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Normal Values for Calprotectin in Stool Samples of Infants from the Population-based Longitudinal Born into Life Study

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Fecal calprotectin is a protein used as a diagnostic marker for inflammatory bowel diseases. We determined upper limits for normal calprotectin values for neonatal, 6, 12 and 24 months old children using a turbidimetric immunoassay in a cohort of Swedish children. The advantage of the method is that, opposite to previously used ELISA method, it enables measuring single samples, and thus, shortens the analysis times significantly. There were 72 samples (41.7 % female) collected neonatally, 63 samples (34.9 % female) at 6 months, 60 samples (40.0 % female) at 12 months and 51 samples (43.1 % female) at 24 months. The upper limits for normal values were 233, 615, 136 and 57 µg mg^{-1} for infants aged 0, 6, 12 and 24 months, respectively.

Keywords: calprotectin, turbidimetric immunoassay, normal values, inflammatory bowel disease, abdominal pain

Introduction

Abdominal pain is one of the most common causes for seeking medical help during childhood and is reported to affect 10% to 20% of schoolchildren at an extent that interferes with normal daily activity [1, 2]. Abdominal pain and diarrhea are both frequently observed in functional gastrointestinal disorders in children, but it may also be the first sign of an inflammatory bowel disease (IBD), which has become more

common among children during the last decades [3, 4]. For long, histopathological analysis of biopsies taken during endoscopy were considered the gold standard for diagnosing IBD, but as this is an invasive and costly procedure, especially with pediatric patients, non-invasive diagnostic tools are sought. New promising markers for diagnostic purposes have been suggested, including measurement of the inflammatory marker calprotectin, which can be analyzed in fecal samples. While fecal calprotectin alone is not sufficient for the initial diagnosis of IBD, it is now considered a reliable marker for active IBD in adults [5] and has been suggested to replace biopsies to predict early relapse of IBD [6]. It has also recently been suggested for the triage of pediatric patients with symptoms suggestive of IBD [7] and can be used as a rule-out test for IBD [8].

Further, detecting abnormal calprotectin levels in infancy could also be used for other diagnostic purposes. For example, high calprotectin values have been linked to the development of other immune-mediated diseases, such as atopic dermatitis and asthma [9]. Further, higher fecal calprotectin levels have been found in children with bacterial gastroenteritis compared to children with viral disease [10].

Calprotectin is a calcium and zinc-binding protein consisting of two heavy and one light polypeptide chains. It is abundant in neutrophils, where it can constitute up to 60 % of the total cytostolic protein content [11], but it is also present in monocytes and macrophages [12]. The protein can be measured in feces, plasma, and other body fluids. In feces it has a homogeneous distribution and is stable for up to 7 days at room temperature [13], making it an ideal diagnostic marker. However, constraints related to sample analysis have hampered the use of calprotectin, as samples were run in batches in 96 well plates using enzyme linked immunosorbent assay (ELISA) and filling

the batch can take a long time. A new method based on particle enhanced turbidimetric immunoassay (PETIA), is available, allowing rapid identification of calprotectin in single fecal samples, enabling fast analysis [14]. The results obtained with this test are comparable to the previously used ELISA method [14].

However, in order to use fecal calprotectin in clinical practice, it is essential to know the upper limit of normal variation among healthy individuals. Such normal values have not been established for the turbidimetric immunoassay. Here we report upper limits for normal values of calprotectin in feces based on a cohort of infants whose calprotectin levels were measured at birth and at the age of 6, 12 and 24 months.

Materials and Methods

The Born into Life Study

The infants included into this study participated in the Born into Life study (Smew et al., submitted), which is a longitudinal cohort study consisting of two cohorts – one of mothers, and the other of their children. The mothers were originally recruited into a larger prospective cohort study LifeGene [15], which consists of index people between 18 to 45 years of age. Female study participants in LifeGene who became pregnant were recruited into Born into Life and answered questionnaires regarding pregnancy, health status and lifestyle at baseline and twice during pregnancy. After gaining parental informed consent, the children were included in the birth cohort upon delivery and all were delivered at the same clinic. Only healthy, full term infants were included in the study. These children were followed with parental questionnaires and biological samples were collected at birth and at 6, 12 and 24 months of age (Table 1).

The health status of the children in the cohort represents well the general population of young children in Sweden in regards to occurrence of food allergies [16] and eczema [17], as well as for antibiotics use[18]. Also the frequency of hospital visits reflects the general population of young children in Sweden [19]. [Table 1 near here]

Samples

Fecal samples were collected at home by the parents from the child's diaper following a standardized protocol the day before visit to the test center, except for the neonatal fecal sample which was collected in conjunction with the routine health check-up 2-4 days after birth. Samples were kept in a freezer and transported to the test center in a Cool Transport Container from Sarstedt in frozen condition. Frozen samples were thawed, weighed and calprotectin was extracted according to manufacturer´s instructions (BÜHLMANN, Schönenbuch, Switzerland). Ethical approval was granted by the Regional Ethics Review Board in Stockholm, Sweden (reference number 2011/192-31/2, 2011/1127-31/3 and 2014/1081-32 with amendments) and complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research. All parents gave informed written consent.

Fecal Calprotectin Assay

Fecal calprotectin was analyzed with fCal Turbo reagent (BÜHLMANN) on a Mindray™ BS-380 analyzer (Mindray Medical International, Shenzhen, China). The fCal Turboreagent kit contains Reaction Buffer (R1) and Immunoparticles (R2) and the total assay time was 10 min. The total coefficient of variation (CV) for the method was 1.9% at 248 μg/g and 0.6% at 1392 μg/g. The fecal calprotectin PETIA shows a good

agreement with the Bühlmann ELISA [14], which in external quality assurance programs belongs to a group of manufacturers that has a calibration above the median in quality assurance programs [20].

Statistical Analyses

As the reliable detection limit for calprotectin with the turbidimetric method is around 5 µg g^{-1}, all values below this, including zeroes, were changed to 0.01. We considered 95 % reference intervals as normal concentrations, but since values of fecal calprotectin that are below detection limit are considered normal, only upper limits are reported together with 90% confidence intervals by bootstrap estimation utilizing the R-package, referenceIntervals (http://CRAN.R-project.org/package=referenceIntervals). Outliers were detected using Horn´s method [21]. Correlation in calprotectin concentration across time was tested using Spearman correlation and the impact of gender to calprotectin levels was tested with t-test. The variation among age groups was tested using Kruskal-Wallis one-way analysis of variance with posthoc test after Nemenyi using R-Package PMCMR (http://CRAN.R-project.org/package=PMCMR). The results were considered significant at $P < 0.05$. All calculations were performed using R (http://www.Rproject.org).

Results

The data include 246 evaluations for 84 individuals across 4 different time points. There were 72 samples (41.7 % female) taken neonatally, 63 samples (34.9 % female) at 6 months, 60 samples (40.0 % female) at 12 months and 51 samples (43.1 % female)

at 24 months. The values decreased over time with the highest concentrations being found at 0 and 6 months, respectively (Figure 1). There was no difference between boys and girls and no correlation was found within individuals over time. The upper limits for normal values for each time point are presented in Table 2. [Table 2 and Figure 1 near here]

Discussion

Our main purpose of the study was to set upper limits for normal variation in fecal calprotectin values during the two first years of life, enabling the use of PETIA as a diagnostic tool. These results confirm that the values are very high in newborns [22, 23], but then decrease fast, apparently stabilizing already at the age of one year. There was no trend within individuals, and variation in levels between different ages more likely reflects changes in other factors, such as introduction of solid food or mild gastrointestinal problems.

In order to decide if a patient´s test values are decreased, normal or elevated, it is important to have appropriate reference values against which the results can be evaluated. Most reference values have been based on healthy adults in the 20-50 year age range and lack of pediatric upper limits for the normal values has restricted the use of fecal calprotectin in children. There is evidence that also dietary factors and the bacterial gut flora may influence the levels of inflammatory markers in feces [24-27]. Both diet and the frequency of gastrointestinal infections vary between countries [28], thus, it is important that the reference material is similar to the patient population that the assay will be used for. Here, the pediatric study population was recruited in

Sweden and is considered representative for Caucasians living in the northern part of Europe.

The main application for fecal calprotectin in adults is to distinguish between irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). As an inexpensive noninvasive assay fecal calprotectin can be used more frequently, allowing a closer treatment monitoring than with colonoscopy. It can thus be used to monitor the effects of different diets on the inflammatory activity [20]. In children, there is also another possible application. A previous study has indicated that fecal calprotectin could be used as a tool for distinguishing between viral and bacterial gastroenteritis with a specificity of 88.9%, and specificity of 76.0% for a cut-off of 710 mg/l [10]. Another large study found strong associations of calprotectin with viral/bacterial diagnosis and also found that the levels of calprotectin were associated with more severe disease in children with more severe gastroenteritis in both the bacterial and viral group separately [29]. However, a third study in 50 children showed no additional benefit of measuring fecal calprotectin above CRP measurement for the discrimination of viral to bacterial gastroenteritis [30]. Further, larger validation studies are needed on this issue, and if shown predictive, rapid determination of fecal calprotectin with normal values would be needed before implementation into the clinical setting.
The limitation of this study is that the number of participants was low. This could impact, for example, the variation within values. For example, one of the values at six months was very high (> 1000 μg mg^{-1}), but due to the small sample size we do not know whether such high values are regularly detected in young healthy infants.

To conclude, determining the upper limits for normal values enables the use of the turbidimetric immunoassay as a diagnostic tool for gastrointestinal disorders in

children under 2 years, facilitating fast and cost efficient monitoring of gastric inflammation.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table 1. Summary of the health questionnaire data for the cohort for the 6, 12 and 24 months check-up.

	6 months	**12 months**	**24 months**
number of respondents/total	53/63	56/60	45/51
Did your child ever have any of the following…			
Abnormal head circumference	4%		
Delayed mental development	0%		
Delayed motoric development	0%		
Hearing problem	0%		
Heart disease	0%		
Hip problem	0%		
RS-virus infection	0%		
Testicles not in scrotum	0%		
Too large weight gain	0%		
Too small weight gain	13%		
Visual impairment	0%		
Did your child ever have…			
Asthma		2%	4%
Colic		4%	0%
Common Cold		93%	98%
Conjunctivitis		13%	27%
Diarrhea		54%	44%
Eczema		25%	20%
Pinworm		0%	2%
Fever seizures		2%	2%
Food allergy		4%	7%
Hives		7%	13%
Otitis		11%	16%
Pneumonia		0%	0%
False Croup		4%	4%
Tonsillitis		4%	4%
Urinary tract infection		2%	2%
Did your child ever…			
Use antibiotics	13%	18%	29%
Visit a hospital for disease	30%	43%	40%

Table 2. Upper limits for normal values for calprotectin in feces of neonates, 6 and 12 month-old infants and 2-year old children determined as 95 % reference intervals with 90 % confidence intervals, and their median values.

Age (months)	N (number of excl. outliers)	Upper limit (µg g^{-1}; 90% confidence interval)	Median (µg g^{-1})
0	71 (2)	324 (274 – 381)	67
6	63 (0)	615 (189 – 1057)	31
12	54 (6)	136 (119 – 179)	19
24	40 (11)	57 (57 – 64)	17

Figure 1. Boxplot of fecal calprotectin concentrations within each of the age classes. Letters illustrate significant differences between groups. One high value (1056 µg g^{-1}) in age group 6 months was excluded from the figure.

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