

Fecal Calprotectin as a Biomarker of Food Allergy and Disease Severity in Children with Atopic Dermatitis without Gastrointestinal Symptoms

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ABSTRACT Fecal calprotectin (FCP) is a biomarker of intestinal inflammation and has recently been proposed as a diagnostic biomarker of food allergy (FA) in children. The aim of this study was to compare FCP level in infants and children under 4 years old with 1) atopic dermatitis (AD) with food allergy (FA) and 2) children with AD and without FA with the results in healthy controls. In total, 46 infants and children (mean age 14 months \pm 12) diagnosed with AD were divided into two groups: G1, children with atopic AD with FA (n=28) and G2, children with AD without FA (n=18). The control group (G3) was made up of healthy children of the same age (n=18). The median FCP was significantly higher in G1 compared with G2 (G1: median 154, IQR 416 μ g/g vs G2: median 41.3, IQR 59 μ g/g; $P=0.0096$). The median FCP in children with AD and FA was significantly higher before elimination diet in comparison with FCP after 3 months of elimination diet (median 154, IQR 416 μ g/g vs median 35, IQR 23 μ g/g; $P=0.0039$). The level of FCP was significantly positively correlated with the SCORAD score ($r=0.5544$, $P=0.0022$). Our study showed a significant difference in level of FCP in patients with AD without FA compared with patients with AD and FA. We also found a positive correlation of FCP with SCORAD score, a biomarker of AD severity. New studies are needed to investigate the role of FCP as a biomarker of FA in children with AD.

KEY WORDS: allergy, atopic dermatitis, inflammatory disorders

INTRODUCTION

Atopic dermatitis (AD) is a chronic, pruritic, inflammatory dermatosis that affects up to 25% of children (1). The prevalence of food allergy (FA) in infants with AD was reported to range from 20% to 80% in various studies, and may be estimated at 30% (2). The prevalence of immunoglobulin E (IgE) -mediated FA

confirmed by double-blind placebo-controlled trials in children with AD, except in patients with a history of anaphylaxis and positive specific IgE (sIgE), ranges from 33-63% (3-5). The early onset of the disease (before the third month of life) and the more severe form of AD are associated with the high values of specific

IgE on milk, eggs and / or peanuts (6). Sensitization is not evidence of clinically relevant allergies, which represents a significant socio-economic and medical problem given the nutritional deficit and the consequent growth disorder to which the children are exposed due to the elimination diet (7). Additionally, food sensitization is also common after the onset of clinical signs of AD, which is associated with the resorption of food allergens through damaged skin (8-10).

Fecal calprotectin (FCP) is a protein most commonly found in neutrophil granulocytes and to a lesser extent in monocytes and reactive macrophages (11-13). FCP gets into the stool via neutrophil granulocytes, and its presence in the stool is usually a reflection of damage to the intestine mucous membrane (14). Concentration of FCP is increased in inflammatory diseases such as inflammatory bowel disease (15), celiac disease (16), gastrointestinal form of graft versus host disease (17), bowel diverticulosis (18), necrotic enterocolitis (19), and acute gastrointestinal infections (20). Concentration of FCP is elevated in children with FA affecting the gastrointestinal tract

(GIT), and FCP was suggested as a non-invasive intestinal inflammatory marker in FA (21,22).

We hypothesized that children with AD (without GIT symptoms) have an increased concentration of FCP compared with healthy subjects. Furthermore, we hypothesized that there is a significant difference in the concentration of FCP in children with AD without FA and without GIT symptoms in comparison with patients who have both AD and FA without GIT symptoms.

The aim of this study was to compare FCP level in infants and children under 4 years of age suffering from atopic dermatitis (AD) with and without food allergy (FA) (and without GIT symptoms for both groups) with FCP level in healthy controls.

SUBJECTS AND METHODS

Subjects

A total of 64 infants and children, 40 (62.5%) boys and 24 (37.5%) girls, age range 1 to 43 months (mean 15 months) were analysed. The 46 subjects were

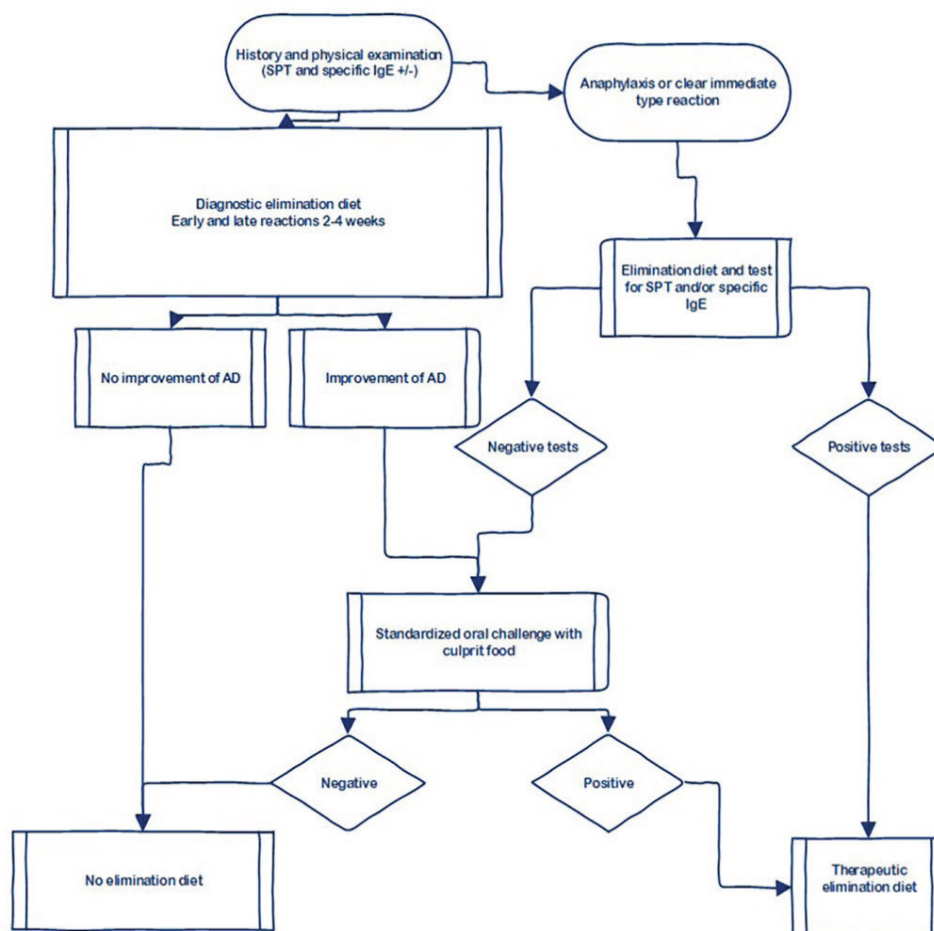


Figure 1. Diagnostic algorithm for food allergy (FA) in children with atopic dermatitis (AD).
SPT: skin prick test; IgE: immunoglobulin E; AD: atopic dermatitis

patients with AD, while the control group consisted of healthy children (n=18). The AD group was divided into two subgroups: G1, children with AD and FA without gastrointestinal symptoms (n=28), and G2, children with AD and without FA (and without gastrointestinal symptoms) (n=18). The characteristics of the subjects are presented in Table 1. The diagnosis of AD was based on the criteria of Hanifin and Rajka (23), and the SCORing of Atopic Dermatitis (SCORAD) index for assessing the severity of AD was evaluated by a pediatric allergist (MN) or dermatologist (NP) (24). The diagnosis of FA was in accordance to European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Guideline: Diagnosis and Management of Cow's Milk Protein Allergy (25) and standardized in-house procedure (Figure 1). We defined FA based on the history of the subject, if they experienced an immediate reaction following ingestion of a specific food and increased serum food-specific IgE levels or positive skin prick test. A standardized oral challenge with food allergen was performed if the specific IgE or skin prick test were negative. A therapeutic elimination diet was provided if the standardized oral challenge with specific food allergen was positive. Patients were considered not to have a diagnosis of FA if the standardized oral challenge was negative. Diagnostic elimination diet was prescribed to patients whose history and physical examination findings were suggestive of the diagnosis of FA (deterioration of skin status after ingestion of certain food allergen without a history of an anaphylaxis or immediate type reaction) and with negative specific IgE or skin prick test. A standardized oral challenge food allergen was performed in patients who improved with regard to clinical symptoms (SCORAD score) after introduction of the elimination diet. A therapeutic elimination diet was

given if the standardized oral challenge was positive. Patients were considered not to have a diagnosis of FA if the standardized oral challenge was negative; in addition, patients were considered not to have a diagnosis of FA if the patients showed no improvement of the clinical symptoms (SCORAD score) during the elimination diet.

Subjects used topical steroids, topical antibiotics, or calcineurin inhibitors for treatment of AD at the time of enrollment.

Diagnostic work-up was performed according to a standardized in-house procedure (according to ESPGHAN guideline (25) and Lieberman *et al.* (26)), and in line with ethical principles (approved by the Institutional Review Board of Children's Hospital Zagreb No. 02-23/14-1-16) and Declaration on Human Rights from Helsinki 1975 and Tokyo amendments 2004-2008 (27). The parents of all subjects consented to the study.

Exclusion criteria included subjects with a respiratory, urinary, or gastrointestinal tract infection during a preceding month, use of corticosteroids or antibiotics during a preceding month, cancer, systemic inflammatory disorders, primary immunodeficiency, inflammatory bowel disease, food allergy with gastrointestinal symptoms, neutropenia, infantile colic, diarrhea, probiotics supplementation, and prematurity.

Healthy children with no allergic disease, food allergy, or gastrointestinal complaints were selected from the Children's Hospital Zagreb Outpatient Clinic and from the children of healthcare workers as the control group.

Methods

The parents completed the questionnaire which included items on age, sex, family history of allergic

Table 1. Characteristics of study subjects

Characteristics	AD (n=46)	G1 (n=28)	G2 (n=18)	Control group (n=18)
Age, mean (SD) [range], months	14.02 (12) [2-43]	7.54 (4.93) [2-21]	24.11 (12.87) [3-43]	17.6 (15.03) [1-42]
Sex, male, No (%)	31 (67.4)	21 (75)	10 (55)	9 (50)
Body weight, mean (SD), kg	9.54 (3.11)	7.90 (1.68)	12.12 (3.14)	8.92 (3.80)
		IgE mediated food allergy, No (%) <ul style="list-style-type: none"> • Mono-sensitized 8 (32) (4 egg, 4 cow's milk) • Poly-sensitized 13 (46,4) (13 egg white, 7 cow's milk, 7 peanut, 7 wheat, 3 hazelnut) Non-IgE mediated food allergy (cow's milk allergy), No (%) 7 (25)		

G1: children with atopic dermatitis (AD) with food allergy (FA); G2: children with AD without FA; SD: standard deviation; IgE: immunoglobulin E

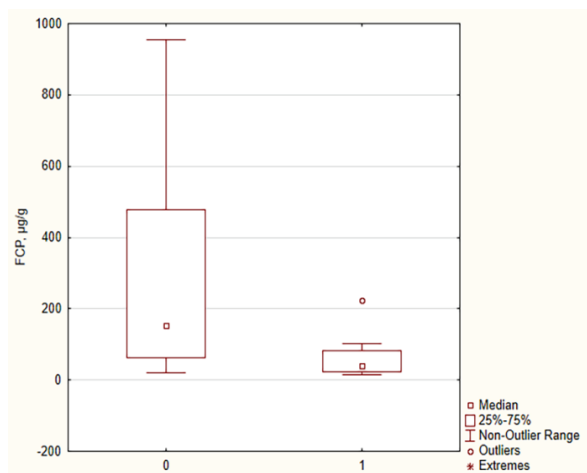


Figure 2. FCP in G1, children with atopic dermatitis (AD) with food allergy (FA) (n=28) and G2, children with AD without FA (n=18). Small boxes represent median values and big boxes represent interquartile range (IQR).

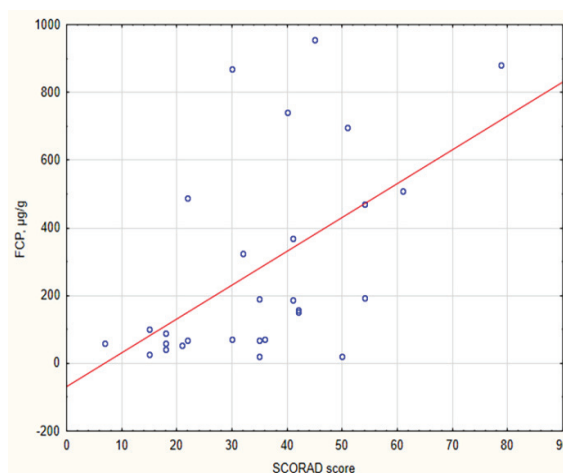


Figure 3. Relationship between fecal calprotectin (FCP) and SCORAD score ($r=0.5544$, $\xi=0.0022$) in children with atopic dermatitis (AD) with food allergy (FA); G1 group, n=28.

diseases, the mode of delivery, birth weight, breast-milk feeding, detailed history after food ingestion, and medication.

Sensitization status was determined by skin prick tests and / or increased level of serum IgE. The skin prick tests (SPT) were performed according to the Lieberman *et al.* (26), with 6 food allergens (Diater, Spain): cow's milk, hen's egg, wheat, peanut, hazelnut, walnut. Negative (saline solution) and positive (histamine 1 mg/mL) controls were used. The tests were considered positive if a mean wheal diameter was ≥ 3 mm compared with a negative control.

Blood sampling was done upon clinical examination between 8.00 a.m. and 12.00 a.m. The following parameters were analyzed in a peripheral blood specimen: white blood cell count (WBC, leukocytes) eosinophilic granulocyte count (Eo), concentration of eosinophil cationic protein (ECP), and concentration of immunoglobulins (Ig) IgA, IgE, IgG, and IgM. The

peripheral blood leukocytes and Eo counts were determined using a Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Kobe Hyogo, Japan). The serum IgA, IgG and IgM concentrations were analysed on the Beckman Coulter AU680 analyzer (Beckman Coulter Inc, Brea, USA) by the turbidimetric immunoassay method. The serum concentrations of total IgE (tIgE), and serum IgE of 6 food allergens (egg white, f1; milk, f2; wheat, f4; peanut, f13; hazelnut, f17; walnut, f256), and ECP concentration were determined using the fluorescence enzyme immunoassay (FEIA) (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden).

The stool samples were obtained from 64 subjects at the time of enrollment and 3 months after elimination diet in the group of children with AD and FA. All stool samples were extracted with the B-CAL-EX Extraction kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland), and FCP concentration was

Table 2. Characteristics and outcomes of children with atopic dermatitis (AD) and FA and healthy children (n=46)

	G1 (m=28)	G3 (m=18)	Statistics*
Gender, male, No (%)	21 (75)	9 (50)	df=1, $P=0.4163$
Age, mean (SD) [range], months	7.54 (4.93) [2-21]	17.6 (15.03) [1-42]	$t=3.2980$, $P=0.0019$
Body weight, mean (SD), kg	7.90 (1.68)	8.92 (3.80)	$t=1.4177$, $P=0.1703$
FCP, median (IQR), µg/g	154 (416)	77.75 (155)	$Z=-1.8231$, $P=0.0679$

G1: children with atopic dermatitis (AD) with food allergy (FA); G3: control group; SD: standard deviation; IQR: interquartile range; FCP: fecal calprotectin

*Group comparisons were performed using the chi 2 -test for sex distribution ($P=0.4163$, $df=1$), t-test for age distribution ($t=3.2980$, $P=0.0019$) and body weight ($t=3.2980$, $P=0.0019$), and Mann Whitney test for FCP ($Z=-1.8231$, $P=0.0679$)

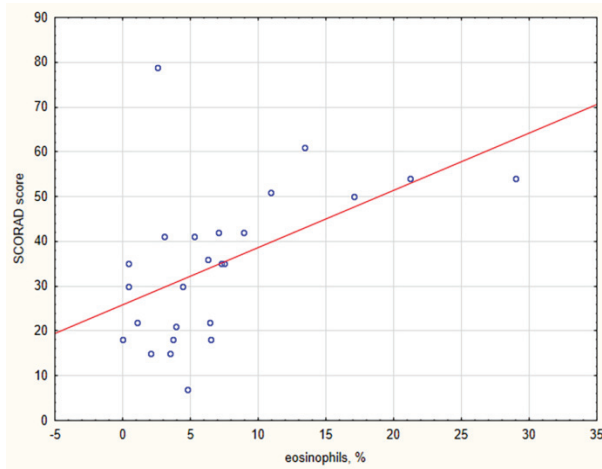


Figure 4. Relationship between SCORAD score and serum eosinophils ($r=0.5735$, $P=0.002$) in children with atopic dermatitis (AD) with food allergy (FA); G1 group, $n=28$.

measured with particle enhanced turbidimetric immunoassay (PETIA) (Bühlmann fCAL Turbo kit, Bühlmann Laboratories AG, Schönenbuch, Switzerland) on the Beckman Coulter AU680 analyzer (Beckman Coulter Inc, Brea, USA). The FCP concentration was expressed as micrograms per gram of feces.

SCORAD scores were assessed in all patients.

Statistical analysis

Data storage and processing for statistical analysis was performed using Microsoft Excel 2013 (Microsoft, USA). Continuous variables were described as mean and standard deviation ($\bar{x} \pm SD$) if they had normal distribution, or median and interquartile range [M (IQR)] for non-normal distribution. The variables that were not normally distributed were normalized using logarithmic transformation before further analysis. Blood cell counts and immunoglobulin concentra-

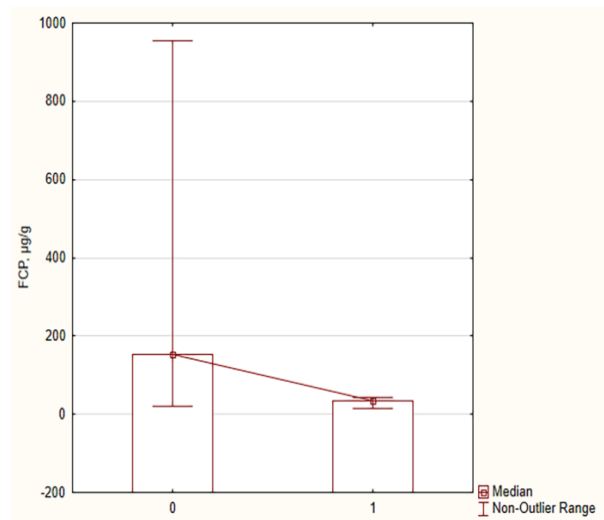


Figure 5. FCP in children with atopic dermatitis (AD) with food allergy (FA) before elimination diet (0) and after 3 months of elimination diet ($n=28$). Small boxes represent median values and big boxes represent interquartile range (IQR).

tions were adjusted for age and expressed as Z-values. Comparisons between groups were made using a Student's t-test for normally distributed variables or Mann Whitney test for non-normally distributed and using χ^2 -test or Fisher exact test for categorical variables.

Analysis of covariance (ANCOVA) was used to compare groups with different age distributions. Age was used as a covariate in the models. Correlation analysis was performed with the Spearman correlation test. The data were analyzed using STATISTICA version 10 software program (StatSoft, Inc. Tulsa, OK) and MedCalc version 12 software program (MedCalc Software, Mariakerke, Belgium). Statistical significance was set to $P<0.05$ for all tests.

Table 3. Characteristics and outcomes of children with atopic dermatitis (AD) without FA and healthy children ($n=36$)

	G2 (N=18)	G3 (N=18)	Statistics**
<drc, male, No (%)	10 (55)	9 (50)	$df=1$, $P=0.8527$
Age, mean (SD) [range], months	24.11 (12.869) [3-43]	17.6 (15.03) [1-42]	$t=-1.3933$, $P=0.1725$
Body weight, mean (SD), kg	12.12 (3.14)	8.92 (3.80)	$t=-1.3755$, $P=0.1905$
FCP, median (IQR), $\mu\text{g/g}$	41.3 (59)	77.75 (155)	$Z=0.7909$, $P=0.4289$

G2: children with AD without FA; G3: control group; SD: standard deviation; IQR: interquartile range; FCP: fecal calprotectin

**Group comparisons were performed using the χ^2 -test for gender distribution ($P=0.8527$; $df=1$), t-test for age distribution ($P=0.1725$, $t=-1.3933$) and body weight ($t=-1.3755$, $P=0.1905$), and Mann Whitney test for FCP ($P=0.4289$, $Z=0.7909$)

Table 4. Comparison of characteristics and outcomes between children with AD and FA before elimination diet and children with AD and without FA (n=46).

	G1 (N=28)	G2 (N=18)	Statistics***
Sex, male, No (%)	21 (75)	10 (55)	df=1, $p=0.5387$
Age, mean (SD) [range], months	7.54 (4.93) [2-21]	24,11 (12.87) (3-43)	$t=-6.1775, P<0.00001$
Body weight, mean (SD), kg	7.90 (1.68)	12.12 (3.14)	$t=-0.3909, P=0.06979$
WBC, mean (SD),	13.36 (4.64)	10.45 (2.88)	$t= 2.299, P=0.0267$
Total IgE, median (IQR), kU/L	49.95 (88.4)	23.9 (95.07)	$Z=1.0060, P=0.3144$
ECP, mean (SD), $\times 10^3/L$	36.99 (26.82)	13.44 (8.83)	$t=2.370, P=0.0307$
IgG, mean (SD), g/L	4.002 (1.90)	7.135 (2.392)	$Z=-1.1003, P=0.2712$
IgA, mean (SD), g/L	0.277 (0.159)	0.647 (0.434)	$Z=0.3043, P=0.7618$
IgM, mean (SD), g/L	0.496 (0.158)	0.845 (0.424)	$Z=-0.4656, P=0.6480$
FCP, median (IQR), $\mu g/g$	154 (416)	41.3 (59)	$F=7.3427, P=0.0096$
SCORAD score, mean (SD)	35.32 (16.26)	26.26(19.06)	$t=1.7241, P=0.0917$

G1: children with atopic dermatitis (AD) with food allergy (FA); G2: children with AD without FA; SD: standard deviation; IQR: interquartile range; WhBC: whole blood count, Ig: immunoglobulin; ECP: eosinophil cationic protein; FCP: fecal calprotectin; SCORAD: SCORing Atopic Dermatitis

***Group comparisons were performed using the chi 2 -test for sex distribution ($P=0.5387, df=1$), t-test for age distribution, body weight ($t=-0.3909, P=0.06979$), and WhBC, ECP, and SCORAD score ($P=0.0267, t= 2.299; P=0.0307, t=2.370; P=0.0917, t=1.7241$, respectively), Mann Whitney test for total IgE, IgG Z-scores, IgA Z-scores, and IgM Z-scores ($P=0.3144, Z=1.0060; P=0.2712, Z=-1.1003; P=0.7618, Z=0.3043; P=0.6480, Z=-0.4656$, respectively) and ANCOVA for FCP ($F=7.3427, P=0.0096$).

RESULTS

Comparison between children with AD and healthy controls

There was no differ in FCP level between children with AD (regardless of presence of FA) and healthy controls (FCP level median [IQR], $\mu g/g$ 70 [152.4], vs 77.75 [155], $Z=-0.8287, P=0.4073$).

There were no statistically significant differences between the G1 and G3 groups in distribution of age, sex, body weight and FCP level (Table 2). There were no statistically significant differences between the G2 and G3 groups for distribution of age, sex, body weight and FCP level (Table 3).

Comparison between children with AD and FA before elimination diet and children with AD and without FA

There were no statistically significant differences between groups in distribution of body wight, sex, WBC counts, total IgE, IgA, IgG, IgM Z-scores and SCORAD score (Table 4). There were significant differences between groups in distribution of age, ECP

level, and FCP level (G2 vs. G3: age, mean [SD], months; 9.5 [4.9] vs 24.11 [12.87], $P\leq 0.0001$; ECP level, mean [SD] 36.99 [26.82] vs. 13.44 [8.83], $P=0.0307$; FCP level median [IQR], $\mu g/g$ 154 [416], vs 41.3 [59], $P=0.0096$; Table 4, Figure 2).

Association of FCP with other inflammatory parameters of AD severity

The level of FCP in children with AD and FA significantly positively correlated with SCORAD score ($r=0.5544, P=0.0022$; Figure 3). SCORAD scores significantly positively correlated with eosinophils ($r=0.5735, p=0.0027$; Figure 4).

Comparison of FCP level before and 3 months after elimination diet

The median FCP in the group of children with AD and FA was significantly higher before elimination diet in comparison with FCP after elimination diet (median 154, IQR 416 $\mu g/g$ vs median 35, IQR 23 $\mu g/g$; Mann Whitney test $Z=2.8850, P=0.0039$; Figure 5).

DISCUSSION

The present study demonstrated that FCP level in patients with AD without FA was lower in comparison with patients who had both AD and FA. FCP level was significantly mildly to highly correlated with SCORAD score, a marker of AD severity in children with AD and FA. Contrary to our hypothesis, there was no difference in FCP level between children with AD (regardless of presence of FA) and healthy controls.

Inflammation of the intestinal mucous membrane that happens without a clear clinical manifestation and without symptoms in the GIT is a predictive factor for the development of atopic diseases in children. Early inflammation of the intestinal mucosa affects the development of inflammatory or atopic skin and respiratory diseases later in life (29). It is believed that inflammation of the intestinal mucosa increases bowel permeability and consequently increases the penetration of insufficiently digested food proteins, which promotes the development of allergy in susceptible individuals (30). Subclinical inflammation of the intestinal mucosa increases the permeability of the intestine and leads to "secondary" sensitization to allergens and ultimately affects the development of atopic diseases later in life.

Increased level of FCP in children with AD and FA is most probably the result of increased intestinal inflammation connected to development of nutritive allergy. However, whether fecal calprotectin directly contributes to the immunopathogenetic mechanisms in children with AD and food allergy remains to be explored. In animal models, FCP, along with other inflammatory factors, promotes Th2 inflammation seen in mild food allergy (31). In 2012, Lack presented a dual-allergen exposure hypothesis that says that exposure to food allergens through the damaged skin can lead to allergy, while consumption of these foods at an early age may actually result in tolerance. It is possible that sensitization to nutritive allergens occurs through damaged skin, and elevated FCP in the gut of such children promotes the continuation of food allergy (32). There was no difference between FCP level in children with AD and healthy controls, so we cannot hypothesize that epithelial barrier dysfunction has an impact on FCP irrespective of FA. Hypothetically, we can distinguish children with AD who have food allergy from those who do not by measuring FCP.

In accordance with Seo *et al.* (33) we found a significant positive association of FCP level and SCORAD score, suggesting FCP as a potential biomarker of AD severity.

Furthermore, in accordance with Beser *et al.* (22) we found a statistically significant difference in FCP

level before and after 3 months of elimination diet in the group of children with AD and FA (mostly IgE-mediated). Thus, FCP may be a useful biomarker of food allergy treatment follow-up in children with FA and AD without GIT symptoms. If these findings are confirmed in a larger number of subjects and studies, they will have a significant impact on reducing oral challenge and unnecessary elimination diets in such children. Thus, FCP could be an inexpensive and non-invasive biomarker of FA activity in children with AD. A correlation between FCP and SCORAD score also speaks in favor of utilizing FCP in this way.

We are aware of certain limitations of the present study, such as the lack of data on other local inflammatory biomarkers of gastrointestinal inflammation. Secondly, we found no significant correlation between fecal calprotectin levels and other inflammatory markers of AD. Furthermore, this was not a longitudinal study with repeated measurements of BMs with consecutive monitoring of AD severity before and after introduction of nutritive allergens. There are also several factors that could confound study results, such as like age, sex, difference in atopic status (IgE-mediated and non-IgE-mediated food allergy, mono-sensitization and poly-sensitization), and nutrition, although the study groups were well-matched for most of these factors.

CONCLUSION

Our results provide evidence supporting the potential utility of a FCP as biomarker of FA in children with AD. However, new studies are required in order to estimate the role of FCP as a biomarker FA in infants and children with AD and FA.

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