

Soluble immunoglobulin A in breast milk is inversely associated with atopic dermatitis at early age: the PASTURE cohort study

L. Orivuori^{1,*}, G. Loss^{2,3,*}, C. Roduit⁴, J.-C. Dalphin⁵, M. Depner⁶, J. Genuneit⁷, R. Lauener^{4,8}, J. Pekkanen⁹, P. Pfefferle¹⁰, J. Riedler¹¹, M. Roponen¹², J. Weber⁶, E. von Mutius⁶, C. Braun-Fahrlander^{2,3}, O. Vaarala¹ and The PASTURE Study Group[†]

¹Department of Vaccination and Immune Protection, National Institute for Health and Welfare, Helsinki, Finland, ²Swiss Tropical and Public Health Institute, Basel, Switzerland, ³The University of Basel, Basel, Switzerland, ⁴Christine Kühne-Center for Allergy Research and Education, University of Zurich Children's Hospital, Zurich, Switzerland, ⁵The Department of Respiratory Disease, Université de Franche-Comté, University Hospital, Besancon, France, ⁶LMU Munich, University Children's Hospital, Munich, Germany, ⁷The Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany, ⁸Children's Hospital of Eastern Switzerland, St. Gallen, Switzerland, ⁹The Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland, ¹⁰Molecular Diagnostics, Institute for Laboratory Medicine and Pathobiochemistry, Philipps-University of Marburg, Marburg, Germany, ¹¹Children and Young Adults' Medicine, Children's Hospital, Schwarzach, Austria and ¹²Department of Environmental Science, University of Eastern Finland, Kuopio, Finland

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Summary

Background The role of breastfeeding for the development of atopic diseases in childhood is contradictory. This might be due to differences in the composition of breast milk and levels of antimicrobial and anti-inflammatory components.

Objective The objective of this study was to examine whether levels of total immunoglobulin A (IgA) or transforming growth factor- β 1 (TGF- β 1) in breast milk were associated with the risk of developing atopic dermatitis (AD), atopic sensitization or asthma at early age taking breastfeeding duration into account.

Methods The birth cohort study PASTURE conducted in Finland, France, Germany and Switzerland provided 610 breast milk samples collected 2 months after delivery in which soluble IgA (sIgA) and TGF- β 1 levels were measured by ELISA. Duration of breastfeeding was assessed using weekly food frequency diaries from month 3 to month 12. Data on environmental factors, AD and asthma were collected by questionnaires from pregnancy up to age 6. Atopic status was defined by specific IgE levels in blood collected at the ages of 4 and 6 years. Multivariate logistic regression models were used for statistical analysis.

Results Soluble IgA and TGF- β 1 levels in breast milk differed between countries, and sIgA levels were associated with environmental factors related to microbial load, for example, contact to farm animals or cats during pregnancy, but not with raw milk consumption. sIgA levels were inversely associated with AD up to the age of 2 years (P -value for adjusted linear trend: 0.005), independent of breastfeeding duration. The dose of sIgA ingested in the first year of life was associated with reduced risk of AD up to the age of 2 (aOR, 95% CI: 0.74; 0.55–0.99) and 4 years (0.73; 0.55–0.96). No clear associations between sIgA and atopy or asthma up to age 6 were observed. TGF- β 1 showed no consistent association with any investigated health outcome.

Conclusion and Clinical Relevance IgA in breast milk might protect against the development of AD.

Keywords asthma, atopic dermatitis, atopy, breast milk, childhood, farming, IgA, TGF- β

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Correspondence:

Laura Orivuori, Immune Response Unit, Department of Vaccination and Immune Protection, National Institute for Health and Welfare, Biomedicum Helsinki 1, P.O. Box 104 FIN-00251 Helsinki, Finland.

E-mail: laura.orivuori@thl.fi

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*These authors contributed equally to this work.

[†]The members of the PASTURE study group are in Appendix (in alphabetical order by study centre).

Introduction

Breastfeeding has a variety of beneficial effects on the development of an infant, but there is conflicting evidence related to its association with the development of allergic disease at early age [1]. Discrepant findings might be due to differences in outcome definitions, assessment of breastfeeding or neglect of considering genetic predisposition [1–3]. Yet, it might also be related to differences in the composition of breast milk that varies greatly between individual mothers [4] and between mothers with and without allergies [5, 6].

Breast milk contains several antimicrobial and anti-inflammatory components, such as cytokines and maternal antibodies against environmental antigens including food antigens and microbes [7]. These components not only protect the child from infections but also educate the infant's immune system to tolerate certain antigens. Components that were identified to possibly underlie a protective breastfeeding effect on allergic disease were soluble IgA (sIgA) in colostrum [8], or mature milk [9], isoforms of transforming growth factor-beta (TGF- β) [4], or soluble CD14 [10], but findings were inconsistent [11, 12] and mechanisms of action remain unclear.

It has been suggested that the reduced risk of developing allergic diseases associated with living on a farm [13] or in a former socialist country could at least in part be mediated through constituents in breast milk, which have been shown to differ between farm and non-farm mothers [14] or mothers living in Estonia or Sweden [15].

The PASTURE study (Protection Against Allergy: Study in Rural Environments) [16] offered the opportunity to prospectively investigate the association of sIgA and TGF- β 1 levels in breast milk and the development of atopic dermatitis (AD), atopic sensitization and asthma up to the age of 6 years taking duration of breastfeeding into account and to evaluate which environmental factors determine sIgA and TGF- β 1 levels in breast milk.

Methods

Study population

PASTURE is a large prospective birth cohort study conducted in rural areas in Austria, Finland, France, Germany and Switzerland. Pregnant women were recruited during the third trimester of pregnancy. Women who lived or worked on family-run farms where any kind of livestock was kept were defined as farmers. The reference group was composed of women from the same rural areas not living on a farm. The study design has been described in detail elsewhere

[17]. Originally, 1133 mothers (64%) from five European countries agreed to participate. For the present analyses, the cohort is limited to participants of four countries as no breast milk samples were available from Austrian mothers, resulting in 913 cohort members. Eight hundred and fifty-three (93.4%) of them provided diary data on breastfeeding up to the age of 1 year. If duration of breastfeeding could not be derived from the diary because mothers had stopped breastfeeding before filling in the first diary (125 of 853) or due to missing structures of diaries (51 of 853), data from the 2-month and 1-year questionnaire were used to determine the duration of breastfeeding. Breast milk samples were collected at the age of 2 months from 622 of 853 (72.9%) mothers who were still breastfeeding. For 610 (98.1%) samples, measurements of both TGF- β 1 and sIgA were available. Of the 231 mothers who did not provide samples, 66 had not been breastfeeding at all. Blood samples were taken from children at the age of 4 ($N = 454$, 74.4%) and 6 years ($N = 455$, 74.6%).

Questionnaires

Extensive questionnaires were administered by interview to the mother of the child within the third trimester of pregnancy, at 2, 12, 18 and 24 months of age and then yearly up to the age of 6 years. Questions were based on previous studies [18–21] and were designed to assess respiratory and other health problems of the mother, agricultural exposures and potential confounders. Mothers kept a weekly diary starting at the third month of the child's life and ending at the first birthday. They recorded breastfeeding behaviour and introduction of complementary foods.

Pregnancy exposures relevant for this analysis were farming (living on a farm vs. not), farm milk consumption, contact to stable/barn (stay in stable/barn at least 15 min per week in one trimester), contact to number of livestock (horse, cow, pig, poultry: 0, 1–2 or 3–4), smoking during pregnancy (in any trimester), maternal/paternal history of asthma, hayfever or AD (doctor's diagnosis or self-reported symptoms). Exposures during the first year of the child's life were farming (child living on a farm during the first year of life), regular visit to farm, regular stay in stable (child stayed in stable at least 15 min per week), current smoking at month 2, duration of any or exclusive breastfeeding (only breast milk, water or tea [22]) continuously in weeks or categorically: never, ≤ 3 months, 3–6 months or > 6 months; and child's farm milk consumption. To describe the introduction of complementary foods during the first year of life, we computed a food diversity score including the food items that were introduced to about 80% of the children or

more during the first year of life [23]. The score included vegetables, fruits, cereals, bread, meat, cake and yogurt.

Health outcomes

Children were defined as having cumulative AD up to the age of 2 or 4 years when the parents reported in the questionnaires that the child had AD diagnosed by a doctor (DD) at least once up to 2 years of age (or 4 years of age, respectively) or with a positive SCORAD [24] score (> 0) assessed at the age of 1 year, during medical examination. In addition, AD with onset within the first year of life was defined as AD up to age 1 (DD or SCORAD) and AD with onset after the first year of life as onset after age 1 up to age 4 (DD) [23]. Atopy at age 4 and 6 was defined as positive test results for specific IgE antibodies (cut-off 0.35 kU/L, 0.7 kU/L, or alternatively 3.5 kU/L) against at least one of the following allergens (*Dermatophagoides pteronyssius*, *Dermatophagoides farinae*, alder, birch, hazel, grass pollen, rye, mugwort, plantain, cat, horse, dog, alternaria, hen's egg, cow's milk, peanut, hazelnut, carrot and wheat flour). Asthma at age of 4 years was defined as a doctor's diagnosis of asthma or of wheezy bronchitis more than once in the past 12 months. Asthma at age 6 was defined as doctor's diagnosis of asthma or of wheezy bronchitis after age of 3 years.

Measurement of breast milk samples

Breast milk samples were stored at -20°C . After thawing, the samples were centrifuged at 10 000 g for 10 min at $+4^{\circ}\text{C}$. Layer of fat was removed, and the clear supernatant was used for analyses. To activate latent TGF- β 1, an activation procedure using hydrochloric acid was performed. Samples were then neutralized with sodium hydroxide containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). TGF- β 1 values were measured using ELISA method (Quantikine Human TGF- β 1 Immunoassay; R&D Systems, Minneapolis, MN, USA). IgA values were measured using ELISA modified from Lehtonen et al. [25]. The antibody used for the primary coating was rabbit anti-human IgA (DakoCytomation, Glostrup, Denmark) 1:1000 in carbonate buffer. The plates were incubated at $+4^{\circ}\text{C}$ overnight and blocked with 1% BSA in PBS, 1 hour at $+37^{\circ}\text{C}$. 0.5% Tween-20 in PBS was used for washing the plates. Peroxidase-conjugated rabbit anti-human IgA (DakoCytomation) 1:5000 in PBS was used as a conjugate and ortho-phenylenediamine (OPD) as a substrate (Kem-En-Tec-laboratories, Taastrup, Denmark). The reaction was stopped with 1 M H_2SO_4 , and optical density was read at 492 nm. The immunoglobulin concentrations were calculated from the control curve, made from standard with known amounts of human IgA (Caltag laboratories, Burlingame, CA, USA).

Statistical analyses

Differences in characteristics of mothers regarding breastfeeding duration were tested by Pearson's chi-square test and expressed as *P*-values. Correlations of continuous variables were expressed as Pearson's coefficient. TGF- β 1 and sIgA variables were log-transformed to obtain approximate normal distribution. Their levels were expressed as geometric means and 95% confidence intervals. To evaluate factors that were associated with levels of breast milk constituents, exposures occurring up to month 2 of age were related to TGF- β 1 and sIgA levels by linear regression and expressed as geometric mean ratios and 95% confidence intervals. Factors that showed a statistically significant association with TGF- β 1 or sIgA in univariate models were then entered in multivariate linear regression models.

The associations of breast milk constituents or breastfeeding duration with AD, atopy or asthma were assessed by multivariate logistic regression models ($N = 610$). Associations between health outcomes and breastfeeding duration were also assessed in the whole cohort with diary data on breastfeeding ($N = 853$). TGF- β 1 and sIgA were categorized into quintiles. In agreement with previous analyses [1, 26], centre, sex, maternal history of allergies and introduction of food during the first year of life were chosen a priori as covariates for multivariate models. As no heterogeneity between centres was found by means of meta-analytical techniques, centre was included as fixed effect. In addition, a general farming variable (living on a farm vs. not) was entered in all models to represent exposure to any farm-related factor. In sensitivity analyses, we also tested whether specific exposures such as contact to stables, barns, raw farm milk consumption and contact to number of livestock at the respective age or continued exposure from pregnancy up to the respective age had a stronger impact on the association between breast milk constituents and health outcomes. To avoid over-adjustment, only one farming variable was entered at a time. Asthma models were calculated with additional adjustment for the child's current atopic status.

To test whether the associations between breast milk constituents and health outcomes might be modified by maternal history of allergic disease, multiplicative interaction terms were included in the final adjusted models. As breastfeeding duration might act (i) as a confounder or (ii) as an effect modifier of the association between levels of breast milk constituents and health outcomes, the variable was (i) added to the final sIgA or TGF- β 1 models and (ii) entered as a multiplicative interaction term to the final adjusted models. Finally, a dose variable for IgA and TGF- β 1 levels was generated by multiplying the levels of each constituent with the duration

of any breastfeeding for each child. These dose variables represented an estimation of the total amount of sIgA or TGF- β 1 ingested by each infant via breast milk in the first year of life. Uni- and multivariate smoothed plots based on generalized additive regression modelling were used to graphically display significant associations of dose variables and health outcomes.

All statistical analyses were performed using STATA/SE 12.1 (STATACorp, College Station, TX, USA), and *P*-values < 0.05 were considered significant.

Ethical approval

The ethical boards of all study centres approved the study, and written informed consent was obtained from the children's parents for questionnaires, blood samples and breast milk analyses.

Results

Among mothers who provided breast milk samples, French mothers and smokers were significantly more likely to breastfeed for shorter duration (Table 1). Mothers with a history of allergic diseases tended to breastfeed more often for over 6 months compared with those with no history of allergic diseases. No significant associations with breastfeeding duration were observed for farming or any other characteristics.

Mothers not providing breast milk samples at month 2 were significantly more likely to be from France, to be less educated, to have no history of allergic diseases, to be smokers and to consume less raw farm milk (Table S1).

Using the full cohort of 853 participants and mothers breastfeeding for more than 6 months as a reference group, children who had not been breastfed were not at an increased risk of suffering from AD, asthma or to be sensitized (Table S2). However, a maternal history of allergic diseases significantly modified the association between breastfeeding duration and asthma at age 6 years (*P* for interaction = 0.021), increasing the odds for asthma among children with a positive maternal history with increasing duration of breastfeeding (aOR for the interquartile range of duration of breastfeeding; 95% CI: 2.34; 1.05–5.21). Results were similar when the analyses were based on exclusive instead of any breastfeeding.

Transforming growth factor- β 1 levels in breast milk measured at the age of 2 months were significantly higher in Finnish mothers, smokers and those who were breastfeeding for a shorter duration (*P*-value for adjusted linear trend < 0.001) (Table 2). Except for smoking, variables remained significant in mutually adjusted models. Levels of sIgA were significantly associated with several of the tested characteristics in unad-

Table 1. Duration of breastfeeding in relation to environmental and farming characteristics of mothers and their children (*N* = 610)

	Duration of breastfeeding			<i>P</i> -value for difference
	> 6 months <i>N</i> (%)	3–6 months <i>N</i> (%)	≤ 3 months <i>N</i> (%)	
Population at birth*	430 (70.5)	153 (25.1)	27 (4.4)	
Female	216 (70.8)	75 (24.6)	14 (4.6)	0.962
Male	213 (70.3)	77 (25.4)	13 (4.3)	
Centre				
Switzerland	143 (77.7)	37 (20.1)	4 (2.2)	< 0.001
France	28 (37.8)	40 (54.1)	6 (8.1)	
Germany	129 (76.3)	33 (19.5)	7 (4.1)	
Finland	130 (71.0)	43 (23.5)	10 (5.5)	
Education				
Low	60 (65.9)	25 (27.5)	6 (6.6)	0.737
Medium	188 (70.7)	66 (24.8)	12 (4.5)	
High	182 (71.9)	62 (24.5)	9 (3.6)	
Maternal history of allergic disease				
No	327 (72.3)	106 (23.5)	19 (4.2)	0.199
Yes	101 (64.7)	47 (30.1)	8 (5.1)	
Siblings				
0–1	281 (70.1)	101 (25.2)	19 (4.7)	0.864
2 or more	149 (71.3)	52 (24.9)	8 (3.8)	
Maternal exposure during pregnancy				
Farming				
Non-farmer	233 (73.0)	71 (22.3)	15 (4.7)	0.239
Farmer	197 (67.7)	82 (28.2)	12 (4.1)	
Contact to stable				
No	213 (74.7)	60 (21.1)	12 (4.2)	0.150
Yes	204 (67.8)	84 (27.9)	13 (4.3)	
Contact to animals (horse, cow, pig, poultry)				
No	157 (71.4)	53 (24.1)	10 (4.5)	0.152
1–2 species	230 (72.8)	71 (22.5)	15 (4.7)	
3–4 species	38 (61.3)	23 (37.1)	1 (1.6)	
Any raw farm milk consumption				
No	275 (68.9)	105 (26.3)	19 (4.8)	0.538
Yes	153 (73.2)	48 (23.0)	8 (3.8)	
Smoking				
Never	307 (76.0)	82 (20.3)	15 (3.7)	< 0.001
Only before pregnancy	88 (65.2)	42 (31.1)	5 (3.7)	
During pregnancy (not at month 2)	20 (48.8)	19 (46.3)	2 (4.9)	
At month 2 assessment	15 (50.0)	10 (33.3)	5 (16.7)	
Child's exposure during first year of life				
Child living on a farm				
No	226 (72.9)	70 (22.6)	14 (4.5)	0.332
Yes	198 (68.0)	81 (27.8)	12 (4.1)	
Regular stay in stable				
No	266 (73.1)	82 (22.5)	16 (4.4)	0.169
Yes	136 (66.0)	61 (29.6)	9 (4.4)	

(continued)

Table 1. (continued)

	Duration of breastfeeding			<i>P</i> -value for difference
	> 6 months <i>N</i> (%)	3–6 months <i>N</i> (%)	≤ 3 months <i>N</i> (%)	
Any raw farm milk consumption				
No	343 (71.3)	115 (23.9)	23 (4.8)	0.271
Yes	79 (67.5)	35 (29.9)	3 (2.6)	

*Minor discrepancies in percentages due to missing variables.

justed analyses including contact to barn (more than 5 hours per week; aOR 95% CI: 1.21 [1.06–1.39]) and contact to one or two species of farm animals (aOR 95% CI: 1.10 [1.01–1.20]) during pregnancy. Contact to cats during pregnancy was associated with higher levels of breast milk sIgA in both crude and mutually adjusted models (aOR 95% CI: 1.10 [1.01–1.19] and 1.01 [1.01–1.20], respectively). After mutual adjustment, sIgA levels also remained inversely associated with duration of breastfeeding (*P*-value for adjusted linear trend < 0.001), decreased in German mothers and increased in mothers smoking during pregnancy or at the time sample was collected, and in those having more than one child. Farming was not significantly associated with sIgA or TGF- β 1 levels (Table 2).

Soluble IgA and TGF- β 1 levels in breast milk were moderately, but significantly correlated (Pearson's coefficient: 0.44, *P* < 0.001).

Soluble IgA levels in breast milk were significantly inversely related to AD up to 2 years of age (Table 3). This association held for adjustment for all potential confounders and was indicative of a dose–response relationship (*P*-value for adjusted linear trend: 0.005). Similar but weaker associations were found for AD up to age 4 (Table 3) and AD with onset within the first year of life (data not shown). Among the children whose mothers provided breast milk samples, shorter duration of breastfeeding tended to increase the risk of AD. Mutual adjustment of duration of breastfeeding and sIgA did not change their relation with AD, and no effect modification by breastfeeding duration was observed.

Transforming growth factor- β 1 showed no clear association with any AD phenotype, and no effect modification by duration of breastfeeding was observed.

Soluble IgA or TGF- β 1 levels in breast milk were not consistently associated with atopy (Table S4). Duration of breastfeeding and sIgA or TGF- β 1 levels in breast milk did not show significant associations with asthma at age 4 or 6 (see Table S3). Maternal history of allergies or duration of breastfeeding did not modify the association of sIgA or TGF- β 1 levels and AD, atopy or asthma.

Dose of sIgA and TGF- β 1 in the first year of life

The estimated dose of sIgA ingested by an infant during the first year of life (product of sIgA level and breastfeeding duration) was significantly inversely associated with AD up to the ages of 2 and 4 years (aOR 95% CI: 0.74 (0.55–0.99) and 0.73 (0.55–0.96), respectively). TGF- β 1 dose showed similar but weaker and non-significant associations with AD up to the ages of 2 and 4 years (aOR 95% CI: 0.86 (0.65–1.14) and 0.83 (0.63–1.08), respectively) (Table S5). When the dose of sIgA and TGF- β 1 was entered in the same final model, the associations were held for sIgA levels with borderline significance. Atopy and asthma were not associated with dose variables.

The crude and adjusted inverse associations of AD up to the age of 2 years with dose of breast milk sIgA in the first year of life are displayed as a smoothed plot in Fig. 1.

Discussion

The results of this cohort study showed that levels of sIgA in mature breast milk were inversely associated with the development of AD up to 2 and 4 years of age among breastfed infants. This association appeared to be based on the dose, the estimated total amount of sIgA that was ingested via breast milk during the first year of life. Asthma and atopic sensitization were not consistently associated with sIgA. TGF- β 1 levels in breast milk showed no consistent association with any of the investigated health outcomes.

Secretory IgA in human milk is an essential mediator of the passive antimicrobial protection provided by breastfeeding. In early infancy, when the infant's intestinal production of secretory IgA is low [27] and the intestinal barrier has not yet developed, the secretory IgA in human milk may prevent an excessive uptake of foreign antigens across the mucosa, thus possibly lowering the risk of allergic sensitization [27, 28].

Our finding that increasing levels of sIgA in breast milk and dose of ingested sIgA during the first year of life are associated with a decreased risk of AD up to age 2 and age 4 adds to the ongoing discussion of allergy-protective effects of breastfeeding. Previous studies found IgA in breast milk to be inversely related to asthma-like symptoms in the first year of life [9] and to atopy at age of 4 years [8], whereas the present study is the first to report an inverse association with AD at early age. Other studies, however, did not find IgA levels in breast milk to be associated with atopic diseases including AD [12, 29]. The latter studies, however, took levels of sIgA at a given time-point and not the total dose into account and were based on relatively small samples.

Table 2. Associations of transforming growth factor- β 1 (TGF- β 1) and IgA levels in breast milk (measured 2 months after giving birth) and exposures expressed as geometric mean ratios[‡]

Exposure	N	Geometric mean (95%-CI)	Crude GMR (95% CI)	Mutually adjusted GMR (95%-CI)
		TGF- β 1 [pg/mL]		
Centre				
Switzerland	184	330.84 (306.72–356.86)	1.00	1.00
France	74	342.44 (309.76–378.57)	1.04 (0.90–1.19)	0.93 (0.81–1.07)
Germany	169	367.19 (339.82–396.76)	1.11 (1.00–1.24)	1.09 (0.98–1.21)
Finland	183	404.52 (372.84–438.89)	1.22 (1.10–1.36)***	1.16 (1.04–1.29)**
Breastfeeding				
Over 6 months	430	342.66 (327.91–358.07)	1.00	1.00
3–6 months	153	396.15 (362.24–433.24)	1.16 (1.05–1.27)**	1.16 (1.05–1.28)**
≤ 3 months	27	561.86 (397.26–794.66)	1.64 (1.34–2.00)***	1.64 (1.34–2.01)***
Farming				
Non-farmer	319	353.93 (333.61–375.50)	1.00	1.00
Farmer	291	373.69 (352.29–396.38)	1.06 (0.97–1.15)	1.05 (0.96–1.14)
Smoking				
Never	404	346.77 (330.26–364.11)	1.00	1.00
Only before pregnancy	135	397.20 (363.97–433.47)	1.15 (1.03–1.27)**	1.11 (1.00–1.23)*
During pregnancy (not at month 2)	41	415.83 (348.33–496.41)	1.20 (1.01–1.42)*	1.14 (0.97–1.35)
At month 2 assessment	30	376.88 (283.47–501.08)	1.09 (0.90–1.32)	1.01 (0.84–1.23)
		IgA [mg/L]		
Centre				
Switzerland	184	183.01 (171.91–194.83)	1.00	1.00
France	74	233.58 (206.30–264.46)	1.28 (1.12–1.46)***	1.13 (0.97–1.31)
Germany	169	165.86 (153.28–179.47)	0.91 (0.82–1.00) [†]	0.91 (0.82–1.01)
Finland	183	188.05 (174.84–202.26)	1.03 (0.93–1.14)	1.00 (0.89–1.12)
Education				
Low	91	200.75 (178.12–226.25)	1.00	1.00
Medium	266	178.13 (168.21–188.64)	0.89 (0.79–1.00)*	0.94 (0.83–1.05)
High	253	186.78 (175.73–198.53)	0.93 (0.83–1.05)	0.98 (0.87–1.12)
Older siblings				
0	193	173.49 (159.92–188.21)	1.00	1.00
1	208	191.57 (180.04–203.85)	1.10 (1.00–1.22)*	1.11 (1.01–1.22)*
2 or more	209	189.42 (177.84–201.75)	1.09 (0.99–1.20)	1.08 (0.98–1.20)
Contact to barn during pregnancy [hours/week]				
0 hour	323	177.48 (167.60–187.94)	1.00	1.00
≤ 5 hours	204	186.73 (175.43–198.76)	1.05 (0.96–1.15)	1.02 (0.92–1.13)
> 5 hours	58	215.19 (189.81–243.96)	1.21 (1.06–1.39)**	1.10 (0.94–1.30)
Cats during pregnancy				
No	332	177.16 (168.21–186.58)	1.00	1.00
Yes	277	194.49 (182.94–206.76)	1.10 (1.01–1.19)*	1.10 (1.01–1.20)*
Contact to animals during pregnancy (horse, cow, pig, poultry)				
No	220	173.24 (162.02–185.24)	1.00	1.00
1–2 species	316	191.34 (181.16–202.09)	1.10 (1.01–1.20)*	1.10 (0.99–1.22) [†]
3–4 species	62	188.72 (165.63–215.03)	1.09 (0.95–1.25)	1.06 (0.91–1.24)
Breastfeeding				
Over 6 months	430	168.01 (161.80–174.46)	1.00	1.00
3–6 months	153	215.30 (197.21–235.04)	1.28 (1.18–1.40)***	1.20 (1.10–1.32)***
≤ 3 months	27	360.46 (258.27–503.08)	2.15 (1.79–2.57)***	2.10 (1.73–2.54)***
Farming				
Non-farmer	319	178.89 (168.96–189.41)	1.00	
Farmer	291	191.80 (181.58–202.59)	1.07 (0.99–1.16)	0.96 (0.86–1.07)

Table 2. (continued)

Exposure	N	Geometric mean (95%-CI)	Crude GMR (95% CI)	Mutually adjusted GMR (95%-CI)
		IgA [mg/L]		
Smoking				
Never	404	175.66 (168.20–183.46)	1.00	1.00
Only before pregnancy	135	189.54 (173.79–206.72)	1.08 (0.98–1.19)	1.04 (0.94–1.14)
During pregnancy (not at month 2)	41	208.77 (170.44–255.72)	1.19 (1.02–1.39)*	1.22 (1.04–1.43)*
At month 2 assessment	30	280.58 (216.69–363.31)	1.60 (1.33–1.91)***	1.40 (1.17–1.69)***

[†]Borderline significance: $P \leq 0.07$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

[‡]Associations of TGF- β 1 and IgA with all variables in Table 1 were tested; only significant associations plus farming variable shown and included in mutually adjusted models.

Table 3. Adjusted[†] associations of IgA and transforming growth factor-beta1 (TGF- β 1) levels in breast milk and duration of breastfeeding and atopic dermatitis in early life (N = 610)

		Atopic dermatitis	
		Up to age 2	Up to age 4
	N	aOR (95% CI)	aOR (95% CI)
IgA: Quintile (Q)			
Q 1	124	1.00	1.00
Q 2	123	0.67 (0.35–1.29)	0.69 (0.37–1.31)
Q 3	123	0.44 (0.22–0.88)*	0.62 (0.32–1.18)
Q 4	123	0.43 (0.22–0.85)*	0.46 (0.24–0.88)*
Q 5	117	0.41 (0.20–0.85)*	0.60 (0.31–1.17)
Breastfeeding			
Over 6 months	430	1.00	1.00
3–6 months	153	1.28 (0.77–2.13)	1.44 (0.89–2.34)
≤ 3 months	27	1.18 (0.41–3.42)	1.95 (0.74–5.13)
Mutually adjusted model of IgA levels and duration of breastfeeding			
IgA: Quintile (Q)			
Q 1	124	1.00	1.00
Q 2	123	0.69 (0.36–1.32)	0.70 (0.37–1.34)
Q 3	123	0.42 (0.21–0.85)*	0.57 (0.30–1.10)
Q 4	123	0.41 (0.21–0.81)*	0.42 (0.22–0.82)*
Q 5	117	0.38 (0.18–0.79)**	0.51 (0.25–1.01)
Breastfeeding			
Over 6 months	430	1.00	1.00
3–6 months	153	1.45 (0.86–2.45)	1.59 (0.96–2.62)
≤ 3 months	27	1.59 (0.52–4.88)	2.40 (0.87–6.59)
TGF-β1: Quintile (Q)			
Q 1	123	1.00	1.00
Q 2	121	0.95 (0.48–1.90)	0.77 (0.40–1.51)
Q 3	126	1.12 (0.58–2.16)	1.13 (0.60–2.12)
Q 4	120	0.64 (0.30–1.34)	0.87 (0.45–1.71)
Q 5	120	1.00 (0.50–1.98)	1.10 (0.57–2.09)

* $P < 0.05$, ** $P < 0.01$.

[†]Logistic regression models adjusted for centre, sex, maternal history of allergies, farming and food score in the first year of life.

We have recently reported that the diversity of introduction of complementary food in the first year of life was associated with a reduction in the risk of having AD with onset after the first year of life [23]. The

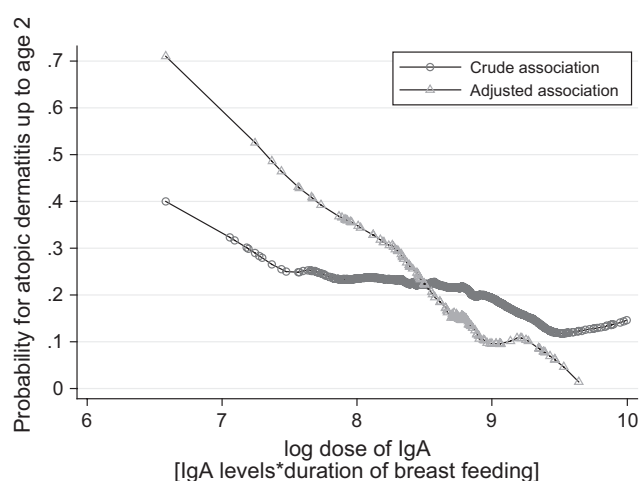


Fig. 1. Smoothed plot of the crude and adjusted association of (IgA) dose and the probability of atopic dermatitis (AD) up to age 2. Adjusted for centre, sex, maternal history of allergies, farming and food score in the first year of life. The estimated dose of soluble IgA (sIgA) ingested by an infant during the first year of life (product of sIgA level and breastfeeding duration) was significantly inversely associated with AD up to age 2.

present analysis thus took the diversity of introduced foods into account and found the inverse association of sIgA and AD to be independent of the effect of complementary food introduction.

The exact mechanism mediating the association of sIgA and AD is unclear. It might be speculated that sIgA-mediated passive antimicrobial protection could influence the colonization or maintenance of a balanced gut microbiota [1]. sIgA might, however, also represent a certain combination of milk ingredients (that was not assessed in this study) contributing to the induction of adaptive immune responses or creating a microenvironment that favours T regulatory cell development [30].

There was an indication that contact to pets and increased number of older children were associated with increased levels of sIgA in breast milk. These factors

may represent increased microbial stimuli for the maternal immune system and might thus induce increased levels of sIgA in breast milk. It is thus possible that sIgA levels in breast milk reflect the environmental microbial load, which modifies the development of allergic diseases, such as AD, rather than sIgA in breast milk. We did not find association between breast milk sIgA and atopy later in life, which suggests that sIgA does not directly affect IgE induction to environmental antigens.

Interestingly, breast milk sIgA was not associated with asthma later in life, suggesting that an effect specific to AD may be involved.

The present study did not find any consistent association between the dose of TGF- β 1 in breast milk and atopic outcomes in the child, nor were there differences in TGF- β 1 levels in breast milk of atopic and non-atopic mothers. Other studies [31], but not all [11], found TGF- β in breast milk to reduce the risk of atopic disease in early life or to be present in lower concentrations in breast milk of mothers with a history of atopic diseases [6, 8]. Interestingly, we observed that long breastfeeding increased the risk of asthma in children of mothers with allergic history. In our study, however, we did not see association between breast milk TGF- β 1 or sIgA and maternal history of allergy. In a large cohort of children with family history of allergy, long exclusive breastfeeding was associated with allergic eczema [32]. However, in some studies, long breastfeeding has been protective against AD, particularly in children of family with allergic history [33]. A recent meta-analysis by Oddy [4] concluded that a majority of studies reported a positive association between immunological parameters and TGF- β 1 or TGF- β 2, indicating protection against allergy-related outcomes in infancy and early childhood. It appeared that the levels of TGF- β 1 measured in breast milk were relatively high in the studies in which the association of TGF- β 1 and atopy was found. Levels of TGF- β 1 in our study were comparable with the low median levels according to Oddy, and therefore, our findings of no association of breast milk TGF- β 1 with atopic diseases may be related to the sensitivity of the method for the detection of TGF- β 1 as suggested by Oddy. However, the number of prospective studies investigating the effect of TGF- β on clinical allergy outcomes is still very limited, and the results are mixed.

A recent study in Italy comparing TGF- β 1 levels in colostrum (day 3) and mature milk (1st month) of 45 farm and 69 urban mothers found significantly higher levels of TGF- β 1 in both types of milk samples among farming mothers [14]. The authors suggested that the higher cytokine concentration in breast milk may influence early modulation of an immune response leading to a reduced prevalence of allergy-related diseases in farm children. However, the present study did not confirm this

assumption. It contrasted rural mothers living or not living on farms, and it may well be that the difference in TGF- β 1 levels might be higher when farm mothers are compared with urban ones. However, the contrast in allergy prevalence of farm and non-farm children has always been found among rural populations [13].

Current smoking at month 2 assessment (which was strongly related to smoking during pregnancy as 88% also smoked while pregnant) was related to increased levels of sIgA in breast milk. Smoking during pregnancy or only before pregnancy was associated with increased levels of TGF- β 1. Cigarette smoke contains toxins and trace amounts of microbial cell components, inducing chronic inflammation at mucosal surfaces and influencing host immunity in a complex way [34]. Yet, the mechanism of how cigarette smoke affects TGF- β 1 and sIgA levels in breast milk is not known.

Significant country differences in the levels of TGF- β 1 and sIgA were found. TGF- β 1 levels were found to be highest in Finland, whereas sIgA levels were highest in France. Country differences in the levels of these breast milk components have previously been reported when breast milk of Swedish and Estonian mothers was compared [15]. Higher sIgA levels, but lower TGF- β levels, were found in mature milk of Estonian as compared to Swedish mothers, but underlying reasons for these differences are not clear.

The strong inverse dose-response relationship between the duration of breastfeeding and levels of sIgA or TGF- β 1 in mature breast milk found in this study has previously been reported by Savilahti et al. [35] with colostrum. It has been shown that bacteria-induced mastitis increased milk TGF- β 1 levels [36]. It is thus possible that TGF- β 1 and IgA in breast milk are induced by microbial stimuli, and the association of high levels of TGF- β 1 and IgA in breast milk with the short duration of breastfeeding is due to subclinical inflammation in the breast tissue. Animal models have also shown that TGF- β can inhibit ductal and alveolar development in breast tissue [37]. It is, however, noteworthy that the inverse association between the dose of sIgA and AD was observed despite the fact that high levels of sIgA measured at month 2 were associated with shorter breastfeeding.

We only had a 1-point measurement of the two breast milk components measured at month 2. However, it has been shown that after a drop in sIgA concentrations in breast milk 10 days post-partum, the levels did not change significantly during the first year of life [38, 39], and TGF- β levels have been shown to be relatively constant at least throughout the first 3 months after birth [40]. It thus can be assumed that the 2-month measurements are representative of the levels the infants consumed during lactation. A further limitation of this study was the lack of information on

current infections during the period of breast milk sampling, thus not allowing to assess their influence on breast milk levels of sIgA or TGF- β 1. Our sample with objective measurements was restricted to mothers who were still breastfeeding during the sampling period, thus restricting the external validity of our findings based on objective measurements to women breastfeeding for longer than 2 months after birth. Methodological limitations may be related to the sensitivity of the detection of breast milk TGF- β 1 as discussed above and on the isoform of TGF- β detected. We did not measure breast milk TGF- β 2, which was shown to be in association with probiotic treatment, that is, microbial exposure [41]. The definition of allergic diseases is always a concern in the follow-up studies when the definition is based not only on doctor's diagnosis, but also on symptoms reported by the parents. The questionnaires used in our study have previously been used for studies with equivalent samples sizes [42, 43], and they were based on the internationally validated ISAAC study questions to have as reliable data as possible.

Strengths of the present study are its sample size and the longitudinal design. This large transnational birth cohort ranging from pregnancy up to the age of 6 years and including repeated standardized [24, 44] and partially objective measures of allergic diseases (such as allergen-specific IgE), objective measurements of breast milk components and a comprehensive assessment of environmental exposures allowed to establish sound

temporal relationships. To our knowledge, this is the first study investigating breastfeeding and atopic diseases that also took the introduction of complementary foods in the first year of life into account and additionally addressed potential confounders adequately [1].

Our study is aimed to dissect the possible factors of breast milk that could be important in the modulation of atopy. The results support the protective effects of breastfeeding on AD, but emphasize the fact that the protective effect is dependent on breast milk composition, such as sIgA, which may explain the variation in the results of the epidemiological studies on breastfeeding in which the duration of breastfeeding is the only determinant. Our results suggest that high sIgA in breast milk may reduce the risk to develop AD at early age. Identification of factors modulating breast milk composition towards higher levels of components promoting immune development such as sIgA might be a contribution to allergy prevention research [45].

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Conflict of interest

The authors declare no conflict of interest.

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The PASTURE Study Group:

Maija-Riitta Hirvonen, Department of Environmental Science, Inhalation Toxicology Laboratory, University of Eastern Finland, P.O Box 1627, Kuopio, Finland

Anne Hyvärinen, The Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland

Anne M. Karvonen, The Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland

Sami Remes, The Department of Pediatrics, Kuopio University Hospital, Kuopio, Finland

Marjut Roponen, The Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland

Pekka Tiittanen, The Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland

Marie-Laure Dalphin, The Department of Respiratory Disease, Université de Franche-Comté, University Hospital, Besancon, France

Vincent Kaulek, The Department of Respiratory Disease, Université de Franche-Comté, University Hospital, Besancon, France

Gisela Büchele, The Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany;

Martin Depner, LMU Munich, University Children's Hospital, Munich, Germany

Markus Ege, LMU Munich, University Children's Hospital, Munich, Germany

Michael Kabesch, Hannover Medical School, Clinic for Paediatric Pneumology and Neonatology, Hannover, Germany

Petra Pfefferle, The Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany

Harald Renz, The Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany

Bianca Schaub, LMU Munich, University Children's Hospital, Munich, Germany

Gert Doekes, The Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht University, Utrecht, the Netherlands

Remo Frei, The University of Zürich, Children's Hospital and the Christine Kühne Center for Allergy Research and Education.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Environmental and farming characteristics of women providing breast milk samples at month 2, not providing breast milk samples and never breastfeeders ($N = 853$).

Table S2. Crude and adjusted associations of duration of breastfeeding and atopic dermatitis, atopy, and asthma.

Table S3. Adjusted[†] associations of TGF- β and IgA levels in breast milk and duration of breastfeeding and asthma at age 4 and 6 ($N = 610$).

Table S4. Adjusted[†] associations of TGF- β and IgA levels in breast milk and duration of breastfeeding and atopy up to age 6 ($N = 610$).

Table S5. Adjusted[†] associations of continuous dose[‡] of IgA or TGF- β 1 levels in breast milk and atopic dermatitis, atopy, and asthma.

Table S1: Environmental and farming characteristics of women providing breast milk samples at month 2, not providing breast milk samples and never breast feeders (N=853).

		Provided sample Month 2	Did not provide sample Month 2	Never breast fed	p-value for difference
		N (%)	N (%)	N (%)	
Population at birth†		622 (72.92)	165 (19.3)	66 (7.7)	
Infant:					
	Female	311 (50.2)	73 (44.2)	30 (45.5)	0.347
	Male	309 (49.8)	92 (55.8)	36 (54.5)	
Center:					
	Switzerland	185 (29.7)	35 (21.2)	4 (6.1)	<0.001
	France	76 (12.2)	67 (40.6)	42 (63.6)	
	Germany	176 (28.3)	42 (25.5)	20 (30.3)	
	Finland	185 (29.7)	21 (12.7)	0 (0.0)	
Education:					
	Low	94 (15.1)	42 (25.5)	29 (43.9)	<0.001
	Medium	272 (43.7)	63 (38.2)	21 (31.8)	
	High	256 (41.2)	60 (36.4)	16 (24.2)	
Maternal history of allergic disease:					
	No	462 (74.5)	106 (64.6)	54 (81.8)	0.010

Yes	158 (25.5)	58 (35.4)	12 (18.2)	
Siblings:				
0-1	407 (65.4)	117 (70.9)	36 (54.5)	0.059
2 or more	215 (34.6)	48 (29.1)	30 (45.5)	
Maternal exposure during pregnancy				
Farming:				
Non farmer	325 (52.3)	89 (53.9)	34 (51.5)	0.915
Farmer	297 (47.7)	76 (46.1)	32 (48.5)	
Contact to stable:				
No	291 (48.7)	75 (47.5)	30 (47.6)	0.958
Yes	307 (51.3)	83 (52.5)	33 (52.4)	
Contact to animals (horse, cow, pig, poultry):				
No	225 (36.9)	70 (45.2)	25 (41.7)	0.127
1-2 species	319 (52.4)	63 (40.6)	28 (46.7)	
3-4 species	65 (10.7)	22 (14.2)	7 (11.7)	
Any raw farm milk consumption:				
No	406 (65.5)	127 (77.0)	47 (72.3)	0.014
Yes	214 (34.5)	38 (23.0)	18 (27.7)	
Smoking:				
Never	414 (66.6)	98 (59.4)	22 (33.3)	<0.001

Only before pregnancy	137	(22.0)	26	(15.8)	23	(34.8)	
During pregnancy (not at month 2)	41	(6.6)	17	(10.3)	5	(7.6)	
At month 2 assessment	30	(4.8)	24	(14.5)	16	(24.2)	
Child's exposure during first year of life:							
Child living on a farm:							
No	316	(51.5)	82	(53.9)	31	(50.8)	0.855
Yes	297	(48.5)	70	(46.1)	30	(49.2)	
Regular stay in stable:							
No	371	(63.7)	87	(58.4)	37	(61.7)	0.478
Yes	211	(36.3)	62	(41.6)	23	(38.3)	
Any raw farm milk consumption:							
No	491	(80.5)	137	(91.3)	49	(81.7)	0.007
Yes	119	(19.5)	13	(8.7)	11	(18.3)	

† Minor discrepancies in percentages due to missings in variables

Table S2: Crude and adjusted associations of duration of breast feeding and atopic dermatitis, atopy, and asthma
(Cohort with data on breast feeding, N=853).

		Atopic Dermatitis			
		Up to age 2		Up to age 4	
Breast feeding		Crude OR (95%-CI)	Adjusted† OR (95%-CI)	Crude OR (95%-CI)	Adjusted† OR (95%-CI)
over 6 months		1.00	1.00	1.00	1.00
3 – 6 months		1.28 (0.81-2.00)	1.05 (0.65-1.70)	1.57 (1.03-2.39)*	1.25 (0.79-1.98)
≤ 3 months		1.66 (1.07-2.58)*	1.15 (0.69-1.89)	1.59 (1.05-2.41)*	1.14 (0.71-1.85)
never		1.40 (0.73-2.68)	0.84 (0.40-1.76)	1.28 (0.68-2.40)	0.83 (0.40-1.69)
		Atopy‡			
		Age 4		Age 6	
over 6 months		1.00	1.00	1.00	1.00
3 – 6 months		1.20 (0.80-1.80)	1.25 (0.81-1.94)	1.12 (0.74-1.69)	1.25 (0.80-1.95)
≤ 3 months		1.00 (0.65-1.54)	1.27 (0.79-2.06)	1.02 (0.67-1.56)	1.30 (0.81-2.09)
never		0.50 (0.24-1.01)	0.77 (0.36-1.65)	0.62 (0.32-1.19)	1.03 (0.50-2.12)
		Asthma			
		Age 4		Age 6	
over 6 months		1.00	1.00	1.00	1.00
3 – 6 months		0.80 (0.31-2.03)	0.79 (0.30-2.06)	0.63 (0.30-1.34)	0.56 (0.25-1.23)
≤ 3 months		1.57 (0.73-3.36)	1.32 (0.57-3.05)	0.98 (0.50-1.90)	0.76 (0.36-1.62)

never	1.06 (0.31-3.69)	1.22 (0.32-4.63)	0.73 (0.25-2.13)	0.69 (0.22-2.21)
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* p<0.05

† Logistic regression models adjusted for: center, sex, maternal history of allergies, farming, food score in the first year of life

‡ cut-off 0.7 kU/l

Table S3: Adjusted† associations of TGF- β and IgA levels in breast milk and duration of breast feeding and asthma at age 4 and 6 (N=610).

		Asthma, aOR (95%-CI)	
		Age 4	Age 6
TGF-β1: Quintile (Q)			
Q 1	1.00		1.00
Q 2	0.57 (0.18-1.84)		0.90 (0.37-2.20)
Q 3	0.43 (0.12-1.50)		0.43 (0.15-1.24)
Q 4	0.53 (0.15-1.87)		0.83 (0.32-2.13)
Q 5	0.65 (0.21-2.02)		0.70 (0.27-1.82)
IgA: Quintile (Q)			
Q 1	1.00		1.00
Q 2	1.01 (0.27-3.80)		2.21 (0.80-6.09)
Q 3	0.63 (0.14-2.81)		0.97 (0.32-3.00)
Q 4	1.81 (0.55-5.98)		1.80 (0.63-5.11)
Q 5	1.77 (0.51-6.18)		1.52 (0.51-4.49)
Breast feeding:			
over 6 months	1.00		1.00
3 – 6 months	0.89 (0.33-2.40)		0.53 (0.23-1.23)
$\leq$ 3 months	1.73 (0.35-8.50)		1.00 (0.21-4.84)

† Logistic regression models adjusted for: center, sex, maternal history of allergies, farming, food score in the first year of life

Table S4: Adjusted† associations of T -β and IgA levels in breast milk and duration of breast feeding and atopy up to age 6 (N=610).

		Atopy					
		Age 4			Age 6		
		(cut-off 0.35 kU/l)	(cut-off 0.7 kU/l)	(cut-off 3.5 kU/l)	(cut-off 0.35 kU/l)	(cut-off 0.7 kU/l)	(cut-off 3.5 kU/l)
		aOR (95%-CI)	aOR (95%-CI)	aOR (95%-CI)	aOR (95%-CI)	aOR (95%-CI)	aOR (95%-CI)
TGF-β1: Quintile (Q)							
Q 1		1.00	1.00	1.00	1.00	1.00	1.00
Q 2		1.10 (0.60-2.01)	1.19 (0.64-2.20)	0.53 (0.23-1.25)	0.75 (0.41-1.35)	0.60 (0.33-1.10)	0.26 (0.11-0.61)**
Q 3		1.03 (0.56-1.91)	1.29 (0.69-2.41)	1.05 (0.48-2.30)	1.04 (0.56-1.93)	0.99 (0.54-1.83)	0.40 (0.18-0.90)*
Q 4		1.46 (0.79-2.70)	1.61 (0.86-3.00)	0.92 (0.41-2.06)	0.84 (0.46-1.53)	0.93 (0.51-1.70)	0.78 (0.39-1.57)
Q 5		1.76 (0.93-3.32)	1.59 (0.85-2.99)	1.12 (0.52-2.45)	1.02 (0.55-1.89)	1.01 (0.55-1.86)	0.64 (0.31-1.32)
IgA: Quintile (Q)							
Q 1		1.00	1.00	1.00	1.00	1.00	1.00
Q 2		1.07 (0.57-2.01)	1.14 (0.60-2.17)	1.03 (0.46-2.34)	1.21 (0.65-2.25)	1.31 (0.71-2.44)	1.15 (0.53-2.50)
Q 3		0.83 (0.44-1.55)	0.84 (0.44-1.59)	0.72 (0.30-1.73)	0.88 (0.48-1.61)	0.94 (0.51-1.73)	0.75 (0.33-1.71)
Q 4		1.42 (0.76-2.67)	1.44 (0.76-2.72)	1.29 (0.58-2.88)	1.34 (0.72-2.51)	1.41 (0.76-2.63)	1.21 (0.55-2.64)
Q 5		1.66 (0.87-3.18)	1.94 (1.01-3.71)*	1.02 (0.44-2.36)	1.29 (0.69-2.42)	1.31 (0.70-2.47)	1.09 (0.49-2.42)

Breast feeding:

over 6 months	1.00	1.00	1.00	1.00	1.00	1.00
3 – 6 months	1.20 (0.75-1.90)	1.12 (0.71-1.77)	1.67 (0.96-2.92)	1.11 (0.69-1.79)	1.23 (0.77-1.97)	1.70 (0.97-2.99)
≤ 3 months	0.90 (0.30-2.66)	0.60 (0.19-1.92)	0.41 (0.05-3.27)	0.37 (0.13-1.08)	0.43 (0.14-1.29)	0.64 (0.14-2.93)

* p<0.05, ** p<0.01

† Logistic regression models adjusted for: center, sex, maternal history of allergies, farming, food score in the first year of life

Table S5: Adjusted† associations of continuous dose‡ of IgA or TGF-β1 levels in breast milk and atopic dermatitis, atopy, and asthma.

Atopic dermatitis		Up to age 2				Up to age 4			
Dose of:									
TGF-β1 (aOR (95%-CI))		0.86	(0.65	-	1.14)		0.83	(0.63	- 1.08)
IgA (aOR (95%-CI))		0.74	(0.55	-	0.99)	*	0.73	(0.55	- 0.96) *
Mutual adjustment for TGF-β and IgA [§]									
Dose of:									
TGF-β1 (aOR (95%-CI))		1.02	(0.73	-	1.44)		0.98	(0.70	- 1.36)
IgA (aOR (95%-CI))		0.73	(0.51	-	1.03)		0.74	(0.52	- 1.04)
Atopy		Age 4				Age 6			
Dose of:									
TGF-β1 (aOR (95%-CI))		1.10	(0.85	-	1.42)		1.05	(0.82	- 1.36)
IgA (aOR (95%-CI))		1.15	(0.88	-	1.49)		1.04	(0.80	- 1.34)
Asthma		Age 4				Age 6			
Dose of :									
TGF-β1 (aOR (95%-CI))		0.77	(0.47	-	1.27)		0.92	(0.62	- 1.38)
IgA (aOR (95%-CI))		1.00	(0.59	-	1.68)		1.06	(0.69	- 1.60)

* p<0.05

† Odds ratios for interquartile range calculated by logistic regression models adjusted for: center, sex, maternal history of allergies, farming, food score in the first year of life.

‡ Dose defined as product of levels in breast milk and duration of any breast feeding

§ Mutual adjustment for IgA and TGF-β in final models

¶ cut-off 0.7 kU/L