the concentrations in two random samples were compared. Seventeen patients delivered 132 pairs of samples from the same number of bowel movements. We found a strong correlation (ICC = 0.79; 95% CI 0.48–0.90) in terms of calprotectin concentrations between the two random samples (Fig. 2).

3.2 Variability of the calprotectin concentrations during the day

The coefficient of variation was calculated for each patient. In Table 2, all the patients' individual results are presented in detail. In summary, median CV was 52% (4–178). The variation was most pronounced in patients and during days with high levels of calprotectin, Fig. 3.

In the literature different cutoff values to identify patients with active ulcerative colitis and to predict mucosal healing have been proposed for calprotectin.[5,12] In the present study, we evaluated if calprotectin in patients with active disease fluctuated over these cutoff values over the day. In six patients the calprotectin values fluctuated below 250 µg/g, in three patients calprotectin values fluctuated below 100 µg/g and in one patient calprotectin values fluctuated below 50 µg/g during the day. In other samples collected during the same day, a high (> 800 µg/g) value of calprotectin was found in all these six patients.

The level of faecal calprotectin correlated statistically significantly with both the time between bowel movements (median r = 0.5, range: − 0.8–0.9; p = 0.013) and the stool consistency (median r = 0.68, range: − 0.68–0.87; p = 0.01). Thus, the longer the time between the bowel movements and the looser the stool consistency, the higher the concentrations of calprotectin. However, the correlation between the presence of blood in the stool and the
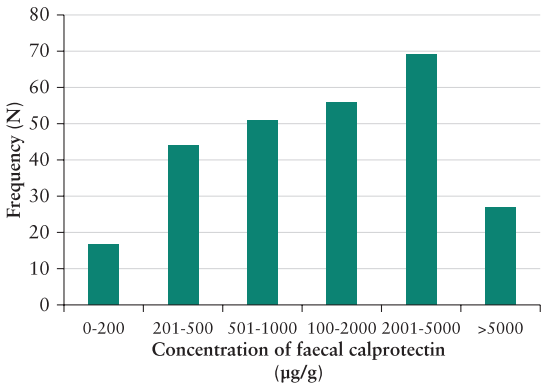

Figure 1. The distribution of all faecal calprotectin in the present study. Stool samples were collected from every bowel movement for two days in 18 patients with active ulcerative colitis.

concentrations of calprotectin did not reach statistical significance (p = 0.057). The variability of calprotectin during the day, both in terms of SD and CV, did not correlate significantly with any of the variables stool consistency, content of blood or time between the bowel movements. Nor did treatment with steroids or thiopurines influence the variability of the faecal calprotectin concentrations.

3.3 Day-to-day variability

We calculated the coefficient of variation based on the mean concentration of calprotectin in the two samples taken at the first bowel movement in the morning of Day 1 and the corresponding mean value on Day 2. The median calprotectin concentrations of Day 1

Figure 2. The plot shows the concentrations of faecal calprotectin in two random samples collected from the same bowel movement (n = 132) in 17 patients with active ulcerative colitis. The diagonal reference line represents the ideal result where Sample 1 = Sample 2. The intraclass correlation coefficient is 0.79.

and Day 2 were 1676 µg/g (232–8560) and 1912 µg/g (254–7947), respectively. The median CV was 40.8% (3.1–127.8). In Fig. 4 the day-to-day variability is displayed for individual patients.

3.4 Stability of calprotectin in faeces kept at room temperature

There was no significant difference in calprotectin concentrations between samples kept at room temperature for one day and three days (Fig. 5). However, after 7 days at room temperature a mean decrease of 28% was found (p < 0.01; 95% CI 0.10–0.47).

3.5 Questionnaire

In all, 17 patients answered the questionnaire. All except two patients had used the stool collection spoon before, while none had experience of the special faeces collection paper. As shown in Table 3, most
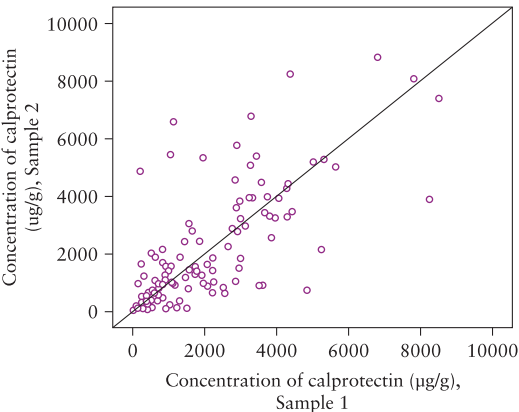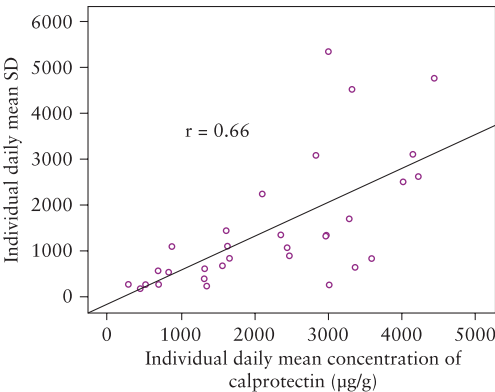
Figure 3. The correlation between the individual daily mean concentrations of calprotectin in patients with mild-moderately active ulcerative colitis, and the corresponding standard deviations (SD), i.e., the correlation between the level of calprotectin and the variability in terms of SD. Days with merely 1 bowel movement were excluded.

Table 2. The individual concentrations of calprotectin in all 18 patients during Day 1 and Day 2.

| Case nr | N[a] | Concentration of calprotectin (µg/g)Day 1 | | CV (%) | N[a] | Concentration of calprotectin (µg/g)Day 2 | | CV (%) |
		Median (range)	Mean; SD			Median (range)	Mean; SD	
1	9	770 (560–12000)	3300; 4500	140	9	4700 (630–8200)	4200; 2600	62
2	2	3700 (3600–3800)	3700; 150	4.0	1	2600	2600; –	–
3	5	3500 (540–5600)	3300; 1700	52	5	2700 (660–4300)	2500; 900	36
4	8	2100 (240–6800)	2400; 1400	58	4	380 (83–4900)	880; 1100	120
5	3	1300	1300; –	–	3	1100 (900–1300)	1100; 190	16
6	4	1200 (920–1900)	1300; 410	31	3	1600 (910–2200)	1600; 680	44
7	5	330 (150–1200)	450; 190	42	4	1200 (210–4800)	1600; 1100	68
8	3	1300 (250–2100)	1327; 620	47	2	960 (3400–9400)	1700; 850	51
9	4	460 (250–1100)	520; 280	53	4	440 (280–1700)	700; 580	83
10	5	3400 (1700–5400)	3600; 850	24	0	–	–	–
11	2	1400 (810–1700)	1400; 240	18	6	960 (160–6600)	1600; 1500	90
12	8	2400 (1300–5300)	3000; 1300	45	9	3500 (1100–8500)	4000; 2500	63
13	6	3700 (390–12000)	4200; 3100	75	6	1400 (470–5200)	2100; 2200	110
14	3	2700 (2600–3900)	3000; 280	9.0	4	3000 (1500–4400)	3000; 1400	46
15	13	210 (24–820)	290; 290	97	13	610 (310–2300)	840; 550	65
16	5	670 (290–1100)	700; 280	41	1	1300	1300; –	–
17	3	4000 (920–8800)	4400; 4800	110	8	880 (96–16000)	3000; 5400	180
18	7	2200 (670–5500)	2400; 1100	44	7	1300 (130–8100)	2800; 3100	110

SD, standard deviation; CV, coefficient of variation.

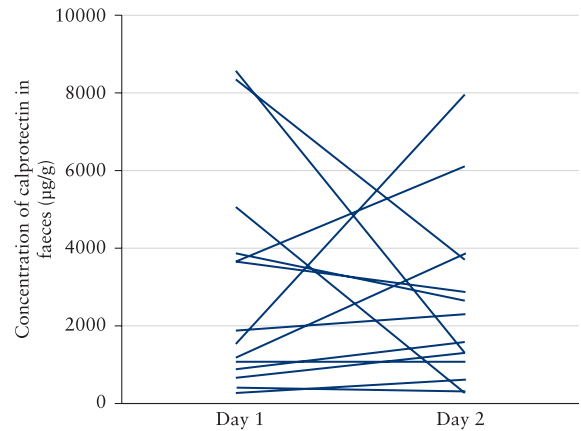[a]Number of bowel movements during Day 1 and Day 2, respectively.

Figure 4. The mean concentration of calprotectin in two stool samples collected at the first bowel movements on two consecutive days in 14 patients with active ulcerative colitis. The median coefficient of variation is 40.8% (range 3.1–127.8).
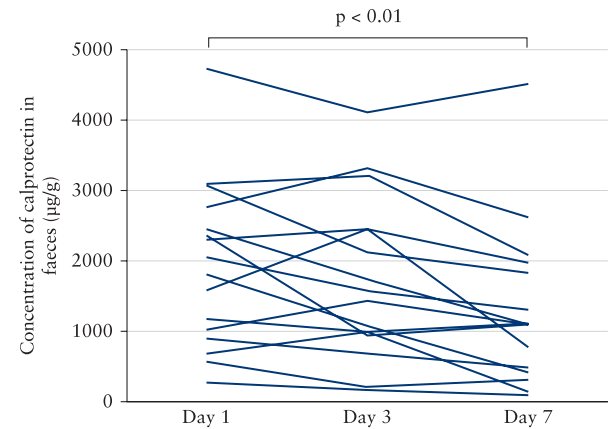

Figure 5. The concentrations of calprotectin in 16 stool samples stored at room temperature for 1, 3 and 7 days. From day 1 to day 7 there was a significant decrease (mean 28%; 95% CI 0.10–0.47) in the concentrations of calprotectin (p < 0.01).

Table 3. The patients' experience of collecting stool samples.

Questions	Answers[c]			
	A	B	C	D
1[a] How did you experience using the faeces collection paper while relieving yourself?	0	4	8	5
2[b] Did you have time to attach the collection paper?	0	1	5	11
3[a] How did you experience loosening the collection paper and flushing it down the toilet?	0	2	3	12
4[a] How did you experience using the spoon for stool sampling?	0	1	5	11

[a]Response options Question 1, and 3–4, A: Very difficult; B: Difficult; C: Acceptable; D: No problem.
[b]Response options Question 2, A: Never; B: Sometimes; C: Almost always; D: Always.
[c]The figures represent the number of patients answering each response option.

randomly collected samples from the same bowel movement was very good. We also state that the time between the bowel movements and the faecal consistency are of importance for the test results.

In clinical practice small random samples of faeces are used to analyse the level of calprotectin. An even distribution of calprotectin in the faeces is crucial to ensure the reliability of such test results. We found a strong correlation between the two samples taken at each bowel movement. This is consistent with the results reported in previous studies, in which the concentrations of calprotectin in three and five small random samples, respectively, were compared with the concentrations in blended stool.[18,19] In the present study, with patients collecting the samples themselves, we did not compare our test results with the concentration in homogenised faeces, which is the method of choice. However, since our results are comparable with those of the previous reports, the use of small, random, patient-collected samples in clinical practice is supported.

A day-to-day variability in the concentrations of faecal calprotectin has been reported in some papers before,[10,16,17] but the variability of calprotectin concentrations during the same day has not been in focus. In our study, patients with active ulcerative colitis were included and the variability in terms of CV was surprisingly high during the day. Since the correlation between two samples from each bowel movement was strong, we consider the variation true and it cannot be explained by sampling errors, the assay used or problems related to laboratory techniques. However, it is important to stress that the variation was greatest in patients with high levels of calprotectin, and the variation increased with higher concentrations of calprotectin. Most of our patients had high levels of calprotectin throughout the day, and a variation in those patients is of little clinical relevance regardless of how large it is. However, due to the great variability in samples from the same day, it is not possible to make an assessment of the severity of the disease activity in the individual patient with IBD based on the levels of calprotectin. Moreover,

patients found it *acceptable* or *without problems* to handle the collection devices. Finally, 11 (65%) patients answered that they would prefer to use the collection paper instead of no collection paper at all at the next time sampling.

4. Discussion

In most studies on faecal calprotectin as a marker of intestinal disease, the stool sampling procedure is only described in general terms and hardly ever in detail. This information is omitted despite a lack of a standardized way to collect stool samples. In our study we have tried to identify and evaluate some factors that potentially affect the test results. In the present study we found that the variability of the faecal calprotectin concentrations, in samples collected during the same day, in patients with active ulcerative colitis, was considerable and in some cases even of clinical importance. However, we found the variability most pronounced in patients with high levels of calprotectin and thus with a low clinical relevance. On the contrary, the correlation between the calprotectin concentrations in two

concerns should be raised when values during the same day fluctuate below the level of anticipated endoscopic remission.[5] Such a fluctuation was found in 33% of our patients.

We used information from the diaries to identify factors related to the calprotectin level and variability. Interestingly, we found that the longer the time between the bowel movements, the higher the concentrations of calprotectin. This information could be clinically useful, especially in patients with no bowel movements during the night and in patients with frequent bowel movements during the morning and then another few during the evening. These patients should preferably collect stool samples at the first defecation in the morning and in the evening, respectively. Furthermore, to be able to compare values over time, the patients should be recommended to always take their samples with approximately the same time interval since the previous bowel movement.

It is reasonable to speculate that leukocyte-derived proteins released into the gut lumen from the inflammatory process, accumulate in the lumen between the bowel movements and are drained during defecation. Consequently, the faecal concentrations of these leukocyte proteins are highest in samples collected when there is long time between the defecations. Our results support this hypothesis and to plan the sampling procedure accordingly is possibly a way to avoid false low results.

We also found a correlation between the stool consistency and the level of calprotectin in faeces. It is reasonable to assume that this is a consequence of the disease activity. Furthermore, we observed that in samples with a lot of mucus, the concentrations of calprotectin were particularly high, but this was not systematically investigated. Still, the amount of mucus in the stool samples can be an explanatory factor to the great intra-individual variability of calprotectin concentrations in patients with IBD.

As day-to-day variations in the concentrations of calprotectin in faeces have been described primarily for patients with active Crohn's disease,[10,17] we asked whether the same was true for patients with active ulcerative colitis. Since the variability within a single day turned out to be high, we preferred to compare the concentrations of calprotectin in the first samples in the morning of Day 1 with the corresponding values on Day 2. Hence, the variability was lower than we found in samples during the same day but similar to what has previously been described for patients with IBD in clinical remission and healthy controls.[16] High degree of variation appears to be most obvious in patients with active IBD, since results from a recent study showed low variability in patients with quiescent Crohn's disease.[24]

There is great consensus regarding the stability of calprotectin in faeces kept at room temperature for 3 days.[18,19,25] However, we found a 28% decrease in the concentrations of calprotectin, when the samples were stored at room temperature for 7 days. Still, this does not impair the possibility to send samples with ordinary mail, but it is not advisable to send the sample so it has to be stored at the post office during the weekend. As far as we know, the stability of calprotectin in faeces kept in a refrigerator has not been assessed for more than 48 h.[18] This is of interest, especially if the laboratories perform the analyses once a week.

Finally, we evaluated how patients experienced the sample procedure itself. The result from our survey clearly shows that this is of minor concern to most patients and adequate equipment is preferred to avoid taking the sample directly from the toilet basin. Different complete faeces collection kits are now available on the market and should be recommended.

Some weaknesses in the study need to be discussed. The study cohort is relatively small, but we believe it is big enough to give clinically relevant and useful information. Furthermore, only patients with active ulcerative colitis were included in this study. Thus, it is not known whether our results are applicable for patients with other intestinal disorders. We have not included a control group in the present study, since the objectives were to evaluate the fluctuation of calprotectin over the day and to find some explanations to the fluctuation, to provide recommendations regarding the stool sampling procedure in these patients.

In conclusion, the present study reveals a great variability in the concentrations of calprotectin in stool samples collected during a single day. Since the levels of calprotectin increased as the time between the bowel movements increased, it seems most appropriate to analyse stool from the first bowel movement in the morning. We also confirm previous studies regarding a day-to-day variation, although the clinical significance of such variability is unsure. Moreover, storage of stool samples at room temperature for more than 3 days is not advisable.

Conflict of interest statement

All authors have made substantial contributions to the manuscript. The specific contribution of each author is listed in the Acknowledgements section.

The authors have no financial conflicts of interest to report.

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Authorship statement

Guarantor of the article: Anders Lasson.

Author contributions: AL, P-OS, LÖ, SI and HS contributed equally to the design of the study. AL collected and analysed the data. SI and MS analysed the stool samples. AL wrote the article and all the other authors contributed with critical revision of the manuscript for important intellectual content and interpretation of the data. All authors read and approved the final version of the manuscript.

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