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Higher circulating EGF levels associate with a decreased risk of IgE sensitization in young children

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Abstract

Background: Decreased exposure to microbial agents in industrialized countries and urban living areas is considered as a risk factor of developing immune-mediated diseases, such as allergies and asthma. Epithelial surfaces in the gastrointestinal and respiratory tracts and in the skin constitute the primary areas in contact with the environmental microbial load.

Methods: We analyzed the levels of 30 cytokines and growth factors in serum or plasma as markers of the immune maturation in the participants in the DIABIMMUNE study from Russian Karelia ($n = 60$), Estonia ($n = 83$) and Finland ($n = 89$), three neighboring countries with remarkable differences in the incidences of allergies, asthma and autoimmune diseases.

Results: We observed an upregulation of T helper cell signature cytokines during the first 12 months of life, reflecting natural development of adaptive immune responses. During the first years of life, circulating concentrations of epidermal growth factor

Abbreviations: EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor.

(EGF) were significantly higher, especially in Russian children compared with Finnish children. The children who developed IgE sensitization showed lower levels of EGF than those without such responses.

Conclusion: Our results suggest that low circulating EGF levels associate with the risk of allergies possibly via the effects on the epithelial integrity and mucosal homeostasis.

KEYWORDS
allergic sensitization, cytokine, epidermal growth factor, epithelial integrity, IgE, T helper cell

1 | INTRODUCTION

The incidence of chronic immune-mediated diseases has been in continuous rise since World War II in the Western world.[1] Several studies have reported increases in the incidence of allergies, asthma, inflammatory bowel diseases, multiple sclerosis, and type 1 diabetes.[1,2] The hygiene hypothesis originally proposed that the decreased number of infections in early life may interfere with the normal maturation and development of the immune system, causing abnormal reactions against harmless antigens, loss of self-tolerance, and onset of immune-mediated diseases such as allergies.[3] Later, the hygiene hypothesis was modified to postulate that a decreased exposure to diverse microbial agents, not necessarily causing infections, in industrialized countries and urban living areas is one of the underlying mechanisms increasing the risk of immune-mediated diseases.[1,4]

Epithelial lining in the gastrointestinal and respiratory tracts and in the skin constitutes the surface areas exposed to the environmental microbial load. Under homeostatic conditions, the epithelial barrier is intact and maintained by different defense and repair mechanisms. Gut immune system and intestinal microbiota are considered as key regulators of the intestinal homeostasis and health of the host. Animal studies have provided mechanistic evidence that gut microbiota regulates intestinal integrity and that gut bacteria induce and regulate peripheral immune responses and establishment of immune tolerance.[5,6] In agreement with animal studies, observations from human studies have demonstrated associations between altered gut colonization in early life and development of aberrant immune responses leading to increased risk of immune-mediated diseases later in life.[7–9]

The importance of environmental factors as modulators of the immune system and the risk of immune-mediated diseases is underlined by remarkable differences in the incidences of allergies, asthma, and autoimmune diseases between neighboring populations in Finland, Estonia, and Russian Karelia.[10] The prevalence of physician-diagnosed asthma has been reported to be 2.9%–5.4% in Estonia[11,12] and 7.2%–11.2% in Finland.[12–14] The prevalence of allergic conditions has been reported to be 3- to 10-fold higher in Finnish young adults than in corresponding Russian subjects.[15] Sensitization rates to any allergens have been reported to be 47.7% in Finland and 15.9% in Russia.[16] In the DIABIMMUNE birth cohort (BC), differences in the gut microbiome composition have been demonstrated between children living in Russian Karelia, Estonia, and Finland,[17] and a link to the risk of allergy responses has also been established.[18] To further elucidate the molecular drivers for the differences in the risk of immune-mediated diseases, we studied the systemic levels of 30 cytokines and growth factors as biomarkers of the maturation of the immune system during the first four years of life in the participants in the DIABIMMUNE study.

Key message

Our study following children from Finland, Russia and Estonia, three countries with different incidences for immune-mediated diseases, indicates that EGF might serve as a protective factor against the development of IgE responses in young children.

2 | MATERIALS AND METHODS

2.1 | Subjects and samples

In the BC of the DIABIMMUNE study, we collected serum samples from Russian (60 children, $n = 144$), Estonian (46 children, $n = 128$), and Finnish (45 children, $n = 126$) children at the age of 3, 6, 12, 18, 24, and 36 months (Figure 1). The number of follow-up samples is shown in Figure 1. The number of children with different amount of follow-up samples is shown in Table S3. In the cohort of young children (YCC) of the DIABIMMUNE study, heparinized peripheral blood samples for plasma isolation were obtained from 44 Finnish and 37 Estonian children at the age of 48 months (Figure 1). Both BC and YCC children were recruited from the same geographically areas. BC children had HLA-conferred susceptibility to type 1 diabetes, whereas YCC children represented the general population in Finland and Estonia. No samples from Russian children were available from the YCC. The current study was approved by the local ethical committees from all three study centers, and written informed consent was obtained from the participating families.

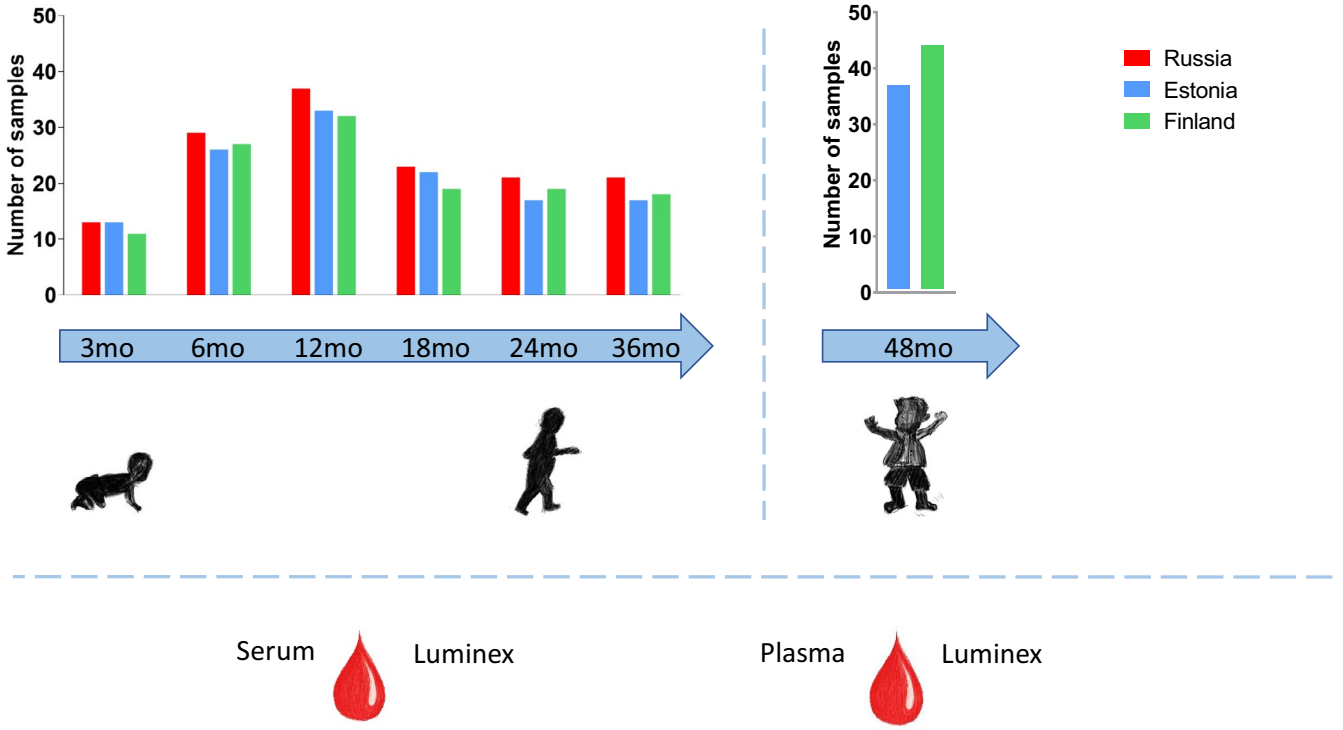

FIGURE 1 Study design: Concentrations of cytokines and growth factors were analyzed during the first four years of life in children from countries with a markedly different incidences of chronic immune-mediated diseases (Finland, Estonia, Russia). Blood samples were collected for cytokine analysis from 3-, 6-, 12-, 18-, 24-, and 36-month-old children from Russia, Estonia, and Finland and from 48-month-old Estonian and Finnish children. The levels of circulating cytokines and growth factors were analyzed with multiplexed Luminex technology. Allergen-specific IgE concentrations were analyzed from serum samples

2.2 | HLA genotyping

The HLA typing of major type 1 diabetes risk DR-DQ haplotypes of the study subjects was performed with a PCR-based lanthanide-labeled hybridization method using time-resolved fluorometry for detection as described before.[19]

2.3 | Allergen-specific IgE analysis and clinical outcomes

Allergen-specific IgE concentrations were analyzed from serum samples at the age of 6, 18, and 36 months in the BC by using the ImmunoCAP fluoroenzyme immunoassay (Thermo Fisher Scientific). In the YCC, samples were analyzed at the age of 48 and 60 months. IgE concentrations to egg, cow's milk, house dust mite, cat, timothy grass, and birch were analyzed at the age of 6 months. Peanut was added to the panel at the age of 18 months and dog at all older ages. Concentrations of at least 0.35 kU/L were considered positive. The treating physician provided allergy diagnosis, and details are shown in Table 1. Detailed characteristics are listed in Table S4. The prevalence of allergy-related symptoms was evaluated by using a modification of the standardized questionnaire by the International Study of Asthma and Allergies in Childhood (ISAAC).[20] For the BC children, the questionnaire was filled out by their parents at the age of 24 and 36 months, and for the YCC children at the age of 48 and 60 months.

2.4 | Quantification of circulating cytokines and growth factors

We analyzed the concentrations of 30 cytokines and growth factors in serum samples from the BC children with a customized 38-plex kit complemented with the Human High Sensitivity T Cell Panel-Immunology Multiplex Assay (Merck, Darmstadt, Germany). The plasma samples from the YCC were analyzed with the Human TH17 Magnetic Bead Panel-Immunology Multiplex Assay complemented with a custom 3-plex assay for epidermal EGF, sCD40L, and TGF-α. See Table S1 for a list of cytokines analyzed in the current study.

Luminex analyses were performed in single reactions. Quantification of the markers was produced with the Bio-plex 200 Luminex instrument and Bio-Plex Manager software (Bio-Rad, Hercules). An 8-point standard curve using five-parameter logistic regression was used for determination of the concentration of the analytes. The samples below minimum detectable concentration (MinDC) were given an arbitrary value of 50% of MinDC. Samples above measurable values were excluded.

TABLE 1 Demographic and clinical characteristics of non-sensitized, sensitized, and allergic children at two time points of the study

	Birth cohort (at 18 months)						Young children's cohort (at 48 months)					
	Physician-diagnosed allergy		sIgE sensitized		Non-sensitized		Physician-diagnosed allergy		sIgE sensitized		Non-sensitized	
	N = 12		*N* = 28		*N* = 28		*N* = 9		*N* = 19		*N* = 71	
	%	sEGF [pg/ml]	%	sEGF [pg/ml]	%	sEGF [pg/ml]	%	pEGF [pg/ml]	%	pEGF [pg/ml]	%	pEGF [pg/ml]
Males (%)	68.8		38.9		48.9		77.8		42		33.3	
Russia	0		21.7	476.3	78.3	535.2	-	-	-	-	-	-
Estonia	38	279.4	19	200.8	81	219.9	1.9	176	18.9	175	81.1	412.6
Finland	21	133	47.4	108.8	52.7	71.4	21.6	268.3	22.2	135.8	77.8	162.7
Clinical outcomes diagnosed by physician												
	%	mean age in months	sEGF [pg/ml]				%	mean age in months	pEGF [pg/ml]			
Asthma	4.8	23	383.3				5.5	35.5	247.6			
Atopic eczema	9.5	6.5	120.5				5.5	34	177			
Bronchitis	1.6	no data	212.8				3.3	29	352.3			
Food allergy	3.2	16	173.2				3.3	3	175			

Note: Values are medians if not otherwise indicated.

Abbreviations: mean age in months, age at first diagnosis; pEGF, plasma EGF; sEGF, serum epidermal growth factor (EGF); sIgE, serum IgE.

2.5 | Statistical analysis

Statistical tests were performed with GraphPad Prism 8.30, IBM SPSS Statistics 25 and R v3.5. Two groups were compared with the non-parametric, Mann–Whitney *U* test. Comparisons between several groups were performed with the Kruskal–Wallis test followed by Dunn's test for comparisons of two groups. Correlations between variables were analyzed with the non-parametric Spearman test. Longitudinal analysis of the serum cytokine levels in different countries was performed with the linear regression model. $p < .05$ was considered statistically significant. The *p* values for differences were not corrected for multiple testing except for the longitudinal analysis of cytokines where the Benjamin-Hochberg method was applied.

3 | RESULTS

3.1 | The maturation of the immune system is characterized by upregulation of circulating cytokines

We observed a clear age-related upregulation of circulating Th1 (IFN-γ), Th2 (IL-5 and IL-13), Th17 (IL-17A), and regulatory T cell (IL-10) type cytokines in the participants from all countries studied (Figure 2). Similar upregulation of key innate immunity cytokines driving the maturation of adaptive immunity, namely, IL-12p70, IL-1b, and IL-6, was observed (Figure 2). The maturation profile for these cytokines was consistent in all countries, and no differences were observed between the countries (See Table S2 for Linear model statistics).

3.2 | EGF levels differed between the countries

Longitudinal analysis of the BC samples revealed that Russian children had significantly increased circulating levels of EGF compared with children from Estonia and Finland during their first 3 years of life (Figure 3 and Table S2). When the concentrations of the growth factors and cytokines were compared at a specific age, Russian children had higher EGF concentration at every time-point in comparison with Estonian and Finnish children. At the age of 24 months, Estonian children had also a higher concentration of EGF when compared to Finnish children (Figure 3). The levels of other cytokines analyzed in the BC samples from Russian, Estonian, and Finnish children are presented in Figure S1. EGF, sCD40L, and CXCL11 (ITAC) showed significantly different levels across the countries. Next, we analyzed EGF concentrations in YCC samples from 48-month-old Estonian and Finnish children and confirmed lower levels of EGF in Finnish children (Median Estonia: 355.8 pg/ml, IQR =516.2 pg/ml–198.5 pg/ml =317.7 pg/ml vs. Finland: 157.1 pg/ml, IQR =236.2 pg/ml–93.8 pg/ml =63.3pg/ml, $p < .0001$). The levels of all other cytokines analyzed are presented in Figure S2.

3.3 | Low circulating EGF associates with atopic sensitization

Next, we wanted to investigate the relation between serum EGF concentration and IgE sensitization to allergens and clinical allergies. The levels of EGF were higher in 18-month-old children without IgE sensitization and no clinical allergy (IgE antibody level below 0.35 U/ml for all allergens analyzed) when compared to children with atopic

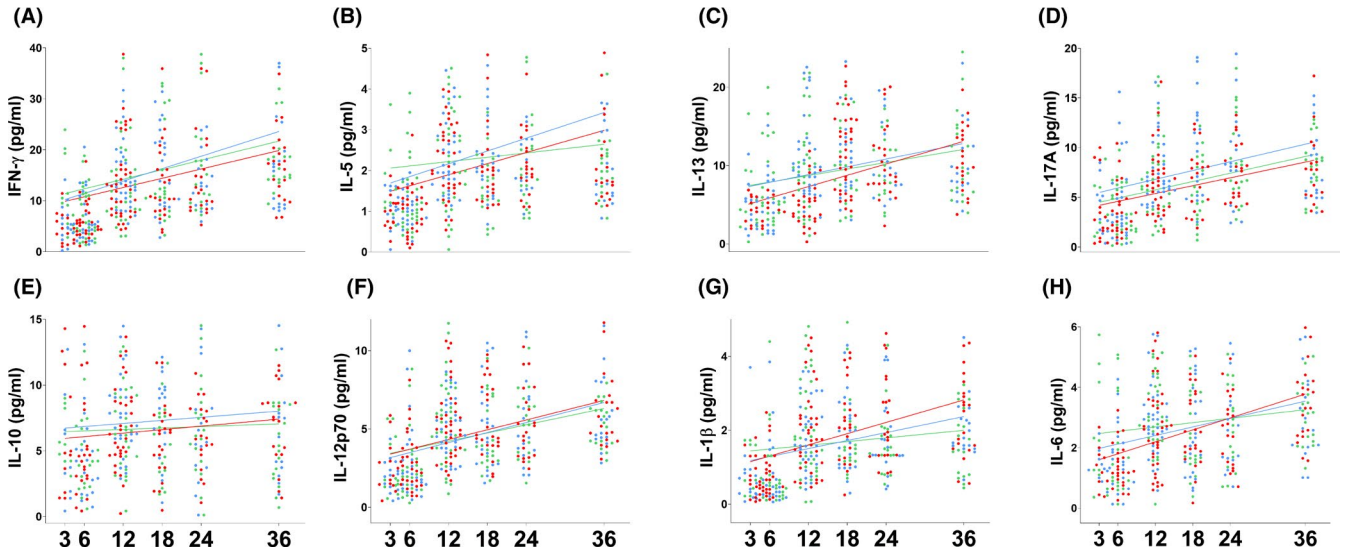

FIGURE 2 (A-E) Longitudinal analysis of circulating Th1 (IFN-γ), Th2 (IL-5 and IL-13), Th17 (IL-17A), and regulatory T cell (IL-10) type cytokines in 3-, 6-, 12-, 18-, 24-, and 36-month-old Russian, Estonian, and Finnish children are illustrated. (F-H) The levels of Th1-supporting IL-12p70 and Th17 supporting IL-1β and IL-6 innate cytokines are shown. Age is a significant driver of the general immune system upregulation in all countries, whereas no longitudinal differences were observed between the countries. Russian children are denoted with red dots and lines, Estonian children with blue dots and lines, and Finnish children with green dots and lines. Longitudinal analyses were performed with linear regression. *p*-values are presented in the Table S2

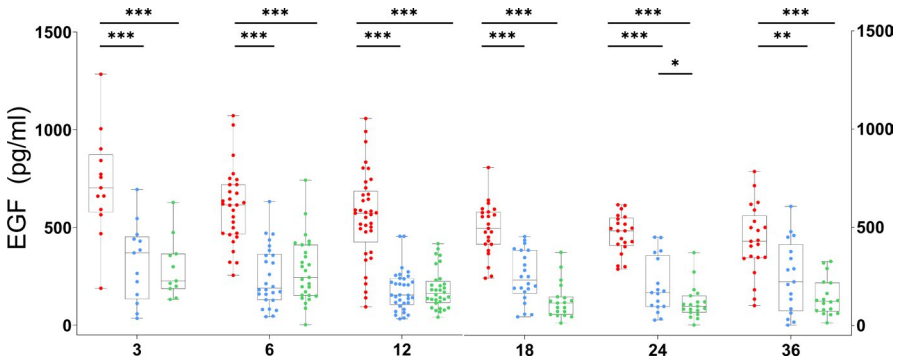

FIGURE 3 Comparisons of serum levels of EGF in Russian, Estonian, and Finnish children of 3, 6, 12, 18, 24, and 36 months of age. Russian children had significantly increased levels of circulating EGF throughout the study period compared with Estonian and Finnish children. Estonian children had elevated EGF levels at the age of 24 months compared with Finnish children. Russian children are denoted with red dots, Estonian children with blue dots, and Finnish children with green dots. Boxes mark the 25th and 75th percentiles, the line in the middle of the box marks the median value, and the whiskers mark the minimum and maximum values. Countries were compared within each time-point with the non-parametric Kruskal–Wallis test followed by Dunn's test for comparison of two countries. (*$p < .05$, **$p < .01$, ***$p < .001$)

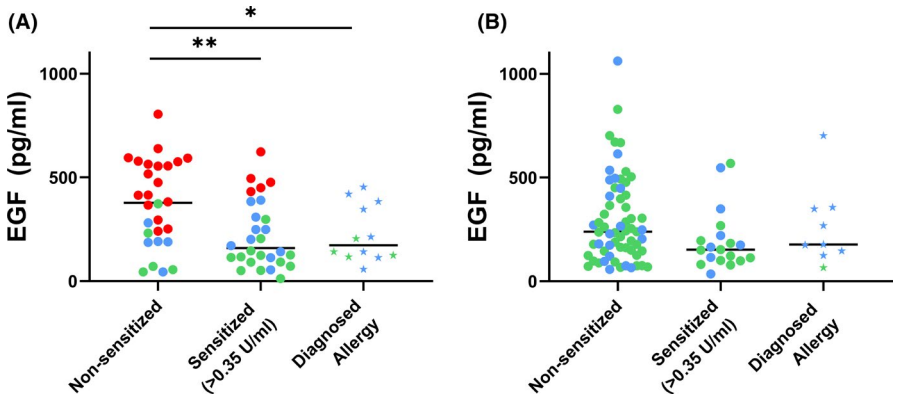

FIGURE 4 The relation between the level of circulating EGF at the age of 18 months and 48 months and atopic sensitization and clinical allergies. (A) The levels of EGF were elevated at the age of 18 months in children without IgE sensitization (IgE antibody level below 0.35 U/ml for all studied allergens) and no clinical allergy when compared to children with atopic sensitization (IgE antibody level equal or above 0.35 U/ml) and clinical allergy. (B) 48-month-old children without atopic sensitization tended to have higher EGF levels compared to children with IgE sensitization. IgE concentrations to egg, cow's milk, house dust mite, cat, dog, timothy grass, peanut, and birch were analyzed. IgE sensitization was defined using the cutoff value of 0.35 U/ml for each allergens tested, and at least one specific IgE antibody had to be ≥0.35 U/ml in a sensitized individual. Russian children are denoted with red dots, Estonian children with blue dots, and Finnish children with green dots. Children with clinically diagnosed allergy are marked with a star. Horizontal lines represent median values. *P*-values were calculated with the non-parametric Mann–Whitney *U* test. (**$p < .01$ and *$p < .05$)

sensitization or diagnosed allergy (Figure 4A). In 48-month-old children, this difference was not statistically significant (Figure 4B).

We wanted to assess whether there was any association between low EGF concentration and a specific allergic disease but found no significant differences. The number of diagnosed allergies furthermore was not related to lower EGF concentration. Since the association between low EGF concentrations seemed to lie with atopic sensitization and not with allergic disease outcome, we further investigated whether there was any relation between EGF concentrations and number of sensitized allergens. There was no significant difference between children that were sensitized to multiple allergens and children who were sensitized to only one allergen.

To verify whether there was a relation between low EGF concentration and specific allergens, we compared concentrations in 18- and 48-month-old children sensitized to different allergens. Non-sensitized children differed in EGF concentration significantly in relation to children sensitized to cow's milk (Figure 5).

4 | DISCUSSION

In the current study, we show differences in circulating EGF concentrations between children living in three neighboring areas with different incidences of immune-mediated diseases. Children from

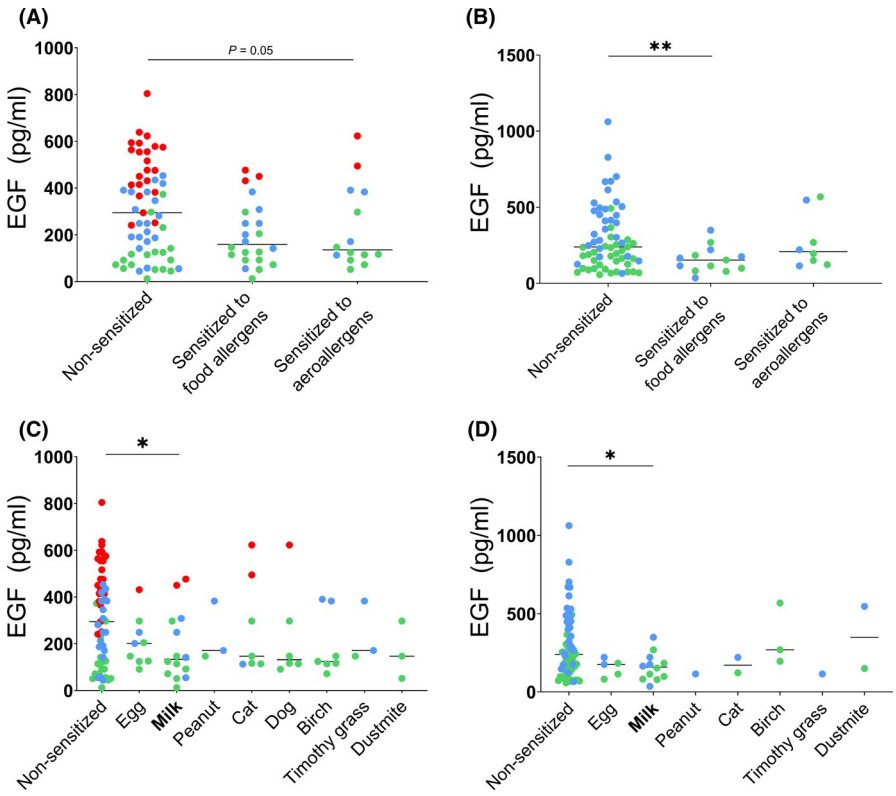

FIGURE 5 (A) Non-sensitized 18-month-old children did not differ significantly ($p = .05$) from children sensitized to the group of food allergens (egg, milk, and peanut) or the group of aeroallergens (cat, dog, birch, timothy grass, and house dust mite) (C) Non-sensitized 18-month-old children had higher EGF concentrations than children sensitized to cow's milk. (B) Non-sensitized 48-month-old children had higher EGF levels than children sensitized to the group of food allergens (egg, milk, and peanut) and children sensitized to cow's milk (D). Russian children are denoted with red dots, Estonian children with blue dots, and Finnish children with green dots. Horizontal lines represent median values. *p*-values were calculated with the non-parametric Mann–Whitney *U* test. (**$p < .01$, *$p < .05$)

Russian Karelia with the lowest rate of immune-mediated diseases showed the highest concentrations of circulating EGF, while Finnish children with the highest incidence of immune-mediated diseases had the lowest levels of systemic EGF. When we investigated this in an independent set of samples collected from 48-month-old children living in Estonia and Finland, a similar pattern of low circulating EGF in Finnish children was observed. Since serum samples were available from BC children and plasma samples from YCC children, some differences in cytokine levels might occur. Serum and plasma levels of most cytokines are roughly concordant, but previously it has been shown that EGF levels were lower in serum than plasma.[21] We did not investigate the role of HLA genotypes in the development of allergy even though the BC inclusion criteria is partly genetic. The YCC children represent the general population in Finland and Estonia. Since the BC children carried HLA-risk genotypes for autoimmunity, their results cannot be generalized for the general population. Contrarily, these risk genotypes are common in Finland and only by a fraction lead to autoimmunity. Recently, Mustonen et al. observed that 3-year-old children with HLA-conferred risk for autoimmunity were somewhat more frequently sensitized compared to the general population and did not have a reduced frequency of atopic and allergic conditions.[22]

EGF is a critical factor regulating cell growth and survival, cell proliferation and differentiation, migration, and apoptosis.[23] EGF plays a significant role during intestinal development in establishing a selective intestinal barrier (reviewed in[24]). The source of circulating EGF could be epithelial cells and/or stem cells of the skin, the respiratory, or intestinal epithelium.[25–29] EGF receptor (EGFR) signaling regulates various processes, including tight junction protein expression, autophagy, and apoptosis of epithelial cells, and further modulates the bacterial and fungal colonization of the epithelium.[30–32]

When we analyzed the relation between EGF and IgE sensitization to allergens, we observed that early-life EGF levels were higher in those children who did not develop allergen-specific IgE responses. This provides a link between low EGF levels and atopic IgE responses, the latter being a risk factor for the development of atopic diseases including dermatitis and asthma.[33] We could not associate the lower early-life EGF concentration to any specific allergic disease outcome since the number of children developing allergies was low but found that lower EGF levels were related to sensitization to cow's milk, in particular, when sensitization to the various allergens tested was compared. The study of Böttcher et al. investigated cytokine responses to allergens in Swedish and Estonian children during the first two years of life. They showed that T-cell responses to birch correlated with the development of atopic disease in Swedish children, with high Th1 responses at 6 and 12 months and high Th2 responses from 12 months onward in children with atopic disease.[34] Recently Kim et al. reported that topical administration of EGF improved the dermatitis score and decreased serum total IgE levels in mice with atopic dermatitis.[35] The levels of various skin barrier-related proteins were upregulated by EGF treatment.[35] In an animal model of atopic dermatitis, EGF attenuated the development and relapse of atopic dermatitis by blunting allergen-induced IL-6 production and Th17 responses suggesting a possible protective role for EGF.[36] We did not find significant correlations between EGF and allergy-induced cytokines. It has to be noted that we studied systemic levels of cytokines and not site-specific interactions.

Importantly, our findings show that systemic EGF levels differ significantly between populations with different risk of immune-mediated diseases, and these results emphasize a possible role of this growth factor and EGFR-mediated regulation of the epithelial lining and barrier mechanisms as a risk factor of allergic diseases in childhood. The specific mechanisms of receptor signaling, especially in the intestine, have not been studied here and require more investigation. Furthermore, our findings regarding specific food allergens direct the focus to the gastrointestinal tract since the circulating EGF levels might reflect differential regulation of epithelial lining and therefore integrity maintenance.

In the BC, we found an age-related upregulation of the cytokines, which are considered as hallmarks of T-cell phenotypes. This indicates the well-known maturation of the adaptive immunity, which takes place during the first years of life.[37] During the follow-up until the age of 3 years, we did not find differences between the children from the different countries in this regard.

Taken together, our results indicate a remarkable difference in the levels of circulating EGF between the children from countries with differential risk of immune-mediated diseases and suggest a role for EGF, especially during early life and in the context of IgE sensitization. We suggest that the decreased expression of EGF in Finnish children may contribute to the disruption of epithelial integrity and homeostasis. The molecular mechanism and factors leading to decreased EGF expression in Finland have not been answered here and are asking for further studies.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Linnea Reinert-Hartwall: Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **Heli Siljander:** Project administration (supporting); Supervision (supporting). **Taina Härkönen:** Formal analysis (supporting); Methodology (supporting); Project administration (supporting); Resources (supporting). **Tommi Vatanen:** Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Jorma Ilonen:** Formal analysis (supporting); Project administration (supporting); Writing-review & editing (supporting). **Onni Niemelä:** Data curation (supporting); Formal analysis (supporting); Methodology (supporting); Writing-review & editing (supporting). **Kristiina Luopajärvi:** Data curation (supporting); Project administration (supporting); Writing-review & editing (supporting). **Natalya Dorshakova:** Project administration (supporting). **Sergei Mokurov:** Project administration (supporting). **Aleksandr Peet:** Data curation (supporting); Project administration (supporting). **Vallo Tillmann:** Project administration (supporting). **Raivo Uibo:** Project administration (supporting); Writing-review & editing (supporting). **Mikael Knip:** Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Supervision (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). **Outi Vaarala:** Conceptualization (lead); Funding acquisition (lead); Project administration (supporting); Supervision (supporting); Writing-original draft (lead); Writing-review & editing (supporting). **Jarno Honkanen:** Conceptualization (lead); Data curation (lead); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Project administration (lead); Supervision (lead); Validation (lead); Visualization (supporting); Writing-original draft (lead); Writing-review & editing (supporting).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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