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ORIGINAL ARTICLE

Reference Intervals for Fecal Calprotectin in Adults Using Two Different Extraction Methods in the Uppsala-SCAPIS Cohort

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SUMMARY

Background: **Fecal calprotectin measurement is generally recommended to exclude inflammatory bowel disease (IBD) in patients with suspected IBD. A problem with the fecal calprotectin assays so far has been the rather long test-turnaround times. Recently a particle enhanced turbidimetric immunoassay (PETIA) for fecal calprotectin with assay times of approximately 10 minutes has been introduced on the European market. The aim of this study was to define reference intervals for adults with this new fecal calprotectin PETIA using two different extraction methods.**
Methods: **Samples were collected from 382 healthy individuals from the Swedish CArdioPulmonary bioImage Study (SCAPIS) Uppsala cohort in the age range 50 - 65 years. 202 samples were processed with CALEX® Cap extraction device (BÜHLMANN, Schönenbuch, Switzerland) and 180 samples were extracted using weighed samples. The extracted samples were analyzed on a Mindray BS-380 using the fCal Turbo PETIA reagent (BÜHLMANN).**
Results: **The calculated reference values for the Calex device were < 199 µg/g for the whole cohort, < 184 µg/g for females, and < 215 µg/g for males, while the corresponding values for weighed samples were < 153 µg/g for the whole cohort, < 141 µg/g for females, and < 215 µg/g for males. There were no significant statistical differences for calprotectin levels in males and females.**
Conclusions: **The CALEX device yielded slightly higher calprotectin values. As there were no significant gender differences, the study indicates gender independent reference intervals of < 199 µg/g feces for the CALEX device and < 153 µg/g feces for weighed samples in patients in the 50 - 65 year age range.**
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INTRODUCTION

Inflammatory bowel disease (IBD) is caused by the interaction of environmental and genetic factors leading to immunological responses and inflammation in the intestines. Crohn's disease and ulcerative colitis are the

best-known forms of IBD. It may be difficult to distinguish IBD patients from patients with irritable bowel syndrome (IBS), especially early in the course of the disease. Traditionally, IBD has been diagnosed with blood markers such as erythrocyte sedimentation rate (ESR) and C-reactive protein in combination with colonoscopy [1,2]. Colonoscopies are costly and burdensome for the patient and the investigation may also worsen the disease state [3]. ESR and CRP are not sufficiently specific for intestinal inflammation and over the last decades stool markers have thus emerged as new diagnostic tools to improve the diagnosis or rule out of IBD. Fecal calprotectin has emerged as the most widely used stool marker for IBD with a higher sensitivity and specificity for IBD than ESR and CRP [4-6]. Calprotectin is a small calcium binding protein consisting of a heterodimer of two S100 proteins, calgranulin A (S100 A8) and calgranulin B (S100A9). It is the most abundant protein in the cytoplasm of neutrophils where it represents approximately 30 - 60% of the protein content [7]. Mucosal inflammation leads to infiltration of granulocytes in the inflamed areas of the gut and may also cause bleeding. Both these mechanisms increase the levels of fecal calprotectin in stool samples [8].

We evaluated a newly developed particle enhanced turbidimetric assay (PETIA) for calprotectin that can be applied on chemistry analyzers available at most clinical chemistry laboratories. Two different extraction methods were compared; CALEX® Cap extraction device and extraction using weighed samples.

The aim of the present study was to establish reference intervals for the fecal calprotectin PETIA using a cohort of healthy individuals in the age range of 50 - 65 years.

MATERIALS AND METHODS

Samples

The study population for this investigation originated from the first 400 participants in the Swedish Cardio Pulmonary bioImage Study (SCAPIS) at the Uppsala site from October 1, 2015 - April 1, 2016. In brief, a random sample of the population in Uppsala municipality aged 50 - 65 years was invited to participate in a detailed health investigation with the aim to include 5,000 participants from 2015 - 2018. The investigations include three onsite visits, and, at the end of the first visit, the participants were given instructions and material to collect a fecal sample at home. The participants were instructed to put the sample in their freezer until they went to the second day of investigations. At arrival to the test center, the fecal samples were barcoded and stored at -80°C until analysis. All participants provided written informed consent. The study was approved by the Regional Ethics Committees in Umeå (2010-228-31M) and in Uppsala (2016/387). Of the 400 first participants, stool samples were available from 385 individuals. Study subjects with self-reported Crohn's disease and ulcerative colitis (n = 3) were excluded from the study, yielding a final study sample of 382 samples.

Sample extraction

Two hundred two samples were extracted with CALEX® Cap extraction device (BÜHLMANN, Schönenbuch, Switzerland) and 180 samples were extracted using weighed samples. CALEX® is a simple one-step extraction where a pin in the sampling tube is used to collect the fecal sample and then reinserted in the tube containing the extraction buffer. The sample tube is then mixed and centrifuged prior to analysis. For the weighed samples, approximately 100 mg feces were weighed and extraction buffer was added to a dilution of 1:50. A metal spring was added to the tubes to facilitate mixing and the tubes were mixed. When the fecal samples were dissolved they were diluted 1:10 with extraction buffer and centrifuged to remove any solids before the analysis.

F-calprotectin assay

Fecal calprotectin was analyzed on a Mindray™ BS-380 (Mindray Medical International, Shenzhen, China) with fCal Turbo reagent (BÜHLMANN, Schönenbuch, Switzerland). The total coefficient of variation for the F-calprotectin assays was 3.2% at 77 and 1.7% at 256 µg/g feces.

Statistical analysis

Calculations of reference intervals and 90% confidence intervals were performed using bootstrap estimation in the program RefVal 4.0 (Department of Clinical Chemistry, Rikshospitalet, N-0027 Oslo, Norway) [9,10]. The determination and evaluation of equality of the reference intervals were performed according to Clinical Laboratory Standards Institute guidelines EP28-A3C. We evaluated if the reference interval for males and females could be combined or should be presented separately by the method described by Lahti et al. [11]. In short, if the proportion of the male or female subgroup distributions outside the combined distribution either exceeds 4.1% or lies below 0.9%, partitioning of reference interval is recommended. Spearman's rank correlations were used to assess correlations of fecal calprotectin with weight, height, and age. The Mann-Whitney *U* Test was used to assess association with gender.

RESULTS

CALEX® Cap extraction device

No significant Spearman's rank correlations were observed between calprotectin concentrations using the CALEX extraction device and weight, height or age. No significant gender effect was observed with the Mann-Whitney *U* Test.

The calculated reference values for the CALEX device were < 199 µg/g feces for the whole cohort and < 184 µg/g for females and < 215 µg/g for males. Evaluation according to the Lahti method suggested a com-

Table 1. Age, weight, and height for the study subjects divided according to gender and extraction method.

CALEX® Cap extraction device		
	Females	Males
Age, years	59 (50 - 65)	57 (50 - 67)
Weight, kg	72 (52 - 121)	87 (56 - 166)
Height, cm	166 (151 - 181)	181 (165 - 196)
Weighed samples		
	Females	Males
Age, years	58 (50 - 65)	57 (50 - 65)
Weight, kg	69 (50 - 108)	84 (62 - 117)
Height, cm	166 (147 - 178)	180 (164 - 199)

The characteristics are presented as median values and range (within brackets).

Table 2. Lower and upper reference limits with 90% confidence intervals for the upper reference limit for males and females extracted with the two methods.

CALEX® Cap extraction device		
	Lower	Upper
All (n = 202)	< 10	199 (133 - 264)
Females (n = 96)	< 10	184 (102 - 265)
Males (n = 104)	< 10	215 (139 - 292)
Weighed samples		
	Lower	Upper
All (n = 180)	< 10	153 (92 - 213)
Females (n = 86)	< 10	141 (114 - 168)
Males (n = 93)	< 10	215 (83 - 347)

The lower detection limit for the calprotectin method was 10 µg/g.

bined reference interval for males and females.

Weighed samples

No significant Spearman's rank correlations were observed between calprotectin concentrations analyzed in weighed samples and weight, height or age. No significant gender effect was observed with the Mann-Whitney *U* Test. The weighed sample cohort was significantly lower than the CALEX extracted samples (Mann-Whitney p = 0.0001). The calculated reference values for the weighed samples were < 153 µg/g feces for the whole cohort and < 141 µg/g for females and < 215 µg/g for males. Evaluation according to the Lahti method suggested a combined reference interval for males and females.

DISCUSSION

Originally, fecal calprotectin was measured with sandwich ELISAs in a microtiter plate format. The assays were performed in batches and the samples were stored at the laboratory until there were sufficient samples collected for a full microtiter plate. This batch mode resulted in long test turnaround times. The development of the fecal calprotectin PETIA significantly reduces turnaround time and also allows more laboratories to perform this test.

In the present study, we obtained reference intervals with the CALEX device of < 199 µg/g feces and a reference interval of < 153 µg/g feces with weighed samples when combining females and males.

This is a bit higher than the 50 - 100 µg/g feces previously used as reference intervals for fecal calprotectin. There may be several explanations for the slightly higher values in the present study. The fecal calprotectin PETIA shows good agreement with the Bühlmann ELISA [11], which in external quality assurance programs belongs to a group of manufacturers that has a calibration above the median in quality assurance programs [12]. Another explanation may be that the study cohort was in the 50 - 65 year range. A number of factors could contribute to higher calprotectin values seen in elderly healthy individuals compared to younger populations. Elderly patients more often have colon diverticulosis, polyps, and hemorrhoids than young persons which could contribute to increased fecal calprotectin values [13]. The mucosa also becomes more fragile with age [14,15]. These factors could lead to higher reference values.

The use of F-calprotectin as part of the work up of patients with suspected IBD is a significant cost reduction in comparison with colonoscopies [16]. Endoscopy in combination with histological biopsy assessments should be used to verify the IBD diagnosis but it should be used selectively as the procedure is invasive, resource intensive, requires patient preparation, and is associated with the inherent risks of invasive procedures. Weighing fecal samples is labor intensive and thus expensive. It also prolongs the time until the test result can be reported as the extraction time is longer than for the CALEX device. The CALEX device eliminates the need for calibrated laboratory balances, it is rapid and reduces labor time in comparison with weighing the samples. The sampling technique with the CALEX device is intended for patient home use. If the patient or the nurse performs the sampling with the CALEX device the extraction will start during the transport of the sample to the laboratory and the extraction will most likely be completed at the time the sample arrives at the laboratory. The laboratory technician thus only needs to inspect the sample tube to verify that the sample has dissolved, centrifuge it, and place it in the chemistry analyzer. The CALEX tube has the same dimension as the standard vacutainer tubes and the sampling device will thus fit directly into the laboratory instruments. In theo-

ry, the only delay prior to the analysis would be the centrifugation time and the test result could be reported within 30 minutes from the arrival to the laboratory.

CONCLUSION

Here we report reference intervals for the new turbidimetric F-calprotectin assay using weighed samples and samples extracted with the CALEX device. The CALEX device yields slightly higher reference intervals than the weighed material. The CALEX device is easy to use and can be used to reduce labor cost while providing very short test turnaround times.

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Declaration of Interest:
The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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