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ARTICLE

Impact of Preanalytical Factors on Calprotectin Concentration in Stool: A Multiassay Comparison

Henrike M. Hamer [a,†] A.H. Leontine Mulder,[b,c,†] Nanne K. de Boer,[d] Femke Crouwel [d] Patrick F. van Rheenen [e] Marlies Spekle,[b] Marloes Vermeer,[f] Lucie Wagenmakers-Huizinga,[g] and Anneke C. Muller Kobold [g,*]

Background: Measuring calprotectin concentration in stool is increasingly important in monitoring disease activity and treatment response in inflammatory bowel disease. This study evaluates the impact of preanalytical storage conditions on reliability of calprotectin testing using 5 different calprotectin immunoassays.

Methods: Aliquots of homogenized fresh fecal samples in untreated or extracted form were stored at room temperature or 4°C. Calprotectin concentration was measured day 0 to 4 and 8. Five different immunoassays and accompanying extraction buffers were used (CALiaGold, Phadia EliA, Bühlmann fCal turbo, ELISA Bühlmann, Inova Quanta Flash). Repeated measurements of change from baseline calprotectin levels over time were analyzed using a mixed model analysis.

Results: Calprotectin concentrations declined over time under all preanalytical conditions with all assays, except for extracted feces stored at 4°C. The rate of decline was greatest in untreated stool kept at room temperature, reaching significant difference from baseline already after 1 day ($P < 0.001$). In extracted feces kept at room temperature, significant difference from baseline was reached after 2 days, and in untreated feces at 4°C, after 4 days. However, the results differed significantly between assays. After 4 days of storage at room temperature, the mean calprotectin decline from baseline differed between 30% and 60%, dependent on the assay used.

Conclusions: Fecal calprotectin concentration in stool samples declines over time, and the rate of decline is greater at higher temperatures. In extracted feces stored at 4°C, calprotectin is most stable. It is assay-dependent how long extracted feces stored at 4°C give reliable test results.

[a]Department of Clinical Chemistry, AGEM Research Institute, Amsterdam UMC, Amsterdam, The Netherlands; [b]Clinical Chemistry, Medlon BV, Enschede, The Netherlands; [c]Clinical Laboratory, Ziekenhuisgroep Twente, Almelo, The Netherlands; [d]Department of Gastroenterology and Hepatology, AGEM Research Institute, Amsterdam UMC, VU University, Amsterdam, The Netherlands; [e]Department of Pediatric Gastroenterology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [f]ZGT Academy, Ziekenhuisgroep Twente, Almelo, The Netherlands; [g]Department of Clinical Chemistry, University of Groningen, University Medical center Groningen, Groningen, The Netherlands.

*Address correspondence to this author at: PO Box 30001, 9700 RB, Groningen, The Netherlands. Email: a.c.muller@umcg.nl.

[†]These authors contributed equally to this study.

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IMPACT STATEMENT

Because calprotectin is generally believed to be resistant to bacterial degradation in the colon and to be stable for 3 to 7 days at room temperature without preservation buffer, many hospitals allow patients to collect fecal samples at home and send them by ordinary mail to the local laboratory. However, recent work has shown that preanalytical and analytical variables can influence the fecal calprotectin measurement. This study shows that the stability of fecal calprotectin depends on the assay system used and whether the sample is stored in an extraction buffer.

INTRODUCTION

In inflammatory bowel disease (IBD), fecal calprotectin measurement is increasingly important in selecting patients for diagnostic endoscopy, monitoring of disease activity, and evaluation of treatment response (1–4).

Calprotectin is a calcium-binding protein mainly produced in neutrophils (5). It is said to be resistant to bacterial degradation in the colon, and the literature almost unanimously states that calprotectin is stable up to 7 days at room temperature without preservation buffer (5–10). These claims result in many laboratories supporting collecting fecal samples at home and sending the samples to a laboratory by regular mail. However, more recent studies raised concerns regarding the stability of calprotectin (11–13), as they demonstrated a decrease in fecal calprotectin levels in aliquots kept at room temperature. One study observed a significant drop in fecal calprotectin concentration after 1 day of storage at room temperature (11). In the international consensus paper on methodological issues in standardization of fecal calprotectin measurements (14), the importance of the preanalytical conditions was addressed as well. Three of 5 research gaps the authors recognized concern the preanalytical phase, one being that the best strategy for stool storage at room temperature should be investigated. Decreased concentrations due to preanalytical conditions could lead to unjustified reassurance and overinterpretation of the patient's response to treatment (11). Direct analysis in the laboratory using automated random access analyzers may overcome the need for sample storage; however, it does not overcome the need for sample transport and sample extraction. The laboratory has to choose between storage of the sample and performance of the extraction batchwise or an immediate extraction upon reception of the sample. Frequently, patients are asked to collect feces at home and bring or send the sample, at room temperature, to the laboratory. Sample transport as well as sample storage and sample extraction are conditions that need to be taken into account when investigating optimal preanalytical conditions. Nowadays, collection tubes for feces that contain extraction buffer are available so that the patient can collect and extract the feces in one step at home. These tubes make storage of either the stool or the extract unnecessary for laboratories with random access analyzers for calprotectin.

To determine fecal calprotectin levels, several methods are available (15–17). Due to the lack of an international reference preparation, calprotectin assays are not standardized, and concentrations differ significantly between assays (15). For the extraction of calprotectin from the fecal sample, each manufacturer has developed a sampling device with an accompanying extraction buffer. The differences between these extraction buffers

and whether these differences affect the stability of calprotectin remain unknown.

The main objective of this multicenter study was to investigate the effect of ambient temperature and storage time on calprotectin levels. We also compare the short-term stability of calprotectin between different assays and their accompanying extraction systems.

MATERIALS AND METHODS

Samples

Prior to sampling, leftover feces samples were homogenized with a disposable stir stick. Subsequently, 45 samples were prepared by splitting 9 homogenized stool samples with calprotectin concentrations that spanned the measuring range of the calprotectin assays (100–2000 μg/g as determined with the local calprotectin assay) into 5 aliquots. The stool samples were not older than 24 h, Bristol stool scale 2–6 (18). Storage and transportation to the participating centers occurred at −20°C.

Study Design

Forty-five frozen anonymized feces aliquots were distributed among the 3 participating centers. The design of the study is depicted in the flowchart in Fig. 1. At the start of the experiment, the samples were thawed and split for either untreated or extracted storage. We assessed the calprotectin concentration over time under 4 conditions, including (a) untreated native stool stored at room temperature (NRT), (b), stool extract stored at room temperature, (c), untreated native stool stored at 4°C, and (d), stool extract stored at 4°C. Aliquots for calprotectin measurement were taken at baseline (t = 0) and at 1, 2, 3, 4, and 8 days, and all samples were subsequently frozen until analysis. To minimize batch-to-batch analytical variation, all analysis of calprotectin was performed as a single measurement in one batch in each center within 2 weeks. In total, 902 out of 945 possible results (95%) were obtained. The missing numbers were due to shortage of fecal sample; in that case, if so, time point 2, 3, or 4 was skipped to obtain at least a result at t = 8.

Calprotectin Assays

In the 3 participating hospitals, a total of 5 different assays were used for calprotectin measurements (Tables 1 and 2).

The Bühlmann fCAL® turbo test (Bühlmann Laboratories AG) is a particle-enhanced turbidimetric immunoassay performed on a COBAS 6000 e501 (Roche Diagnostics).

The Bühlmann fCAL® enzyme-linked immunosorbent assay (ELISA; Bühlmann Laboratories AG) is a sandwich-based ELISA performed and analyzed using a DS2 Dynex ELISA robot.

The CALliaGold® test (Sentinel CH) is a particle-enhanced turbidimetric immunoassay (PETIA). Analysis was performed using a SENTiFIT 270 Analyzer.

EliA™ Calprotectin test (Thermo Fisher Scientific) is a fluorescence enzyme immunoassay. Analysis was performed using the Phadia 250.

QUANTA Flash Calprotectin (Inova Diagnostics) is a chemiluminescent immunoassay. Analysis was performed on BIO FLASH®. An overview of the characteristics of the 5 test systems is given in Table 1.

For each test method, the stool sample was extracted with the extraction device and extraction buffer as recommended by its manufacturer.

To compare the results between assays, calprotectin levels determined using different assays were expressed as μg/g feces and as percentage recovery based on the calprotectin level at t = 0. Since timepoint t = 0 was identical for all conditions, this sample was analyzed only once at each center for each assay. The concentration of calprotectin at t = 0 was set at 100%.

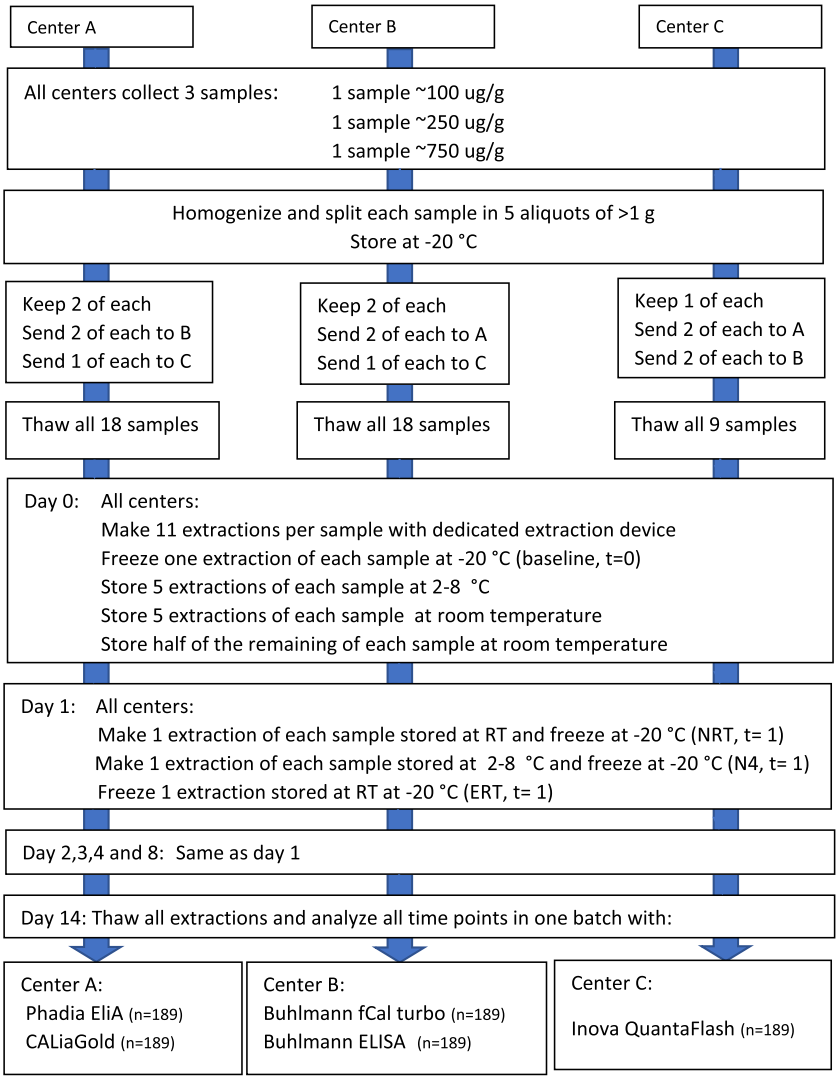

Fig. 1. Study design.

Statistics

Intra-assay CV from all assays described in the Methods section were determined by each laboratory using the CLSI EP15 guideline.

Repeated measurements of calprotectin levels over time were analyzed using mixed model analysis, where group (i.e., preanalytical condition or assay type) and measurement (calprotectin recovery) were both added as fixed variables. The interaction term group × measurement was added to analyze differences between groups over time. For all comparisons, a P value < 0.05 was regarded as significant. Data were analyzed using the software program SPSS for Windows, version 24 (IBM). Data were visually checked for normal distribution with histograms. Homogeneity of variances was checked with residual plots.

Power analysis was performed based on an expected correlation in calprotectin levels between

Table 1. Characteristics of the fecal calprotectin assays used in this study.

Calprotectin assay	Test principle	Weight of feces for extraction, mg	Fecal extraction dilution	Measuring range, µg/g	Antibody system used (as indicated by manufacturer)
Sysmex CALiaGold	Particle-enhanced turbidimetric immunoassay	10	1:170	22–2200	Polyclonal avian
ThermoFisher Phadia EliA	Fluorescence enzyme immunoassay	30	1:50	3.8–6000	Monoclonal native (capture) Monoclonal mouse (detection)
Bühlmann fCal turbo	Particle-enhanced turbidimetric immunoassay	10	1:50	26–8000[a]	Polyclonal avian
Bühlmann ELISA	Enzyme-linked immunosorbent assay	10	1:50	10–1800[a]	Monoclonal mouse (capture) Monoclonal mouse (detection)
Inova Quanta Flash C.	Chemiluminescence immunoassay	56	1:50	16.1–3500	Polyclonal rabbit (capture) Monoclonal mouse (detection)

[a]After dilution.

Table 2. Stability of native and extracted feces according to the manufacturer's inserts.

	Native feces	Extracted feces
CALiaGold	3 days at 20°C to 25°C or 6 days at 2°C to 8°C	3 days at 28°C and 6 days at 2°C to 8°C. Frozen (−20°C): stable up to 12 months
Phadia EliA	3 days at 2°C to 8°C; otherwise, freeze at −20°C	3 days at room temperature, 7days at 2°C to 8°C, 3 months at −20°C
Bühlmann fCal turbo	Transport at room temperature or refrigerated, arrival within 3 days. Upon arrival, refrigerate at 2°C to 8°C and extract within 3 days. Do not store samples at elevated temperatures	Stable at room temperature (23°C) for 7 days, at 2°C to 8°C for 15 days and at −20°C for at least 23 months
Bühlmann ELISA	At least 6 days at 2°C to 8°C	Stable for at least 7 days at 2°C to 8 C and for at least 24 months at −20°C
Inova Quanta Flash	Up to 4 days at 2°C to 8°C; otherwise, freeze at −20°C	Stable for up to 72 h at room temperature (20–25°C); refrigerated (2–8°C), up to 21 days; frozen (−15°C to−25°C), up to 3 months

the time points of 0.80, using data from a previous study (11). An effect size of 0.25, a power of 0.95, and an alpha of 0.05 were used, which resulted in a minimum sample size of 20. We chose to include 45 samples.

ETHICAL CONSIDERATIONS

All samples were leftover anonymized fecal samples. The Medical Ethics Review Board of the University Medical Centre Groningen concluded

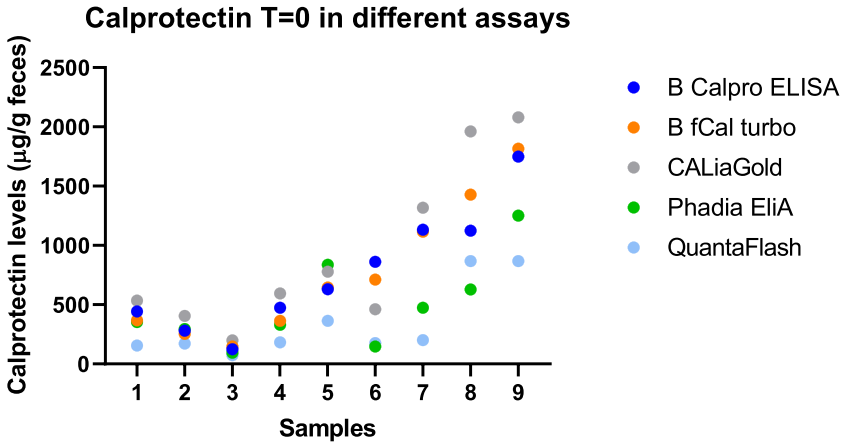

Fig. 2. Fecal calprotectin levels (µg/g feces) of 9 samples (x-axis) measured with 5 different assays.

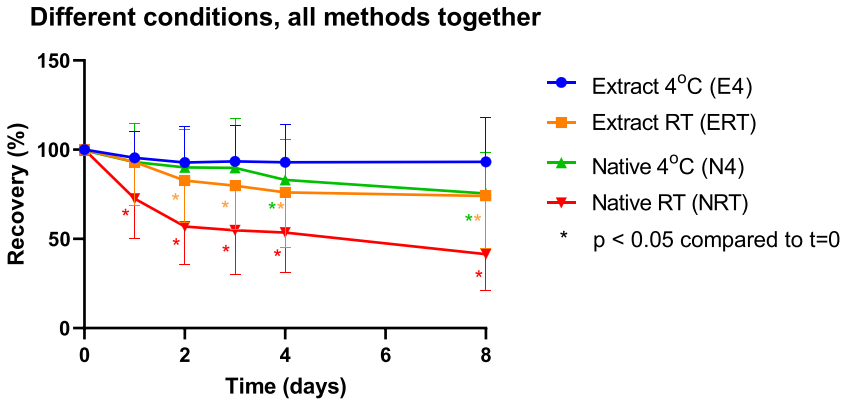

Fig. 3. Calprotectin recovery (%, y-axis). Pooled results of 5 different assays. For statistical significance of differences between timepoints, see Table 2. The bars represent the standard error of the mean.

that this study was not considered as clinical research with human subjects (METc 2019/372) and waived the need for ethical approvement or consent.

RESULTS

Calprotectin Concentrations of Different Samples

Baseline calprotectin levels of the 9 samples measured with the different assays are shown in Fig. 2. Calprotectin concentrations varied considerably depending on the assay used, and the variation between assays was larger at higher calprotectin concentrations.

Change from Baseline Calprotectin of Different Preanalytical Conditions over Time

The pooled calprotectin change from baseline for the 4 preanalytical conditions is shown in Fig. 3 and Table 3. Calprotectin concentrations in stool extracts kept at 4°C remained stable until day 8. In the other 3 storage conditions, calprotectin concentrations declined from baseline. The largest decline was

Table 3. Time to reach significant change from baseline for each preanalytical condition: pooled results of 5 different assays.

	P value[a]																	
	Extract stored at 4°C				Extract stored at room temperature				Native sample stored at 4°C				Native sample stored at room temperature					
	t = 0	t = 1	t = 2	t = 3	t = 4	t = 0	t = 1	t = 2	t = 3	t = 4	t = 0	t = 1	t = 2	t = 3	t = 4	t = 0	t = 1	t = 2	t = 3	t = 4
t = 1	NS[b]					NS					NS					<0.001				
t = 2	0.052	NS				<0.001	NS				NS	NS				<0.001	<0.001			
t = 3	NS	NS	NS			<0.001	NS	NS			NS	NS	NS			<0.001	<0.001	NS		
t = 4	NS	NS	NS	NS		<0.001	NS	NS	NS		<0.001	NS	NS	NS		<0.001	<0.001	NS	NS	
t = 8	NS	NS	NS	NS	NS	<0.001	NS	<0.05	NS	NS	<0.001	<0.001	<0,05	NS	NS	<0.001	<0.001	<0.001	<0.001	<0.001

[a] $P < 0.05$ is statistically significant.
[b] Not significant.

observed in untreated feces kept at room temperature, which already showed a significant difference on day 1 compared to baseline. After 4 days of storage at room temperature, the mean calprotectin decline from baseline differed between 30% and 60%, depending on the assay used (Fig. 3). Samples with high calprotectin levels did not yield different results compared to samples with low calprotectin levels.

Effect of Different Preanalytical Conditions and Assays

Change from baseline calprotectin was stratified according to the assay in relation to the preanalytical conditions (Fig. 4). For all individual assays, samples extracted and stored at 4°C had the best recovery, while lowest recovery was found for NRT. Statistical analysis showed that this difference between NRT and extracted samples stored at 4°C was significant for all assays except the CaliaGold assay (Table 4).

When comparing assays within the same preanalytical conditions, we found only significant differences for extracts stored at 4°C. QUANTA Flash recovery was significantly lower compared to the CALiaGold assay (Phadia Elia Calprotectin test, $P = 0.03$; fCAL turbo Bühlmann, $P = 0.009$; Bühlmann Calpro ELISA, $P = 0.008$). For the other conditions, no significant differences were found between the assays (data not shown).

DISCUSSION

In this study, we investigated the effect of different preanalytical conditions on fecal calprotectin recovery, using different assay systems. We demonstrated that extracted feces kept at 4°C for up to 8 days resulted in the least change from baseline of calprotectin, independent of the used assay. NRT were the least stable, and a significant decrease was already observed after 1 day.

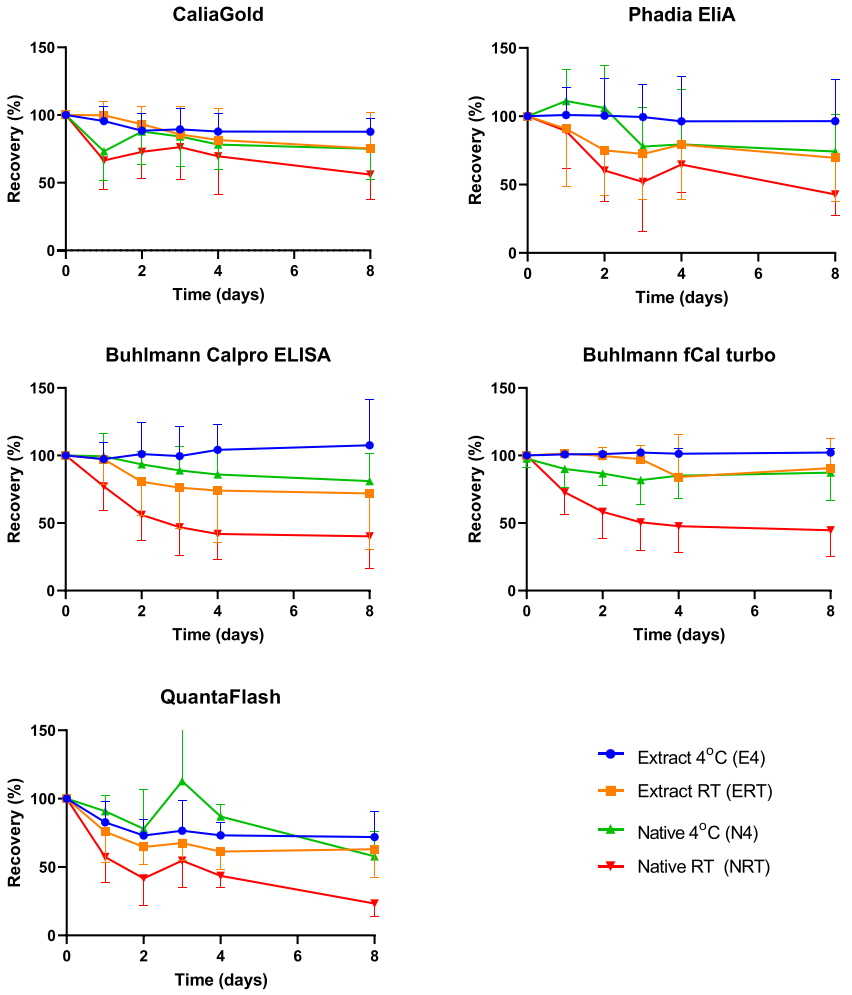

Fig. 4. Calprotectin recovery (%, y-axis) in time (days, x-axis). Results are shown for CALiaGold, Phadia ELIA, Bühlmann Calpro ELISA, Bühlmann fCal turbo, and Innova QuantaFlash. For statistical significance of differences between conditions, see Table 3. The bars represent the standard error of the mean.

In general, in IBD literature, calprotectin is considered a stable biomarker (5–10). Most publications refer to an initial publication by Roseth et al., published in 1992, which stated that calprotectin is stable for 7 days at room temperature (5). However, recent literature has shown that there is loss of immunoreactive calprotectin over time (11–13). The present study additionally demonstrates that calprotectin recovery over time differs depending on storage temperature, the type of assay used, and whether or not samples are extracted immediately.

All manufacturers advise the storage of feces or extract in the assay inserts (Table 2). Buhlmann and Sysmex state that native feces can be stored for several days at room temperature before extraction. Our study shows that the decline of the calprotectin is already significant after 1 day of storage of native stool at room temperature. Inova claims stability of extracted feces for at least 72 h stored at

Table 4. Comparison of the effect of the preanalytical condition for each assay.

	P value[a]														
	CALiaGold			Phadia			Bühlmann ELISA			Bühlmann fCal turbo			QuantaFlash		
	E4[b]	ERT[c]	N4[d]	E4	ERT	N4	E4	ERT	N4	E4	ERT	N4	E4	ERT	N4
ERT	NS[f]			NS			NS			NS			<0.05		
N4	NS	NS		NS	NS		NS	NS		0.05	NS		NS	<0.05	
NRT[e]	0.09	NS	NS	<0.05	NS	NS	<0.05	0.07	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

[a]$P < 0.05$ is statistically significant.
[b]Extracted feces stored at 4°C.
[c]Extracted feces stored at room temperature.
[d]Native feces stored at 4°C.
[e]Native feces stored at room temperature.
[f]Not significant.

room temperature; however, our study shows significant decline for all timepoints (t = 1 to t = 8) compared with t = 0 if extracted samples stored at room temperature were analyzed with Inova Quanta Flash (data not shown). Our data show that the information in the kit inserts is not always accurate. The new upcoming European In Vitro Diagnostics Regulation may hopefully result in more reliable information from the manufacturers.

Changes in calprotectin recovery may be explained by differences in immunoreactivity of the antibodies used in the different assays. Assays using monoclonal antibodies depend on one stable epitope, whereas assays using polyclonal antibodies, detecting several epitopes, may allow for more epitope variation. Our study was unfortunately not powered to be able to investigate this. Furthermore, no information was available from the assay manufacturers concerning type of monoclonal antibody used and/or epitope recognized by these antibodies. The loss of reactivity was also seen in samples that were stored as extracts, although to a much lesser extent. Thus, the storage of samples as extracts allows for longer storage times prior to analysis, although the exact time depends on the assay used. Of all the assays tested, the QUANTA Flash assay showed the highest loss of reactivity of the extracted samples, starting from day 1.

Calprotectin is a noncovalent heterodimeric protein complex (S100A8/S100A9). The 2 proteins that assemble as the calprotectin heterodimer are not equally susceptible to proteolysis (19). Thus, for the fecal calprotectin recovery, it may also be important toward which protein subunit the antibodies in the assays are directed.

A major strength of our approach is that we comprehensively investigated calprotectin stability using different assays in a multicenter design. This study is the first to demonstrate that the magnitude of the effect of preanalytical conditions on calprotectin recovery depends on the type of assay used.

Some limitations also need to be considered. The stability experiments were not performed using completely fresh fecal material. To minimize the effect of ongoing calprotectin degradation, only feces samples collected within 24 h were included. According to the kit inserts, this would not influence the results. A last and important issue to be raised is the fact that calprotectin assays suffer from a lack of international standardization. Although this study was not designed to investigate assay agreement, we observed that the amount of calprotectin at t = 0 varied by a factor of 3.4 (range 2–6) depending on the assay that was used. In most, but not for all samples, the CALiaGold assay produced the highest calprotectin levels, and the QUANTA

flash assay, the lowest calprotectin levels. This confirms the recommendation of D'Amico et al. that standardization of the method is needed (14) and emphasizes the importance of using the same test during the follow-up of individual patients.

In monitoring the disease activity in patients with IBD, fecal calprotectin has gained an important clinical role. One of the major reason for this success is based on the general assumption that fecal calprotectin is a stable biomarker, even to the extent that in many medical centers, patients are instructed to collect a fecal sample at home and send it via regular mail to the laboratory for analysis. Based on our results, this strategy is not feasible, and physicians should include a safety margin when interpreting calprotectin results. Such a safety margin, also termed a grey zone, has been suggested previously (11, 20, 21). A grey zone of 100 to 250 μg/g is advised for an adult patient with known IBD, a grey zone of 250 to 500 μg/g for children with IBD, and a grey zone of 50 to 100 μg/g is advised for patients without known IBD. Calprotectin results within these values should be reevaluated by repeated testing of a fresh fecal sample. In our study, the number of results falling within the grey zone or a result below this grey zone was significantly larger when feces was stored as NRT, compared to when feces was stored as extracted feces at 4°C, indicating the clinical importance of choosing the right preanalytical conditions. We interpret calprotectin values in children < 250 μg/g as IBD in biochemical remission and calprotectin values > 500 μg/g as active disease that requires treatment intensification. We ask patients with calprotectin values in the intermediate grey zone to retest in 1 month before progressing to a therapeutic decision. The intermediate grey zone is where calprotectin instability potentially leads to underdiagnosis of active disease and undertreatment. Test results outside the grey zone will not lead to management errors.

CONCLUSION

This study has shown that preanalytical conditions have a significant impact on fecal calprotectin recovery and thus may also impact clinical management of a patient. Furthermore, the stability also depends on the assay system and whether the sample is stored in an extraction buffer. We demonstrated that extracted feces kept at 4°C resulted in the most optimal recovery of calprotectin, although storage of native samples at 4°C was feasible for some assays. Therefore, each laboratory should optimize the preanalytical conditions according to the assay used. When interpreting the calprotectin result, the preanalytical conditions need to be considered.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved*.

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