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Reference values of fecal calgranulin C (S100A12) in school aged children and adolescents

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Abstract

Background: Calgranulin C (S100A12) is an emerging marker of inflammation. It is exclusively released by activated neutrophils which makes this marker potentially more specific for inflammatory bowel disease (IBD) compared to established stool markers including calprotectin and lactoferrin. We aimed to establish a reference value for S100A12 in healthy children and investigated whether S100A12 levels can discriminate children with IBD from healthy controls.

Methods: In a prospective community-based reference interval study we collected 122 stool samples from healthy children aged 5–19 years. Additionally, feces samples of 41 children with suspected IBD (who were later confirmed by endoscopy to have IBD) were collected. Levels of S100A12 were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) (Inflamark®). The limit of detection was 0.22 µg/g.

Results: The upper reference limit in healthy children was 0.75 µg/g (90% confidence interval: 0.30–1.40). Median S100A12 levels were significantly higher in patients with IBD (8.00 µg/g [interquartile range (IQR) 2.5–11.6] compared to healthy controls [0.22 µg/g (IQR < 0.22); $p < 0.001$]). The best cutoff point based on receiver operating characteristic curve was 0.33 µg/g (sensitivity 93%; specificity 97%).

Conclusions: Children and teenagers with newly diagnosed IBD have significantly higher S100A12 results

compared to healthy individuals. We demonstrate that fecal S100A12 shows diagnostic promise under ideal testing conditions. Future studies need to address whether S100A12 can discriminate children with IBD from non-organic disease in a prospective cohort with chronic gastrointestinal complaints, and how S100A12 performs in comparison with established stool markers.

Keywords: adolescent; child; inflammatory bowel disease; reference value; S100A12 protein; S100 proteins.

Introduction

Fecal markers are increasingly used as a screening test to select children with high suspicion of inflammatory bowel disease (IBD) for diagnostic endoscopy [1]. Fecal calprotectin (S100A8/A9) is the most studied fecal marker for intestinal inflammation. According to a recently published meta-analysis (nine studies, describing 853 patients), fecal calprotectin has a high overall sensitivity of 97% (95% confidence interval [CI] 92%–99%) and a moderate specificity of 70% (95% CI 59%–79%) for diagnosing IBD [2]. A calprotectin test result in the reference range will thus rule out IBD. This can easily be remembered with the mnemonic SnNOut – (i.e. when performing a test with high sensitivity [Sn], a negative result [N] rules out [Out] the target disease). The downside of using calprotectin as screening test is that a considerable proportion of the children with increased fecal calprotectin values and negative stool cultures (22%) do not have IBD, and will be unnecessary selected for endoscopy and biopsy [3, 4].

Fecal calgranulin C

Fecal calgranulin C (S100A12) is significantly less investigated [5] and largely unknown among clinicians as a screening test for IBD. Both S100A12 and calprotectin are member of the S100 calcium-binding protein family and are released from the inflamed mucosa into the gut lumen [5]. S100A12 acts independently from calprotectin

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[6], and is exclusively released by activated neutrophils, while calprotectin is released from a multitude of activated and damaged cells including granulocytes, monocytes, and epithelial cells. As infiltration of neutrophils into the intestinal mucosa is one of the most prominent histological features in IBD, we think that S100A12 is possibly more specific for IBD-associated inflammation than calprotectin [7, 8]. Both markers are stable for 3–7 days at room temperature, enabling stool collection at home and easy transportation to the hospital laboratory [9, 10]. Calprotectin as well as S100A12 concentrations in stools of healthy volunteers show a downward trend with age from birth and reach stable values by the age of 5 [11–17].

Study aim

In this study we aim to establish a reference value for S100A12 in healthy children aged 5 and above. Secondly, we investigated if the S100A12 stool test can discriminate children with newly diagnosed IBD from healthy controls.

Materials and methods

Healthy participants

We collected stool samples of healthy school-aged children and teenagers in a prospective community-based reference interval study that ran between June 2015 and March 2016. Teenagers were recruited from a secondary school in Groningen (The Netherlands), while representatives of the younger age group were enrolled via colleagues and friends. Participants were eligible for inclusion when they had no history of chronic gastro-intestinal disease, and no acute diarrhea or use of non-steroidal anti-inflammatory drugs in the week before stool collection. Girls were advised not to collect a stool sample during their menstrual period.

IBD patients

Children and teenagers with newly diagnosed IBD who had sent in a feces sample <6 weeks prior to the confirmatory endoscopy were used for comparison. The diagnosis IBD was based on the criteria of the European Society for Pediatric Gastroenterology Hepatology and Nutrition [18]. The stool samples of these patients were stored for the CACATU-study. This trial is registered under identifier NCT02197780 in ClinicalTrials.gov, and entails a prospective diagnostic study that evaluates the test accuracy of fecal calprotectin and S100A12 in children with suspected IBD. The stool samples were used with permission of the patients and their legal guardians for the current study.

Stool collection and analysis

Participants defecated onto a stool collection sheet (Alere Health BV, The Netherlands) held above their own toilet and collected one sample with a classical screw top container with spoon, which was then sent to the department of Laboratory Medicine in the University Medical Centre Groningen in a plastic postage-paid return envelope. Transportation time varied between 1 and 7 days, after which the samples were stored at -80°C until analysis. Maximum storage time was 6 months. All samples were measured between August 2015 and June 2016 by one experienced lab technician (LW), who was blinded for clinical symptoms of patients. S100A12 analyses were performed with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Inflamark[®], CisBio Bioassays Codolet, France) on a Dynex DS2 Automated ELISA System (Alpha Labs, Easleigh, UK).

Prior to extraction, fecal samples were thawed at room temperature and 100 mg of the homogenized feces was suspended in 1:50 extraction buffer. After vigorous vortex mixing for 30 s and incubating on a tube rotator for 25 min, we transferred ~1–2 mL of homogenate to an Eppendorf type tube and centrifuged it at 17,100 g for 5 min and subsequently diluted the samples 50 times. One hundred microliters aliquots in duplicate of the supernatant were then added to the wells coated with anti-S100A12 monoclonal antibody of bovine serum albumin. After incubation for 30 min at 600 rpm, the plates were washed three times with 300 μL /well of washing buffer (3 mL Tween 20 in 1 L distilled water). Then 100 μL of a second monoclonal antibody (anti-S100A12 coupled to horse radish-peroxidase) was added, and the plate was again incubated for 30 min at 600 rpm and washed. Next 100 μL of tetramethylbenzidine substrate was added to initiate the colorimetric reaction. After 10 min the reaction was stopped by adding 100 μL of sulfuric acid. The absorbance was read at 450 nm. The ELISA was calibrated with purified human S100A12 protein. The calibrator was ready to use after reconstitution with 0.5 mL distilled water. For each duplicate, the mean optical density was calculated and a calibration curve was constructed. The curve was plotted as a cubic regression with DS-matrix software, version 1.23.

Manufacturer's performance claims are presented in Supplementary Data 1. We verified analytical sensitivity (limit of detection, LOD), between-test variation and within-test variation with the automated DS2 in our laboratory. We calculated the analytical sensitivity by measuring the extraction fluid 10-times (limit of blank, LOB) in one ELISA run and calculated the LOD at 2 standard deviations (SDs) of the LOB. We determined the between-run variation using the duplicates of the kit control (20 runs) and by selecting three feces pools around the same levels as the manufacturers' claims and determined the variation between five ELISA runs (each pooled sample measured in duplicate). For the within-run variation we used also three feces pools (low, intermediate, high). First, we measured one extract 20-times in one ELISA plate, and then we repeated the extraction from each pool 10-times and measured the duplicates on one plate.

Data collection and statistics

Demographic information and stool results were recorded electronically using SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA)

and are presented with GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA, USA). Standard descriptive statistics were used. Not normally distributed variables are presented as median and interquartile range (IQR) and tested using the Mann-Whitney U-test. All tests were two sided and the level of significance was set at a p-value <0.05. Reference values were calculated by using the simple nonparametric method presented in the Clinical and Laboratory Standards Institute (CLSI) guideline C28-A2 [19]. Outliers were detected by performing Dixon's test and interpreted according to Reeds' criteria: absolute difference between extreme observation and the next largest observation (D), divided by the range of all observations (R) [20]. If the difference D was equal to or greater than one-third of the range of R, the extreme value was deleted [21].

The upper reference limit in healthy subjects was defined as the 97.5th percentile of observations (rank 118.95) [19]. A 90% confidence interval (CI) around the upper reference limit was determined using the 115 and 121 rank number.

We evaluated the diagnostic accuracy of S100A12 for IBD with a receiver operating characteristic (ROC) curve analysis. Sensitivity and specificity for the best cut-off point were calculated with their 95% CIs.

Human patients protection

This study was performed according to the Declaration of Helsinki. The Medical Ethics Review Committee of the University Medical Centre Groningen confirmed that this study and the earlier mentioned CACATU-study were not subject to the Dutch Medical Research Involving Human Subjects Act. The data were collected and recorded by the investigators in such a manner that subjects could not be identified, neither directly nor through identifiers linked to the subjects. The legal guardians from all participants, as well as the children aged 12 and above, gave informed consent for participation.

Results

We tested 122 stool samples from healthy children and 41 from patients. The baseline characteristics are presented in Table 1.

Table 1: Characteristics of participants.

	Healthy (n = 122)	Newly diagnosed with IBD (n = 41)	p-Value
Gender (boys), n (%)	57 (47%)	21 (51%)	0.59
Age (median, IQR), years	12 (9–14)	14 (12–16)	0.001 ^a
Age group ^b			0.04 ^a
5–11 years old, n (%)	53 (44%)	10 (24%)	
12–19 years old, n (%)	68 (56%)	31 (76%)	

^aStatistically significant difference. ^bOne healthy participant did not state age.

Reference value calgranulin C (healthy children)

One outlier was detected according to Dixon and Reed criteria for outliers [21]. This value (5.02 µg/g) was excluded from further analysis. The distribution of the remaining 121 S100A12 measurements is shown in Figure 1. One hundred and ten (91%) children presented with a level below the LOD. The upper reference limit (97.5 percentile) was 0.75 µg/g (90% CI 0.30–1.40).

None of the healthy children had IBD-affected first-degree relatives. Sixteen participants (13%) reported the use of medication that is unlikely to influence the test results. Among them were 10 who incidentally used inhalation therapy for allergic rhinitis or asthma, while others used zopiclon, methylphenidate, incidentally polyethylene

Figure 1: Distribution of fecal calgranulin C (S100A12) test results in healthy children and children with newly diagnosed IBD.

Figure 2: Receiver operating characteristic (ROC) curve of fecal calgranulin C (S100A12). Area under the curve (AUC) is 0.97 (95% CI 0.93–1.00).

Table 2: Overview of literature on fecal S100A12.

Age of participants	Assay	Number of healthy controls	Fecal S100A12 level healthy controls	Number of (active) IBD patients	Fecal S100A12 level IBD patients	Cut-off value	Sensitivity, %	Specificity, %
Reference value study								
Question: What is the normal S100A12 value in healthy controls?								
Day et al. [17]	0.16–13.8 years As described in Sidler et al. [7]	49	Median 0.5 mg/kg (range 0.39–25)	–	–	–	–	–
Phase I diagnostic accuracy study: proof of concept study								
Question: Do patients with IBD have a higher fecal S100A12?								
Nylund et al. [22]	8–18 years As described in Foell et al. [23]	15	Median 0.07 ug/g (range 0.06–0.10)	27	Median 5.05 ug/g (range 0.10–16.25)	–	–	–
Phase I–II diagnostic accuracy study: explanatory study								
Question: Can fecal S100A12 discriminate under ideal circumstances?								
De Jong et al. [10]	18 months–18 years As described in Yang et al. [24]	25	Median 0.69 mg/kg (range 0.39–17.72 mg/kg)	23	Median 95.40 mg/kg (range 6.19–349.9 mg/kg)	10 mg/kg	96%	92%
Kaiser et al. [25]	17–43 years As described in Foell et al. [23]	24	Median 0.006 ± 0.03 mg/kg	50	Median 2.45 ± 1.15 mg/kg	0.8 mg/kg	81% CD, 91% UC	100%
Phase III diagnostic accuracy study: real world study								
Question: Does fecal S100A12 discriminate in routine practice (patients with suspected IBD)?								
Sidler et al. [7]	2–16 years As described in Yang et al. [24]	30 ^a	Median 1.2 mg/kg (range 0.5–28.3 mg/kg)	31	Median 55.5 mg/kg (range 8.9–500)	10 mg/kg	97%	97%

glycol, oral contraceptives (2×), or growth hormone. A sensitivity analysis comparing the reference values of patient with and without medication was not significantly different (data not shown). We did not observe any difference in mean (SD) S100A12 results when the cohort was divided into the age categories 5–11 years and 12–19 years, (respectively, $0.25 \pm 0.18 \mu\text{g/g}$ and $0.24 \pm 0.11 \mu\text{g/g}$ [$p=0.63$]). Differences in fecal S100A12 levels between boys and girls were not found (data not shown).

Diagnostic accuracy calgranulin C

Median fecal S100A12 level in children with IBD was $8.02 \mu\text{g/g}$ (IQR 2.3–11.4) and was significantly higher compared to healthy children (Figure 1). Twenty-one children were diagnosed with Crohn's disease, 19 with ulcerative colitis and one with IBD-unclassified. Median S100A12 levels were highest in children with ulcerative colitis compared to children with Crohn's disease (respectively, $11.0 \mu\text{g/g}$ [IQR 7.5–28.7] and $6.7 \mu\text{g/g}$ [IQR 1.3–10.4, $p=0.03$]).

The ROC curve depicted in Figure 2 shows that the ideal cut-off point to distinguish children with IBD from healthy controls leads to a sensitivity of 93% (95% CI 81–99) and a specificity of 97% (95% CI 92–99). This cut-off point corresponds with a S100A12 value of $0.33 \mu\text{g/g}$. The earlier estimated upper reference limit ($0.75 \mu\text{g/g}$) leads to a sensitivity of 86% (95% CI 71–95) and a specificity of 98% (95% CI 93–100).

Discussion

Main findings

In this paper we present for the first time the normal value of S100A12 using a commercially available testkit. We found that the majority of healthy children had S100A12 levels below the detection limit. We hypothesize that in the absence of excessive recruitment and accumulation of activated neutrophils in the intestinal lumen, which is observed under pathological conditions such as IBD, S100A12 can hardly be found in the stool [26]. Secondly, we found excellent diagnostic power to distinguish children with newly diagnosed IBD from healthy controls.

Comparison with other literature

Diagnostic test development can be divided into four different phases [27]. In Table 2 we summarized the literature

on fecal S100A12 with respect to these four phases. We found one study that described S100A12 levels in a cohort of healthy Australian infants and New Zealander children [17]. Although this cohort was too small to report reliable reference values according to the CLSI guidelines [19], it showed a trend towards consistently low levels of S100A12 in children older than 5 years, with more divergent levels of S100A12 below this age, similar to reference values of stool calprotectin [13, 16, 17].

The best cutoff point to distinguish healthy children from those with newly diagnosed IBD in our study population ($0.33 \mu\text{g/g}$) was substantially lower than previously reported cutoff points ($0.8 \mu\text{g/g}$ [25] and $10 \mu\text{g/g}$ [10]). Differences are likely to be explained by differences in used assays and selection of patients. At all events, the studies agreed on the excellent diagnostic accuracy of the S100A12 stool test to distinguish patients with IBD from controls [7, 10, 25].

Limitations

The performance of the S100A12 testkit is potentially biased due to the case-control design. We compared a preselected group of patients with an established diagnosis and healthy individuals (rather than testing a group of patients merely suspected of IBD). It tells us that the S100A12 test shows diagnostic promise under ideal conditions. By establishing the pediatric reference range for fecal S100A12 biomarker, we have taken an important first step toward harnessing the full potential of S100A12 in the pediatric population. Future studies need to address whether S100A12 can discriminate children with IBD from non-organic disease in a prospective cohort with chronic gastrointestinal complaints, and how S100A12 performs in comparison with established stool markers like fecal calprotectin.

Conclusions

The upper reference value of fecal S100A12 in healthy children aged 5 and above measured with a commercially available assay is $0.75 \mu\text{g/g}$. S100A12 shows diagnostic promise under ideal testing conditions with an ideal cut-off of $0.33 \mu\text{g/g}$.

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Article note: An interim analysis of this study was orally presented at the ESPGHAN Annual Meeting in Athens in 2016.