Original article

Association of T helper type 2 cytokines with sensitization to food in pediatric atopic dermatitis patients

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Background: *childhood atopic dermatitis (AD) most commonly presents with sensitization to environmental allergens. Presence of food allergen-specific IgE is common in childhood and does not always correlate with clinical symptoms, which in children usually affect the skin and may exacerbate the course of AD. Exposure to an allergen in the gastrointestinal tract activates Th2 immune reactions.* ***Objective:*** *with this study we wanted to compare blood and stool Th2 cytokine concentrations and fecal calprotectin (FC) value in pediatric AD with (eAD) and without (iAD) sensitization to food.* ***Methods:*** *51 children with AD were enrolled in the study. 57% (n=29) had food allergen specific IgE and comprised eAD group, 43% (n=22) – iAD group. Blood and stool were tested for IL-4, IL-5, IL-13 concentrations using an enzyme linked immunosorbent assay. Stool samples were tested for FC concentrations.* ***Results:*** *iAD had significantly higher blood and stool IL-4 values than eAD: 2.82 pg/ml vs. 0, p=0.005; 2.98 pg/ml vs. 0, p=0.007, respectively. There was no difference in IL-5 and IL-13 blood and stool concentrations between the groups. Children with AD had significantly higher FC values, compared to healthy controls: 36.5 mg/kg vs. 6.45 mg/kg, p=0.018. FC was slightly higher in eAD group than iAD, but the difference was not significant: 38.5 mg/kg vs. 25.0 mg/kg, p=0.861.* ***Conclusions:*** *sensitization to food is not significantly associated with Th2 cytokines in pediatric AD patients. Increase in FC values is characteristic to AD, but not sensitization to food.*

Keywords: : interleukin-4, interleukin-5, interleukin-13, fecal calprotectin, atopic dermatitis, food allergen specific IgE.

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INTRODUCTION

Atopic diseases in children continue to be a serious health problem worldwide and their prevalence in some countries is still increasing.[1] Atopic dermatitis (AD) is a chronic, relapsing, inflammatory skin disease, mediated by activated and expanded different T helper subsets and their cytokines.[2] According to the presence of allergen-specific immunoglobulin E (sIgE) in the blood, AD can be classified into intrinsic (iAD) and extrinsic types (eAD).[3] eAD type, with sensitization to food, is more common in childhood.[4] Presence of food allergen sIgE in the blood is a possible predictor of food allergy (FA) which, in children, most commonly presents with skin reactions.[5] Studies demonstrate, that food allergy (FA) has a strong association with atopic dermatitis (AD), its severity and chronicity.[6] A greater risk for developing FA have children with AD, who are younger (≤2 y.), had an early onset of AD (before 3 months of age) and have a more severe form of AD.[7] Some studies demonstrate, that nearly 16% of infants with AD develop at least one food allergy.[8]

Most common sensitizing factors in children with AD are cow's milk, hen's egg, peanut, wheat, soy, nuts, and fish. Sensitization to certain products is age dependent: cow's milk, hen's egg, peanuts and soy are more common to small children.[9] Studies demonstrate, that sensitization to food is common in childhood.[10] Presence of allergen sIgE in the blood does not always correspond to clinical reactions, but they may occur in the absence of allergen sIgE due to the cross-reactivity.[11] The most common route for sensitization to oral allergens is through the gastrointestinal tract. Nonoral routs, like transcutaneous sensitization is also possible, especially in patients with AD.[12]

A contact with an allergen in an allergic person triggers an activation and differentiation of naïve T-cells into Th2 (T helper cell type 2) subset. Th2 inflammation is orchestrated by Interleukin-4 (IL-4), IL-5, IL-13. These cytokines promote class switching and IgE synthesis in B-cells,

eosinophilia, mast cell hyperplasia. A repeated exposure to the allergen promotes a quick activation and expansion of the certain Th2 cell clone.[13] Food allergens sustain Th2 type inflammatory state, which is important in the pathogenesis of atopic diseases.[14] Studies show that intestinal exposure to allergens results in accumulation of immune cells and low grade inflammation.[15] Calprotectin, also known as S100A8/A9 protein heterodimer, is abundant in the cytoplasm of neutrophils and comprises approximately half of their total cytoplasmic protein content.[16] Calprotectin is released from activated neutrophils, increased fecal concentration represents inflammatory activity in the intestine.[17]

The gastrointestinal tract is the main gateway for food allergens. Allergen sIgE testing lacks correlation with clinical symptoms, especially in children.[10] Early diagnosis and proper interpretation of clinical tests, concerning allergic sensitization is important to prevent clinical manifestations, exacerbations and associated complications. Stool testing for Th2 type cytokines and fecal calprotectin (FC) could give an insight of immune reactions ongoing in the gut mucosa. With this study we wanted to evaluate blood and stool levels of IL-4, IL-5, IL-13 and FC in pediatric AD with and without sensitization to food. The aim of this study was to test if sensitization to food is associated with an activation of Th2 type immune responses in pediatric atopic dermatitis patients.

METHODS
Study population and ethical considerations
We studied 51 patients with atopic dermatitis, hospitalized in Children's Hospital, Affiliate of Vilnius University Hospital Santaros Klinikos, Lithuania. Diagnosis was assessed according to clinical symptoms by pediatric allergologist and clinical immunologist. Diagnostic criteria of Hanifin and Rajka were applied.[18,19] Atopic dermatitis severity scoring index (SCORAD) was used to evaluate the severity. Atopic dermatitis with SCORAD ≤24 was classified as mild, 25 – 50 – moderate, >50 – severe. According to the presence of food allergen sIgE results test subjects were divided into eAD and iAD groups. 17 healthy control subjects, who had no history of atopic diseases, nor any current inflammatory diseases made up the control group. Participants parents or legal guardians provided their agreement in participating in the study by signing a written informed consent form. Ethics approval for the research study was obtained from Vilnius Regional Biomedical Research Ethics Committee.

Sample collection and preparation
Blood and stool samples from test subjects and stool samples from control subjects were analysed. Based on clinical history single-food IgE test, or food allergy panel testing was performed with Phadia Immunocap 100 analyzer (Phadia, Uppsala Sweden). Sensitization to cow's milk, hen's egg, peanut, hazelnut, soybean, wheat, corn, codfish results were obtained. Allergen specific IgE result of ≥0.35 kUA/l was considered postive.[20] Absolute eosinophil counts were measured from venous blood using automated hematology analyzer (Sysmex XT 4000i, Roche Germany). Total blood IgE concentration was tested with (Cobas e411, Roche Germany). The rest of the blood and stool samples were stored at -80°C for further cytokine and fecal calprotectin testing.

Serum and stool IL-4, IL-5, IL-13 measurements
Frozen stool and serum samples were completely defrosted prior testing. Suspensions were prepared from stool samples: 0.1g stool was suspended in 1 ml phosphate saline buffer (PBS, pH=7.2). Suspensions were thoroughly vortexed, left to sit at room temperature for 15 min, then once again vortexed and centrifuged (10000 x g, 20 min). Supernatants were used for the test procedure. Supernatants and serum samples were tested for IL-4 using Human Interleukin-4 (Hu IL-4) ELISA kit (Thermo Fisher Scientific, USA), IL-5 – Human Interleukin-5 (Hu IL-5) ELISA kit (Thermo Fisher Scientific, USA), IL-13 – Human Interleukin-13 ELISA kit (Elabscience, China). Assay procedures were performed according to manufacturer's recommendations. Detection range for IL-4, provided by the manufacturers, was 0 – 500 pg/ml, IL–5: 0–750 pg/ml, IL–13: 0–1000 pg/ml.

Fecal calprotectin measurement
Stool samples were completely defrosted prior testing. Ready to use stool extraction kit tubes were used for the extraction (EliА™, Fecal extraction device, Thermo Fisher Scientific, USA). Defrosted stool samples were homogenised with wortex, left to sit for 10 minutes at room temperature, then centrifuged (3000 x g, 5 min.). Obtained supernatants was transferred to new tubes and used for further testing. Fecal calprotectin analysis was performed with fluorescence enzyme immunoassay, using Phadia Immunocap 100 analyser (Phadia, Uppsala, Sweden). Manufacturers provided measuring range: 0 - ≥3000.0 mg/kg.

Statistical analysis

MS Office Excel, MedCalc software were used for data management and statistical analysis. Nonparametric data were expressed with a median and range. Two groups of variables were compared with Mann-Whitney U test. Kruskal-Wallis test was used to compare groups of variables with an ordinal dependent variable. Categorical data were expressed with a number and percentage, difference was determined using Chi-Square test. Difference between the groups was considered significant when p<0.05.

RESULTS

A total of 51 children with AD was enrolled in the study. 57% (n=29) comprised eAD group, 43% (n=22) – iAD group. eAD group was slightly older (27.0 mo. vs. 12.5 mo. p=0.044). There was no significant difference according to gender. Based on SCORAD, 54% in iAD group had mild, 32% - moderate, 14% - severe AD. In eAD group: 52% - mild, 31% - moderate, 17% - severe AD. eAD patients had slightly higher absolute blood eosinophil numbers and total IgE, compared to iAD, but the difference was not significant. Detailed patient characteristics are provided in table 1.

Blood IL-4 values in iAD ranged from 0 – 3.56 pg/ml, median – 2.82 pg/ml. eAD: 0 – 2.95 pg/ml, median – 0. The difference was significant between the groups: 2.82 pg/ml vs. 0, p=0.005. Stool IL-4 values in iAD were from 0 – 3.27 pg/ml, median – 2.98 pg/ml. eAD: 0 – 0.44 pg/ml, median – 0. The difference was significant between the groups: 2.98 pg/ml vs. 0, p=0.007. Blood IL-5 concentrations were similar between the groups: iAD: 0 – 8.66 pg/ml, median – 0; eAD: 0 – 38.67 pg/ml, median – 0. There was no significant difference between the groups. Stool IL-5 in iAD ranged from 0 to 17.21 pg/ml, median – 0; eAD: 0 – 33.17 pg/ml, median – 2.92 pg/ml. The difference was not significant between the groups: 0 vs. 2.92 pg/ml, p=0.272. Blood IL-13 concentrations in iAD ranged from 0 to 144.95 pg/ml, median – 0.1 pg/ml; eAD: 0 – 135.79 pg/ml, median – 0. The difference was not significant between the groups: 0.1 pg/ml vs. 0, p=0.197. Stool IL-13 levels in iAD ranged from 0 to 407.60 pg/ml, median – 27.24 pg/ml. eAD: 0.38 pg/ml – 178.41 pg/ml, median – 29.83 pg/ml. The difference was not significant between the groups, 27.24 pg/ml vs. 29.83 pg/ml, p=0, 0.765. Summary provided in table 2. We also wanted to test, if Th2 type cytokine concentrations associate with the severity of AD. IL-4, IL-5 and IL-13 blood and stool values, obtained in our study, showed no difference in association with severity of atopic dermatitis in iAD and eAD groups.

Table 1. Patient characteristics.

Characteristics	Intrinsic AD n=22	Extrinsic AD n=29	p value
Age (mo) (range)	12.5 (1 – 51)	27.0 (5 – 77)	0.044*
Gender (male/female) (%)	11/11 50%/50%	25/4 86%/14%	0.652§
SCORAD	mild AD: 54% (n=12) moderate AD: 32% (n=7) severe AD: 14% (n=3)	mild AD: 52% (n=15) moderate AD: 31% (n=9) severe AD: 17% (n=5)	
Blood eosinophils median (cells/μl) (range)	0.28 (0.04 – 1.18)	0.48 (0.08 – 0.811)	0.234*
Total IgE median (IU/ml) (range)	59.85 (18.5 – 381.0)	257.9 (55.1 – 4320.0)	0.191*
Sensitization to food profile n (% total)		hen's egg: 21 (72.4%) cow's milk: 15 (51.7%) corn: 7 (24.1%) hazelnut: 7 (24.1%) peanut: 6 (20.7%) soy bean: 6 (20.7%) cod fish: 4 (13.8%) wheat: 4 (13.8%) shrimp: 2 (6.9%)	

* - Mann-Whitney test for independent samples; § - Chi-square test.

**Table 2.** Th2 cytokine results for intrinsic and extrinsic atopic dermatitis groups.

	Intrinsic AD	Extrinsic AD	p value
blood IL-4 (pg/ml) median (range)	2.82 (0 – 3.56)	0 (0 – 2.95)	0.005*
stool IL-4 (pg/ml) median (range)	2.98 (0 – 3.27)	0 (0 – 0.44)	0.007*
blood IL-5 (pg/ml) median (range)	0 (0 – 8.66)	0 (0 – 38.67)	0.492*
stool IL-5 (pg/ml) median (range)	0 (0 – 17.21)	2.92 (0 – 33.17)	0.272*
blood IL-13 (pg/ml) median (range)	0.1 (0 – 144.95)	0 (0 – 135.79)	0.197*
stool IL-13 (pg/ml) median (range)	27.24 (0 – 407.60)	29.83 (0.38 – 178.41)	0.765*

* - Mann-Whitney test for independent samples

**Table 3.** Fecal calprotectin results for atopic dermatitis patients and healthy controls

		Fecal calprotectin (mg/kg) median (range)		Controls (mg/kg) median (range)	p value
AD	iAD	36.5 (0 – 1743.0)	25.0 (0 – 996.0)	6.45 (0 – 70.0)	0.018*
	eAD		38.5 (0 – 1743.0)		

* - Mann-Whitney test for independent samples performed on total AD (n=51) vs. controls (n=17).
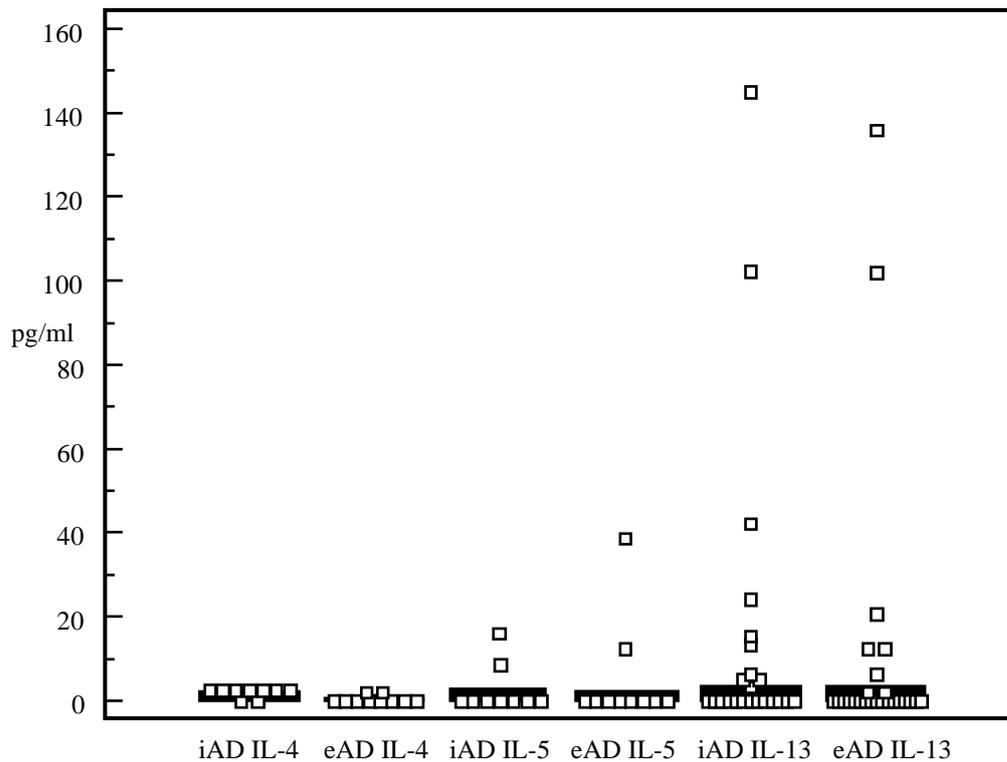
**Figure 1.** Blood IL-4, IL-5, IL-13 concentrations

Blood IL-4, IL-5, IL-13 cytokine concentrations between intrinsic (iAD, n=22) and extrinsic (eAD, n=29) atopic dermatitis groups. Comparisons were performed using Mann-Whitney U test. Medians are marked with black bars.
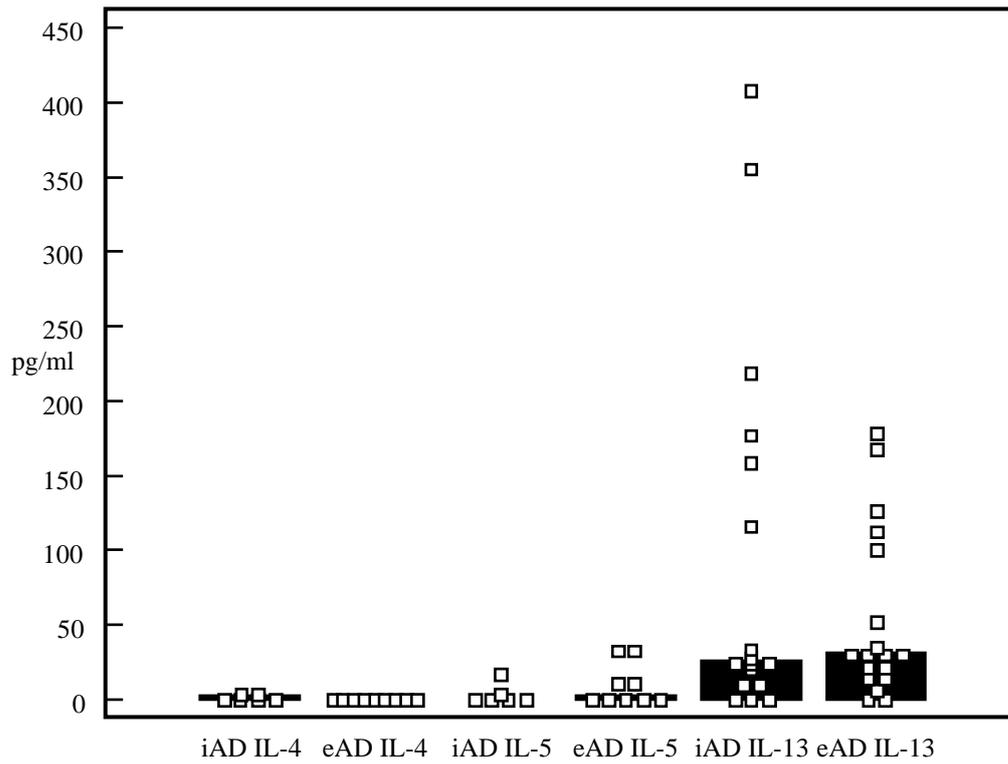
Figure 2. Stool IL-4, IL-5, IL-13 concentrations

Stool IL-4, IL-5, IL-13 cytokine concentrations between intrinsic (iAD, n=22) and extrinsic (eAD, n=29) atopic dermatitis groups. Comparisons were performed using Mann-Whitney U test. Medians are marked with black bars.

Fecal calprotectin values in children with AD were from 0 to 1743.0 mg/kg, median – 36.5 mg/kg. The difference was significant, compared to healthy controls: 36.5 mg/kg vs. 6.45 mg/kg, p=0.018. eAD group had slightly higher FC, compared to iAD, but the difference was not significant: 38.5 mg/kg vs. 25.0 mg/kg, p=0.861. See table 3 and figure 3.
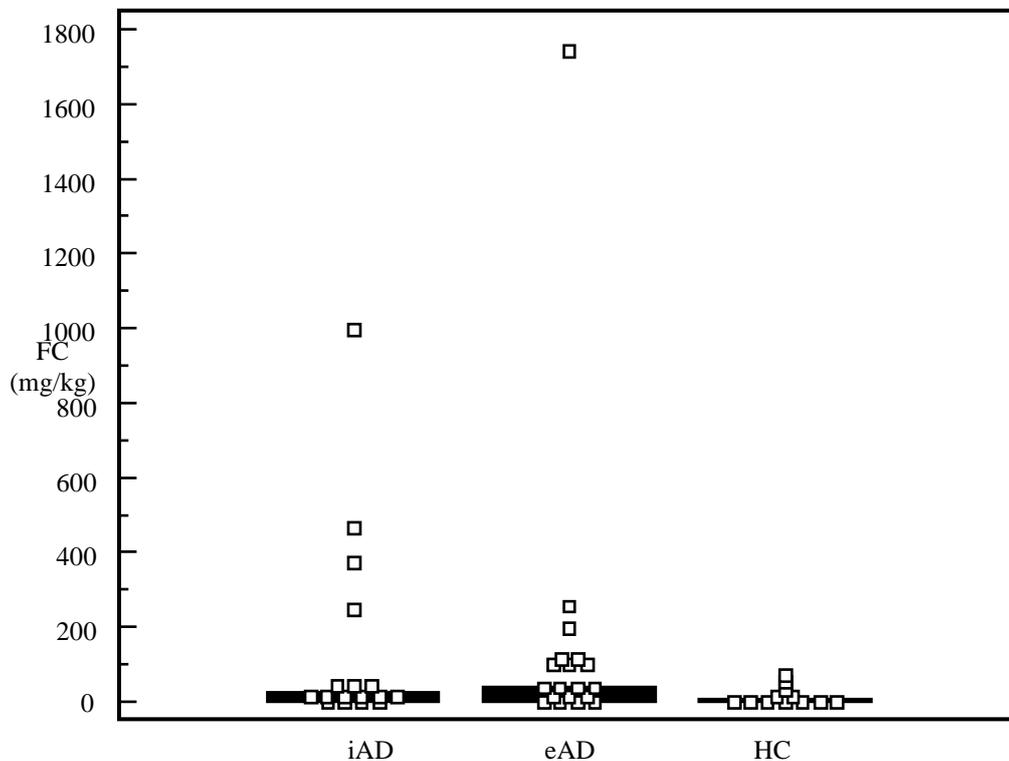
Figure 3. Fecal calprotectin results

Comparison of fecal calprotectin values between intrinsic (iAD, n=22), extrinsic (eAD, n=29) atopic dermatitis groups and healthy controls (n=17). Medians are marked with black bars.

DISCUSSION

Overactivation of Th2 immune responses with an increased secretion of cytokines plays an important role in the pathogenesis of AD.[21] Pediatric AD is highly associated with other atopic diseases, like FA.[22] Mucosal encounter to an allergen triggers Th2 type immune inflammation with an increased IL-4, IL-5, IL-13 secretion, neutrophil and other immune cell recruitment.[23] Childhood AD most commonly presents with sIgE to any allergen, however food is the most common cause of sensitization in younger children.[4, 10] In our study 57% of the test subjects had food allergen sIgE in their blood. Studies demonstrate, that hen's egg and cow's milk allergens are most likely to cause sensitization to food in younger children.[24] These food allergens were the most common cause of sensitization in our study group. The gastrointestinal tract is considered to be the main route for sensitization to oral allergens.[13] In our study blood and stool samples were tested for Th2 cytokines. We expected higher stool Th2 cytokine concentrations in eAD group due to the main route of an allergen encounter, however this was not the case. Besides oral routs, alternative ways for sensitization are also possible. Studies demonstrate, that infants can get sensitized to food through breast-feeding.[25] H.A. Brough *et al.* demonstrates, that damaged skin barrier in AD is a potential risk factor for developing sensitization to certain food products, e.g. peanuts.[26] Irrespective of the sensitization route, sensitized individuals develop a Th2 mediated intestinal inflammation, after oral allergen ingestion.[27] We did not find any information concerning stool testing for Th2 cytokines. This might not be a proper method for the detection of Th2 mediated immune inflammation in the gastrointestinal tract mucosa. Our study does not show any significant differences in IL-5 and IL-13 levels between the eAD and iAD groups. An experimental murine model demonstrates, that following allergen stimulation, sensitized mice produced high quantities of IL-5 and IL-13 compared to naïve.[28] *In vitro* stimulation of allergen-specific lymphocytes, derived from gastrointestinal tract mucosa, showed an increased production of IL-5 and IL-13.[29] However, studies concerning humans are inconclusive. N. Novak *et al.* found significantly higher IL-5 in eAD and IL-13 in iAD patients.[30] Our study shows, that iAD group had significantly higher blood (2.74 pg/ml vs. 0, p=0.022) and stool (2.98 pg/ml vs. 0, p=0.007) IL-4 levels, compared to eAD. This was unexpected, because according to literature IL-4 is the main cytokine required for allergic sensitization and initiation of Th2 immune responses.[31] However studies, concerning humans are inconclusive. M

Kimura *et al.* showed, that IL-4 levels demonstrated the best correlation with allergen sIgE synthesis in atopic children.[32] According to N. Novak *et al.* blood IL-4 concentrations were similar in iAD and eAD groups.[30] K.V. Barros *et al.* reports that infants with cow's milk protein allergy had a higher plasma IL-4 and IL-13 concentrations, which were significantly lower after the elimination diet.[33]

Experimental animal models, investigating intestinal allergic sensitization process reveal, that following allergen encounter, intestinal epithelial basement membrane was disrupted and an increased influx of immune cells, including neutrophils, was observed.[34] According to our data children with AD had significantly higher FC, compared to healthy controls (36.5 mg/kg vs. 6.45 mg/kg, p=0.018). S. Seo *et al.* found significantly greater FC levels in children with severe AD.[35] In our study eAD group had slightly higher FC concentration, compared to iAD, but the difference was not significant (38.5 mg/kg vs. 25.0 mg/kg, p=0.861). Low grade intestinal inflammation is associated with food allergen encounter in a sensitized person. Experimental models of food hypersensitivity showed, that neutrophils were also activated during food allergen processing, which led to an increased FC level during Th2 mediated intestinal inflammation.[36] Ö.F. Beşer *et al.* reports that children with IgE-mediated cow's milk protein allergy had significantly lower FC concentration after the elimination diet, comparing to FC before the diet.[37]

CONCLUSION

Sensitization to food is not associated with Th2 type cytokine activation in pediatric atopic dermatitis patients. FC is elevated in children with AD, but not sensitized to food AD patients.

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