Development of atopic dermatitis and its association with prenatal and early life exposures

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Caroline Roduit

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Prof. Charlotte Braun-Fahrländer

Prof. Henriette Smit

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Prof. Dr. Jörg Schibler
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

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Summary

Over 20% of children in industrialized countries are affected by atopic dermatitis. From epidemiological studies, it is quite obvious that the worldwide prevalence of atopic dermatitis has considerably increased over the past decades and constitutes a major public health problem. Atopic dermatitis is a chronic inflammatory skin disease that occurs in very early life and frequently precedes the development of asthma and allergic rhinitis during the first several years of life. Although a large numbers of researches were conducted, today there is no good evidence that measures for primary prevention of atopic dermatitis are effective.

The aim of this work was to better understand which environmental factors and their timing of exposure might influence the development of atopic dermatitis in childhood, using data from a longitudinal study, the PASTURE/EFRAIM birth cohort. We focused on prenatal and early life exposures, as atopic dermatitis frequently occurs in the first years of life. We further analyzed which farm-related exposures during pregnancy and early life were associated with gene expression of innate immunity receptors in early life and whether there were differences in gene expression of those receptors at birth between children developing atopic dermatitis and the one who did not develop the disease.

We found that contact to farm animals and to cats were negatively associated with atopic dermatitis already when these exposures occur during pregnancy. We also observed a dose-response of this protective effect with an increasing number of different farm animal species the mother had contact to during pregnancy.

From the hygiene hypothesis, it was suggested that a reduced microbial stimulation of the innate immune system in early life may lead to the development of allergic diseases. In our study, we found that maternal farming during pregnancy was associated with an up-regulation of gene expression of innate immunity receptors at birth. An increased gene expression of those receptors measured at one year of age was positively associated with child's consumption of raw milk during the first year of life. Moreover, children with a lower expression of innate immune receptors at birth

had an increased risk of developing atopic dermatitis in the two first years of life compared to children with a higher expression of those receptors.

Then, we could also show that, among early life exposures, infant's feeding practices plays an important role on the development of atopic dermatitis. For those analyses, we excluded children with onset of the disease within the first year of life, in order to take into account the reverse causality. Introduction of milk products, especially yogurt, was negatively associated with atopic dermatitis. Furthermore, children with an increased food diversity introduced in the first year had a reduced risk of developing atopic dermatitis and also asthma, food allergy and sensitization.

Taking together the results of this thesis, it can be concluded that the protective "farm effect" may also be effective on atopic dermatitis, especially when the exposure occurs during pregnancy. The role of the diversity of environmental exposures on atopic dermatitis has also been highlighted in this work. These results may contribute to the development of new strategies for primary prevention of atopic dermatitis among children.

Chapter 1

1. Introduction:

1.1 The Allergy Epidemic

Allergic diseases affect approximately one billion people in the world and represent one of the **most common of chronic non-communicable diseases, with the earliest onset.**[1] The prevalence of allergic diseases continues to increase worldwide in both industrialized and developing countries with a significant socio-economic impact.[2] This increase is especially problematic in children with currently more than 30% of children affected by an allergic disease. The most common allergic conditions in children are atopic dermatitis, food allergies and asthma. This increase in the prevalence in epidemic proportions started in the 1960s in industrialized countries with respiratory allergies which seems to have reached a peak in urbanized regions.[3] The "allergy epidemic" started later in developing countries and the rise in prevalence is still ongoing. Already about 20% of the world's population is affected by one or more allergic conditions including eczema, asthma, allergic rhinitis and food allergies. Specific IgE sensitization rates to one or more common allergens among school children are currently approaching 50% in industrialized countries. Reasons for the increase in the prevalence of allergic diseases are not known, but are thought to be closely linked to environmental factors that affect the regulation of tolerance in the immune system.[1] Evidences from various studies suggested that "the hygiene hypothesis" plays a large role in the allergy epidemic.

Currently there are no cures for allergic diseases and symptomatic therapies are often administered for prolonged periods. Likely the best long term solution to the allergy epidemic is prevention. Primary prevention of allergy is much debated and most researches have failed to develop effective prevention strategies.[4]

1.2 Atopic dermatitis

Atopic dermatitis is a chronic, highly pruritic, inflammatory skin disease and one of the most common skin disorders. Over 20% of children in industrialized countries are affected by atopic dermatitis.[3, 5, 6] In more than 60% of the children, the disease started within the first 2 years of age.[7] In infancy, the first lesions usually emerge on the cheeks and the scalp and causes crusted erosions (fig. 1). During childhood, lesions often involve flexures, the neck and also wrists and ankles. In adolescence and adulthood lichenified plaques may appear.

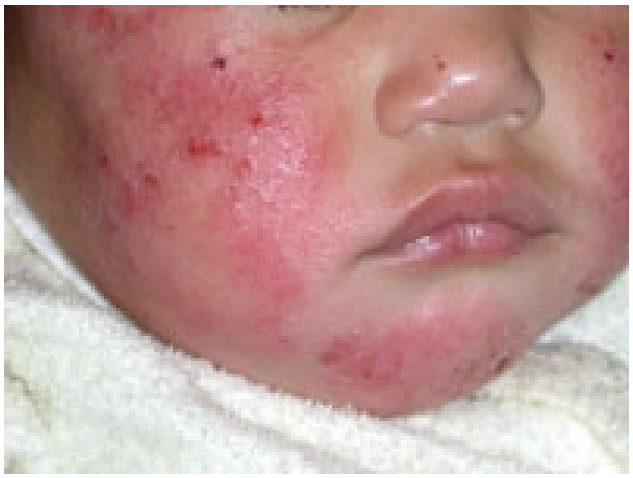

Fig. 1 Infantile atopic dermatitis

Parental atopy, in particular atopic dermatitis is significantly associated with early atopic dermatitis in children and its severity. Moreover, several candidate genes, involved in the epidermal barrier function and in the regulation of the innate and adaptive immunity, have been identified in atopic dermatitis.[8] Several loss-of-function mutations of the filaggrin gene (*FLG*), involved in the skin barrier function, have been reported in patients with atopic dermatitis.[9, 10] Mutations of *FLG* gene occur mainly in early-onset atopic dermatitis and was shown to be associated with the severity of the disease.[10] The discovery of the filaggrin gene has highlighted the importance of skin barrier function and also of the gene-environment interaction effect in the pathogenesis of atopic dermatitis. It was shown that the number of older siblings interact positively with *FLG* gene mutation on the risk of developing atopic

dermatitis.[11] Other studies showed an increased risk for the disease in children with the mutation, who were exposed to a cat during the first year of life.[12, 13] However, more than 50% of patients suffering of atopic dermatitis do not carry a *FLG* mutation and other genetic factors may be involved. Mutations of the filaggrin genes can lead to defect in the epidermal barrier and thus have been suggested to initiate or increase sensitization to allergen.[14, 15] This is supported by experimental data in mouse models.[16, 17]

Sensitization to food allergens (cow's milk and hen's eggs) is associated with infantile atopic dermatitis and related to disease severity. Food allergen sensitization is also predictive for persistence of symptoms throughout childhood.[18] Even though many children will outgrow the disease (about 40% before adolescence), by others it will persist.[19] Moreover, it was shown that children with atopic dermatitis are at high risk of allergic asthma and allergic rhinitis. Among those with atopic dermatitis during the first 2 years of life, 50% will develop asthma during subsequent years.[20] The severity of the disease and sensitization to food allergens were shown to be major determinants to increase this risk.[18, 21]

However, until present the mechanism which determines whether atopic dermatitis will persist or spontaneously disappear in a child, or whether this child will continue to develop allergic airway diseases, such as asthma or allergic rhinitis, is unknown. Early onset of atopic dermatitis is considered to be one of the first manifestations in the atopic march, which describes the typical sequence of clinical symptoms of allergic diseases with begin in early life (fig. 2). Atopic dermatitis, as well as food allergy, starts within the first year of life, and is followed by the development of asthma and allergic rhinitis during the first several years of life.

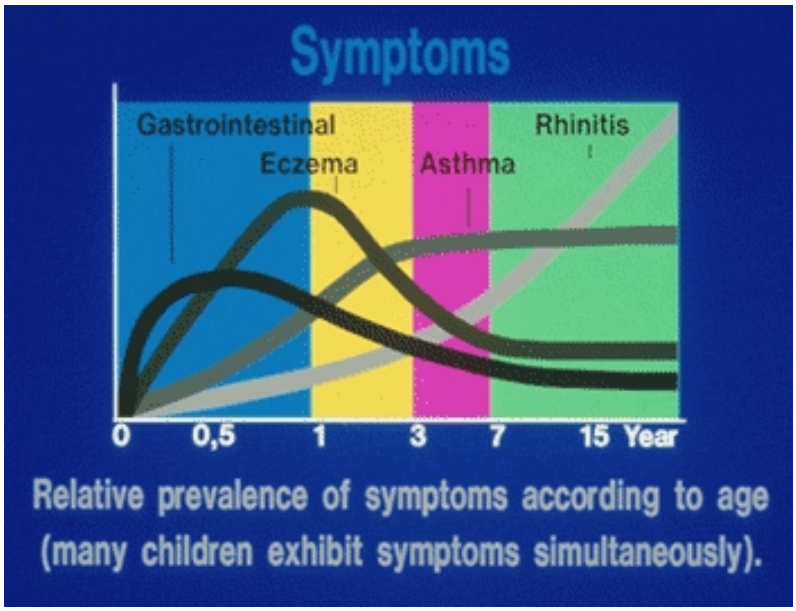

Fig. 2: the Atopic March

Current measures for primary prevention in atopic dermatitis are not conclusive[4] and treatment are only based on symptomatic therapies. Moreover, as mentioned before, atopic dermatitis frequently manifests within the first months of life. Better understanding and defining which environmental factors may protect against the development of atopic dermatitis and other allergic diseases will help to develop new strategies in primary prevention. As atopic dermatitis mainly occurs in early life, the timing of exposures is of importance. Prenatal and early life periods are a critical window for strategies in primary prevention.

1.3 Prevalence of atopic dermatitis

The prevalence of atopic dermatitis has doubled or tripled in industrialized countries during the past three decades, with today 15 to 30% of children affected.[22]
According to the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence of symptoms of atopic dermatitis in children six or seven years of age during a one-year period varied from less than 2% in Iran and China to approximately 20% in Australia, England, and Scandinavia (fig. 3). [5] In Switzerland the prevalence

of atopic dermatitis is about 17%.[23] Within the same country, like in Germany and Switzerland, rural areas show relatively low prevalence compared to urbanized areas.[24]

The ISAAC study analyzed also the change in prevalence of allergic diseases and could show that the greatest increase was for atopic dermatitis in the young age group (6-7 year-age group).[3]

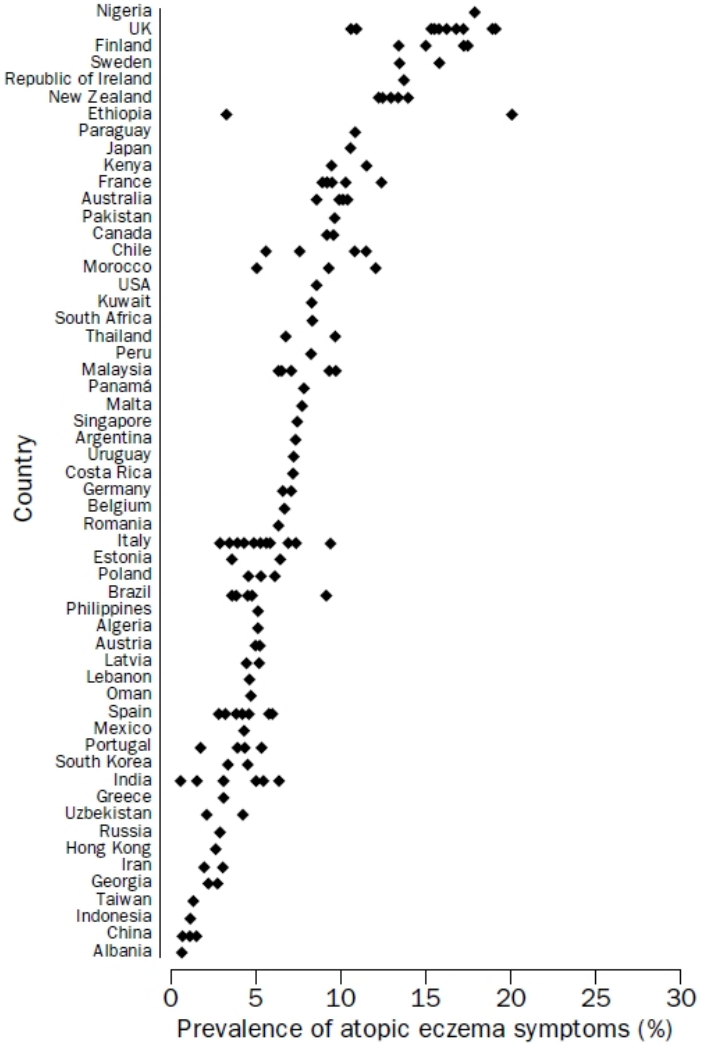

Fig. 3: 12-month prevalence of atopic eczema symptoms[5]

Even though family history has clearly been demonstrated to play a role in the risk of allergic diseases, the strong increase in prevalence during the last decades of allergic diseases can not be explained by genetic factors. Several epidemiological studies have shown that environmental factors most likely play a role in the rising rate of atopic dermatitis and allergic diseases. The farming environment, rich in microbial compounds, was strongly investigated and showed a consistent protective effect on the risk of developing allergic diseases in childhood.[25-28] These results are in agreement with the so-called "hygiene hypothesis", suggesting that lack of microbial exposure might be a risk factor for allergic diseases. Nevertheless, its association with atopic dermatitis remains unclear.[25, 29-31]

Other environmental exposures, such as nutritional factors, have been suggested to be involved in the increasing prevalence of allergic diseases in the last decades. The western diet characterized by high intakes of red meat, refined grain and high fat foods, was shown to be a risk factor for the development of atopic dermatitis.[32] In addition, an inverse relationship was seen between prevalence of atopic dermatitis and the intake of vegetables, protein from cereals and nuts, as well as a potential protective effect of increased fruit consumption.[33] Moreover, a reduction of the risk of atopic dermatitis was associated with a high fish intake during late infancy.[34, 35]
Studies of the farming environment have shown that consumption of unprocessed farm milk was associated with fewer allergic diseases.[26, 36-38]

1.4 Hygiene hypothesis and atopic dermatitis

As mentioned above, atopic dermatitis was shown to be more prevalent in western than in developed countries.[5] Up to 30% of children are affected by atopic dermatitis in industrialized countries.[22]

First described by Strachan, the so-called hygiene hypothesis was based on the observation that children with an increased number of siblings had less allergic rhinitis and atopic dermatitis.[39] A lot of epidemiological results support the interaction between microbial burden and the prevalence of allergy. However, most of the research on allergic diseases and their relation to the hygiene hypothesis have

focused on asthma. Epidemiologic evidence supporting this association with atopic dermatitis remains inconsistent.[39-42] Nevertheless, a few studies have shown that day care attendance, the number of older siblings, farm environment and exposure to pets might be protective factors against atopic dermatitis.[39, 43-45]

Regarding the influence of farming environment, most of the studies did not find a significant association between farm environment and atopic dermatitis, even though a decreased risk of asthma and hay fever was shown.[25, 26, 30, 46] One limitation of those studies might be their cross-sectional design. Studies with longitudinal design are needed to analyze the association between environmental factors and atopic dermatitis with focus on early life, as the disease frequently first occurs within the first year of life. One Swedish large cohort study showed a small reduction of the risk of developing atopic dermatitis with living on a farm.[31] In addition, one cross-sectional study from New Zealand showed a protective farm effect on atopic dermatitis and that this effect could already be effective during pregnancy.[47]

Exposure to endotoxins (lipopolysaccharides found in the outer cell membrane of Gram-negative bacteria) have been suggested as an explanation why pets or farm environmental factors may have a protective effect on allergic diseases.[48] A birth cohort study could show a negative association between exposure to high levels of endotoxin and atopic dermatitis, among children with parental history of asthma or allergies.[49] A birth cohort study from Germany (LISA study) has suggested an up to 50% reduction of the risk of atopic dermatitis in the first 6 months of life associated with endotoxin exposure measured in dust from mothers' mattresses.[50]

As extension of the hygiene hypothesis, probiotics supplementation has been tested as prophylaxis against atopic dermatitis. Probiotics are supplements containing microorganisms with the intention to provide health benefits when consumed. Different probiotics, such as strains of lactobacteria and bifidobateria, have been extensively studied in randomized clinical trials (RCTs). However, substantial heterogeneity between those studies was described. Cochrane meta-analysis concluded that “although there was a reduction in clinical atopic dermatitis in infants, this effect was not consistent between studies” and that “there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease”.[51]

The suggested immunological basis of the hygiene hypothesis is that a reduced microbial stimulation of receptors of the innate immune system in early life leads to a shift towards Th-2 responses against allergens and therefore could induce the development of allergic diseases.[52] In fact, researches on the molecular mechanisms of the hygiene hypothesis have reported that Toll-like receptors (TLRs) have the ability to modulate allergic responses.[53]

1.5 The innate immune system

The innate immune system constitutes the first line of defense to foreign molecules, such as microbes, and directs the adaptive immune response by T helper cell activation. The development of innate immunity is determined by a combination of genetic and environmental factors and most likely a combination of both. As mentioned above, it was suggested that the innate immune system is involved in the signals delivered by the high levels of microbial components associated with farming environment and that an altered stimulation of the innate immune system might influence the development of allergic disease.

Activation of the innate immune system is mediated by pattern recognition receptors (PRRs), such as TLRs and CD14, which are present on immune cells and recognize pathogen-associated molecular patterns (PAMPs). At least 10 different TLRs have been described in humans; each TLR is associated with the recognition of certain groups of PAMPs (bacterial, fungal or viral structures).[54] It was suggested that genetic alterations in the innate immunity, especially in receptors of PAMPs, may modify the risk of allergic diseases. Direct associations between single nucleotide polymorphisms (SNPs) in *TLRs* and allergies have been shown.[55, 56] *TLR9* and *CD14* promoter polymorphisms were associated with atopic dermatitis.[57, 58] Moreover, several findings indicate that environmental factors associated with high levels of microbial components may interact with existing polymorphisms in *TLRs* on the development of allergic disease. Also direct associations between single nucleotide polymorphisms (SNP) in *TLRs* and allergies have been shown.[59, 60] Furthermore, previous studies have suggested that gene expression of these receptors is up-regulated by exposure to environmental factors rich in microbial compounds, such as farming, and already when exposure occurs during pregnancy.[61, 62] However the

direct correlation between the presence of allergic diseases in children and TLRs or CD14 expression was not made. Defining whether there is a difference in gene expression would facilitate a better understanding of the determinants of atopic dermatitis.

1.6 Atopic dermatitis and infant's diet

Nutrition is an important environmental factor in early life, which influences the development of the child's immune system. The role of nutrition during infancy on the development of allergies later on in childhood remains controversial.

During the early postnatal period, the infant gut is first exposed to different food antigens and these exposures might influence the development of immune tolerance. Mechanisms could include the acquisition of the microbiota through the diet or diet-microbiota interactions.[63-65] It has been suggested that modern changes in the postnatal environment, such as dietary exposure in infancy, might not optimally support induction of immune tolerance, as the incidence of allergic diseases during the last decades shows a strong increase.[66] Interestingly and as already mentioned above, the western diet was shown to be a risk factor for the development of atopic dermatitis.[32] However, current guidelines no longer recommend food allergen avoidance or delaying introduction in the infant's diet in order to prevent allergic diseases as no clear benefit has been shown.[67] First introduction of complementary food in an infant's life and its association with allergic diseases has raised much controversy. Some recent studies even showed that early introduction of complementary food, like the introduction of fish before one year of age or early exposure to cow's milk, might have a protective effect on allergic diseases.[34, 68-72] One study found a protective effect of the introduction of any complementary food within the first four months on atopic dermatitis, but only among children with allergic parents.[73] Therefore, more evidence is needed with respect to the role of early nutritional exposures in the development of atopic dermatitis and allergic diseases. One major concern regarding studies on the association between early life exposure to foods and allergic diseases, especially atopic dermatitis, is the reverse causality effect. Among children with early symptoms and/or those with allergic parents, feeding practices will be different, with the tendency to delayed introduction of

complementary food. Therefore early introduction of food may wrongly appear to result in less allergic diseases.

Studies of the farming environment have shown that consumption of unprocessed farm milk was associated with fewer allergic diseases although there was some heterogeneity of the effects, especially with atopic dermatitis. This evidence was based on cross-sectional studies of school-aged children only.[29, 37, 38]

1.7 Atopic dermatitis and breastfeeding

The relation between breastfeeding and its association with allergic diseases remains conflicting.[74, 75] A meta-analysis of 27 prospective studies failed to show a statistically significant benefit with exclusive breastfeeding (pooled OR: 0.89, 95%CI 0.76-1.04) and concluded that there is no strong evidence of a protective effect of exclusive breastfeeding for at least three months against atopic dermatitis, even among children with a positive family history.[76] One reason might be the differences in the composition of breast milk which vary greatly between individual mothers and between mothers with and without allergies.[77-79] Moreover, it has been shown that constituents in breast milk differ between farm and non-farm mothers.[80] Components that were identified to possible play a role in a protective effect of breast feeding on allergic diseases are soluble immunoglobulin A (sIgA), isoforms of transforming growth factor beta (TGF-β) and soluble CD14. However findings remain inconsistent.[81-85]

1.8 Methods

Concerning the research questions of this work, we used data from the PASTURE/EFRAIM (Protection against Allergy-Study in Rural Environments/ Early Farm-Related anti-Allergy Immune Mechanisms), a birth cohort study conducted in five European countries (Austria, Germany, France, Finland and Switzerland). This study was designed to evaluate, from early life, risk factors and preventive factors in the development of atopic diseases.[86] Pregnant women were recruited during the third trimester of pregnancy between August 2002 and March 2005 and divided in

two groups. Women who lived on family-run farms where any kind of livestock was kept were assigned to the farm group. Women from the same rural areas not living on a farm were in the reference group. In total, 1133 children were included in this birth cohort.

The questionnaires developed within the PASTURE study group used questions on various exposures and outcomes from the Asthma Multicenter Infants Cohort Study (AMICS)[87], the Allergy and Endotoxin (ALEX) study[26], and the Prevention of Allergy Risk factors for Sensitization in children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study.[27] Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18, 24 months of age and then yearly up to six years of age. Feeding practices and the occurrence of itchy rash were reported by parents between the 3rd and 12th month of life in monthly and weekly diaries, respectively.

Children were labeled having atopic dermatitis when the parents reported in the questionnaires that the child had atopic dermatitis diagnosed by a doctor at least once and/or with positive Scorad score (>0) assessed during medical examination at the age of 1 year.

Blood samples were used to perform genotyping (including SNPs in innate immunity receptor genes) and to measure gene expression of Toll-like receptors 1-9 and CD14.

The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents.

1.9 Aims of this thesis

- In **chapter 2,** we investigated in a longitudinal way the association between prenatal farm-related exposures and the development of atopic dermatitis in early life. Further, we looked for differences in gene expression of receptors of the innate immunity at birth between children who will develop atopic dermatitis or not in early childhood.

- The hygiene hypothesis has proposed that a reduced microbial stimulation of the immune system may lead to the development of allergic diseases. In

chapter 3, we analyzed which farm-related exposures during pregnancy and early life are associated with gene expression of innate immunity receptors, measured at birth and one year of age.

- The potential association between infant feeding practices and allergic diseases remains a topic of debate. In **chapter 4,** we evaluated the influence of early postnatal exposures, farm-related and nutrition, on the development of atopic dermatitis later on.

- In **chapter 5,** we analyzed whether differences in breast milk components are associated with atopic dermatitis, sensitization or asthma.

- In **chapter 6,** we further evaluated the role of infant feeding practices, already mentioned in chapter 4, and its association with other allergic diseases.

Chapter 2

Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis

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Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis

Caroline Roduit, MD, MPH,[a] Johanna Wohlgensinger, PhD,[a] Remo Frei, PhD,[a] Sondhja Bitter, MD,[b,c] Christian Bieli, MD, PhD,[a] Susanne Loeliger,[a] Gisela Büchele, MPH,[e] Josef Riedler, MD,[f] Jean-Charles Dalphin, MD, PhD,[g] Sami Remes, MD,[h] Marjut Roponen, PhD,[i] Juha Pekkanen, MD,[i] Michael Kabesch, MD,[j] Bianca Schaub, MD,[k] Erika von Mutius, MD, MSc,[k] Charlotte Braun-Fahrländer, MD,[b,c] Roger Lauener, MD,[a,d] and the PASTURE Study Group *Zurich, Basel, and Davos, Switzerland, Ulm, Hannover, and Munich, Germany, Schwarzach, Austria, Besançon, France, and Kuopio, Finland*

Background: Cross-sectional studies have suggested that prenatal farm exposures might protect against allergic disease and increase the expression of receptors of the innate immune system. However, epidemiologic evidence supporting the association with atopic dermatitis remains inconsistent.
Objective: To study the association between prenatal farm-related exposures and atopic dermatitis in a prospective study. We further analyzed the association between the expression of innate immune genes at birth and atopic dermatitis.
Methods: A total of 1063 children who participated in a birth cohort study, Protection against Allergy-Study in Rural Environments, were included in this study. Doctor diagnosis of atopic dermatitis was reported by the parents from 1 to 2 years of age by questionnaire. Gene expression of Toll-like receptors (TLRs) and CD14 was assessed in cord blood leukocytes by quantitative PCR.
Results: Maternal contact with farm animals and cats during pregnancy had a significantly protective effect on atopic dermatitis in the first 2 years of life. The risk of atopic dermatitis was reduced by more than half among children with mothers having contact with 3 or more farm animal species during pregnancy compared with children with mothers without contact (adjusted odds ratio, 0.43; 95% CI, 0.19-0.97). Elevated expression of TLR5 and TLR9 in cord blood was associated with decreased doctor diagnosis of atopic dermatitis. A significant interaction between polymorphism in *TLR2* and prenatal cat exposure was observed in atopic dermatitis.
Conclusion: Maternal contact with farm animals and cats during pregnancy has a protective effect on the development of atopic dermatitis in early life, which is associated with a lower expression of innate immune receptors at birth. (J Allergy Clin Immunol 2011;127:179-85.)

Key words: *Prenatal, farm animal, Toll-like receptors, atopic dermatitis, gene-environment interaction*

Atopic dermatitis is a chronic, inflammatory, pruritic skin disease, often occurring in early infancy, that affects up to 20% of the children in industrialized countries.[1] Asthma develops in approximately 30% of children with atopic dermatitis, and allergic rhinitis in 35%.[2] According to the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence of symptoms of atopic dermatitis in children 6 or 7 years of age during a 1-year period varied from less than 2% in Iran and China to approximately 20% in Australia, England, and Scandinavia.[1] The etiology of atopic dermatitis is complex and involves an interaction between genetic and environmental factors and the immune system. The hygiene hypothesis has contributed to the understanding of allergic diseases, suggesting that lack of microbial exposure might be a risk factor. A protective effect of microbial exposure on asthma has been described.[3-5] However, epidemiologic evidence supporting these associations with atopic dermatitis remains inconsistent.[6-8]

From [a]the University of Zurich, Children's Hospital, and Christine Kühne-Center for Allergy Research and Education; [b]the Swiss Tropical and Public Health Institute, Basel; [c]the University of Basel; [d]the Children's Allergy and Asthma Hospital, Hochgebirgsklinik, and Christine Kühne-Center for Allergy Research and Education, Davos; [e]the Institute of Epidemiology, University of Ulm; [f]Children's Hospital Schwarzach; [g]the Department of Respiratory Disease, University Hospital of Besançon; [h]the Department of Pediatrics, Kuopio University Hospital; [i]the Department of Environment Health, National Institute for Health and Welfare, Kuopio; [j]the Center for Pediatrics, Clinic for Paediatric Pneumology and Neonatology, Hannover Medical School; and [k]University Children's Hospital Munich.

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The PASTURE Study Group: Gertraud Weiß, Ellen Üblagger, Claudia Humer, Manuela Rußegger (Austria); Raija Juntunen, Reetta Tiihonen, Pekka Tiittanen, Kati Huttunen, Suvi Virtanen, Timo Kauppila, Aino Nevalainen, Tomi-Pekka Tuomainen, Anne Karvonen, Mikko Lappalainen (Finland); Marie-Laure Dalphin, Dominique Vuitton, Renaud Piarroux, Gabriel Reboux, Sandrine Roussel, Bertrand Sudre (France); Susanne Schmid, Sabina Illi, Nicola Korherr, Jon Genuneit, Richard Peter, Serdar Sel, Nicole Blümer, Petra Pfefferle, Harald Renz (Germany); Bert Brunekreef, Ulrike Gehring, Gert Dockes (The Netherlands); Felix H. Sennhauser (Switzerland).

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Reprint requests: Caroline Roduit, MD, MPH, Kinderspital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. E-mail: Caroline.Roduit@kispi.uzh.ch.

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Abbreviations used
EFRAIM: Mechanism of Early Protective Exposures on Allergy Development
GEE: General estimating equation
GMR: Geometric mean ratio
ISAAC: International Study of Asthma and Allergies in Childhood
OR: Odds ratio
PASTURE: Protection against Allergy-Study in Rural Environments
SNP: Single nucleotide polymorphism
TLR: Toll-like receptor

In addition, recent cross-sectional studies have shown that the protective effect of farm exposures on allergic diseases could already be effective during pregnancy.[9,10]

Although it has been suggested that an altered stimulation of the innate immune system might influence the development of allergic disease, the underlying immunologic mechanisms of these protective effects of farm exposures remain unclear. Activation of the innate immune system is mediated by pattern recognition receptors, such as Toll-like receptors (TLRs), which are present on immune cells and recognize pathogen-associated molecular patterns. Previous studies have suggested that gene expression of these receptors is upregulated by exposure to environmental factors rich in microbial compounds.[9,11] Moreover, several findings indicate that environmental factors associated with high levels of microbial components may interact with existing polymorphisms in *TLRs* on the development of allergic disease, and direct associations between single nucleotide polymorphisms (SNPs) in *TLRs* and allergies have been shown.[12-15] Recently an association between a *TLR9* promoter polymorphism and atopic eczema has been reported.[16] However the direct correlation between the presence of allergic diseases in children and TLRs or CD14 expression was not made. Defining whether there is a difference in gene expression would facilitate a better understanding of the determinants of atopic dermatitis.

In this study, we longitudinally analyzed the effect of prenatal environmental exposures related to the hygiene hypothesis on the development of atopic dermatitis in the first 2 years of life within a prospective birth cohort, the Protection against Allergy-Study in Rural Environments (PASTURE)/Mechanism of Early Protective Exposures on Allergy Development (EFRAIM) study.[17] Because gene expression of innate immunity receptors reflects both genetic and environmental influences, we further examined whether TLRs and CD14 expression in cord blood samples from these children was associated with the development of atopic dermatitis. In addition, we investigated whether the interaction between polymorphisms in *TLRs* and prenatal exposures has an impact on gene expression and on atopic dermatitis.

METHODS

Study design

The PASTURE/EFRAIM study is a prospective birth cohort study involving children from rural areas in 5 European countries (Austria, Finland, France, Germany, and Switzerland), designed to evaluate risk factors and preventive factors for atopic diseases. The design of this cohort has been described in detail elsewhere.[17] Briefly, pregnant women were recruited during the third trimester of pregnancy and divided into 2 groups. Women who lived or worked on family-run farms where any kind of livestock was kept were assigned to the farm group. The reference group was composed of women from the same rural areas not living on a farm. In total, 1133 children were included in this birth cohort. The questionnaires developed within the PASTURE study group used questions on various exposures from ISAAC,[18] the Allergy and Endotoxin (ALEX) study,[3] and the Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle (PARSIFAL) study.[5] Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18, and 24 months of age. The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents.

Study population

Children from the PASTURE/EFRAIM birth cohort with data available on atopic dermatitis at least once between 1 and 2 years of age and on farming status (n = 1063) were included. Among these children, 905 have data available on mRNA analysis in cord blood samples and between 961 and 987 on polymorphisms in *TLRs*, depending on the SNP.

Definitions

Children were labeled as having doctor's diagnosis of atopic dermatitis when the parents reported at least once in the questionnaire at the age of 12, 18, or 24 months that the child had been diagnosed with atopic dermatitis by a doctor. Farmer children were defined as children whose parents answered positively to the question, "Does your child live on a farm?" and whose family ran the farm. Maternal farm-related and pet exposures during pregnancy were obtained from the self-reported questionnaires at the third trimester of pregnancy. Prenatal exposures to barn or stable were defined as an exposure of at least a quarter of an hour per week. Contact with farm animal species was assumed if the mother reported contact at least several times per month in 1 of the pregnancy trimesters. Consumption of farm milk was defined as a consumption of at least on average 10 mL farm milk per day. Data on potential confounders, such as smoking during pregnancy, sex, mode of delivery, birth weight, gestational age, maternal education, and breast-feeding, were obtained from the self-reported questionnaires at the third trimester of pregnancy, at 2 months, and 1 year of age. Maternal atopy was defined as ever having asthma or hay fever. This information was self-reported.

Expression of Toll-like receptors and CD14

Blood samples were collected from the umbilical cord at birth. For the assessment of mRNA, the blood was collected in a PAXgene Blood RNA tube containing an RNA-stabilizing solution (PreAnalytiX/Qiagen, Hilden, Germany) and then frozen to −80°C within 24 hours.[19] In a central laboratory (Zurich, Switzerland), the RNA was isolated by using the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The mRNA was reverse-transcribed into cDNA by using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif). Quantitative real-time PCR was performed on the 7900HT Fast Real-Time PCR System by using the Micro fluidic card TaqMan Array system of Applied Biosystems. The data presented are normalized values for the endogenous controls (18S ribosomal RNA and β-2-microglobulin) using the comparative (delta delta cycle threshold, ΔΔCt) method according to the manufacturer's instructions (Applied Biosystems). TLR3 expression was excluded from the analyses because the expression level was less than the detection limit on most of the cord blood samples.

Genotyping

Genotyping was performed by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as described previously.[20] Derived genotype frequencies were compared with the expected allelic population equilibrium based on the Hardy-Weinberg equilibrium test to control for technical genotyping errors. cDNA was amplified in duplicate by using an iCycler (Bio-Rad, Hercules, Calif), with 18s as a reference gene.

TABLE I. Characteristics of children

Characteristics	Total study population N = 1063 n (%)	Farmer n = 508 n (%)	Nonfarmer n = 555 n (%)
Girl	517 (48.7)	249 (49.0)	268 (48.5)
Center			
Austria	209 (19.6)	99 (19.5)	110 (19.8)
Switzerland	226 (21.3)	101 (19.9)	125 (22.5)
France	191 (18.0)	91 (17.9)	100 (18.0)
Germany	233 (21.9)	109 (21.5)	124 (22.4)
Finland	204 (19.2)	108 (21.3)	96 (17.3)
Mother with atopy history*	310 (29.2)	118 (23.3)	192 (34.6)
Mode of delivery: cesarean section	185 (17.6)	86 (17.0)	99 (18.0)
Birth weight			
<2500 g	16 (1.5)	9 (1.8)	7 (1.3)
2500-4500 g	1019 (96.0)	484 (95.3)	535 (96.7)
>4500 g	26 (2.5)	15 (2.9)	11 (2.0)
No. of siblings*			
0	383 (36.0)	129 (25.4)	254 (45.8)
1-2	567 (53.4)	291 (57.3)	276 (49.7)
≥3	113 (10.6)	88 (17.3)	25 (4.5)
Mother smoking during pregnancy* (at least in 1 trimester)	140 (13.2)	43 (8.5)	97 (17.5)
Prenatal farm exposures			
Work on a farm during pregnancy*	406 (38.3)	364 (71.8)	42 (7.6)
Farm milk during pregnancy*	445 (42.0)	372 (73.5)	73 (13.2)
Farm milk during pregnancy: unboiled*	358 (33.8)	296 (58.5)	62 (11.2)
Work in stable during pregnancy* (at least 15 min/wk in 1 of the trimesters)	541 (53.2)	443 (89.3)	98 (18.8)
Work in barn during pregnancy* (at least 15 min/wk in 1 of the trimesters)	402 (39.6)	342 (69.1)	60 (11.5)
Contact with farm animals during pregnancy* (horse, cow, pig, or poultry, at least several times per month)			
3-4 species	120 (11.7)	96 (20.0)	24 (4.4)
1-2 species	508 (49.7)	345 (71.9)	163 (30.1)
0 species	394 (38.6)	39 (8.1)	355 (65.5)
Contact with any pets during pregnancy*	666 (62.7)	419 (82.5)	247 (44.5)
Contact with cats during pregnancy*	472 (44.4)	349 (68.8)	123 (22.2)
Contact with dogs during pregnancy*	344 (32.5)	241 (47.7)	103 (18.6)

*$P < .05$, based on χ^2 test between farmer and nonfarmer.

Polymorphisms in *TLRs* were selected as previously described.[21] These SNPs were as follows: *TLR1/C-2299T* (rs5743594), *TLR1/T-2192C* (rs5743595), *TLR1/A742G* (rs4833095), *TLR2/T596C* (rs3804099), *TLR2/T-16934A* (rs4696480), *TLR4/C8851T*, *TLR4/A8551G*, *TLR4/T-1607C*, *TLR5/T1845C* (rs5744174), *TLR5/C1173T* (rs5744168), *TLR5/A1774G* (rs2072493), *TLR6/T-2079A* (rs5743789), *TLR7/A1796T* (rs179008), *TLR7/C12318T*, *TLR8/C10907A*, *TLR8/A-4824G* (rs3761624), *TLR9/T-2622C* (rs5743836), and *TLR9/T-2871C* (rs187084).

Statistical analysis

Data analysis was conducted by using SAS software version 9.2 (SAS Institute, Inc, Cary, NC).

General estimating equations (GEEs) were used to investigate the longitudinal effects of prenatal farm exposures, gene expression of TLRs and CD14 in cord blood, and genotypes of SNPs in *TLRs* on atopic dermatitis from age 1 to 2 years, taking into account the correlation between repeated measures in the same individual. The associations between the different SNPs in *TLR* and atopic dermatitis were corrected for multiple testing by using the false discovery rate (Benjamini procedure; $P \leq .05$ is significant).[22] To test for effect modification, we calculated terms for interactions between farm exposures and TLR expression and the different centers in the GEE model, and they were not significant. For gene expression, geometric mean ratios (GMRs) with 95% CIs were calculated by exponentiation of the regression coefficients and their 95% CIs. Estimates of cumulative incidence of atopic dermatitis in the first 2 years of age were calculated by the discrete time survival analysis among 3 groups of children categorized by the tertile of gene expression. The discrete time hazard is the conditional probability that a child will develop an atopic dermatitis onset in a period given that atopic dermatitis was not reported in an earlier period. Linear regression was performed to analyze the association between prenatal exposure and mRNA gene expression in cord blood, and when heterogeneity between study centers was significant, the model was adjusted for center as a random effect estimate, and as a fixed effect estimate when there was no heterogeneity. To test for gene-environment interactions, interaction terms were included in the models. Adjustment was made for the following potential confounders: farming status, maternal atopy, sex, number of older siblings, smoking during pregnancy, and study center. Birth weight, mode of delivery, maternal education, exposure to stable in the first year of life, breast-feeding, and consumption of farm milk in the first year of life were added to the model but did not change the results, so they were not kept in the final model. A P value below .05 was considered statistically significant.

RESULTS

General characteristics

Among the 1063 children included in this study, there were 508 farmers and 555 nonfarmers. The proportion of children having a doctor diagnosis of atopic dermatitis at least once between 1 and 2 years of age among the total study population was 17.8%. The characteristics of the study population showed no differences of the distribution of farmer and nonfarmer children among the 5 centers (Table I). Among children in the nonfarmer group, the frequency of mothers with allergy and those smoking during

pregnancy were higher compared with the children in the farmer group. Among farmer children, the number of siblings was higher compared with nonfarmer children. For the other characteristics, mode of delivery and birth weight, there were no differences depending on the farming status. We observed that a small number of mothers (n = 42) from the nonfarmer group were working on a farm (from neighbors or relatives) or were in contact with a farm animal during pregnancy. The maternal contact with pets was higher among the farmer than the nonfarmer group.

There was no difference with respect to farming status between the total study population and the subgroup of children with data available on gene expression of TLRs and CD14 at birth (data not shown).

Association between prenatal farm exposures and atopic dermatitis

The cumulative prevalence of atopic dermatitis was lower in farmer children than nonfarmer children between 1 and 2 years of age, even though it was not significant (Fig 1, *A*). Among children with mothers working on a farm during pregnancy, this cumulative prevalence was significantly lower compared with children with mothers not working on a farm (14.4% and 20.0% at 2 years of age, respectively; Fig 1, *B*). The longitudinal analyses accounting for repeated measurements (GEE) showed that prenatal farm animal contact was associated with a lower risk of developing atopic dermatitis in the first 2 years of life (Table II). The consumption of farm milk or unboiled farm milk during pregnancy was not associated with atopic dermatitis. Contact during pregnancy with horses, cows, pigs, and poultry, for which we observed a negative association with atopic dermatitis, was included to calculate a score of the number of species the mother had contact with during pregnancy. The risk of developing atopic dermatitis was reduced by a factor of 0.7 among children with mothers having contact with 1 to 2 species and by a factor of 0.4 with prenatal contact with 3 to 4 species compared with the reference group with no prenatal contact with farm animals. For each additional farm animal species the mother had contact with, we observed a reduction of 20% in the development of atopic dermatitis (adjusted odds ratio [OR], 0.80; 95% CI, 0.65-0.99; Fig 2). Moreover, we found a significant negative association between prenatal contact with cats and the presence of atopic dermatitis in the first 2 years of life (adjusted OR, 0.68; 95% CI, 0.46-1.00).

There was no significant association between the different SNPs in *TLRs* and atopic dermatitis after correction for multiple testing (data not shown). The protective effect of prenatal contact with farm animals and cats might differ between the genotypes of SNPs in *TLRs* (see this article's Table E1 in the Online Repository at www.jacionline.org). The analyses for a gene-environment interaction showed a significant ($P = .02$) effect modification of 1 SNP in *TLR2* (*TLR2/T-16934A*) on the association between prenatal exposure to cats and the development of atopic dermatitis early in life and a marginally significant effect modification ($P = .09$) for prenatal exposure to farm animals. We observed a strong protective effect of the prenatal animal (farm animals and cats) exposures only among children with genotype AA in *TLR2/T-16934A* (Fig 3). We did not observe a significant gene-environment interaction for the other SNPs in *TLRs*. However, the significant protective effect of contact with farm animals and cats during pregnancy on atopic dermatitis was more

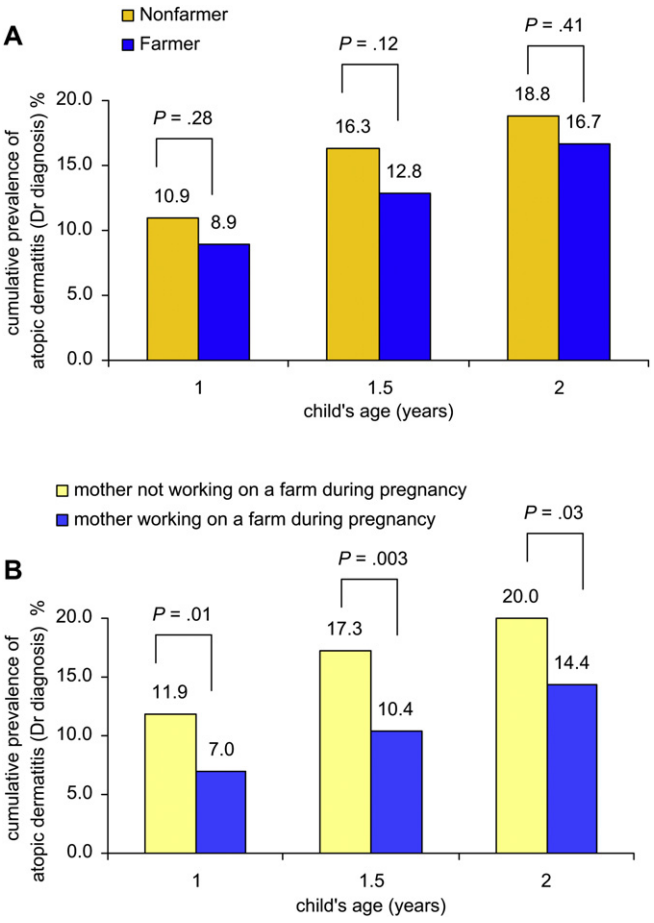

FIG 1. Cumulative prevalence of atopic dermatitis between farmers and nonfarmers **(A)** and between mothers working and not working on a farm during pregnancy **(B)**. *P* value based on the χ^2 test. *Dr,* Doctor.

pronounced among certain genotypes. Because we found differences between the distribution of genotypes between Finland and the other centers, we repeated the same analyses excluding the Finnish population, and we observed strongly comparable results (data not shown).

Association between gene expression and atopic dermatitis

We observed a negative association between the gene expression of TLRs and CD14 assessed in cord blood and the development of atopic dermatitis in the first 2 years of life (Fig 4). Gene expression of TLR5 and TLR9 in cord blood was significantly reduced among children with atopic dermatitis compared with children with no dermatitis (adjusted GMR, 0.66; 95% CI, 0.48-0.89; and adjusted GMR, 0.73; 95% CI, 0.56-0.96, respectively). The same trend was observed for TLRs 1, 2, 4, 6, 7, and 8 and CD14, even though it was not significant. None of the potential confounding factors (maternal atopy, sex, siblings, maternal smoking during pregnancy, study center, or farming status) had a major influence on the results. Survival analyses showed that children with a level of TLR5 or TLR9 expression in the upper tertile had a risk reduced by around 50% of developing atopic dermatitis compared with children with an expression in the lower

TABLE II. Association between prenatal exposures and the development of atopic dermatitis in the first 2 years of life

Prenatal exposures	Crude ORs	95% CI	Multivariate adjusted ORs*	95% CI
Farming status	0.83	0.59-1.17	0.85	0.59-1.21
Work on a farm during pregnancy	**0.65**	**0.45-0.94**	0.83	0.55-1.25
Farm milk during pregnancy	0.72	0.50-1.02	0.86	0.56-1.33
Farm milk during pregnancy: unboiled	0.72	0.50-1.04	0.93	0.62-1.41
Work or stay in stable during pregnancy	0.76	0.54-1.08	0.84	0.47-1.51
Work or stay in barn during pregnancy	0.85	0.60-1.23	1.09	0.71-1.66
Contact with farm animal during pregnancy				
Horse	0.65	0.39-1.07	0.64	0.39-1.06
Cow	0.74	0.53-1.04	0.79	0.49-1.27
Pig	**0.43**	**0.21-0.89**	0.54	0.26-1.11
Sheep	1.01	0.61-1.65	1.15	0.68-1.95
Hare	0.84	0.53-1.32	0.96	0.61-1.53
Poultry	0.66	0.43-1.03	0.76	0.47-1.21
Horse, cow, pig, or poultry				
3-4 species	**0.40**	**0.19-0.82**	**0.43**	**0.19-0.97**
1-2 species	0.71	0.49-1.01	0.71	0.45-1.15
0 species, reference	1.00		1.00	
Contact with any pets during pregnancy	**0.63**	**0.45-0.89**	**0.65**	**0.44-0.96**
Contact with cat during pregnancy	**0.58**	**0.41-0.82**	**0.68**	**0.46-1.00**
Contact with dog during pregnancy	0.78	0.54-1.13	0.71	0.48-1.07

*Adjusted for center, atopic mother, sex, smoking during pregnancy, and farming status (not adjusted for farming status for, as exposures: farming status and working on a farm during pregnancy). Interaction test with center: not significant for all of the exposure variables. Boldface values: $P < .05$

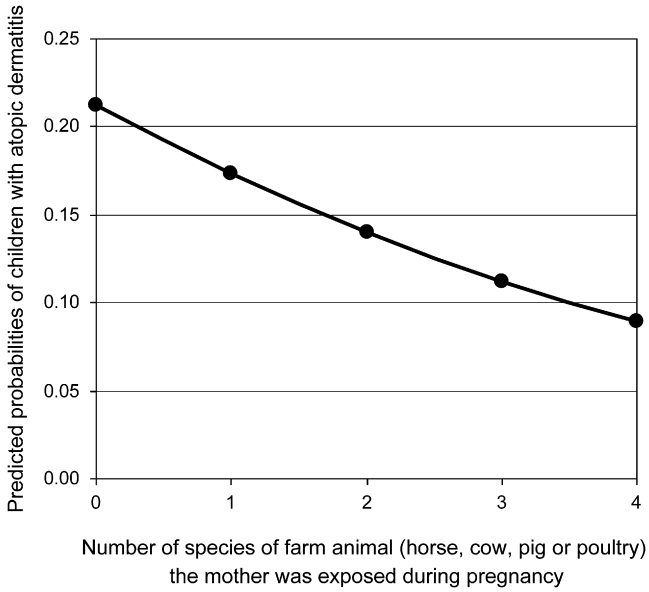

FIG 2. Association between prenatal farm animal exposures and atopic dermatitis. OR, 0.80 (95% CI, 0.65-0.99) for atopic dermatitis with each additional farm animal species, $P = .04$, adjusted for center, atopic mother, sex, smoking during pregnancy, and arming status (GEE analysis). Test of interaction between center and prenatal exposure to farm animal was non-significant; $P = .81$.

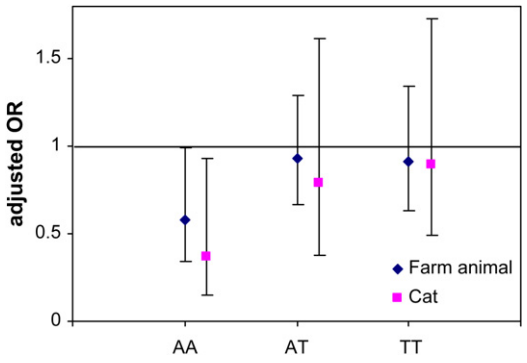

FIG 3. The effect of prenatal contact with farm animals and cat on atopic dermatitis by genotypes of *TLR2_min16934*. Reference group: no prenatal contact with animals. OR adjusted for study center, mother with atopy, sex, smoking during pregnancy, and farming status. $P = .09$ for interaction term between *TLR2_min16934* and prenatal farm animal contact. $P = .02$ for interaction term between *TLR2_min16934* and prenatal cat contact.

tertile (TLR5: adjusted OR, 0.56; 95% CI, 0.37-0.83; TLR9: adjusted OR, 0.53; 95% CI, 0.36-0.80; Fig 5).

Association between prenatal exposures and gene expression

There were no significant associations between contact with farm animals or pets during pregnancy and gene expression of TLRs and CD14 measured at birth after adjustment for farming status, maternal atopy, sex, and study center (data not shown). However, we observed a slight trend of an enhanced gene expression of TLR5 and TLR9 with the increasing number of species of farm animals the mother was exposed to during pregnancy, even though it was not significant (GMR for TLR5 expression, 1.25; 95% CI, 0.92-1.70; GMR for TLR9 expression, 1.13; 95% CI, 0.80-1.59; ratio of prenatal contact with 4 species compared with no contact). The different SNPs in *TLRs* did not reveal a gene-environment effect on the gene expression of these receptors assessed at birth. When TLR5 or TLR9 expressions were included in the model of the association between exposure and atopic dermatitis, we observed a reduction of the direct effect of exposure on atopic dermatitis. This indicated that an effect of exposure on atopic dermatitis might be mediated by TLR5 or TLR9 expressions.

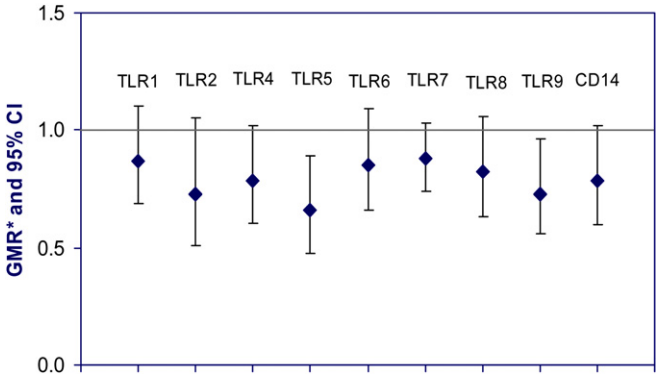

FIG 4. Association between the development of atopic dermatitis in the first 2 years of life and the gene expression of TLRs and CD14 at birth. *GMR,* Ratio of the TLRs geometric mean in children with atopic dermatitis compared with children without atopic dermatitis. *Adjusted for farmer, mother with atopy, sex, older siblings, smoking during pregnancy, and study center.

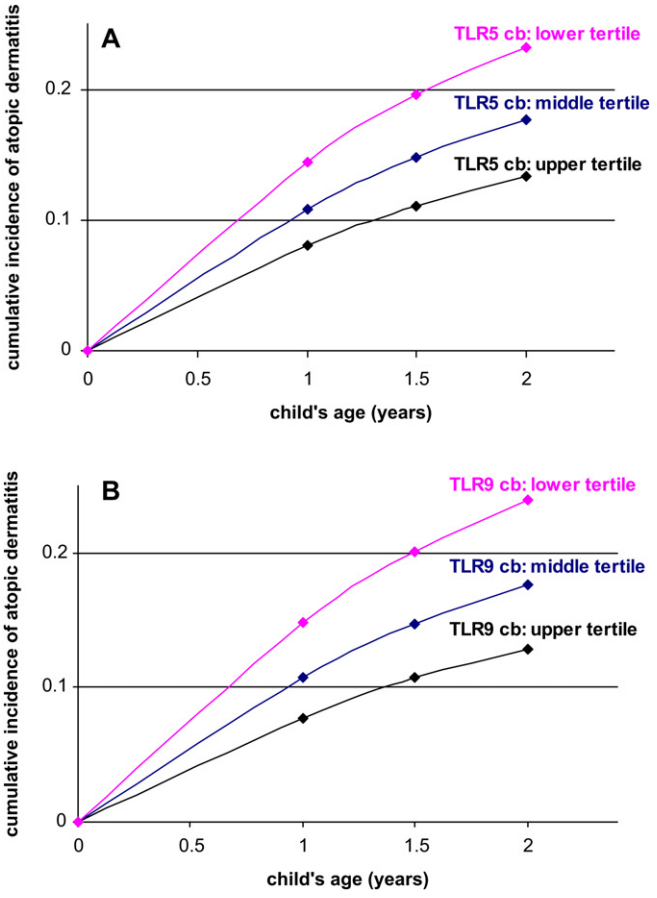

FIG 5. Cumulative incidence of atopic dermatitis, stratified between tertiles of gene expression of TLR5 **(A)** and TLR9 **(B)** at birth. *A,* TLR5. The cumulative incidences of atopic dermatitis by age 2 years were significantly different between the tertiles of TLR5: $P = .003$. *B,* TLR9. The cumulative incidences of atopic dermatitis by age 2 years were significantly different between the tertiles of TLR9: $P < .001$. *cb,* Cord blood.

DISCUSSION

This study shows that children with mothers having contact with farm animals and cats during pregnancy have a reduced risk of developing atopic dermatitis in the first 2 years of life. The interaction between polymorphisms in *TLRs* and environment on atopic dermatitis reveals a slight effect with a SNP in *TLR2*. We also show that children with higher gene expression of TLR5 and TLR9 at birth have a decreased risk of atopic dermatitis compared with children with lower expression of these receptors. These results suggest a role of the innate immune system in mediating the protective effect of prenatal exposures on the development of atopic dermatitis in children, along the line of recent data obtained in the mouse model.[23]

Several epidemiologic studies have shown that environmental factors rich in microbial compounds such as the farming environment have a protective effect on allergic disease.[3,24] Nevertheless, in the previous studies on allergic disease and living on a farm, the association with atopic dermatitis remains unclear.[4,25-27] In our study, we observed a protective effect on atopic dermatitis in children when the mother was working on a farm during pregnancy and especially when she was exposed to an increasing number of different farm animal species. These results from longitudinal analyses support the previous cross-sectional findings of a protective effect of prenatal exposure to a farm environment on atopic sensitization and on atopic dermatitis, asthma, and hay fever.[9,10]

Moreover, our findings support that gene-environment interactions play a role in the development of atopic dermatitis in children and suggest a biological underlying mechanism for this relationship. Because TLRs are acting as the first line of defense against microbial compounds, the level of gene expression of these receptors might be a marker of the influence of the genes and environment on the development of atopic dermatitis.

As previously suggested in studies showing that the expression of innate immunity receptors was increased in children exposed to environments rich in microbial components, these results show that the innate immune system might modulate the development of allergic diseases.[11,28] Moreover, in a cross-sectional study, Ege et al[9] observed a positive association between maternal contact with farm animals during pregnancy and the gene expression of receptors of the innate immune system in children 5 to 13 years of age. Similarly, 1 previous study reported a decreased TLR2 and TLR4 expression in cord blood in relation to maternal allergy but did not examine allergic outcomes.[29] In our study, the negative association between the level of gene expression assessed in cord blood samples and atopic dermatitis was not influenced by adjusting for maternal atopy.

The strength of this study is the prospective design, which avoids recall bias and gives the opportunity to study the effect of timing of exposures and the influence of innate immunity at birth on the development of atopic dermatitis.

The development of atopic dermatitis occurs usually in the first year of life.[30] For this reason, we defined children having the disease when diagnosed in the 2 first years of life. Because a definition of atopic dermatitis based on symptoms could lead to an overestimation of the prevalence of the disease, we used doctor diagnosis of atopic dermatitis. Moreover, we repeated the analysis with a definition based on symptoms as the outcome and observed a similar tendency of an association with the gene expression of TLRs at birth (data not shown).

Toll-like receptors, part of innate immunity, are the first line of defense against microbial compounds, and are therefore biomarkers of enhanced exposure to microbial substances. TLR5 and TLR9 are both receptors for bacterial components. TLR5 recognizes bacterial flagellin,[31] found on nearly all motile

bacteria, and TLR9 recognizes oligodeoxynucleotide containing unmethylated CpG motifs present in bacterial DNA.[32] It was shown that TLR5 activation and TLR9 induce maturation of antigen-presenting cells to a T_H1-biased immune response.[33,34]

Several SNPs in genes encoding for TLR have been shown to be associated with allergic diseases, especially with asthma.[12-14] In 2 studies, associations between SNPs in *TLR2* and *TLR9* genes and atopic dermatitis have been reported.[16,35] However, previous studies on the gene expression of these receptors related to the development of allergic disease had mainly shown an association between the level of expression and exposure to environmental factors rich in microbial substances but not with allergic diseases. A previous study demonstrated that a polymorphism in *TLR2* was associated with the frequency of asthma and allergies among farmer children.[12] Our results showed a slight effect of gene-environment interaction on atopic dermatitis, with the same SNP in *TLR2*.

Maternal contact with farm animals and cats during pregnancy and a higher expression of the receptors of innate immunity at birth have a protective effect on the development of atopic dermatitis in the first 2 years of life.

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Clinical implications: Prenatal exposures influence the development of atopic dermatitis in children.

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TABLE E1. Associations between prenatal contact with animals and the development of atopic dermatitis in the first 2 years of age, stratified by genotype

				Contact with farm animals: horse, cow, pig, poultry		Contact with cats	
				Atopic dermatitis		Atopic dermatitis	
Gene	SNP	Genotype	Frequency % (N)	OR*	95% CI	OR*	95% CI
TLR1	tlr1_cmin2299t	CC	69.8 (683)	0.84	0.64-1.08	0.61	0.36-1.04
	(rs5743594)	CT	27.6 (270)	0.91	0.59-1.39	0.94	0.45-1.97
		TT	2.6 (25)	n/a		n/a	
	tlr1_tmin2192c	TT	63.8 (629)	0.75	0.55-1.03	**0.55**	0.34-0.91
	(rs5743595)	TC	30.8 (304)	0.74	0.47-1.16	1.28	0.60-2.73
		CC	5.4 (53)	n/a		n/a	
	tlr1_a742g	AA	53.9 (531)	**0.69**	0.49-0.97	**0.50**	0.29-0.86
	(rs4833095)	AG	38.0 (375)	0.81	0.55-1.20	1.1	0.51-2.35
		GG	8.1 (80)	n/a		n/a	
TLR2	tlr2_t596c	TT	31.5 (303)	0.85	0.60-1.22	0.65	0.35-1.19
	(rs3804099)	TC	50.1 (481)	**0.70**	0.50-0.99	0.67	0.36-1.27
		CC	18.4 (177)	1.05	0.70-1.59	0.61	0.20-1.84
	tlr2_tmin16934a†	AA	28.2 (276)	**0.58**	0.34-0.99	**0.37**	0.15-0.93
	(rs4696480)	AT	45.9 (449)	0.93	0.67-1.29	0.79	0.38-1.62
		TT	25.9 (253)	0.91	0.63-1.34	0.9	0.49-1.73
TLR4	tlr4_c8851t	CC	86.9 (855)	0.81	0.64-1.02	0.68	0.43-1.10
		CT	12.6 (124)	0.86	0.52-1.43	0.62	0.26-1.48
		TT	0.5 (5)	n/a		n/a	
	tlr4_a8551g	AA	88.2 (863)	**0.77**	0.62-0.98	0.68	0.42-1.08
		AG	11.3 (111)	1.00	0.56-1.80	0.78	0.32-1.93
		GG	0.5 (5)	n/a		n/a	
	tlr4_tmin1607c	TT	72.9 (707)	0.87	0.67-1.12	0.60	0.37-1.00
		TC	24.2 (235)	0.77	0.50-1.20	0.95	0.43-2.12
		CC	2.9 (28)	n/a		n/a	
TLR5	tlr5_a1774g	AA	71.5 (691)	0.82	0.63-1.07	0.74	0.45-1.21
	(rs2072493)	AG	26.6 (257)	0.77	0.55-1.09	0.57	0.23-1.40
		GG	1.9 (18)	n/a		n/a	
	tlr5_c1173t	CC	88.7 (874)	**0.76**	0.59-0.96	0.66	0.41-1.06
	(rs5744168)	CT	10.8 (106)	1.05	0.62-1.78	0.82	0.23-2.84
		TT	0.5 (5)	n/a		n/a	
	tlr5_t1845c	TT	32.7 (318)	0.75	0.40-1.41	0.92	0.37-2.25
	(rs5744174)	TC	50.0 (486)	0.78	0.59-1.05	**0.51**	0.30-0.88
		CC	17.3 (168)	0.82	0.53-1.27	0.85	0.31-2.29
TLR6	tlr6_tmin2079a	TT	63.9 (621)	0.74	0.54-1.02	**0.54**	0.33-0.88
	(rs5743789)	TA	30.3 (294)	0.9	0.60-1.33	1.41	0.59-3.38
		AA	5.8 (56)	n/a		n/a	
TLR7	tlr7_a17961t	AA	69.1 (679)	0.86	0.65-1.13	0.78	0.47-1.29
	(rs179008)	AT	15.8 (155)	0.76	0.49-1.16	0.64	0.24-1.73
		TT	15.1 (148)	0.66	0.35-1.24	0.5	0.16-1.53
	tlr7_c12318t	CC	85.4 (836)	0.8	0.63-1.02	0.76	0.48-1.20
		CT	8.4 (82)	0.93	0.52-1.66	0.70	0.18-2.70
		TT	6.2 (61)	n/a		n/a	
TLR8	tlr8_c10907a	CC	53.7 (527)	0.92	0.68-1.25	0.72	0.39-1.32
		CA	24.1 (236)	**0.63**	0.43-0.94	0.58	0.22-1.22
		AA	22.2 (218)	0.8	0.47-1.35	0.80	0.36-1.80
	tlr8_amin4824g	AA	65.1 (643)	0.8	0.58-1.11	0.67	0.38-1.18
	(rs3761624)	AG	20.2 (199)	0.91	0.63-1.31	0.47	0.21-1.04
		GG	14.7 (145)	n/a		n/a	
TLR9	tlr9_tmin2622c	TT	76.4 (750)	0.83	0.66-1.04	**0.55**	0.35-0.87
	(rs5743836)	TC	22.3 (219)	0.75	0.42-1.35	0.93	0.35-2.49
		CC	1.3 (13)	n/a		n/a	
	tlr9_tmin2871c	TT	30.8 (300)	1.08	0.75-1.54	1.03	0.50-2.13
	rs187084	TC	50.8 (495)	**0.65**	0.44-0.94	0.61	0.35-1.07
		CC	18.4 (180)	0.8	0.57-1.12	0.38	0.14-1.09

n/a, Not applicable.

Boldface values: $P < .05$.

Reference group: no prenatal contact with animals.

*Adjusted for study center, mother with atopy, sex, smoking during pregnancy, and farming status.

†Interaction test with $P < .1$ (P values for interaction term between tlr2_tmin16934a and farm animal contact, .09; and between tlr2_tmin16934a and cats contact, .02).

Chapter 3

Prenatal and early-life exposures alter expression of innate immunity genes: The PASTURE cohort study

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Prenatal and early-life exposures alter expression of innate immunity genes: The PASTURE cohort study

Georg Loss, MSc,[a,b]* Sondhja Bitter, MD,[a,b]* Johanna Wohlgensinger, PhD,[c] Remo Frei, PhD,[c] Caroline Roduit, MD, MPH,[c] Jon Genuneit, MD,[d] Juha Pekkanen, MD,[e,f] Marjut Roponen, PhD,[e] Maija-Riitta Hirvonen, PhD,[g] Jean-Charles Dalphin, MD, PhD,[h] Marie-Laure Dalphin, MD,[h] Josef Riedler, MD,[i] Erika von Mutius, MD, MSc,[j] Juliane Weber, MD,[j] Michael Kabesch, MD,[k] Sven Michel, MSc,[k] Charlotte Braun-Fahrländer, MD,[a,b] Roger Lauener, MD,[c,l] and the PASTURE study group‡ *Basel, Zurich, and Davos-Wolfgang, Switzerland, Ulm, Munich, and Hannover, Germany, Kuopio, Finland, Besancon, France, and Schwarzach, Austria*

Background: There is evidence that gene expression of innate immunity receptors is upregulated by farming-related exposures.
Objective: We sought to determine environmental and nutritional exposures associated with the gene expression of innate immunity receptors during pregnancy and the first year of a child's life.
Methods: For the Protection Against Allergy: Study in Rural Environments (PASTURE) birth cohort study, 1133 pregnant women were recruited in rural areas of Austria, Finland, France, Germany, and Switzerland. mRNA expression of the Toll-like receptor (TLR) 1 through TLR9 and CD14 was assessed in blood samples at birth (n = 938) and year 1 (n = 752). Environmental exposures, as assessed by using questionnaires and a diary kept during year 1, and polymorphisms in innate receptor genes were related to gene expression of innate immunity receptors by using ANOVA and multivariate regression analysis.
Results: Gene expression of innate immunity receptors in cord blood was overall higher in neonates of farmers (*P* for multifactorial multivariate ANOVA = .041), significantly so for *TLR7* (adjusted geometric means ratio [aGMR], 1.15; 95% CI, 1.02-1.30) and *TLR8* (aGMR, 1.15; 95% CI, 1.04-1.26). Unboiled farm milk consumption during the first year of life showed the strongest association with mRNA expression at year 1, taking the diversity of other foods introduced during that period into account: *TLR4* (aGMR, 1.22; 95% CI, 1.03-1.45), *TLR5* (aGMR, 1.19; 95% CI, 1.01-1.41), and *TLR6* (aGMR, 1.20; 95% CI, 1.04-1.38). A previously described modification of the association between farm milk consumption and *CD14* gene expression by the single nucleotide polymorphism *CD14/C-1721T* was not found.
Conclusion: Farming-related exposures, such as raw farm milk consumption, that were previously reported to decrease the risk for allergic outcomes were associated with a change in gene expression of innate immunity receptors in early life. (J Allergy Clin Immunol 2012;130:523-30.)

Key words: *Innate immunity, Toll-like receptors, CD14, prenatal, childhood, farming, farm milk, nutrition*

Innate immunity is the pivotal system that facilitates interactions with microbes at the interfaces of an organism with the environment.[1] These immune responses are mediated in large part by Toll-like receptors (TLRs) and CD14, a group of transmembrane and intracellular proteins that recognize pathogen-associated molecular patterns.[1,2]

The development of innate immunity is determined based on genetic and environmental factors and possibly a combination of both. Environmental exposures rich in microbes encountered during pregnancy or early life have been shown to be associated with upregulating mRNA expression of innate immunity receptors[3,4] and with a decreased risk for allergic diseases.[5,6] Variations in innate immunity receptor genes can influence the mRNA expression of these genes[7] or receptor-mediated cytokine production and have been shown to be associated with asthma and allergic disease.[1,7-11] Furthermore, it has been reported that polymorphisms in innate immunity receptor genes modified the effect of environmental exposure, such as contact with animals or farm

From [a]the Swiss Tropical and Public Health Institute, Basel; [b]the University of Basel; [c]the University of Zürich, Children's Hospital and the Christine Kühne Center for Allergy Research and Education; [d]the Institute of Epidemiology and Medical Biometry, Ulm University; [e]the Department of Environmental Health, National Institute for Health and Welfare, Kuopio; [f]Public Health and Clinical Nutrition, the University of Eastern Finland, Kuopio; [g]the Department of Environmental Science, Inhalation Toxicology Laboratory, the University of Eastern Finland, Kuopio; [h]Université de Franche-Comté, Department of Respiratory Disease, University Hospital, Besancon; [i]the Children's Hospital Schwarzach, Schwarzach; [j]LMU Munich, University Children's Hospital, Munich; [k]the Clinic for Paediatric Pneumology and Neonatology, Hannover Medical School; and [l]the Christine Kühne Center for Allergy Research and Education, Hochgebirgsklinik Davos, Davos-Wolfgang.

*These authors contributed equally to this work.

‡Members of the Protection Against Allergy: Study in Rural Environments study group are listed in this article's acknowledgements section.

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Corresponding author: Georg Loss, MSc, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, 4002 Basel, Switzerland. E-mail: georg.loss@unibas.ch.

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Abbreviations used
aGMR: Adjusted geometric means ratio
Ct: Cycle threshold
MANOVA: Multivariate ANOVA
PASTURE: Protection Against Allergy: Study in Rural Environments
SNP: Single nucleotide polymorphism
TLR: Toll-like receptor

milk consumption, on allergic disease occurrence.[7,11,12] However, these previous investigations were limited to cross-sectional analyses and often lacked reproducibility.[13]

Studies also found that introduction of complementary foods, such as fish and cow's milk, or the diversity of foods introduced early was inversely related to allergic outcomes, proposing that exposure to a variety of antigens, including but not limited to nutritional sources, early in life might be essential for the development of immune tolerance.[14,15]

The development of asthma and allergic disease might be mediated by the innate immune system and its orchestration of complex immune cascades.[1] The first stages of life seem critical for the maturation of the innate immune system,[16] but little is known about the development of gene expression over time and the relevant environmental exposures influencing it.

The Protection Against Allergy: Study in Rural Environments (PASTURE) study[17] offered the opportunity to prospectively investigate the development of gene expression of innate immunity receptors from birth to year 1, taking into account polymorphisms in receptor genes, and to analyze the environmental and nutritional exposures influencing gene expression.

METHODS

Study population

PASTURE is a large prospective birth cohort study conducted in rural areas of Austria, Finland, France, Germany, and Switzerland. The study team contacted 2871 women, of whom 1772 (61.7%) were identified as eligible for participation (Fig 1). Potential participating families were contacted in the third trimester of pregnancy. Exclusion criteria were living on a farm without livestock, maternal age of less than 18 years, premature delivery, genetic disease in the offspring, no telephone connection, and insufficient knowledge of the country's language. Women living on a farm where livestock was held or whose partners actively run a farm were considered farming women. A subset of the population living in close neighborhoods in likewise rural environments not occupationally involved in farming activities were selected as the comparison group (nonfarmers). For more details, see the Methods section in this article's Online Repository at www.jacionline.org.

Those 1133 (63.9%) subjects willing to participate were included in the study (530 farming and 603 nonfarming women). For mRNA analyses, 938 (82.3%) cord blood samples and 752 (72.8%) blood samples from year 1 were available. The study population and the populations with available mRNA measurements at birth and year 1 did not differ in respect to farming status, but slightly more Finnish than French women provided blood samples. No differences were seen with respect to age of pregnant mothers; educational level; number of older siblings; smoking status; pet ownership; family history of asthma, hay fever, and eczema; or prevalence of farm milk consumption.

Questionnaires

Extensive questionnaires were administered by means of interview to the mother of the child within the third trimester of pregnancy and 2 and 12 months after the birth of the study child. Questions were based on previously published studies[18-21] and designed to assess respiratory and other health issues of the mother, agricultural exposures, and potential confounders, such as active and passive smoking, parental education, and family size. In addition to the extensive questionnaires, the mothers kept a weekly diary from month 3 to year 1 of the child's life to record, among other items, the introduction of a variety of food items. Relevant pregnancy variables were farming (living on a farm vs not), maternal farm work (mother working on a farm during pregnancy), contact with a stable/barn (stay in stable/barn during pregnancy at least 15 minutes per week in 1 trimester), contact with a number of farm animals (horse, cow, pig, and poultry: 0, 1-2, or 3-4), maternal/paternal history of asthma or hay fever (doctor's diagnosis and self-reported symptoms for both outcomes), smoking during pregnancy (in any trimester), and farm milk consumption during pregnancy (never, only boiled farm milk, or any unboiled farm milk). Variables during the first year were farming (child living on a farm during first year of life), regular visits to a farm, regular stays in a stable/barn (child stayed in stable/barn at least 15 minutes per week), smoking during lactation, duration of breast-feeding (never, ≤3 months, 3-6 months, or >6 months), duration of exclusive breast-feeding (never, ≤3 months, or >3 months), and child's farm milk consumption (never, only boiled farm milk, or any unboiled farm milk during year 1).

Measurement of mRNA expression in cord blood and at year 1

Blood samples were collected from the umbilical cord at birth and at the age of 1 year. For the assessment of mRNA, the blood was collected in a PAXgene Blood RNA tube containing an RNA-stabilizing solution (PreAnalytiX/Qiagen, Hilden, Germany) and then frozen to −80°C within 24 hours.[22] At the central laboratory of the Children's Hospital of Zurich, the RNA was isolated with the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The mRNA was reverse transcribed into cDNA by using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif). Quantitative real-time PCR was performed on the 7900HT Fast Real-Time PCR System using the Micro fluidic card TaqMan Array system (Applied Biosystems). The data presented are normalized values for the endogenous controls (*18S* rRNA and β_2-microglobulin *[B2M]*) by using the comparative (ΔΔ cycle threshold [ΔΔCt]) method, according to the manufacturer's instructions (Applied Biosystems). *TLR3* expression was excluded from the analyses because the expression level was less than the detection limit in most of the cord blood samples. Extensive quality control measures have been incorporated in the PASTURE cohort study, particularly for laboratory work but also for field work.[17] Genotyping was described in detail elsewhere.[11] For detailed methods, see the Methods section in this article's Online Repository.

Statistical analyses

Differences in environmental and farming characteristics between farmers and nonfarmers in pregnancy (mothers) and during year 1 (children) were tested by using the Pearson χ^2 test.

To quantify the results obtained by using real-time RT-PCR of mRNA of CD14 and *TLR1*, *TLR2*, and *TLR4* through *TLR9*, the comparative threshold method of Giulietti et al[23] was used. This method expresses the measured number of PCR cycles of the participants relative to 1 participant. We chose a nonfarmer with results of greater than the detection limit for all mRNA measurements in cord blood as a reference. The results provide a multiple of amount of mRNA in comparison with the reference. Because the distribution of the gene expression levels were skewed, the calculated variables were log transformed (natural logarithm), resulting in an approximately normal distribution.

The transformed data were used in linear regression models to calculate associations between mRNA expression and single nucleotide polymorphisms (SNPs) in innate immunity genes and exposures in pregnancy and the first year of life (expressed as geometric mean ratios and *P* values). Maternal exposure during pregnancy and child's exposure in the first year of life were combined to also test the effects of continued exposure of farming, farm milk consumption, contact with a stable, contact with pets, and smoking on mRNA expression at age 1 year. A solid food score was developed to test the influence

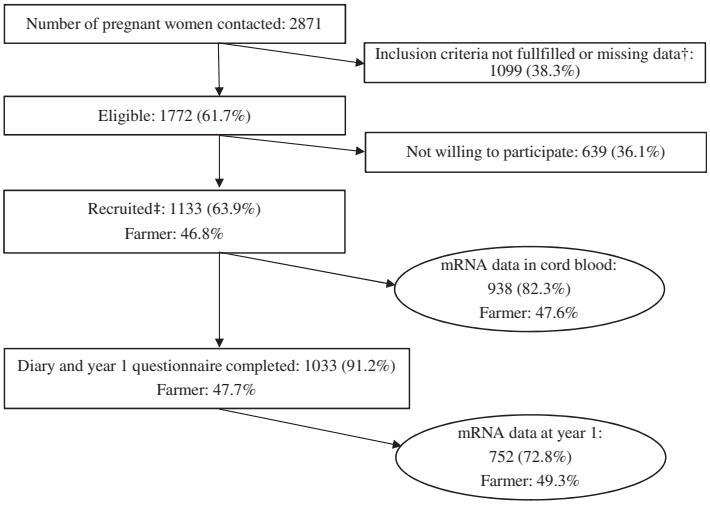

FIG 1. Selection of PASTURE study population and participants for mRNA analyses. †Inclusion criteria: living on a farm with livestock, maternal age greater than 18 years, term delivery, no genetic disease in offspring, telephone connection, and sufficient knowledge of the country's language. ‡Selection criteria: pregnancy questionnaire was completed.

of early introduction of a variety of solid foods on mRNA expression. The time of first food introduction was subdivided into 4 periods (introduced in months 3-6, 7-9, or 10-12 or never in the first year of life). Early introduction of a food item was defined as the period when at least 25% of the children received the respective food to generate dichotomous variables with sufficient numbers for analysis. The crude association of early introduction of each solid food item with mRNA expression was then tested and, if significant for at least 1 receptor, added to the solid food score (the final score included yogurt, butter, vegetable, fruit, meat, nut, fish, chocolate, and cereal with and without gluten).

Multivariate models were developed as follows. Pregnancy exposures were related to mRNA expression in cord blood, and exposures during year 1 were related to expression at year 1 in crude regression models. Maternal history of asthma or hay fever, sex, and center were chosen as covariates *a priori* and were included in all multivariate analyses (paternal history was also tested but did not change models). Variables significantly associated with mRNA expression of 2 or more receptor genes or significantly associated with expression of 1 receptor gene and significantly associated with mRNA expression in simple multivariate analysis of variance (MANOVA) were included in a final model: the pregnancy model included maternal smoking during pregnancy and farming (unboiled farm milk consumption was not included because of colinearity with farming), and the year 1 model included maternal smoking during breast-feeding, education, the solid food score, child's farm milk consumption, and duration of breast-feeding. Heterogeneity between centers was tested by means of meta-analytic techniques. If heterogeneity was present, final models were additionally adjusted for center with a random effect estimate, and if not, center was included as a fixed effect.

To avoid spurious findings because of testing multiple TLRs, the overall association of exposures on mRNA expression was additionally calculated in a MANOVA, adjusting for the same covariates as the regression models. MANOVAs provided omnibus tests that inherently correct for multiple comparisons. Levels of significance in all ANOVAs were evaluated based on Wilks lambda. To assess the development of mRNA expression from birth to year 1, the difference of normalized mRNA expression from cord blood and year 1 was calculated (diff-mRNA), and regression models were developed accordingly. Finally, interaction terms were included in the final models to test for gene-environment interactions of child's farm milk consumption and all assessed genetic variations of innate immune receptors on the effect on mRNA expression. All statistical analyses were performed with STATA/SE 10.1 software (StataCorp, College Station, Tex), and *P* values of less than .05 were considered significant.

Ethical approval

The ethical boards of the 5 study centers approved the study, and written informed consent was obtained from the children's parents for questionnaires, blood samples, and genetic analyses.

RESULTS

Farming mothers were significantly more exposed to stables, barns, and farm animals and more often consumed farm milk during pregnancy than nonfarming mothers (Table I). Parental history of hay fever or asthma and maternal smoking was more common among nonfarmers, whereas farm families more often kept a cat or dog and tended to have a higher number of children. A higher proportion of nonfarmers breast-fed for longer than 6 months, and they were less likely to never breast-feed. Similar results were found for exclusive breast-feeding. At year 1, farm and nonfarm children showed similar differences in environmental exposures as observed for their mothers during pregnancy. More farmers introduced unboiled farm milk within the first year of life (29.0%) compared with nonfarmers (4.5%), and they introduced a higher number of different solid foods early within the first year of life.

Pregnancy exposures

In univariate analyses maternal farming in pregnancy was significantly positively associated with cord blood mRNA expression of several receptor genes and in a simple MANOVA (Table II). Significant and positive associations were also found for maternal consumption of unboiled farm milk; however, this variable was strongly correlated with farming. Smoking during pregnancy and male sex decreased the expression of the receptor genes. Multifactorial MANOVA showed that mRNA expression was significantly greater in neonates of farmers compared with that seen in neonates of nonfarmers ($P = .041$). For individual receptor genes, a significantly higher mRNA expression was found in farmers for *TLR7* (adjusted geometric means ratio [aGMR], 1.15; 95% CI, 1.02-1.30; $P = .021$) and *TLR8* (aGMR, 1.15; 95% CI, 1.04-1.26; $P = .005$; Fig 2).

TABLE I. Environmental and farming characteristics of pregnant women and children in the first year of life by farming status

	Farmer, no. (%)		Nonfarmer, no. (%)		*P* value for difference
	No.	Percent	No.	Percent	
Population at birth	530	46.8	603	53.2	
Male sex	266	51.4	294	51.4	.988
Center					
Austria	105	47.7	115	52.3	.389
Switzerland	107	44.2	135	55.8	
France	94	46.3	109	53.7	
Germany	112	44.1	142	55.9	
Finland	112	52.3	102	47.7	
Education					
Low	116	21.9	86	14.3	<.001
Medium	234	44.2	253	42.0	
High	180	34.0	264	43.8	
Maternal history of asthma	38	7.2	61	10.1	.080
Maternal history of hay fever	108	20.4	196	32.5	<.001
Maternal farming exposure during pregnancy*					
Contact with stable	464	89.6	107	18.9	<.001
Contact with barn	362	70.0	65	11.5	<.001
Contact with >2 farm animals	208	39.2	64	10.7	<.001
Contact with cats and/or dogs	430	81.3	233	38.6	<.001
Farm milk consumption	406	76.6	98	16.3	<.001
Only boiled farm milk	94	17.8	27	4.5	<.001
Any unboiled farm milk	310	58.7	70	11.6	
Smoking	46	8.7	112	18.6	<.001
Child's farming exposure during first year of life*†					
Population at year 1	493	47.7	540	52.3	
Child living on a farm	486	98.6	10	1.9	<.001
Regular visit to farm	487	99.0	77	14.4	<.001
Regular stay in stable	332	71.7	40	7.6	<.001
Contact with cats and/or dogs	402	81.5	188	34.8	<.001
Farm milk consumption	283	57.8	51	9.5	<.001
Only boiled farm milk	141	28.8	27	5.1	<.001
Any unboiled farm milk	142	29.0	24	4.5	
Unboiled farm milk after month 10	78	16.0	15	2.8	<.001
Unboiled farm milk before month 10	60	12.3	8	1.5	
Early introduced solid food items (food score)					
0	51	10.3	102	18.9	<.001
1-3	191	38.7	214	39.6	
4-6	174	35.3	157	29.1	
7-11	77	15.6	67	12.4	
Smoking during breast-feeding	18	(3.8)	35	(6.8)	.034
Any breast-feeding					
>6 mo	240	48.7	285	52.8	.027
3-6 mo	118	23.9	96	17.8	
≤3 mo	89	18.1	120	22.2	
Never	46	9.3	39	7.2	
≥2 Siblings	235	47.7	111	20.6	<.001

*There are minor discrepancies in percentages because of missing values in variables.
†Percentage of population at year 1.

Exposures during the first year of life

Children's consumption of unboiled farm milk during the first year of life showed the strongest association with mRNA expression at year 1, upregulating mRNA expression of *CD14*, *TLR4*, *TLR5*, *TLR6*, and *TLR7* when compared with no farm milk consumption, whereas other farming-related exposures during year 1 showed no significant associations (Table III). Early introduction of several food items was associated with mRNA expression of individual receptors (for details, see Table E1 in this article's Online Repository at www.jacionline.org). When summarized as solid food score, an increasing number of items was significantly associated with *TLR4* mRNA expression (Table III).

After adjustment for all potential confounders, mRNA expression of *TLR4* (aGMR, 1.22; 95% CI, 1.03-1.45; $P = .020$), *TLR5* (aGMR, 1.19; 95% CI, 1.01-1.41; $P = .034$), and *TLR6* (aGMR, 1.20; 95% CI, 1.04-1.39; $P = .015$) was statistically significantly upregulated by unboiled farm milk consumption compared with no farm milk consumption (Fig 3), whereas the association of the food score and mRNA expression was no longer significant (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org).

We also examined relations of prenatal exposures to mRNA expression at year 1, but no significant associations were observed (data not shown).

The correlations of mRNA expression of single innate immunity receptors between cord blood and year 1 were poor, with the highest and only significant correlations for *TLR8* (Pearson correlation = 0.35, $P < .001$) and *TLR1* (Pearson correlation = 0.31, $P < .001$). When exposures were related to the difference in mRNA expression between year 1 and cord blood, results were similar to findings with only year 1 expression as the outcome, although they were less pronounced (data not shown). Among the tested continued exposures from pregnancy to age 1 year, only raw farm milk consumption was found to be significantly associated with increased gene expression of 1 receptor *(TLR6)* in unadjusted models.

Polymorphisms in *TLR1*, *TLR4*, *TLR6*, and *TLR8* were significantly associated with gene expression of the respective receptors at birth and similarly at year 1 (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). We also tested whether polymorphisms modified the association between a child's unboiled farm milk consumption and gene expression of innate immunity receptors, yet only 2 SNPs in *TLR8 (TLR8/C9008T)* and *TLR9 (TLR9/T−2622C)* showed significant interactions (P for both interactions = .007).

DISCUSSION

This study shows that farming status of pregnant mothers was associated with increased gene expression of innate immunity receptors at birth (overall and individually with *TLR7* and *TLR8*), whereas increased gene expression at year 1 was most strongly associated with child's consumption of raw farm milk during the first year of life (*TLR4*, *TLR5*, and *TLR6*). Several genetic variations in genes of the innate immunity receptors were associated with expression of the respective receptors, but only 2 SNPs in *TLR8* and *TLR9* significantly modified the association of unboiled farm milk and mRNA expression of the respective receptor at year 1. Changes in gene expression of innate immunity receptors caused by farming exposure and unboiled farm milk consumption might be involved in explaining the reported protective effects of farming-related exposures on the development of allergic disease in children.[5,6,21]

In contrast to previous cross-sectional studies,[3,4] the present analyses allowed us to prospectively relate a variety of exposures during pregnancy and the first year of life to the expression of innate immunity genes. Early life is a critical time window because

TABLE II. Crude association* of exposures during pregnancy and mRNA expression at birth (n = 938, only significant associations are shown)

Exposure during pregnancy	mRNA expression, GMR (95% CI)									*P* value of simple MANOVA
	CD14	*TLR1*	*TLR2*	*TLR4*	*TLR5*	*TLR6*	*TLR7*	*TLR8*	*TLR9*	
Farming			1.08† (1.00-1.16)		1.09† (1.00-1.18)		1.17† (1.04-1.31)	1.16‡ (1.06-1.28)		.041
Farm milk consumption										
No										.047
Only boiled farm milk										
Any unboiled farm milk					1.10† (1.01-1.20)			1.14† (1.03-1.26)		
Maternal farm work										.301
Contact with stable								1.11† (1.01-1.22)		.174
Contact with barn										.376
Contact with number of farm animals										
0										.206
1-2										
3-4										
Cats or dogs										.450
Smoking						0.85†,§ (0.75-0.97)		0.86† (0.74-0.99)		.279
Male sex		0.89†,§ (0.81-0.98)	0.90†,§ (0.85-0.98)							.002
Center										<.001

*Geometric mean ratios *(GMRs)* and 95% CIs were calculated by using regression models.
†$P < .05$.
‡$P < .01$.
§Associations were also significant after farming adjustment.

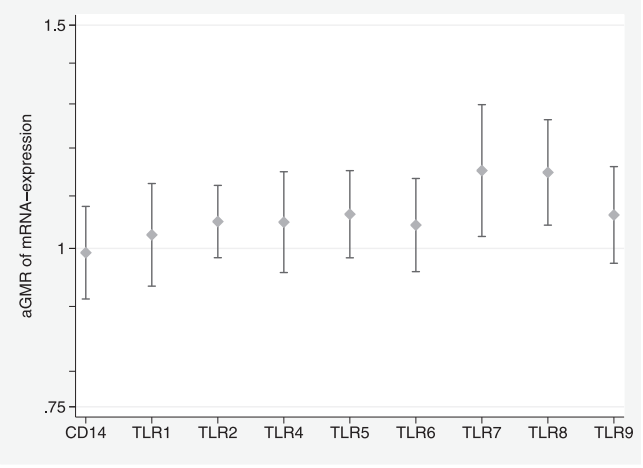

FIG 2. Adjusted association of farming/nonfarming during pregnancy and mRNA expression at birth expressed as geometric mean ratios and 95% CIs adjusted for maternal history of asthma or hay fever, sex, center (random), and maternal smoking during pregnancy. Fully adjusted multifactorial MANOVA of mRNA expression and farming *P* value = .041.

it has been shown that the neonatal TLR system undergoes rapid and differential development during the first month of life.[24]

Maternal involvement in farming during pregnancy was an exposure associated with expression of several innate immunity receptors in cord blood, whereas specific activities, such as working in stables or barns or contact with farm animals, were not. Maternal farming might thus be an overall indicator of other activities, including consumption of farm milk during pregnancy. In previous cross-sectional studies[3,4] higher gene expression of innate immunity receptors at school age in farm compared with nonfarm children has clearly been observed and might reflect continuous exposure of children growing up on a farm over several years. However, the previously reported association between prenatal farm animal exposure and innate immunity gene expression[12] was not found in this prospective study. The gene expression of individual receptors significantly upregulated by farming varied between studies,[3] likely reflecting the different composition of the respective environments. TLR-binding ligands were mainly ascribed to microbial origin.[1] Recent evidence suggests that the diversity of the microbial environment and not individual microbes is important to confer protection against asthma,[5] and TLR-mediated innate response pathways are believed to be important in promoting regulatory pathways that inhibit the allergic immune response.[25] Nonmicrobial ligands were recently shown to trigger TLR4 signaling. The major house dust mite allergen Der p 2 functionally mimicked MD-2, the LPS binding component of TLR4, triggering TLR4 signaling in the absence of MD-2,[26] whereas the heavy metal Ni^{2+} directly activated human TLR4.[27]

The expression of TLRs and CD14 at birth and at year 1 was not closely correlated, suggesting that environmental exposures encountered by the infant induce substantial changes in innate immunity during the first year of life. Food is a main source of the infant's new exposure during early life. Breast-feeding has recently been shown to modulate innate immunity responses during the neonatal period.[16] In the present study the infant's consumption of raw farm milk during the first year of life was the exposure most strongly upregulating the expression of TLRs, whereas duration of breast-feeding had no significant effect. Also, the child's contact with stables, farm animals, or pets was not associated with receptor gene expression. It is possible

TABLE III. Crude association* of exposures during the first year of life and mRNA expression at year 1 (n = 752)

Exposure during first year of life	mRNA expression, GMR (95% CI)									*P* value of simple MANOVA
	CD14	*TLR1*	*TLR2*	*TLR4*	*TLR5*	*TLR6*	*TLR7*	*TLR8*	*TLR9*	
Farming										.947
Farm milk consumption										
No										.079
Only boiled farm milk										
Any unboiled farm milk	1.15† (1.02-1.31)			1.27‡ (1.09-1.47)	1.19† (1.04-1.37)	1.24§ (1.10-1.40)	1.24‡ (1.06-1.46)			
Regular visit to farm										.848
Regular stay in stable										.943
Cats or dogs										.772
Smoking during lactation	1.24† (1.00-1.54)			1.29† (1.00-1.67)						.406
Duration of breast-feeding										
>6 mo										.198
3-6 mo	1.15† (1.02-1.29)									
≤3 mo										
Never									0.77† (0.62-0.97)	
Exclusive breast-feeding										
>3 mo										.229
≤3 mo										
Never									0.75† (0.60-0.94)	
No. of solid food items introduced early										
0										.018
1-3										
4-6				1.25‡ (1.06-1.48)						
7-11				1.27† (1.04-1.54)						

All significant associations are shown.
*Geometric mean ratios *(GMRs)* and 95% CIs were calculated by using regression models.
†$P < .05$, ‡$P < .01$, and §$P < .001$: all associations with gene expression of individual receptors were also significant after farming adjustment.

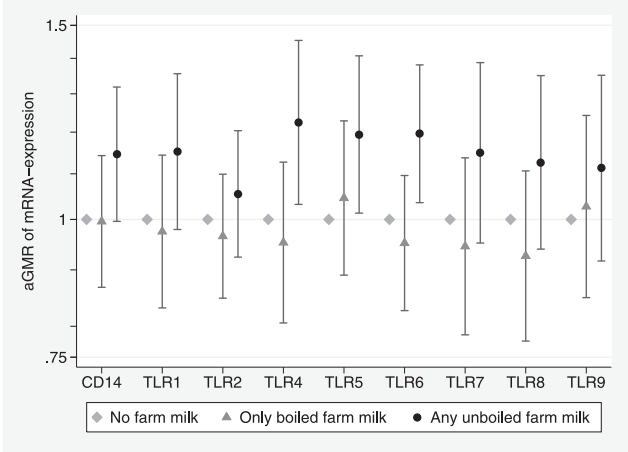

FIG 3. Adjusted association of child's farm milk consumption during first year of life and mRNA expression at year 1 expressed as geometric mean ratios and 95% CIs adjusted for farming, maternal history of asthma or hay fever, sex, center, maternal smoking during breast-feeding, solid food score, education, and duration of breast-feeding.

that the intensity of exposure to stable microbes is too low to increase expression of innate immunity receptors as long as children do not walk around themselves in stables or barns.

Raw farm milk is rich in (mostly) nonpathogenic microorganisms.[6,28] It is conceivable that the microorganisms ingested with raw milk influence the composition of the gut flora and stimulate expression of innate immunity receptor genes.[29] Whether increased expression of TLRs associated with raw milk consumption reflects a relevant pathway underlying allergic disease development or whether it is merely an indicator of exposure to microbes remains an open question. A recent cross-sectional study found whey protein levels but not the actual counts of bacteria in farm milk to be associated with less asthma in children.[6] However, the actual level of microorganisms in farm milk consumed at school age might not be a precise indicator of the child's regular exposure to a diversity of microorganisms in raw milk, which could be involved in inducing tolerance and protecting against chronic inflammation.[29]

Several SNPs in innate immunity receptor genes were reported to modify the risk for asthma or atopy, but results were often not reproducible.[1] Bieli et al[7] described a modification of the association between farm milk consumption at school age and *CD14* gene expression by the SNP *CD14/C-1721T*; however, this could not be confirmed in the present study, in which modifications by SNPs in *TLR8* and *TLR9* were found. Given the many SNPs tested, spurious findings cannot be excluded, and further investigations are needed. A recent genome-wide association study could not reproduce previously

reported interactions of SNPs and farming-related factors on allergic outcomes in childhood and concluded that common genetic polymorphisms are unlikely to modify the protective influence of the farming environment on childhood asthma and atopy.[13] A similar conclusion might apply to interactions of SNPs and farming-related factors on gene expression of innate immunity receptors, given the low reproducibility of such results.

As a further limitation in interpreting the results presented in this article, it should be considered that mRNA was measured in whole-blood samples, and the observed effects cannot be ascribed to a distinct cell type.

The farming-related factors that we identified to alter gene expression of innate immunity genes (farming and raw farm milk consumption) were also associated with a decreased risk for allergic disease in previous studies.[6,30] Regulation of the innate immune system might be relevant to explain the protective effects of these microbe-rich farming exposures. The present study does not allow us to answer whether the upregulation of innate immune receptors directly modulates the development of allergic disease or whether it is a marker for the effect of genes and the environment on allergic disease. Future analyses of this cohort will allow us to disentangle whether the timing of exposure or repeated exposure are more relevant in inducing the protective effects observed in cross-sectional studies at school age.

Farming-related exposures, such as raw farm milk consumption, that were previously reported to decrease the risk for allergic outcomes were associated with a change in gene expression of innate immunity receptors in early life.

The PASTURE study group members (in alphabetical order by study center):

Anne Hyvärinen,[a] Anne M. Karvonen,[a] Sami Remes,[b] Pekka Tiittanen[a] (Finland); Vincent Kaulek[c] (France); Gisela Büchele,[d] Martin Depner,[e] Markus Ege,[e] Petra Pfefferle,[f] Harald Renz,[f] Bianca Schaub[e] (Germany); and Gert Doekes[g] (The Netherlands)

From [a]the Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland; [b]the Department of Pediatrics, Kuopio University Hospital, Kuopio, Finland; [c]the Department of Respiratory Disease, Université de Franche-Comté, University Hospital, Besancon, France; [d]the Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany; [e]LMU Munich, University Children's Hospital, Munich, Germany; [f]the Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany; and [g]the Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht University, Utrecht, The Netherlands.

Key messages

- **Farming status of pregnant mothers was associated with increased gene expression of innate immunity receptors at birth (overall and individually with *TLR7* and *TLR8*).**
- **The child's consumption of raw farm milk during the first year of life was associated with increased gene expression of innate immunity receptors at year 1 (*TLR4*, *TLR5*, and *TLR6*).**
- **A previously described modification of the association between farm milk consumption and *CD14* gene expression by the SNP *CD14/C-1721T* was not found.**

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METHODS

Study population

PASTURE is a large prospective birth cohort study conducted in rural areas in Austria, Finland, France, Germany, and Switzerland. The study team contacted 2871 women, of whom 1772 (61.7%) were identified as eligible for participation. Initially, farming and nonfarming pregnant women living in the 5 rural areas were identified in the third trimester of pregnancy. Contact with pregnant women was achieved either by study staff visiting birth preparatory courses (Austria and Germany); by lists of pregnant women received from hospitals (Finland) or insurance companies (France); by advertising in hospitals, doctors' offices, and shops in rural areas (Switzerland); or by involving the midwives in distributing study information material at any type of contact they had with pregnant women. Furthermore, articles in regional newspapers and farmers' journals and on the Internet, as well as spots on the radio or television, were used to make the project better known among the rural population.

Pregnant women contacted were asked to fill in a short recruitment questionnaire, assessing eligibility and possible nonparticipation bias. Eligible women were then contacted by telephone and asked to participate.

To be eligible, pregnant women had to fulfill the following inclusion criteria:

A. A woman was considered to be a "farming woman" if at the time of recruitment she and her family lived on a farm where livestock was held. No distinction was made between full-time and part-time farmers. This criterion was also fulfilled when a family lived on a farm only as a tenant without being involved in farm work at all. If the family had moved away from the farm by the time of the child's birth, the status of the family was changed to "nonfarmer." Because it was clear from the beginning that the number of farming women included in the study would not be very high (only approximately 400-500), the type of farming was restricted as far as possible, and crop farms, for example, were not included to avoid too much heterogeneity in exposure, which could lead to small numbers in any subgroup analysis.
B. The nonfarming women lived in the same areas as the farming women and were recruited at the same hospitals. To reduce differences in other lifestyle factors, they were not taken from an urban hospital (eg, Munich or Salzburg). As a limit for the size of the town in which a nonfarming participant could live, women were included from towns of less than 30,000 inhabitants only. However, women from smaller towns but with relevant (heavy) industry were not recruited either. In addition, in the Bavarian study center, where the large city of Munich was quite near and easy to reach, all families in which either the mother or the father travelled to Munich every day for work (commuters) were not considered eligible.
C. Farming families not living on a farm where livestock was held (or running such a farm) but just on a farm where exclusively poultry was held or on an exclusive crop farm were not considered eligible either as farmers or as nonfarmers (intermediate exposure).

In addition, the following exclusion criteria were defined:

- women less than 18 years of age;
- twin pregnancy/siblings of a child already included in the study;
- mother who intended delivery at home;
- families who intended to move away from the area where the study was done;
- families without telephone connection; and
- insufficient knowledge of the country's language.

Furthermore, after delivery, the following participants were excluded:

- premature delivery (before the 37th week of pregnancy, n = 14) and
- serious genetic illnesses (eg, Down syndrome; n = 2).

Those 1133 (63.9%) subjects willing to participate were included in the study (530 farming and 603 nonfarming women). For mRNA analyses, 938 (82.3%) cord blood samples and 752 (72.8%) blood samples of year 1 were available. The study population and the populations with available mRNA measurements at birth and year 1 did not differ in respect to farming status, but slightly more Finnish than French women provided blood samples. No differences were seen with respect to age of pregnant mothers; educational level; number of older siblings; smoking status; pet ownership; family history of asthma, hay fever, and eczema; or prevalence of farm milk consumption.

Questionnaires

Extensive questionnaires were administered by interview to the mother of the child within the third trimester of pregnancy and 2 and 12 months after birth of the study child. Questions were based on previously published studies[E1-E4] and designed to assess respiratory and other health issue of the mother, agricultural exposures, and potential confounders, such as active and passive smoking, parental education, and family size. In addition to the extensive questionnaires, the mothers kept a weekly diary from month 3 to year 1 of the child's life to record, among other things, the introduction of a variety of food items. Relevant pregnancy variables were farming (living on a farm vs not), maternal farm work (mother working on a farm during pregnancy), contact with a stable/barn (stay in stable/barn during pregnancy at least 15 minutes per week in 1 trimester), contact with a number of farm animals (horse, cow, pig, or poultry: 0, 1-2, or 3-4), maternal/paternal history of asthma or hay fever (doctor's diagnosis and self-reported symptoms for both outcomes), smoking during pregnancy (in any trimester), and farm milk consumption during pregnancy (never, only boiled farm milk, or any unboiled farm milk). Variables during the first year were farming (child living on a farm during first year of life), regular visit to a farm, regular stay in a stable/barn (child stayed in stable/barn at least 15 minutes per week), smoking during lactation, duration of breast-feeding (never, $\leq$3 months, 3-6 months, or >6 months), duration of exclusive breast-feeding (never, $\leq$3 months, or >3 months), and child's farm milk consumption (never, only boiled farm milk, or any unboiled farm milk during year 1). The time of exposure to stables or barns was assessed by questionnaire in days per week and minutes per day for farmers and in hours per month for nonfarmers. This separate information was combined in a variable for both farmers and nonfarmers (time of exposure in minutes per week). A mother or child staying in a stable/barn for at least 15 minutes per week was defined as exposed. The cutoff (at least 15 minutes per week) was based on the distribution of exposure in the whole population to provide sufficient numbers of exposed subjects for statistical models.

Measurement of mRNA expression in cord blood and at year 1

Blood samples were collected from the umbilical cord at birth and at age 1 year. For the assessment of mRNA, the blood was collected in a PAXgene Blood RNA tube containing an RNA-stabilizing solution (PreAnalytiX/Qiagen) and then frozen to -80°C within 24 hours.[E5] At the central laboratory of the Children's Hospital of Zurich, the RNA was isolated with the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The concentration and purity of the RNA was assessed by Nanodrop (Thermo Scientific, Waltham, Mass). Samples with a concentration of at least 20 ng/μL and a purity quotient (absorbance at 260 nm/absorbance at 280 nm) of between 1.8 and 2 were used for quantitative real-time PCR. Immediately after RNA isolation, it was reverse transcribed into cDNA by using the TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed on the 7900HT Fast Real-Time PCR System with the Micro fluidic card TaqMan Array system (Applied Biosystems). The data presented are calculated by using the comparative ($\Delta\Delta$Ct) method according to the manufacturer's instructions (Applied Biosystems).[E6,E7] In brief, the Ct values of the target genes were normalized to the geometric mean of the housekeeping genes *18S* rRNA and β_2-microglobulin *(B2M)* to normalize the PCR for the amount of RNA added to the reaction because there is variability in quantification and reverse transcription (ΔCt). In a second step a nonfarming child was used as a reference to assess relative quantification describing the change in expression of a target gene (eg, *TLR2*) of a distinct child relative to the expression of the same gene of the reference child ($\Delta\Delta$Ct). To

get the numeric value, we then used the $2^{-\Delta\Delta Ct}$ calculation. *TLR3* expression was excluded from the analyses because the expression level was less than the detection limit in most of the cord blood samples. Extensive quality control measures have been incorporated in the PASTURE cohort study, particularly for laboratory work but also for field work.[E8]

Genotyping

Genotyping was performed by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as described previously.[E9] Derived genotype frequencies were compared with the expected allelic population equilibrium based on the Hardy-Weinberg equilibrium test to control for technical genotyping errors. cDNA was amplified in duplicate by using an iCycler (Bio-Rad Laboratories, Hercules, Calif), with *18S* as a reference gene. Polymorphisms in TLRs were selected as previously described.[E10] These SNPs were as follows: *TLR1/C−2299T* (rs5743594), *TLR1/T−2192C* (rs5743595), *TLR1/A742G* (rs4833095), *TLR2/T1349C* (rs3804100), *TLR2/T596C* (rs3804099), *TLR2/T−16934A* (rs4696480), *TLR4/C8851T* (rs4986791), *TLR4/A8551G* (rs4986790), *TLR4/G−2570A* (rs2737190), *TLR4/T−1607C* (rs10759932), *TLR5/A1774G* (rs2072493), *TLR5/T1845C* (rs5744174), *TLR5/C1173T* (rs5744168), *TLR6/T−1928C* (rs5743792), *TLR6/T−2079A* (rs5743789), *TLR7/A17961T* (rs179008), *TLR7/C12318T* (rs1620233), *TLR8/C10907A* (rs3747414), *TLR8/C9008T* (rs2159377), *TLR8/A−4824G* (rs3761624), *TLR9/T-2622C* (rs5743836), *TLR9/T−2871C* (rs187084), and *CD14/C-1721T* (rs2915863).

Statistical analyses

The overall association of exposures on mRNA expression was additionally calculated in MANOVA (adjusting for the same covariates as the regression models) to avoid spurious findings caused by testing multiple TLRs. MANOVA provided omnibus tests, which inherently correct for multiple comparisons. MANOVA can protect against type I errors that might occur if multiple ANOVAs were conducted independently. Repeated univariate measures can dramatically increase type I errors. Furthermore, multiple univariate measures do not equal a multivariate measure because they do not take into account colinearity (correlations among dependent variables). Therefore a MANOVA acts as an inherent Bonferroni correction by keeping the experiment-wide probability of making type I error less than 5%. Levels of significance in all ANOVAs were evaluated based on Wilks lambda.

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TABLE E1. Crude association* of food exposures during the first year of life and mRNA expression at year 1 (n = 752)

Early food introduction vs late or never	mRNA expression						*P* value of simple MANOVA
	CD14	*TLR2*	*TLR4*	*TLR6*	*TLR7*	*TLR8*	
Other milk products (exclusive cow's milk)					1.15† (1.02-1.30)		.385
Yogurt			1.18† (1.02-1.37)				.110
Butter	0.89† (0.81-0.98)						.046
Vegetable			1.18† (1.06-1.32)				.033
Fruit			1.16† (1.04-1.29)				.010
Meat	1.13† (1.02-1.25)		1.20‡ (1.07-1.35)		1.20‡ (1.05-1.36)		<.001
Nut		0.82† (0.70-0.96)					.019
Fish			1.16† (1.04-1.29)				.163
Chocolate						1.17† (1.01-1.35)	.692
Cereal (gluten)		0.91† (0.83-1.00)		0.90† (0.81-0.99)			<.001
Cereal (no gluten)	1.13† (1.02-1.26)						.064

All significant associations are shown.
*Geometric mean ratios and 95% CIs were calculated by using regression models.
†$P < .05$ and ‡$P < .01$, all associations with gene expression of individual receptors were also significant after farming adjustment.

TABLE E2. Adjusted associations* of exposures during pregnancy and mRNA expression at birth (n = 938)

	mRNA expression					
Exposure during pregnancy	***TLR1***	***TLR2***	***TLR5***	***TLR6***	***TLR7***	***TLR8***
Farming		1.05 (0.98-1.12)	1.06 (0.98-1.15)		1.15† (1.02-1.30)	1.15‡ (1.04-1.26)
Smoking				0.85† (0.75-0.97)		0.87 (0.76-1.01)
Male sex	0.90† (0.82-0.99)	0.90† (0.85-0.97)				

Results from final adjusted models in Fig 2 are shown for variables with significant crude associations with mRNA expression in Table II.

*Geometric mean ratios and 95% CIs were calculated by using regression models adjusted for maternal history of asthma or hay fever, sex, center (random), and maternal smoking during pregnancy.

†$P < .05$.

‡$P < .01$.

TABLE E3. Adjusted associations* of exposures during first year of life and mRNA expression at year 1 (n = 752)

	mRNA expression					
Exposure during first year of life	***CD14***	***TLR4***	***TLR5***	***TLR6***	***TLR7***	***TLR9***
Farm milk consumption						
No						
Only boiled farm milk						
Any unboiled farm milk	1.15 (1.00-1.32)	1.22† (1.03-1.45)	1.19† (1.01-1.41)	1.20† (1.04-1.38)	1.15 (0.95-1.39)	
Smoking during lactation	1.04 (0.85-1.29)	1.11 (0.86-1.44)				
Duration of breast-feeding						
>6 mo						
3-6 mo	1.10 (0.98-1.24)					
≤3 mo						
Never						0.74† (0.56-0.98)
No. of solid food items introduced early						
0						
1-3						
4-6		1.10 (0.92-1.33)				
7-11		1.10 (0.88-1.37)				

Results from final adjusted models in Fig 3 are shown for variables with significant crude associations with mRNA expression in Table III.
*Geometric mean ratios and 95% CIs were calculated by using regression models adjusted for farming, maternal history of asthma or hay fever, sex, center, maternal smoking during breast-feeding, solid food score, education, and duration of breast-feeding.
†$P < .05$.

TABLE E4. Crude associations of genotypes and mRNA expression in cord blood and at age 1 year

		mRNA expression in cord blood				mRNA expression at age 1 y			
Receptor/SNPS	**Genotype**	**No.**	**GMR**	**95% CI**	***P* value**	**No.**	**GMR**	**95% CI**	***P* value**
TLR1/C−2299T (rs5743594)	CC	597	1.00			483	1.00		
	CT	242	1.21	1.09-1.35	<.001	193	1.19	1.06-1.34	.003
	TT	22	1.41	1.04-1.92	.028	19	1.55	1.13-2.13	.006
TLR1/T−2192C (rs5743595)	TT	547	1.00			453	1.00		
	TC	271	0.68	0.62-0.74	<.001	208	0.61	0.56-0.68	<.001
	CC	45	0.20	0.17-0.25	<.001	38	0.26	0.21-0.32	<.001
TLR1/A742G (rs4833095)	AA	462	1.00			385	1.00		
	AG	337	0.74	0.67-0.81	<.001	263	0.69	0.62-0.76	<.001
	GG	67	0.34	0.29-0.40	<.001	52	0.32	0.27-0.39	<.001
TLR2/T1349C (rs3804100)	TT	746	1.00			601	1.00		
	TC	119	1.01	0.92-1.12	.824	97	0.98	0.88-1.09	.713
	CC	0				1			
TLR2/T596C (rs3804099)	TT	267	1.00			218	1.00		
	TC	422	0.95	0.88-1.03	.225	353	1.00	0.92-1.09	.914
	CC	158	0.94	0.85-1.04	.220	115	1.01	0.90-1.13	.910
TLR2/T−16934A (rs4696480)	AA	239	1.00			202	1.00		
	AT	401	0.93	0.86-1.01	.093	314	1.06	0.97-1.16	.192
	TT	219	0.97	0.89-1.07	.589	182	1.00	0.90-1.10	.938
TLR4/C8851T (rs4986791)	CC	758	1.00			604	1.00		
	CT	106	1.42	1.23-1.64	<.001	94	1.23	1.04-1.44	.013
	TT	4	1.11	0.56-2.19	.762	2	1.40	0.50-3.94	.525
TLR4/A8551G (rs4986790)	AA	762	1.00			611	1.00		
	AG	97	1.43	1.24-1.66	<.001	83	1.29	1.09-1.53	.004
	GG	4	1.11	0.56-2.20	.763	2	1.41	0.50-3.96	.518
TLR4/G−2570A (rs2737190)	GG	89	1.00			71	1.00		
	GA	352	0.86	0.73-1.00	.056	270	1.04	0.85-1.26	.733
	AA	367	0.78	0.66-0.91	.002	294	1.02	0.84-1.25	.816
TLR4/T−1607C (rs10759932)	TT	625	1.00			503	1.00		
	TC	201	0.95	0.85-1.07	.413	171	0.94	0.82-1.07	.336
	CC	26	1.20	0.91-1.58	.198	20	1.21	0.87-1.69	.259
TLR5/A1774G (rs2072493)	AA	610	1.00			503	1.00		
	AG	225	1.01	0.92-1.11	.859	174	0.92	0.82-1.04	.189
	GG	17	1.20	0.90-1.61	.216	11	0.95	0.62-1.45	.814
TLR5/T1845C (rs5744174)	TT	276	1.00			222	1.00		
	TC	426	0.94	0.86-1.03	.214	357	0.88	0.78-0.99	.030
	CC	152	0.90	0.80-1.01	.085	111	0.93	0.79-1.10	.397
TLR5/C1173T (rs5744168)	CC	766	1.00			621	1.00		
	CT	95	0.98	0.86-1.11	.753	76	1.04	0.88-1.23	.629
	TT	4	1.05	0.58-1.89	.883	2	1.80	0.67-4.81	.241
TLR6/T−1928C (rs5743792)	TT	806	1.00			661	1.00		
	TC	50	1.26	1.05-1.52	.014	34	0.88	0.71-1.09	.238
	CC	1				0			
TLR6/T−2079A (rs5743789)	TT	539	1.00			449	1.00		
	TA	264	0.94	0.86-1.04	.227	204	0.90	0.82-1.00	.046
	AA	50	0.75	0.62-0.91	.003	37	0.92	0.75-1.13	.437
TLR7/A17961T (rs179008)	AA	599	1.00			475	1.00		
	AT	137	1.18	1.00-1.41	.054	121	0.97	0.83-1.14	.731
	TT	127	0.89	0.75-1.06	.204	103	0.98	0.82-1.16	.803
TLR7/C12318T (rs1620233)	CC	738	1.00			594	1.00		
	CT	70	1.12	0.89-1.41	.331	63	1.15	0.93-1.42	.202
	TT	52	0.88	0.67-1.14	.325	40	1.12	0.86-1.45	.401
TLR8/C10907A (rs3747414)	CC	468	1.00			361	1.00		
	CA	208	0.98	0.87-1.10	.705	173	0.95	0.82-1.09	.460
	AA	187	0.79	0.69-0.89	<.001	163	0.81	0.70-0.93	.003
TLR8/C9008T (rs2159377)	CC	658	1.00			531	1.00		
	CT	128	0.85	0.74-0.97	.015	105	0.71	0.61-0.83	<.001
	TT	74	0.40	0.34-0.47	<.001	61	0.44	0.36-0.54	<.001
TLR8/A−4824G (rs3761624)	AA	562	1.00			461	1.00		
	AG	181	0.71	0.64-0.79	<.001	143	0.67	0.59-0.76	<.001
	GG	125	0.32	0.29-0.37	<.001	97	0.29	0.25-0.33	<.001

(Continued)

TABLE E4. *(Continued)*

		mRNA expression in cord blood				mRNA expression at age 1 y			
Receptor/SNPS	**Genotype**	**No.**	**GMR**	**95% CI**	***P* value**	**No.**	**GMR**	**95% CI**	***P* value**
TLR9/T−2622C (rs5743836)	TT	662	1.00			532	1.00		
	TC	189	0.94	0.85-1.05	.274	158	1.00	0.87-1.16	.957
	CC	12	0.98	0.67-1.43	.899	10	0.84	0.50-1.41	.504
TLR9/T−2871C (rs187084)	TT	265	1.00			220	1.00		
	TC	437	1.01	0.91-1.12	.870	346	0.97	0.84-1.11	.633
	CC	155	0.95	0.83-1.08	.415	130	1.10	0.91-1.31	.320
CD14/C-1721T (rs2915863)	CC	130	1.00			102	1.00		
	CT	380	0.98	0.86-1.11	.721	296	0.99	0.86-1.14	.898
	TT	294	1.04	0.91-1.19	.553	233	0.98	0.84-1.14	.778

GMR, Geometric mean ratio.

TABLE E5. Adjusted associations of genotypes and mRNA expression in cord blood and at age 1 year

		mRNA expression in cord blood				mRNA expression at age 1 y			
Receptor/SNPS	**Genotype**	**No.**	**aGMR***	**95% CI**	***P* value**	**No.**	**aGMR†**	**95% CI**	***P* value**
TLR1/C−2299T (rs5743594)	CC	597	1.00			483	1.00		
	CT	242	1.22	1.09-1.36	<.001	193	1.19	1.06-1.35	.004
	TT	22	1.46	1.08-1.98	.014	19	1.59	1.16-2.18	.004
TLR1/T−2192C (rs5743595)	TT	547	1.00			453	1.00		
	TC	271	0.68	0.62-0.75	<.001	208	0.62	0.56-0.69	<.001
	CC	45	0.21	0.17-0.25	<.001	38	0.26	0.21-0.31	<.001
TLR1/A742G (rs4833095)	AA	462	1.00			385	1.00		
	AG	337	0.74	0.68-0.82	<.001	263	0.70	0.63-0.77	<.001
	GG	67	0.34	0.29-0.40	<.001	52	0.31	0.26-0.38	<.001
TLR2/T1349C (rs3804100)	TT	746	1.00			601	1.00		
	TC	119	1.01	0.91-1.11	.892	97	0.98	0.87-1.09	.671
	CC	0				1			
TLR2/T596C (rs3804099)	TT	267	1.00			218	1.00		
	TC	422	0.95	0.88-1.03	.184	353	1.00	0.92-1.09	.981
	CC	158	0.94	0.85-1.04	.211	115	0.98	0.87-1.11	.796
TLR2/T−16934A (rs4696480)	AA	239	1.00			202	1.00		
	AT	401	0.93	0.86-1.01	.105	314	1.06	0.97-1.17	.182
	TT	219	0.98	0.89-1.08	.695	182	1.00	0.90-1.11	.946
TLR4/C8851T (rs4986791)	CC	758	1.00			604	1.00		
	CT	106	1.44	1.25-1.66	<.001	94	1.22	1.03-1.44	.020
	TT	4	0.98	0.50-1.93	.950	2	1.42	0.50-3.99	.506
TLR4/A8551G (rs4986790)	AA	762	1.00			611	1.00		
	AG	97	1.47	1.27-1.70	<.001	83	1.26	1.06-1.50	.010
	GG	4	0.98	0.49-1.93	.949	2	1.44	0.51-4.04	.491
TLR4/G−2570A (rs2737190)	GG	89	1.00			71	1.00		
	GA	352	0.87	0.74-1.02	.089	270	1.08	0.87-1.33	.495
	AA	367	0.78	0.67-0.92	.002	294	1.13	0.91-1.39	.273
TLR4/T−1607C (rs10759932)	TT	625	1.00			503	1.00		
	TC	201	0.95	0.85-1.06	.376	171	0.90	0.79-1.03	.138
	CC	26	1.21	0.91-1.60	.191	20	1.02	0.73-1.44	.905
TLR5/A1774G (rs2072493)	AA	610	1.00			503	1.00		
	AG	225	1.01	0.92-1.11	.813	174	0.92	0.81-1.05	.210
	GG	17	1.27	0.94-1.71	.125	11	0.93	0.61-1.41	.719
TLR5/T1845C (rs5744174)	TT	276	1.00			222	1.00		
	TC	426	0.93	0.85-1.02	.126	357	0.89	0.79-1.01	.072
	CC	152	0.90	0.79-1.01	.072	111	0.92	0.78-1.09	.334
TLR5/C1173T (rs5744168)	CC	766	1.00			621	1.00		
	CT	95	0.98	0.86-1.11	.716	76	1.01	0.85-1.21	.881
	TT	4	1.11	0.61-2.02	.727	2	1.59	0.59-4.28	.359
TLR6/T−1928C (rs5743792)	TT	806	1.00			661	1.00		
	TC	50	1.30	1.08-1.56	.006	34	0.86	0.69-1.07	.176
	CC	1				0			
TLR6/T−2079A (rs5743789)	TT	539	1.00			449	1.00		
	TA	264	0.93	0.85-1.03	.165	204	0.90	0.81-1.00	.058
	AA	50	0.74	0.61-0.90	.002	37	0.92	0.74-1.13	.419
TLR7/A17961T (rs179008)	AA	599	1.00			475	1.00		
	AT	137	1.18	0.98-1.43	.086	121	0.99	0.83-1.19	.954
	TT	127	0.88	0.73-1.05	.165	103	0.93	0.77-1.12	.423
TLR7/C12318T (rs1620233)	CC	738	1.00			594	1.00		
	CT	70	1.11	0.87-1.41	.398	63	1.22	0.97-1.53	.087
	TT	52	0.92	0.70-1.20	.539	40	1.20	0.91-1.59	.202
TLR8/C10907A (rs3747414)	CC	468	1.00			361	1.00		
	CA	208	0.96	0.83-1.10	.525	173	0.98	0.83-1.15	.805
	AA	187	0.80	0.70-0.91	<.001	163	0.79	0.68-0.92	.002
TLR8/C9008T (rs2159377)	CC	658	1.00			531	1.00		
	CT	128	0.84	0.73-0.97	.018	105	0.71	0.60-0.84	<.001
	TT	74	0.41	0.34-0.49	<.001	61	0.46	0.37-0.57	<.001
TLR8/A−4824G (rs3761624)	AA	562	1.00			461	1.00		
	AG	181	0.70	0.62-0.79	<.001	143	0.69	0.60-0.80	<.001
	GG	125	0.33	0.29-0.38	<.001	97	0.29	0.25-0.34	<.001

(Continued)

TABLE E5. *(Continued)*

		mRNA expression in cord blood				mRNA expression at age 1 y			
Receptor/SNPS	**Genotype**	**No.**	**aGMR***	**95% CI**	***P* value**	**No.**	**aGMR†**	**95% CI**	***P* value**
TLR9/T−2622C (rs5743836)	TT	662	1.00			532	1.00		
	TC	189	0.94	0.84-1.05	.265	158	1.02	0.88-1.20	.765
	CC	12	0.90	0.62-1.32	.601	10	1.00	0.55-1.80	.997
TLR9/T−2871C (rs187084)	TT	265	1.00			220	1.00		
	TC	437	1.02	0.92-1.13	.670	346	0.96	0.83-1.11	.566
	CC	155	0.94	0.82-1.07	.339	130	1.09	0.90-1.31	.368
CD14/C-1721T (rs2915863)	CC	130	1.00			102	1.00		
	CT	380	0.97	0.85-1.10	.628	296	0.97	0.84-1.12	.632
	TT	294	1.01	0.88-1.15	.900	233	0.96	0.83-1.12	.603

*Adjusted for center, farming, sex, maternal history of asthma and hay fever, siblings, and smoking during pregnancy.
†Adjusted for center, child's farming status at year 1, sex, maternal history of asthma and hay fever, maternal smoking during lactation, siblings, and solid food score.

Chapter 4

Development of atopic dermatitis according to age of onset and the association with prenatal and early life exposures

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Development of atopic dermatitis according to age of onset and association with early-life exposures

Caroline Roduit, MD, MPH,[a] Remo Frei, PhD,[b] Georg Loss, PhD,[c] Gisela Büchele, MPH,[f] Juliane Weber, MD,[g] Martin Depner, PhD,[g] Susanne Loeliger, MLS,[a] Marie-Laure Dalphin, MD,[h] Marjut Roponen, PhD,[i] Anne Hyvärinen, PhD,[j] Josef Riedler, MD,[k] Jean-Charles Dalphin, MD, PhD,[l] Juha Pekkanen, MD,[j] Erika von Mutius, MD, MSc,[g] Charlotte Braun-Fahrländer, MD,[c,d] Roger Lauener, MD,[a,e] and the Protection Against Allergy–Study in Rural Environments study group* *Zurich, Basel, and Davos, Switzerland, Ulm and Munich, Germany, Besançon, France, Kuopio, Finland, and Schwarzach, Austria*

Background: Environmental factors can affect the development of atopic dermatitis, and this was described to be already effective during pregnancy and in early life. An important early postnatal exposure is nutrition, although its association with allergic disease remains unclear.

Objective: We sought to determine prospectively whether early postnatal exposures, such as the introduction to complementary food in the first year of life, are associated with the development of atopic dermatitis, taking into account the reverse causality.

Methods: One thousand forty-one children who participated in the Protection Against Allergy–Study in Rural Environments birth cohort study were included in the current study. Atopic dermatitis was defined by a doctor's diagnosis reported by the parents of children up to 4 years of age, by questionnaires, and/or by positive SCORAD scores from 1 year of age and according to the age of onset within or after the first year of life. Feeding practices were reported by parents in monthly diaries between the 3rd and 12th months of life.

Results: The diversity of introduction of complementary food in the first year of life was associated with a reduction in the risk of having atopic dermatitis with onset after the first year of life (adjusted odds ratio for atopic dermatitis with each additional major food item introduced, 0.76; 95% CI, 0.65-0.88). The introduction of yogurt in the first year of life also reduced the risk for atopic dermatitis (adjusted odds ratio, 0.41; 95% CI, 0.23-0.73).

Conclusion: As early-life exposure, the introduction of yogurt and the diversity of food introduced in the first year of life might have a protective effect against atopic dermatitis. (J Allergy Clin Immunol 2012;130:130-6.)

Key words: *Atopic dermatitis, diversity, complementary food*

Increasing evidence suggests that prenatal and early-life environmental exposures can influence immune responses and the development of allergic diseases. Atopic dermatitis is a chronic inflammatory skin disease, and in 60% of children, the onset of disease occurs during the first year of life.[1]

As early-life exposure, nutrition is a major environmental factor that might have an effect on the immune system and could be a factor that would lead to the prevention of allergic diseases. Studies of the farming environment have shown that consumption of unprocessed farm milk was associated with fewer allergic diseases, although there was some heterogeneity of the effects, especially with atopic dermatitis. In addition, this evidence was based on cross-sectional studies of school-aged children only.[2-6] First introduction of complementary food in an infant's life and its association with allergic diseases is another aspect of nutrition raising much controversy. Food allergen avoidance during pregnancy or infancy has provided no consistent evidence of allergy prevention[7,8] and is no longer recommended.[9] A systematic review of 13 studies of the relationship between early introduction of solid food and the development of allergies concluded that the evidence of this relation is inconsistent and conflicting.[10] Some recent studies even showed that early introduction of complementary food, like the introduction of fish before 1 year of age or early exposure to cow's milk, might have a protective effect against

From [a]the University of Zurich, Children's Hospital, and the Christine Kühne-Center for Allergy Research and Education, Zurich; [b]the Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, and the Christine Kühne-Center for Allergy Research and Education, Zurich; [c]the Swiss Tropical and Public Health Institute, Basel; [d]the University of Basel; [e]Children's Allergy and Asthma Hospital, Hochgebirgsklinik, and the Christine Kühne-Center for Allergy Research and Education, Davos; [f]the Institute of Epidemiology, University of Ulm; [g]University Children's Hospital Munich; [h]the Department of Pediatrics, University Hospital of Besançon; [i]the Department of Environmental Science, University of Eastern Finland, Kuopio; [j]the Department of Environment Health, National Institute for Health and Welfare, Kuopio; [k]Children's Hospital Schwarzach; and [l]the Department of Respiratory Disease, UMR/CNRS 6249 Chrono-environnement, University Hospital of Besançon.

*Members of the Protection Against Allergy–Study in Rural Environments study group are listed in the acknowledgments.

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Corresponding author: Caroline Roduit, MD, MPH, Kinderspital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. E-mail: Caroline.Roduit@kispi.uzh.ch.

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Abbreviations used
OR: Odds ratio
PASTURE: Protection Against Allergy–Study in Rural Environments
SCFA: Short-chain fatty acid

allergic diseases.[11-15] One study found a protective effect of the introduction of any complementary food within the first 4 months on atopic dermatitis but only among children with allergic parents.[16] Therefore more evidence with respect to the role of early nutritional exposures is needed.

The birth cohort study Protection Against Allergy–Study in Rural Environments (PASTURE) offered the opportunity to evaluate the effect of prenatal and postnatal exposures on the development of allergic diseases.[17] We previously reported an inverse association between prenatal contact with animals and atopic dermatitis up to 2 years of age.[18] In the present analysis we longitudinally evaluated whether early postnatal exposures, especially food introduction and its diversity, were associated with the development of atopic dermatitis, with data available up to 4 years of age. One major concern with postnatal exposures, especially with the association between feeding practices and atopic dermatitis, is the potential bias caused by the reverse causality effect. This source of bias arises when the reason for introducing or not introducing a certain type of food is strongly associated with the outcome. Among children with early symptoms of the disease, those with allergic parents, or both, introduction of certain complementary food, especially allergenic food, tends to be delayed. We thus focused our analyses on children with atopic dermatitis occurring after the first year of life, ensuring that exposure occurred before onset of the disease.

METHODS

Study design

PASTURE is a prospective birth cohort study involving children from rural areas in 5 European countries (Austria, Finland, France, Germany, and Switzerland) designed to evaluate risk factors and preventive factors for atopic diseases. The design of this cohort has been described in detail elsewhere.[17] Briefly, pregnant women were recruited during the third trimester of pregnancy and divided into 2 groups. Women who lived or worked on family-run farms on which any kind of livestock was kept were assigned to the farm group. The reference group was composed of women from the same rural areas not living on a farm. In total, 1133 children were included in this birth cohort. The questionnaires developed within the PASTURE study group used questions on various exposures and outcomes from the Asthma Multicenter Infants Cohort Study,[19] the Allergy and Endotoxin study,[2] and the Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle study.[20] Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy; when the children were 2, 12, 18, and 24 months of age; and then yearly up to 4 years of age. Feeding practices and the occurrence of itchy rash were reported by parents between the 3rd and 12th months of life in monthly and weekly diaries, respectively. The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents.

Study population

Children from the PASTURE birth cohort with data available on atopic dermatitis up to 4 years of age, farming status, parental allergic history, and feeding practices in the first year of life (n = 1041) were included.

Definitions

Children were labeled as having atopic dermatitis when the parents reported in the questionnaires that the child had atopic dermatitis diagnosed by a doctor at least once between 12 months and 4 years of age, positive SCORAD scores (>0) assessed at the age of 1 year during medical examination, or both. Among the 144 children defined as having atopic dermatitis at the age of 1 year, 44 had only a positive SCORAD score, 50 had only a doctor's diagnosis, and 50 had both. Children with no atopic dermatitis but missing information at 1 or more time points were defined as "missing" (n = 129) when the prevalence of atopic dermatitis up to 4 years was calculated. In most children atopic dermatitis occurs early in life, and to be able to evaluate exposures occurring before the disease, we used 2 different definitions of atopic dermatitis depending on the occurrence of the disease: atopic dermatitis with onset within the first year of life and atopic dermatitis with onset after the first year of life. Farmer's children were defined as children who were living on a farm on which livestock was held and whose family ran the farm according to parental reports. Maternal farm-related exposures during pregnancy were obtained from the self-reported questionnaires at the third trimester of pregnancy. Prenatal contact with farm animal species was assumed if the mother reported contact at least several times per month in one of the pregnancy trimesters. Postnatal exposures to stables during the first year of life were defined as the child's exposure for at least a quarter of an hour per week. Feeding practices were reported by parents in monthly diaries between the 3rd and 12th months of life. Parents indicated for each food item whether it was given to the child in the last 4 weeks and, if so, how often. A diversity score was calculated, including the major food items, which were defined as the ones introduced in the first year of life to approximately 80% of the children or more. The score included vegetables or fruits, cereals, bread, meat, cake, and yogurt. The same food items were used to define the diversity score within the first 6 months of life. For the association between single food items and atopic dermatitis, we used as reference children for whom the item was not introduced in the first year of life. When it was introduced for less than 15% of the children, cutoffs to earlier time points were used. Introduction of cow's milk was defined as either exclusive consumption of milk purchased in a shop (shop milk) or introduction of milk produced or purchased directly from a farm exclusively or in combination with shop milk and irrespective of whether the milk was boiled or unboiled (any farm milk).

Data on potential confounders, such as smoking during pregnancy, sex, mode of delivery, birth weight, gestational age, maternal education, and duration of breast-feeding were obtained from the self-reported questionnaires at the third trimester of pregnancy and at 2 months and 1 year of age. Duration of breast-feeding was categorized according to the number of months children were breast-fed (not exclusively). Parental history of allergies was defined as ever had asthma, hay fever, or atopic dermatitis, which was self-reported.

Statistical analysis

Data analysis was conducted with SAS software, version 9.2 (SAS Institute, Inc, Cary, NC).

The χ^2 test was used to evaluate the differences between the prevalences of atopic dermatitis depending on parental allergic status and also to compare the diversity score among subgroups of children. Generalized estimating equations were used to investigate the longitudinal effects of prenatal and postnatal exposures on atopic dermatitis with onset after the first year of life, taking into account the correlation between repeated measures (age 18, 24, 36, and 48 months) in the same subject. For the associations between exposures and atopic dermatitis with onset within the first year of life, we used logistic regression because only 1 time point (age 12 months) was taken into account. From these analyses, odds ratios (ORs) with 95% CIs were reported. To avoid reverse causality, with timing of introduction of food in the first year of life, we limited the analyses to children with atopic dermatitis first occurring after the first year of life. For the analysis comparing introduction of food before or after 6 months of age, the analysis was restricted to children without skin symptoms (itchy rash and diary data) within the first 6 months of life. To evaluate the relation between the diversity score and atopic dermatitis, we performed nonparametric smoothing regression analysis. Family history of

TABLE I. Prevalence of atopic dermatitis, according to time of onset and parental allergic status

	Total (n = 912), % (n)	No allergic parents (n = 426), % (n)	Only 1 allergic parent (n = 385), % (n)	Two allergic parents (n = 101), % (n)	*P* value*
Atopic dermatitis up to age 4 y	27.1 (247)	21.8 (93)	28.3 (109)	44.6 (45)	<.001
Atopic dermatitis with onset within the first year of life	15.9 (144)	12.9 (55)	15.0 (57)	32.0 (32)	<.001
Atopic dermatitis with onset after the first year of life†	10.7 (97)	8.7 (37)	12.6 (48)	12.0 (12)	.18

*Based on χ^2 test between parental allergic status and atopic dermatitis.
†Missing information for the first year of life for 6 children.

allergies is a dominant predictive factor of allergic diseases, particularly atopic dermatitis. Therefore all models were adjusted for parental history of allergy (ever eczema, hay fever, or asthma). For the association between food exposures and atopic dermatitis, we stratified the analyses by this variable. To test for effect modification between food item exposures and parental history of allergy, we calculated terms for interactions in the generalized estimating equation model.

All models were adjusted for study centers as a fixed effect because we did not find heterogeneity between the centers (tested by means of meta-analytic techniques). Multivariate models were further adjusted for farming and duration of breast-feeding in the first year of life because these variables are well known as potential confounders. Smoking during pregnancy and maternal education were added to the model but did not change the results, and therefore they were not kept in the final model. A *P* value of less than .05 was considered statistically significant.

RESULTS

Prevalence of atopic dermatitis

In total, 1041 children were included in this study. The proportion of farmer's children was 47.8%, and 558 (53.6%) had at least 1 allergic parent; among them, 39.1% (218/558) were farmer's children. The general characteristics of this study population were described in our previous analysis.[18] The cumulative prevalence of children with atopic dermatitis in the first 4 years of life was 27.1% (Table I). This prevalence was significantly higher in children with 2 allergic parents than among children with nonallergic parents (44.6% and 21.8%, respectively). For 59.8% (144/241) of these children, the disease appeared in the first year of life. The influence of parental allergy was more pronounced in these children than in those with disease onset after the first year of life (Table I).

Association between early postnatal exposures to farm animals and atopic dermatitis

We did not observe an association between early postnatal contact with farm animals (presence of the child in the stable in the first year of life) and atopic dermatitis with onset after the first year of life. After adjustment for prenatal exposures (adjusted OR, 0.97; 95% CI, 0.53-1.75), unadjusted results were very similar (data not shown). To separate the influence of prenatal and postnatal exposures to farm animals, a variable with 4 mutually exclusive categories was computed: children with both exposures, those with only prenatal or only postnatal exposure, and those not exposed. The negative association between prenatal contact with farm animals and atopic dermatitis was observed only when the disease onset occurred during the first year of life (see Table E1 in this article's Online Repository at www.jacionline.org). By contrast, postnatal exposure was inversely associated with atopic dermatitis with onset after the first year of life, but this analysis was based on small numbers and not statistically significant.

Feeding practices in the first year of life

At 2 months of age, exclusive breast-feeding was observed among 66.0% of the children, and 18.6% were not breast-fed. About half of the children (46.4%) were breast-fed for more than 6 months (not exclusively), and no difference with respect to the parental history of allergy was observed (data not shown).

For only 18 (1.7%) children, no complementary food was introduced in the first year of life (Table II). These children did not differ from those with complementary food introduced in terms of farming status, parental allergic history, maternal education, or duration of breast-feeding (data not shown). In the first year of life, cow's milk was introduced to half of the study population (Table II). Of these children, 43.8% consumed only shop milk, and 56.2% consumed any farm milk, and 118 (37%) of the farm milk drinkers consumed unboiled farm milk.

About 80% of the children consumed vegetables or fruits, cereals, bread, meat, cake, and yogurt during the first year of life. A diversity score including these 6 major food items was calculated, and more than two thirds of the children consumed all 6 items (Table III). Significantly fewer food items were introduced among nonfarmer's children and those with at least 1 parent with a history of allergy. The diversity score showed no difference between children breast-fed for more or less than 6 months. As expected, among children with allergic parents, the allergenic food items, such as dairy products, egg, nut, and soy, were introduced later (data not shown).

Association between complementary food introduction and atopic dermatitis

The analyses of atopic dermatitis with onset within the first year of life showed an inverse association with the introduction in the first year of life for most of the food items, especially the allergenic foods (see Table E2 in this article's Online Repository at www.jacionline.org). This negative association is most likely due to delayed introduction of certain foods in children with early symptoms. Analyses were restricted to children having no atopic dermatitis in the first year of life but who had it later to avoid this form of bias. The introduction of yogurt and shop milk within the first year of life showed an inverse association with the development of atopic dermatitis with onset after the first year of life compared with no introduction, indicating a protective effect (adjusted OR, 0.41; 95% CI, 0.23-0.73 and adjusted OR, 0.52; 95% CI, 0.30-0.92, respectively); unadjusted results were very similar (data not shown). Analyses stratified by parental history of allergy showed similar associations (Fig 1 and see Table E3 in this article's Online Repository at www.jacionline.org). The consumption of farm milk in the first year of life had a tendency to decrease the risk of having atopic dermatitis but only among children with no allergic parents (adjusted OR, 0.49; 95% CI,

TABLE II. Time of first introduction of different food items in the first year of life (n = 1041)

	3-6 mo, % (n)	7-9 mo, % (n)	10-12 mo, % (n)	In total introduced in the first year of life, % (n)
Any food items (15 items)	72.2 (752)	25.6 (267)	0.4 (4)	98.3 (1023)
Any cow's milk	7.2 (75)	20.1 (209)	26.9 (280)	54.2 (564)
Only shop milk (n = 724)*	2.2 (16)	10.6 (77)	21.3 (154)	34.1 (247)
Any farm milk (n = 794)*	6.4 (51)	16.0 (127)	17.5 (139)	39.9 (317)
Yogurt	14.1 (147)	38.4 (400)	26.8 (279)	79.3 (826)
Other milk products	6.7 (70)	30.5 (317)	36.4 (379)	73.6 (766)
Eggs	3.2 (33)	28.1 (292)	36.1 (376)	67.3 (701)
Nuts	0.6 (6)	6.2 (65)	17.6 (183)	24.4 (254)
Vegetables or fruits	71.1 (740)	26.5 (276)	0.6 (6)	98.2 (1022)
Cereals	32.9 (342)	44.3 (461)	10.7 (111)	87.8 (914)
Bread	16.5 (172)	59.1 (615)	18.0 (187)	93.6 (974)
Meat	25.6 (266)	55.6 (579)	11.8 (123)	93.0 (968)
Fish	4.8 (50)	28.7 (299)	23.4 (244)	57.0 (593)
Soy	0.9 (9)	2.0 (21)	2.3 (24)	5.2 (54)
Margarine	8.6 (90)	28.1 (292)	22.0 (229)	58.7 (611)
Butter	6.0 (62)	33.6 (350)	30.2 (314)	69.7 (726)
Cake	11.2 (117)	47.1 (490)	27.9 (290)	86.2 (897)
Chocolate	3.8 (39)	15.2 (158)	27.3 (284)	46.2 (481)

*Reference group: children who did not consume any cow's milk in the first year of life.

TABLE III. Diversity score with major food items introduced in the first year of life among all study populations and subgroups

	Diversity score*			
	0-3 items, % (n)	4-5 items, % (n)	6 items, % (n)	*P* value†
All study population	5.4 (56)	31.9 (332)	62.7 (653)	
Farmer				
Yes	2.8 (14)	27.9 (139)	69.3 (345)	<.001
No	7.7 (42)	35.5 (193)	56.7 (308)	
Allergic parents (≥1)				
Yes	6.8 (38)	34.4 (192)	58.8 (328)	.007
No	3.7 (18)	29.0 (140)	67.3 (325)	
Breast-feeding >6 mo‡				
Yes	6.3 (30)	31.9 (153)	61.9 (297)	.31
No	4.2 (23)	31.4 (172)	64.4 (352)	

*Diversity score with major food items: vegetables or fruits, any cereals, meat, bread, cake, and yogurt.
†*P* value based on χ^2 test.
‡Missing information for 14 children.

0.21-1.18). Tests for interaction between parental history of allergy and farm milk showed a *P* value of .08. Introduction of vegetables or fruits in the first 6 months reduced the risk of atopic dermatitis (adjusted OR, 0.56; 95% CI, 0.31-1.00).

A smoothed plot of the prevalence of atopic dermatitis in relation to the diversity score (0-6) was performed to evaluate whether the diversity of foods consumed during the first year of life was associated with atopic dermatitis occurring after the first year of life, showing a decrease in prevalence of the disease with an increasing score (Fig 2). Dividing the diversity score into 3 different categories with the largest as the reference category, we observed a dose-response association with atopic dermatitis (Table IV). For each additional major food item introduced in the first year of life, we observed a significant reduction of 25% in the development of atopic dermatitis. Similar results were obtained in separate analyses stratified by parental history of allergy (adjusted OR for each food item introduced among children with parents with no allergy, 0.70; 95% CI, 0.56-0.87; among those with at least 1 parent with allergy: adjusted OR, 0.81; 95% CI, 0.65-1.01). The score was recalculated excluding yogurt to evaluate the influence of the yogurt item in this score and showed the same association with atopic dermatitis (Table IV). Moreover, yogurt remained significantly associated with atopic dermatitis after adjustment for the reduced score. Smoothed plots with a diversity score including all 15 food items also showed a decrease in prevalence of the disease with an increasing score most strongly with up to 6 items (see Fig E1 in this article's Online Repository at www.jacionline.org). When the major food items were excluded from the score, no association was observed (see Fig E2 in this article's Online Repository at www.jacionline.org).

Additionally, we evaluated the association between the introduction of food in the first 6 months of life and atopic dermatitis with onset within the first year of life in the subgroup of children and no symptoms of atopic dermatitis (itchy rash) within the first 6 months (60.3% of the children with atopic dermatitis with early onset). We could also observe a decreased risk of atopic dermatitis with increasing numbers of major food introduced in the first 6 months, indicating a dose-response effect (Table V). We also observed similar results in separated analyses stratified by the parental history of allergy, as well as in unadjusted analyses (data not shown).

DISCUSSION

Our study shows a strong association between the family history of allergies and atopic dermatitis with early onset but not with onset occurring after the first year of life. The previously reported protective prenatal effect of exposure to farm animals was limited to atopic dermatitis occurring during the first year of life. The postnatal exposure to farm animals was not significantly associated with atopic dermatitis.

Feeding practices in the first year of life seem to be associated with atopic dermatitis. We showed that the diversity of food items introduced in the first year of life reduces the risk of atopic dermatitis later in life. Introduction of yogurt in the first year of life showed a strong protective effect against atopic dermatitis with onset after the first year of life independently of the diversity of food.

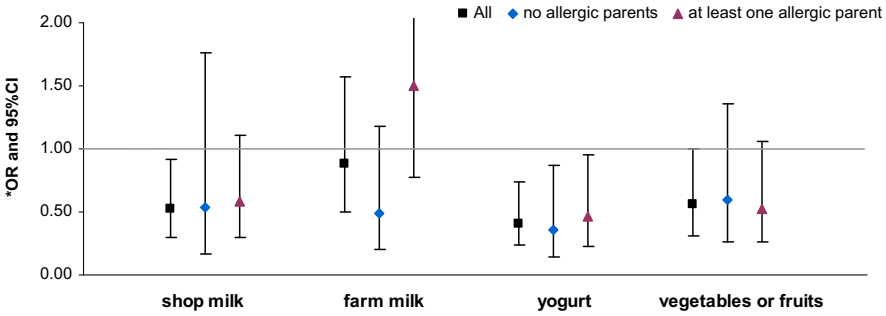

FIG 1. Association between food item introduction in the first year of life and atopic dermatitis with onset after the first year of life stratified by parental allergies. *Adjusted for farmer, center, breast-feeding, and parental history of allergies (ever eczema, hay fever, or asthma). For dairy products: introduction in the first year of life compared with no introduction before 1 year of age. For vegetables or fruits: introduction in the first 6 months of life compared with no introduction before 6 months of age.

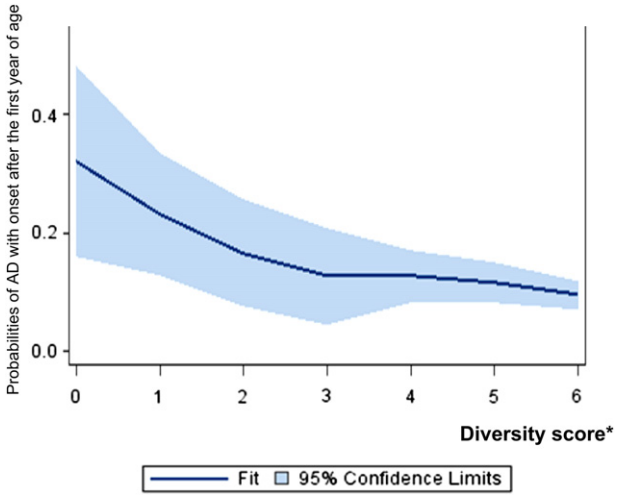

FIG 2. Association between increasing numbers of different major food items (n = 6) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Diversity score with major food items: vegetables or fruits, any cereals, meat, bread, cake, and yogurt.

Our results suggest that the association between genetic factors and prenatal exposures is stronger with atopic dermatitis with onset early in life than with late-onset atopic dermatitis. In a Dutch birth cohort study it was shown that filaggrin gene mutations were associated with atopic dermatitis when the occurrence of the disease was in the first year of life but not after[21] and that cat exposure increased the effect of filaggrin gene mutations on atopic dermatitis, indicating a gene-environment interaction.[22] Epigenetic mechanisms in the development of allergic diseases have recently raised much interest.[23] Our finding that the protective effect of prenatal exposures was not observed in children with atopic dermatitis occurring after the first year of life challenges the idea of epigenetic mechanisms underlying the association between prenatal contact with animals and atopic dermatitis with late onset. It might be that atopic dermatitis depending on the age of onset represents 2 different phenotypes of the disease and that genetic and epigenetic mechanisms influence mainly the early onset. However, our study was not sufficiently powered to evaluate the independent role of postnatal exposure to farm animals on atopic dermatitis occurring after the first year of life.

Our results support recent studies showing an inverse association between early introduction of complementary food, such as fish and cow's milk, and allergic diseases and highlight the role of the diversity of environmental exposures for the development of allergic diseases. We have previously shown that the diversity of maternal exposure to different farm animal species during pregnancy had a protective effect on atopic dermatitis early in life.[18] Moreover, recent findings have shown that the diversity of microbial exposures might play a role in the protective effect with regard to asthma.[24] Bacterial diversity of the intestinal flora has also been suggested to be associated with atopic diseases, even though its association with atopic dermatitis remains unclear.[25] Food is a major environmental factor, especially for infants who encounter large quantities of new food components during their first year of life. Diversity of food seems to be protective against the development of atopic dermatitis. This was observed only when the diversity score was composed of major food items. This might be due to the fact that the number of children exposed to the other items was too small to show an effect or that these items had no effect. Our findings on the diversity of food introduced in the first year of life might support the hypothesis that exposure to a variety of antigens, such as food protein, during a specific time window early in life might be essential for the development of immune tolerance.[26]

The strengths of this study are the prospective design and the detailed data collection of feeding practices in the first year of life. Focusing the analyses on atopic dermatitis occurring after the first year of life allowed us to avoid the reverse causality effect. However, this focus also reduced the number of affected children because the onset of this disease occurred in the first year of life for most of the children, thus limiting the power of the present analysis. Food exposures early in life might differently affect the disease with early or late onset. However, when we restricted our analyses to children without symptoms of atopic dermatitis during the first 6 months but atopic dermatitis with onset within the first year of life, similar associations with introduction of food in the first 6 months were found as in patients with late onset of the disease.

Yogurt is produced by bacterial fermentation of milk by lactic acid bacteria. Many of these bacterial strains have been selected as probiotics. The strong protective effect of consumption of yogurt in the first year of life on atopic dermatitis could be due to these bacteria. In the present study, unfortunately, no information on children's consumption of yogurt with or without live

TABLE IV. Association between the diversity score with major items introduced in the first year of life and atopic dermatitis with onset after the first year of life

	Atopic dermatitis with onset after the first year of life			
	No.	OR*	95% CI	*P* value†
Diversity score with major food items‡ (0-6)				
No. of items introduced in the first year				
0-3	56	**2.87**	**1.26-6.56**	.01
4-5	332	**1.72**	**1.06-2.80**	.03
6, reference	653	1	–	
For each major food item introduced§	1041	**0.76**	**0.65-0.88**	<.001
Diversity score with major food items, without yogurt (0-5)				
For each major food items introduced§	1041	**0.75**	**0.62-0.91**	.003

Boldface values are significant ($P < .05$).
*Adjusted for farmer, center, duration of breast-feeding, and parents with atopy (eczema, asthma, or hay fever).
†*P* value based on generalized estimating equation analysis.
‡Diversity score with major food items: vegetables or fruits, any cereals, meat, bread, cake, and yogurt.
§OR for atopic dermatitis with each additional food item introduced in the first year of life.

TABLE V. Association between the diversity score with major items introduced in the first 6 months and atopic dermatitis with onset within the first year of life but no symptoms in the first 6 months

	Atopic dermatitis with onset within the first year of life but no symptoms (itchy rash) in the first 6 mo			
	No.	OR*	95% CI	*P* value†
Diversity score with major food items‡ (0-6)				
No. of items introduced in the first 6 mo				
0-1	532	**2.12**	**1.12-4.03**	.02
2	190	1.76	0.85-3.69	.16
≥3, reference	319	1	–	
For each major food item introduced§	1041	0.88	0.73-1.05	.16
Diversity score with major food items, without yogurt (0-5)				
For each major food item introduced§	1041	0.85	0.70-1.04	.12

Boldface values are significant ($P < .05$).
*Adjusted for farmer, center, duration of breast-feeding, and parents with atopy (eczema, asthma, or hay fever).
†*P* value based on logistic regression analysis.
‡Diversity score with major food items: vegetables or fruits, any cereals, meat, bread, cake, and yogurt.
§OR for atopic dermatitis with each additional food items introduced in the first 6 months of life.

bacterial cultures was available. One of the first randomized controlled trials, which was conducted by Kalliomäki et al[27] in pregnant women with a family history of allergy, showed a 50% reduction in clinical eczema among the probiotic group. Even though several other studies and meta-analyses on probiotics and the prevention of allergic diseases have been conducted, most of them concluded that there is insufficient evidence to recommend probiotics for prevention. Recently, a Finnish study observed that probiotics were protective for allergic disease only among children born by means of cesarean section.[28] In our study we observed the same protective effect of the introduction of yogurt on atopic dermatitis when analyses were restricted to children born by means of vaginal delivery, arguing against effect modification by mode of delivery in the present study (data not shown).

Levels of metabolites produced by intestinal microbiota, such as short-chain fatty acids (SCFAs), have been shown to be increased in fecal and plasma samples after yogurt consumption.[29,30] Moreover, it has been suggested that SCFAs might have anti-inflammatory properties,[31,32] and thus it has been proposed that factors that influence the intestinal microbiota and the production of SCFAs might have an effect on immune and inflammatory responses. Interestingly, more fecal SCFAs were found among children from rural Africa compared with those from urban Europe, providing indirect evidence of a role of SCFAs. As among children from Africa, a lower prevalence of allergic diseases was observed compared with the prevalence seen in children from western Europe.[33,34]

The protective effect of cow's milk in the present study was mainly related to consumption of shop milk. Previously, cross-sectional studies on farm milk consumption and allergies suggested a protective effect of farm milk consumption on asthma, whereas the association with atopic dermatitis was more controversial.[5,6,35] In the present study the relationship between farm milk consumption in the first year of life and atopic dermatitis tended to be modified by parental history of allergies, which has not been observed in the cross-sectional studies.[6,35] A tendency toward a decreased risk of atopic dermatitis with consumption of farm milk was observed only among children with parents without allergies. These results could be explained by a gene-environment interaction effect, which is supported by a previous study showing that a polymorphism in the gene encoding CD14 modified the effect of farm milk consumption on allergic diseases and CD14 gene expression.[36] With other complementary foods, we did not have evidence for effect modification by parental allergies in the association with atopic dermatitis.

Children exposed to complementary foods, especially yogurt, and an increased diversity of foods within the first year of life have

a reduced risk of atopic dermatitis independently of parental history of allergies.

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Clinical implications: The diversity of food introduced in the first year of life might decrease the risk of atopic dermatitis in children.

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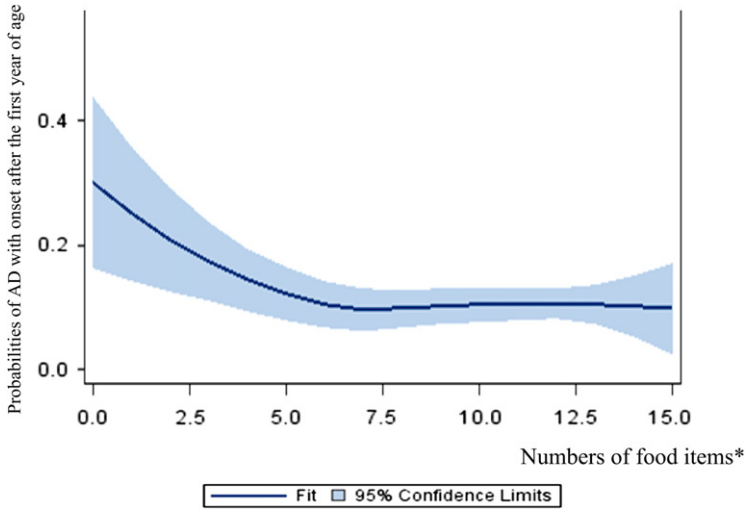

FIG E1. Association between increasing numbers of all different food items (n = 15) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Food items (n = 15): any cow's milk, yogurt, other milk products, eggs, nuts, vegetables or fruits, cereals, bread, meat, fish, soy, margarine, butter, cake, and chocolate.

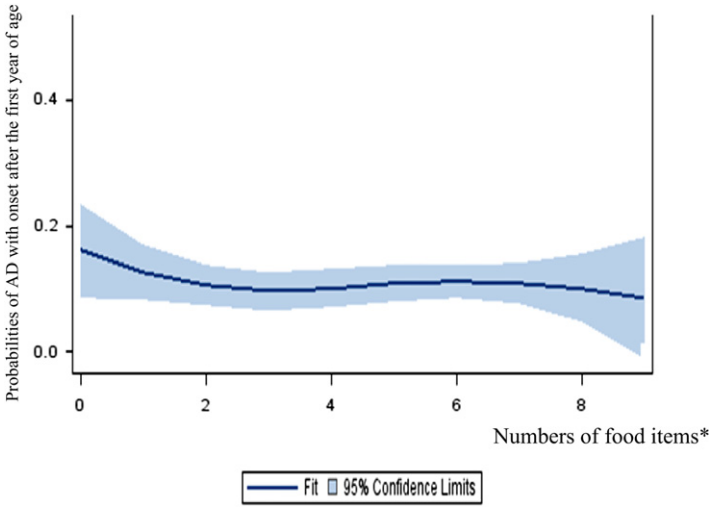

FIG E2. Association between increasing numbers of all different food items excluding major foods (n = 9) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Food items (n = 9): any cow's milk, other milk products, eggs, nuts, fish, soy, margarine, butter, and chocolate.

TABLE E1. Association between prenatal and postnatal exposures to farm animals and atopic dermatitis with different time of onset

		Atopic dermatitis with onset within the first year of life		Atopic dermatitis with onset after the first year of life	
	Percent (n)	OR*	95% CI	OR*	95% CI
Contact with farm animal					
Prenatal and postnatal combined					
Prenatal and postnatal	37.7 (345)	0.51	0.26-1.00	1.20	0.53-2.71
Only prenatal	22.8 (209)	0.61	0.35-1.08	1.09	0.56-2.14
Only postnatal	2.9 (26)	2.01	0.78-5.15	0.34	0.03-3.75
No contact, reference	36.6 (335)	1			

Postnatal: presence of the child in the stable within the first year of life.
*Adjusted for farmer, center, parents with atopy, smoking during pregnancy, and duration of breast-feeding.

TABLE E2. Association between introduction of food items in the first year of life and atopic dermatitis up to 4 years of age and with onset within the first year of life

	Atopic dermatitis up to 4 y of age		Atopic dermatitis with onset within the first year of life		Atopic dermatitis with onset within the first year of life but no symptoms of itchy rash in the first 6 mo	
	OR*	95% CI	OR*	95% CI	OR*	95% CI
Dairy items						
Cow's milk						
3-12 mo	**0.46**	**0.32-0.65**	**0.60**	**0.41-0.87**	0.65	0.40-1.05
Not in first year, reference†	1.00	–	1.00		1.00	
Only shop's milk						
3-12 mo	**0.31**	**0.20-0.49**	**0.54**	**0.33-0.87**	0.57	0.31-1.07
Not in first year, reference†	1.00	–	1.00		1.00	
Any farm's milk						
3-12 mo	**0.61**	**0.39-0.95**	0.63	0.38-1.03	0.67	0.36-1.24
Not in first year, reference†	1.00		1.00		1.00	
Yogurt						
3-12 mo	**0.31**	**0.20-0.46**	**0.52**	**0.34-0.82**	0.60	0.33-1.07
Not in first year, reference	1.00		1.00		1.00	
Other milk products (eg, cheese and quark)						
3-12 mo	**0.62**	**0.41-0.94**	**0.60**	**0.40-0.90**	**0.58**	**0.35-0.96**
Not in first year, reference	1.00		1.00		1.00	
Other food items						
Nuts						
3-12 mo	0.99	0.62-1.57	**0.54**	**0.33-0.90**	**0.51**	**0.26-0.99**
Not in first year, reference	1.00		1.00		1.00	
Eggs						
3-12 mo	0.76	0.51-1.12	**0.55**	**0.38-0.80**	0.61	0.38-1.00
Not in first year, reference	1.00		1.00		1.00	
Fish						
3-12 mo	0.45	0.29-0.69	**0.52**	**0.35-0.77**	**0.51**	**0.30-0.87**
Not in first year, reference	1.00		1.00		1.00	
Meat						
3-8 mo	0.75	0.49-1.14	0.71	0.47-1.08	0.77	0.46-1.31
Not in first 8 mo, reference	1.00		1.00		1.00	
Cereals						
3-8 mo	0.95	0.62-1.44	1.14	0.74-1.74	1.29	0.75-2.21
Not in first 8 mo, reference	1.00		1.00		1.00	
Vegetables or fruits						
<6 mo	0.46	0.31-0.70	0.81	0.53-1.25	0.67	0.38-1.15
Not in first 6 mo, reference	1.00		1.00		1.00	
Bread						
3-8 mo	0.82	0.57-1.17	0.80	0.55-1.16	0.63	0.39-1.02
Not in first 8 mo, reference	1.00		1.00		1.00	
Soy						
3-12 mo	0.78	0.35-1.75	1.13	0.53-2.41	1.16	0.43-3.13
Not in first year, reference	1.00		1.00		1.00	
Margarine						
3-12 mo	0.72	0.48-1.07	0.76	0.51-1.14	0.75	0.45-1.26
Not in first year, reference	1.00		1.00		1.00	
Butter						
3-12 mo	0.89	0.60-1.30	0.78	0.52-1.16	0.83	0.50-1.41
Not in first year, reference	1.00		1.00		1.00	
Cake						
3-8 mo	0.70	0.47-1.02	0.72	0.49-1.07	0.66	0.40-1.10
Not in first 8 mo, reference	1.00		1.00		1.00	
Chocolate						
3-12 mo	0.53	**0.36-0.77**	**0.66**	**0.45-0.98**	0.95	0.58-1.54
Not in first year, reference	1.00		1.00		1.00	

Boldface values are significant ($P < .05$).

*Adjusted for farmer, center, breast-feeding (duration), and parental history of allergies (ever eczema, hay fever, or asthma).

†Reference = no introduction of any cow's milk in the first year of life.

TABLE E3. Association between introduction of food items in the first year of life and atopic dermatitis with onset after the first year of life stratified by parents with or without allergies

	Entire study population			Parents without allergy			At least 1 parent with allergy		
		Atopic dermatitis, with onset after the first year of life			Atopic dermatitis, with onset after the first year of life			Atopic dermatitis, with onset after the first year of life	
	No.	OR*	95% CI	No.	OR*	95% CI	No.	OR*	95% CI
Dairy items									
Cow's milk									
3-12 mo	564	0.68	0.44-1.05	289	0.49	0.24-1.01	275	0.86	0.51-1.45
Not in first year, reference†	477	1.00	–	194	1.00		283	1.00	
Only shop's milk									
3-12 mo	247	**0.52**	**0.30-0.92**	98	0.54	0.17-1.77	149	0.58	0.30-1.10
Not in first year, reference†	477	1.00	–	194	1.00		283	1.00	
Any farm's milk‡									
3-12 mo	317	0.88	0.49-1.57	191	0.49	0.21-1.18	126	1.50	0.77-2.89
Not in first year, reference†	477	1.00		194	1.00		283	1.00	
Yogurt									
3-12 mo	826	**0.41**	**0.23-0.73**	**411**	**0.36**	**0.15-0.87**	**415**	**0.46**	**0.23-0.95**
Not in first year, reference	215	1.00		72	1.00		143	1.00	
Other milk products (eg, cheese and quark)									
3-12 mo	766	1.07	0.59-1.91	373	1.25	0.47-3.34	393	1.00	0.47-2.13
Not in first year, reference	275	1.00		110	1.00		165	1.00	
Other food items									
Nuts									
3-12 mo	254	1.35	0.79-2.31	135	1.49	0.66-3.37	119	1.28	0.62-2.61
Not in first year, reference	787	1.00		348	1.00		439	1.00	
Eggs									
3-12 mo	701	1.02	0.63-1.66	353	1.08	0.46-2.53	348	1.01	0.55-1.85
Not in first year, reference	340	1.00		130	1.00		210	1.00	
Fish									
3-12 mo	593	0.73	0.43-1.24	288	1.09	0.43-2.75	305	0.58	0.31-1.09
Not in first year, reference	448	1.00		195	1.00		253	1.00	
Meat									
3-8 mo	697	0.87	0.50-1.54	333	1.01	0.42-2.42	364	0.81	0.37-1.77
Not in first 8 mo, reference	344	1.00		150	1.00		194	1.00	
Cereals									
3-8 mo	700	0.72	0.42-1.25	304	0.79	0.35-1.80	396	0.70	0.36-1.37
Not in first 8 mo, reference	341	1.00		179	1.00		162	1.00	
Vegetables or fruits									
<6 mo	462	0.56	0.31-1.00	218	0.59	0.26-1.36	244	0.52	0.26-1.06
Not in first 6 mo, reference	579	1.00		265	1.00		314	1.00	
Bread									
3-8 mo	787	0.84	0.52-1.35	378	0.70	0.36-1.35	409	0.96	0.51-1.81
Not in first 8 mo, reference	254	1.00		105	1.00		149	1.00	
Soy									
3-12 mo	54	0.92	0.39-2.17	18	0.88	0.12-6.65	36	0.92	0.38-2.21
Not in first year, reference	987	1.00		465	1.00		522	1.00	
Margarine									
3-12 mo	611	0.69	0.41-1.16	267	0.62	0.24-1.62	344	0.74	0.40-1.34
Not in first year, reference	430	1.00		216	1.00		214	1.00	
Butter									
3-12 mo	726	1.00	0.64-1.55	358	0.87	0.41-1.83	368	1.09	0.63-1.89
Not in first year, reference	315	1.00		125	1.00		190	1.00	
Cake									
3-8 mo	444	0.75	0.47-1.21	239	0.75	0.37-1.51	205	0.76	0.41-1.44
Not in first 8 mo, reference	597	1.00		244	1.00		353	1.00	
Chocolate									
3-12 mo	481	0.73	0.47-1.15	251	0.86	0.41-1.82	230	0.67	0.38-1.17
Not in first year, reference	560	1.00		232	1.00		328	1.00	

Boldface values are significant ($P < .05$).

*Adjusted for farmer, center, breast-feeding (duration), and parental history of allergies (ever eczema, hay fever, or asthma).

†Reference = no introduction of any cow's milk in the first year of life.

‡Interaction term between parents with atopy and food item ($P = .08$).

Chapter 5

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

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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. Roduit[4], J.-C. Dalphin[5], M. Depner[6], J. Genuneit[7], R. Lauener[4,8], J. Pekkanen[9], P. Pfefferle[10], J. Riedler[11], M. Roponen[12], J. Weber[6], E. von Mutius[6], C. Braun-Fahrländer[2,3], O. Vaarala[1] and The PASTURE Study Group[†]

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

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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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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-β1 (TGF-β1) 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-β1 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-β1 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 of age 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-β1 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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*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-β1 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-β1 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-β1 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°C. Layer of fat was removed, and the clear supernatant was used for analyses. To activate latent TGF-β1, an activation procedure using hydrochloric acid was performed. Samples were then neutralized with sodium hydroxide containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). TGF-β1 values were measured using ELISA method (Quantikine Human TGF-β1 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°C overnight and blocked with 1% BSA in PBS, 1 hour at + 37°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-β1 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-β1 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-β1 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-β1 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-β1 models and (ii) entered as a multiplicative interaction term to the final adjusted models. Finally, a dose variable for IgA and TGF-β1 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-β1 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-β1 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			
	> 6 months *N* (%)	3–6 months *N* (%)	≤ 3 months *N* (%)	*P*-value for difference
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			
	> 6 months *N* (%)	3–6 months *N* (%)	≤ 3 months *N* (%)	*P*-value for difference
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-β1 levels (Table 2).

Soluble IgA and TGF-β1 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-β1 showed no clear association with any AD phenotype, and no effect modification by duration of breastfeeding was observed.

Soluble IgA or TGF-β1 levels in breast milk were not consistently associated with atopy (Table S4). Duration of breastfeeding and sIgA or TGF-β1 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-β1 levels and AD, atopy or asthma.

Dose of sIgA and TGF-β1 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-β1 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-β1 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-β1 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-β1 (TGF-β1) 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-β1 [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) IgA [mg/L]	Crude GMR (95% CI)	Mutually adjusted GMR (95%-CI)
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-β1 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-β1) 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.

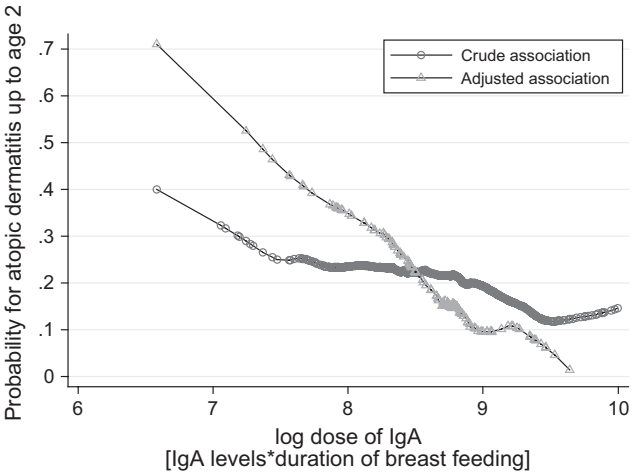

Fig. 1. Smoothed plot of the crude and adjusted association of (IgA) dose and the probability of atopic dermatitis (AD) up to age 2. Adjusted for centre, sex, maternal history of allergies, farming and food score in the first year of life. The estimated dose of soluble IgA (sIgA) ingested by an infant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

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 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-β1 in breast milk and atopic outcomes in the child, nor were there differences in TGF-β1 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-β1 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-β1 or TGF-β2, indicating protection against allergy-related outcomes in infancy and early childhood. It appeared that the levels of TGF-β1 measured in breast milk were relatively high in the studies in which the association of TGF-β1 and atopy was found. Levels of TGF-β1 in our study were comparable with the low median levels according to Oddy, and therefore, our findings of no association of breast milk TGF-β1 with atopic diseases may be related to the sensitivity of the method for the detection of TGF-β1 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-β1 levels in colostrum (day 3) and mature milk (1st month) of 45 farm and 69 urban mothers found significantly higher levels of TGF-β1 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-β1 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-β1. 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-β1 and sIgA levels in breast milk is not known.

Significant country differences in the levels of TGF-β1 and sIgA were found. TGF-β1 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-β1 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-β1 levels [36]. It is thus possible that TGF-β1 and IgA in breast milk are induced by microbial stimuli, and the association of high levels of TGF-β1 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-β1. 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-β1 as discussed above and on the isoform of TGF-β detected. We did not measure breast milk TGF-β2, 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-β1 levels in breast milk and atopic dermatitis, atopy, and asthma.

Chapter 6

Increased food diversity in the first year of life is inversely associated with allergic diseases.

This paper has been published:

Caroline Roduit, Remo Frei, Martin Depner, Bianca Schaub, Georg Loss, Jon Genuneit, Petra Pfefferle, Anne Hyvärinen, Anne M Karvonen, Josef Riedler, Jean-Charles Dalphin, Juha Pekkanen, Erika von Mutius, Charlotte Braun-Fahrländer, Roger Lauener, and the PASTURE study group

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Increased food diversity in the first year of life is inversely associated with allergic diseases

Caroline Roduit, MD, MPH,[a] Remo Frei, PhD,[b] Martin Depner, PhD,[c] Bianca Schaub, MD,[c] Georg Loss, PhD,[d] Jon Genuneit, MD,[e] Petra Pfefferle, PhD,[f] Anne Hyvärinen, PhD,[g] Anne M. Karvonen, PhD,[g] Josef Riedler, MD,[h] Jean-Charles Dalphin, MD, PhD,[i] Juha Pekkanen, MD,[g] Erika von Mutius, MD, MSc,[c] Charlotte Braun-Fahrländer, MD,[d,k] Roger Lauener, MD,[j] and the PASTURE study group*

Zurich, Basel, and Davos, Switzerland, Munich, Ulm, and Marburg, Germany, Kuopio, Finland, Schwarzach, Austria, and Besançon, France

Background: The role of dietary factors in the development of allergies is a topic of debate, especially the potential associations between infant feeding practices and allergic diseases.
Previously, we reported that increased food diversity introduced during the first year of life reduced the risk of atopic dermatitis.
Objective: In this study we investigated the association between the introduction of food during the first year of life and the development of asthma, allergic rhinitis, food allergy, or atopic sensitization, taking precautions to address reverse causality.
We further analyzed the association between food diversity and gene expression of T-cell markers and of Cε germline transcript, reflecting antibody isotype switching to IgE, measured at 6 years of age.
Methods: Eight hundred fifty-six children who participated in a birth cohort study, Protection Against Allergy Study in Rural Environments/EFRAIM, were included. Feeding practices were reported by parents in monthly diaries during the first year of life. Data on environmental factors and allergic diseases were collected from questionnaires administered from birth up to 6 years of age.
Results: An increased diversity of complementary food introduced in the first year of life was inversely associated with asthma with a dose-response effect (adjusted odds ratio with each additional food item introduced, 0.74 [95% CI, 0.61-0.89]). A similar effect was observed for food allergy and food sensitization. Furthermore, increased food diversity was significantly associated with an increased expression of forkhead box protein 3 and a decreased expression of Cε germline transcript.
Conclusion: An increased diversity of food within the first year of life might have a protective effect on asthma, food allergy, and food sensitization and is associated with increased expression of a marker for regulatory T cells. (J Allergy Clin Immunol 2014;133:1056-64.)

Key words: *Asthma, food allergy and sensitization, food diversity, children*

Nutrition is an important environmental factor in early life that influences the development of the child's immune system. The role of nutrition during infancy on the development of allergies later in childhood remains controversial. Moreover, reverse causality is always a matter of concern.

From [a]the University of Zurich, Children's Hospital, and Christine Kühne-Center for Allergy Research and Education, Zurich; [b]the Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich and Christine Kühne-Center for Allergy Research and Education, Zurich; [c]Dr von Hauner Children's Hospital, Ludwig-Maximilians-Universität, Munich; [d]the Swiss Tropical and Public Health Institute and the University of Basel; [e]the Institute of Epidemiology and Medical Biometry, Ulm University; [f]the Institute for Laboratory Medicine, Pathobiochemistry and Molecular Diagnostics, Philipps University of Marburg; [g]the Department of Environment Health, National Institute for Health and Welfare, Kuopio; [h]Children's Hospital Schwarzach; [i]the Department of Respiratory Disease, UMR/CNRS 6249 Chrono-environment, University Hospital of Besançon; [j]Children's Hospital of Eastern Switzerland St Gallen and the Christine Kühne-Center for Allergy Research and Education, Davos; and [k]the University of Basel.

*The members of the PASTURE study group are (in alphabetical order by study center): Maija-Riitta Hirvonen, Sami Remes, Marjut Roponen, and Pekka Tiittanen (Finland); Marie-Laure Dalphin and Vincent Kaulek (France); Gisela Büchele, Markus Ege, Michael Kabesch, and Harald Renz (Germany); and Gert Doekes (The Netherlands).

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Corresponding author: Caroline Roduit, MD, MPH, Kinderspital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. E-mail: Caroline.Roduit@kispi.uzh.ch.

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Abbreviations used
Foxp3: Forkhead box protein 3
OR: Odds ratio
PASTURE: Protection Against Allergy Study in Rural Environments
T-bet: T-box transcription factor

During the early postnatal period, the infant gut is first exposed to different food antigens, and these exposures might influence the development of immune tolerance. Mechanisms could include the acquisition of the microbiota through the diet or diet-microbiota interactions.[1-3] It has been suggested that modern changes in the postnatal environment, such as early dietary exposure, might not optimally support induction of immune tolerance because the incidence of allergic diseases during the last decades shows a strong increase.[4] However, current guidelines no longer recommend food allergen avoidance or delaying introduction in the infant diet to prevent allergic diseases because no clear benefit has been shown.[5] Recent studies have suggested a protective effect of early introduction of complementary food on allergic diseases, even though the underlying mechanism on the maturation of the mucosal immune system remains unknown.[6-12] However, it is widely accepted that oral tolerance mechanisms include regulatory T cells. Forkhead box protein 3 (Foxp3) is a key transcription factor for regulatory T cells, and it was shown that depletion of $Foxp3^+$ cells inhibited oral tolerance.[13] Our recent findings on the protective effect of an increased diversity of food introduced in the first year of life on atopic dermatitis are in agreement with the hypothesis that exposure to a variety of food antigens during early life might be important for the development of immune tolerance.[14]

Here we studied whether complementary food introduced in the first year of life was associated with asthma, food allergy, allergic rhinitis, and atopic sensitization up to 6 years of age in the prospective birth cohort study Protection Against Allergy Study in Rural Environments (PASTURE/EFRAIM).[15] Similar to our previous analyses on atopic dermatitis, we took into account potential reverse causality. We further examined whether food diversity introduced in the first year of life had an effect on gene expression of the T-cell markers T-box transcription factor (T-bet) and Gata-3, transcription factors related to the development of T_H1 and 2 T_H2 cells, respectively, and Foxp3, a transcription factor driving the development of regulatory cells T, and expression of Cε germline transcript, a marker for antibody isotype switching to IgE, at 6 years of age.

METHODS

Study design and population

The PASTURE/EFRAIM study is a prospective birth cohort involving children from rural areas in 5 European countries (Austria, Finland, France, Germany, and Switzerland) designed to evaluate risk factors and preventive factors for atopic diseases.[15] Pregnant women were recruited during the third trimester of pregnancy between August 2002 and March 2005 and divided into 2 groups. Women who lived on family-run farms where any kind of livestock was kept were assigned to the farm group. Women from the same rural areas not living on a farm were in the reference group. In total, 1133 children were included in this birth cohort. The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents.

Children with data available on allergic diseases up to 6 years of age, farming status, parental allergic history, maternal educational status, number of siblings, and feeding practices in the first year of life (n = 856) were included in the current study.

Definitions

Questionnaires were administered in interviews or self-administered to the mothers within the third trimester of pregnancy and when the children were 2, 12, 18, and 24 months of age and then yearly up to age 6 years. Children were defined as having asthma when the parents reported at least once that the child had either doctor-diagnosed asthma or at least 2 doctor-diagnosed episodes of obstructive bronchitis in the last 12 months in the year 4, 5, or 6 questionnaires independent of a diagnosis reported in the first 3 years of life. Obstructive bronchitis is commonly used to define the first occurrence of asthmatic symptoms. Food allergy was defined when the parents reported up to age 6 years that the child had at least once been given a diagnosis of food allergy by a doctor. Allergic rhinitis was defined by the presence of symptoms (itchy, runny, or blocked nose without a cold and associated with red itchy eyes) or doctor-diagnosed allergic rhinitis ever reported in the 6-year questionnaire. Levels of allergen-specific IgE antibodies (*Dermatophagoides pteronyssius*, *Dermatophagoides farinae*, alder, birch, hazel, grass pollen, rye, mugwort, plantain, cat, horse, dog, *Alternaria* species, hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat flour) were measured in blood among children at age 4.5 and 6 years, as well as their mothers and fathers. Sensitization was defined as a specific IgE level of 3.5 kU/L or greater and as being strongly associated with allergic diseases.

Parents indicated the food item that was given to the child in the last 4 weeks in each monthly diary between the 3rd and 12th months of life. For the introduction of complementary food, we used the same diversity score, as previously described, based on major food items, which were defined as the items introduced in the first year of life to at least 80% of the children.[14] The food diversity score is a total count of the number of different food items included in the child's diet. Diversity scores were calculated as follows: (1) with major food items introduced in the first year of life (n = 6, including vegetables or fruits, cereals, bread, meat, cake, and yogurt); (2) with the same major food items but introduced in the first 6 months of life; and (3) with all food items introduced in the first year of life (n = 15, including any cow's milk, yogurt, other milk product, eggs, nuts, vegetables or fruits, cereals, bread, meat, fish, soy, margarine, butter, cake, and chocolate) reported in the monthly diary. The latter was calculated to include potentially allergenic food items.

Farmer children were defined as children who were living on a farm where livestock was held and whose family ran the farm, according to parental reports. Information on parental atopic status, maternal education, smoking during pregnancy, mode of delivery, birth weight, gestational age, sex, number of siblings, and duration of breast-feeding was recorded in questionnaires during pregnancy, 2 months after birth, and at 1 year of age. Positive parental history of allergies was defined as ever having asthma, allergic rhinitis, or atopic dermatitis.

Gene expression of T-cell markers

Blood samples were collected at birth (cord blood) and at 1, 4.5, and 6 years of age for assessment of mRNA expression. The method for these measurements was described in detail elsewhere.[16] The data presented are normalized values for the endogenous controls (18S rRNA and β_2-microglobulin) determined by using the comparative ($\Delta\Delta$ cycle threshold) method, according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif).

Statistical analysis

Differences in characteristics of children regarding the diversity food score were tested by using the χ^2 test. Logistic regression was used to investigate the association between food exposure and asthma, food allergy, allergic rhinitis, and atopic sensitization. We performed and compared different models. Model 1 was the crude model, and model 2 included adjustment for the potential confounders: farmer, center, duration of breast-feeding (categorized according to the number of months children were breast-fed, not exclusively), parents with allergy, maternal education, sex, and number of siblings. To take into account reverse causality, we used model 3, which included the same adjustment as model 2 but additionally excluded children with doctor-diagnosed food allergy within the first year of life. For asthma, children

with at least 1 episode of doctor-diagnosed obstructive bronchitis or asthma within the first year of life were also excluded. Sensitivity analysis with additional exclusions for wheeze, atopic dermatitis, or both within the first year of life was performed. Parental allergic status is potentially a strong confounder because it might influence infant feeding practices; therefore we performed additional analysis using different definitions based on atopic sensitization and/or history of allergies among the mother, father, or both. Atopic dermatitis might also be a confounder and potentially on the causal pathway between food diversity and asthma, and therefore we performed analyses with additional adjustment to control for this. Sensitivity analyses were performed with modified food scores, removing 1 of the items to determine the influence of a single item in this score. Smooth plots were performed with generalized additive models by using R statistical software to graphically display the dose-response effect of the food diversity score on allergic diseases. Test for linear trend between the food diversity score and health outcomes was performed by using the Cochran-Armitage trend test.

The gene expression data were analyzed by using the comparative threshold method of Giulietti et al.[17] This method expresses the measured number of PCR cycles of the participants relative to 1 participant. We chose as a reference the cord blood values for a nonfarmer child whose expression of all genes was greater than the detection limit. The results are expressed as a gene expression multiplication factor compared with the reference. Because the distribution of the gene expression levels was skewed, the variables were log transformed (natural logarithm), resulting in an approximately normal distribution. We used linear regressions to calculate associations between food diversity scores and mRNA expression (expressed as geometric mean ratios). Interaction terms were included in the multivariate models to test for effect modification between the diversity food score and parental history of allergy, center, and farming status on allergic diseases. Data analysis was conducted with SAS software, version 9.2 (SAS Institute, Cary, NC).

RESULTS

Characteristics and prevalence of allergic diseases

Among the 856 children included in this study, 51.5% were farmer children, and 53.6% had at least 1 allergic parent (Table I). The description of food diversity was mentioned in our previous study.[14] Farmer children received a higher number of different food items in the first year of life compared with nonfarmer children. Differences between centers were observed regarding the diversity score, with a higher proportion of French and German children having a low score. A higher proportion of children with at least 1 parent with a history of atopy had a low score compared with children with parents with no history of atopy. No association between the diversity score and sex, number of siblings, duration of breast-feeding, and maternal education was found. Approximately half of the children (47.4%) were breast-fed for more than 6 months (not exclusively), and no association between duration of breast-feeding and asthma, allergic rhinitis, food allergy, and atopic sensitization was observed (data not shown). Characteristics of children excluded from the analysis because of missing data (n = 277) did not differ from those of the included children, except for a higher proportion of nonfarmer children (58.5%) among the excluded children.

There were 848 and 809 subjects with available data for allergic rhinitis and food allergy, respectively. The cumulative prevalence of asthma between 3 and 6 years of age was 8.6%, the cumulative prevalence of allergic rhinitis up to 6 years of age was 7.6%, and the cumulative prevalence of food allergy up to 6 years was 7.4%. These proportions were significantly higher in children with 2 allergic parents than among children with nonallergic parents (asthma, 10.7% vs 6.3%; allergic rhinitis, 11.0% vs 3.6%; and food allergy, 10.6% vs 3.7%, respectively).

Data on atopic sensitization were available for 596 children at age 4.5 years, 6 years, or both. Sensitization to any allergen was present in 25.5% of children, sensitization to food allergens was present 10.7%, and sensitization to inhalant allergens was present in 22.1%, as measured at 4.5 or 6 years.

Association between food diversity score and allergic diseases and atopic sensitization

Dividing the diversity score into 3 categories with the highest (all 6 food items) as reference, we observed a significant inverse dose-response association with asthma in crude analysis (model 1) and after adjustment for potential confounders (model 2, Table II). The test for linear trend was significant for asthma, food allergy, and food sensitization ($P < .001$, $P < .001$, and $P = .006$, respectively). We observed a significant reduction of 26% for the development of asthma, with each additional food item introduced in the first year of life. This inverse association between the food diversity score and asthma remained stable after exclusion of children with a doctor's diagnosis of food allergy and defined as having asthma, obstructive bronchitis, or both within the first year of life (model 3). Sensitivity analyses with additional exclusion of children with wheezing (n = 223 [26.1%]), atopic dermatitis (n = 119 [14.4%]), or both within the first year of life were performed and showed similar results (crude analysis only among children with no wheeze and no atopic dermatitis in the first year of life: odds ratio [OR] for 0-3 vs 6 items of 3.83 [95% CI, 1.02-14.41] and OR for 4-5 vs 6 items of 1.63 [95% CI, 0.74-3.61]). Moreover, analysis excluding children (n = 30) among those parents who reported that they avoided introducing food within the first year of life because of child's allergy showed similar associations.

Additional adjustment for atopic dermatitis showed similar results (see Table E1 in this article's Online Repository at www.jacionline.org), and the negative association between food diversity score and asthma was stronger for children having both diseases (n = 29) compared with those having neither disease (OR for food diversity score, continuous: 0.61 [95% CI, 0.49-0.76]). For children with asthma but no atopic dermatitis up to 6 years (n = 40), a similar association was observed with an increasing diversity food score (OR for food diversity score, continuous: 0.80 [95% CI, 0.62-1.04]). Additional analyses were performed for children defined as having asthma based only on a doctor's diagnosis of asthma between 3 and 6 years of age (n = 36), which showed similar results (see Table E2 in this article's Online Repository at www.jacionline.org). Recently published results from this birth cohort showed that late-onset and persistent wheeze phenotypes were best correlated with clinical phenotypes of asthma.[18] We observed that the strongest association between the food diversity score and different phenotypes of wheeze was with late-onset and persistent wheeze phenotypes (OR for transient wheeze, 1.08 [95% CI, 0.86-1.35]; OR for intermediate wheeze, 0.91 [95% CI, 0.68-1.21]; OR for late-onset wheeze, 0.75 [95% CI, 0.53-1.08]; and OR for persistent wheeze, 0.76 [95% CI, 0.55-1.07]).

A smoothed plot of the relationship between the food diversity score and asthma was performed, which showed a decrease in the log odds of asthma with an increasing score (Fig 1, *A*).

TABLE I. Characteristics of the study population and prevalence of allergic diseases

	All		Food diversity score						
			0-3 items		4-5 items		6 items		
	No.	Percent	No.	Percent	No.	Percent	No.	Percent	*P* value*
Characteristics									
All	856	100	37	4.3	263	30.7	556	65.0	
Farmer									<.001
Yes	415	51.5	9	24.3	112	57.4	294	52.8	
No	441	48.5	28	75.7	151	42.6	263	47.2	
Center									.01
Austria	161	18.8	1	2.7	58	22.1	102	18.3	
Switzerland	187	21.9	9	24.3	57	21.7	121	21.8	
France	150	17.5	12	32.4	48	18.2	90	16.2	
Germany	203	23.7	12	32.4	63	23.9	128	23.0	
Finland	155	18.1	3	8.1	37	14.1	115	20.7	
Sex									.66
Girls	424	49.5	20	54.0	125	47.5	279	50.2	
Boys	432	50.5	17	46.0	138	52.5	277	49.8	
Siblings									.10
0	310	36.2	9	24.3	100	38.0	201	36.1	
1-2	459	53.6	27	73.0	140	53.2	292	52.6	
≥3	87	10.2	1	2.7	23	8.8	63	11.3	
Parents with atopy history									.005
Yes	459	53.6	26	70.3	156	59.3	277	49.8	
No	397	46.4	11	29.7	107	40.7	279	50.2	
Breast-feeding									.70
Never	80	9.4	5	13.5	26	9.9	49	8.8	
>0-2 mo	132	15.4	5	13.5	40	15.2	87	15.7	
3-6 mo	238	27.8	6	16.2	75	28.5	157	28.2	
7-9 mo	180	21.0	9	24.3	48	18.3	123	22.1	
≥10 mo	226	26.4	12	32.4	74	28.1	140	25.2	
Maternal education									.16
Low	140	16.4	11	29.7	40	15.2	89	16.0	
Mid	370	43.2	10	27.0	109	41.4	251	45.1	
Mid-high	251	29.3	10	27.0	82	31.2	159	28.6	
High	95	11.1	6	16.2	32	12.2	57	10.3	
Outcome prevalences									
Asthma (doctor-diagnosed asthma and/or ≥2 obstructive bronchitis episodes 3-6 y)	74/856	8.6	7	18.9	33	12.6	34	6.1	<.001
Allergic rhinitis (doctor-diagnosed hay fever or allergic rhinitis OR symptoms [nasal AND eye] ever at age 6 y)	64/848	7.6	4	10.8	24	9.2	36	6.6	.31
Food allergy up to 6 y (doctor-diagnosed food allergy)	60/809	7.4	7	21.9	23	9.2	30	5.7	.001
Any sensitization at 4.5 or 6 y (cutoff: 3.5 kU/L)	152/596	25.5	12	46.2	44	24.2	96	24.7	.05
Food sensitization at 4.5 or 6 y (cutoff: 3.5 kU/L)	62/580	10.7	7	26.9	23	13.1	32	8.5	.006
Inhalant sensitization 4.5 or 6 y (cutoff: 3.5 kU/L)	131/594	22.1	9	34.6	39	21.4	83	21.5	.29

*Based on the χ^2 test.

The analysis restricted to children for whom no food avoidance was reported within the first year of life because of child's allergy showed the same pattern (Fig 1, *B*). To also include allergenic foods, we additionally analyzed the association between another food diversity score with all the food items (n = 15) and asthma. The same inverse dose-response effect was observed (Fig 1, *C*). The same analyses were performed with food allergy (Fig 2), and similar patterns were observed. Additionally, we calculated a diversity score with food items introduced within the first 6 months of life and observed the same tendency of a negative association between a higher score compared with a lower score on asthma risk (OR for food diversity score, continuous: 0.83 [95% CI, 0.68-1.03]), although with a test for linear trend of borderline significance (P = .08, see Fig E1 in this article's Online Repository at www.jacionline.org).

The children with a low food diversity score (with the major food items) had an increased risk of food allergy up to 6 years of age and sensitization to food allergens at age 4.5 or 6 years compared with children with the highest score (Table II). The analysis with children having doctor-diagnosed food allergy combined with positive food sensitization (n = 18) showed an even stronger negative association with food diversity (OR for food diversity score, continuous: 0.55 [95% CI, 0.40-0.76]). After exclusion of children with food allergy within the first year of life, the associations were no longer statistically significant, even though estimates for the continuous variable remained similar (model 3). There was a tendency toward a negative association between the food diversity score and allergic rhinitis or sensitization to inhalant allergens, although this was not statistically significant. There was no interaction between the food diversity score and parental allergies or farming status on

TABLE II. Association between diversity score within the first year of life and allergic diseases and atopic sensitization

	Model 1			Model 2			Model 3		
	No.	OR	95% CI	No.	OR	95% CI	No.	OR	95% CI
Asthma									
Food diversity score within 1st year									
0-3 items	7/37	**3.58**	**1.47-8.75**	7/37	**3.15**	**1.24-8.05**	5/32	**3.77**	**1.23-11.53**
4-5 items	33/263	**2.20**	**1.33-3.64**	33/263	**2.01**	**1.24-3.46**	16/215	1.60	0.80-3.20
6 items, reference	34/556	1		34/556	1		21/487	1	
Diversity score, continuous	74/856	**0.73**	**0.61-0.87**	74/856	**0.74**	**0.61-0.89**	42/734	**0.76**	**0.60-0.96**
Allergic rhinitis									
Diversity score major food within 1st year									
0-3 items	4/37	1.73	0.58-5.15	4/37	1.78	0.56-5.67	2/34	0.94	0.21-4.26
4-5 items	24/261	1.45	0.84-2.48	24/261	1.35	0.77-2.38	20/252	1.14	0.63-2.06
6 items, reference	36/550	1		36/550	1		36/547	1	
Diversity score, continuous	64/848	0.83	0.68-1.01	64/848	0.80	0.64-1.00	58/833	0.93	0.71-1.22
Doctor-diagnosed food allergy									
Food diversity score within 1st year									
0-3 items	7/32	**4.65**	**1.86-11.61**	7/32	**4.43**	**1.62-12.10**	4/29	2.61	0.77-8.88
4-5 items	23/249	1.69	0.96-2.97	23/249	**1.85**	**1.02-3.35**	13/239	1.13	0.56-2.31
6 items, reference	30/528	1		30/528	1		26/524	1	
Diversity score, continuous	60/809	**0.71**	**0.58-0.85**	60/809	**0.70**	**0.57-0.86**	43/792	0.79	0.60-1.03
Sensitization to food allergens at 4.5 or 6 y									
Food diversity score within 1st year									
0-3 items	7/26	**3.99**	**1.56-10.19**	7/26	**5.47**	**1.91-15.67**	6/23	**5.22**	**1.70-16.04**
4-5 items	23/176	1.63	0.92-2.87	23/176	1.52	0.83-2.76	22/167	1.53	0.83-2.84
6 items, reference	32/378	1		32/378	1		31/376	1	
Diversity score, continuous	62/580	**0.76**	**0.62-0.93**	62/580	**0.72**	**0.57-0.90**	59/566	**0.72**	**0.56-0.91**
Sensitization to inhalant allergens at 4.5 or 6 y									
Food diversity score within 1st year									
0-3 items	9/26	1.93	0.83-4.49	9/26	1.79	0.74-4.37	7/23	1.50	0.57-3.95
4-5 items	39/182	1.00	0.65-1.53	39/182	0.87	0.56-1.37	36/172	0.84	0.53-1.34
6 items, reference	83/386	1		83/386	1		83/384	1	
Diversity score, continuous	131/594	0.94	0.78-1.13	131/594	0.97	0.80-1.18	126/579	1.01	0.82-1.25

Diversity scores with major food items are shown. Boldface values are significant ($P < .05$).
Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, sex, breast-feeding, siblings, and maternal education; *Model 3*, model 2 plus exclusion of food allergy at 1 year (n = 17) and only with asthma: exclusion of at least 1 episode of obstructive bronchitis and/or asthma, both doctor diagnosed and reported at 1 year (n = 102).

allergic diseases. Moreover, similar results were obtained in separate analyses stratified by parental history of allergy or by farming status, as well as after additional adjustment for consumption of unboiled farm milk (data not shown).

The analysis with different definitions of asthma, both atopic and nonatopic (defined with or without positive sensitization to inhalant allergens at 4.5 or 6 years of age: n = 23 and n = 30, respectively) showed the same tendencies (adjusted OR for each additional food items introduced in the first year of life, 0.70 [95% CI, 0.52-0.94] and 0.77 [95% CI, 0.59-1.00], respectively). Analysis for a subgroup excluding children with a low score of food diversity showed similar results (see Table E3 in this article's Online Repository at www.jacionline.org). Sensitivity analyses with modified scores, excluding 1 of the food items to evaluate the influence of the excluded item in this score, showed similar associations between reduced scores and allergic diseases (data not shown).

To analyze the association between single food items and allergic outcomes, we also performed different models to take into account the reverse causality effect. The results from the analyses between single food items and asthma showed a strong negative association with milk products, such as yogurt and butter, introduced within the first year of life, compared with reference children who did not consume these foods in the first year of life (see Table E4 in this article's Online Repository at www.jacionline.org). The analysis among a subgroup excluding children with food allergy or asthma within the first year of life (model 3) showed similar results, although these were weaker for introduction of yogurt. The analysis with the 2 predictor variables of yogurt and butter in the model showed the same protective effect, as well as stratified analysis by parental history of allergy (data not shown). The risk of food allergy was reduced by a factor of 0.5 among children who consumed fish within the first year of life compared with children who did not (see Table E5 in this article's Online Repository at www.jacionline.org). This effect remained significant after adjustment for potential confounders, such as atopic dermatitis, and after exclusion of children with food allergy within the first year of life. The stratified analysis showed an inverse association only among the children with allergic parents (data not shown). The negative association between fish introduced within the first year of life and sensitization to food allergens was only significant in the crude analysis (see Table E6 in this article's Online Repository at www.jacionline.org).

Association between food diversity score and gene expression for marker of IgE antibody isotype switching and for T-cell markers

Among children with a low food diversity score within the first year of life, we observed a significantly increased level of Cε germline transcript at age 6 years by a factor of 1.8 compared with

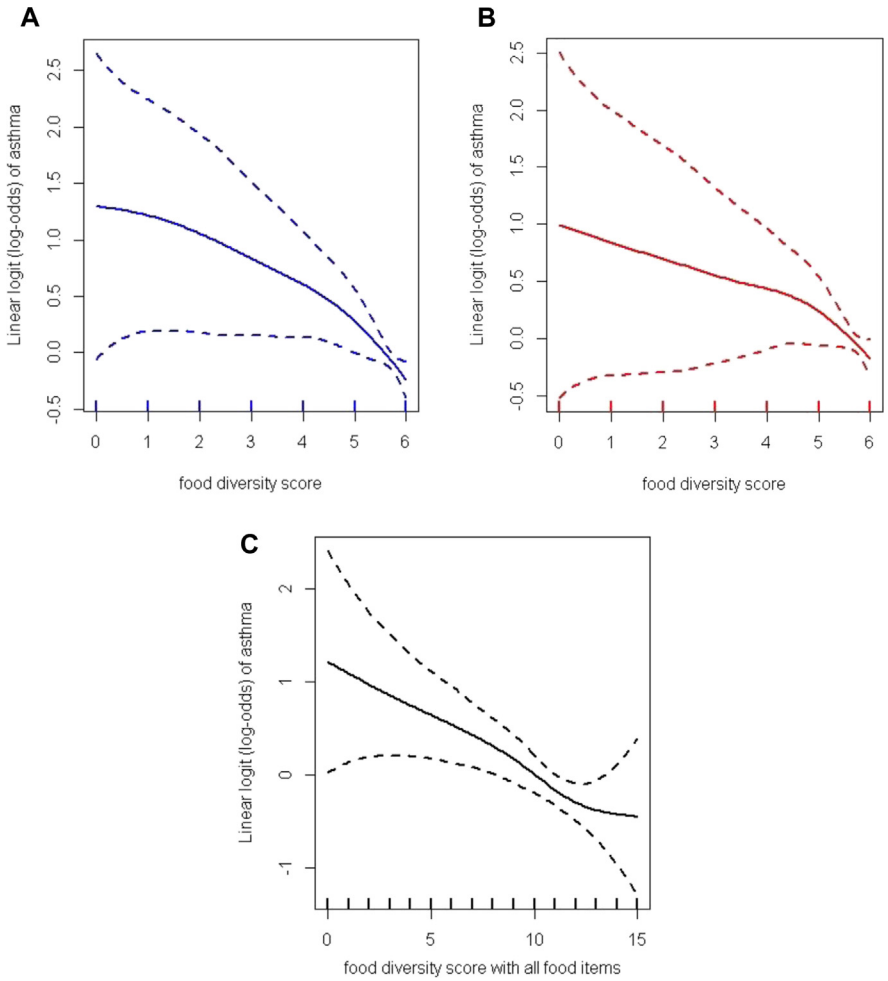

FIG 1. Association between increasing diversity of food introduced within the first year of life and asthma. **A,** Diversity score with major food for the entire study population. **B,** Diversity score with major food restricted to children without food avoidance within the first year of life because of allergies. **C,** Diversity score with all different food items for the entire study population. The *solid line* represents the predicted value of asthma as a function of the score, and *dashed lines* represent the CI. The *y-axis* is the linear logit of asthma, and the values are centered on 0 (50/50 odds) and extended to both positive and negative values. All models are adjusted for farmer, center, duration of breast-feeding, parents with allergy, maternal education, sex, and siblings.

children with the highest score (Table III). Moreover, those children with a low food diversity score showed a significantly lower level of gene expression for Foxp3, a transcription factor driving development of regulatory T cells, measured at 6 years after adjustment for potential confounders and the value measured in cord blood (Table IV). Analysis stratified by parental allergic status showed similar results. We did not find an indirect effect of Foxp3 or Cε germline transcript expression on the associations between food diversity and allergic diseases or sensitization (data not shown). We did not observe an association between the food diversity score and mRNA expression of T-bet and Gata-3, transcription factors related to the development of T_H1 and T_H2 cells, respectively.

DISCUSSION

Our data show that an increased diversity of food introduced in children's diet within the first year of life is negatively associated with the development of asthma and food allergy up to 6 years of age and on sensitization to food allergens at 4.5 or 6 years of age. Moreover, among children with a low food diversity score, we found an increased expression of marker for antibody isotype switching to IgE and a reduced expression of the regulatory T cell–associated gene Foxp3 measured at 6 years of age.

To our knowledge, the present findings are the first showing an inverse association of an increased diversity of exposures to food antigens in the first year of life on the development of allergic diseases later in childhood. Previous studies on the diversity of food introduced in infants' diets and allergies focused on the first 4 or 6 months of life.[19-21] Only 1 study reported an increased risk of doctor-diagnosed eczema up to 6 years of age in association with an increased diversity of solid food introduced within the first 4 months.[20] However, this effect was seen neither with eczema up to 4 years nor with a definition based on symptoms, and a tendency of protective effect on asthma up to 6 years was observed with an increased diversity of food introduced within the first 4 months. In our study a diversity score with food items introduced within the first 6 months of life was calculated, and

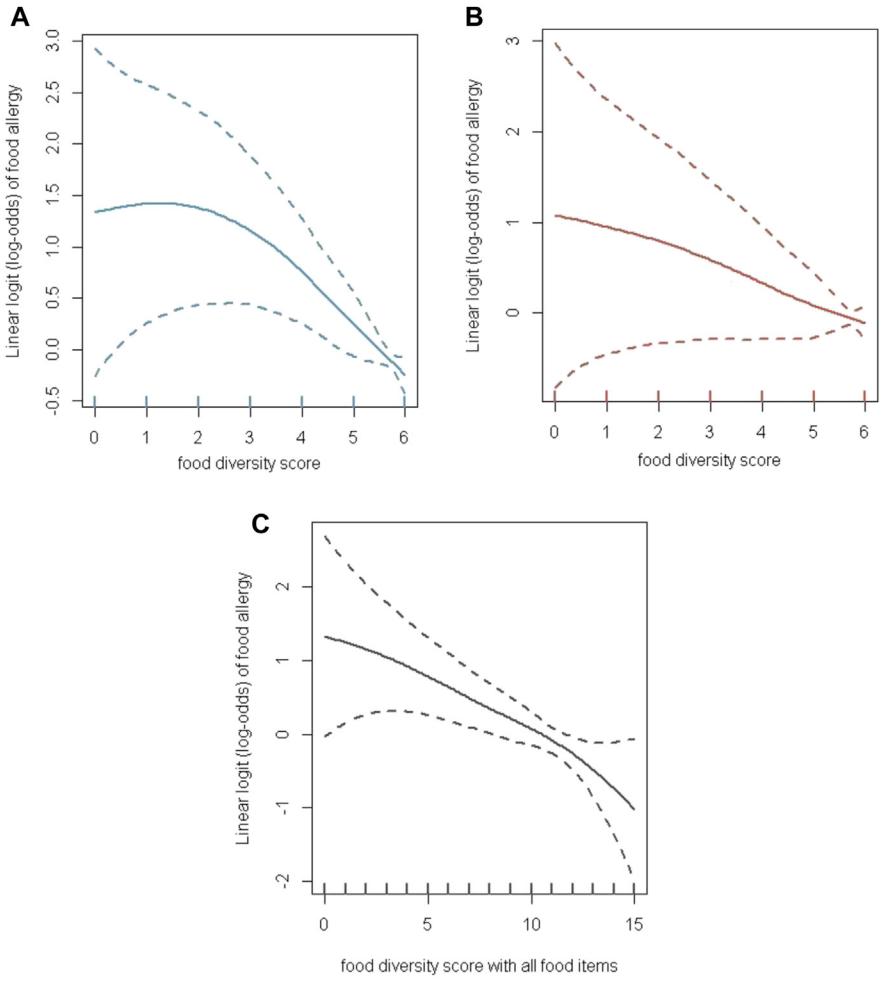

FIG 2. Association between increasing diversity of food introduced within the first year of life and food allergy. **A,** Diversity score with major food for the entire study population. **B,** Diversity score with major food restricted to children without food avoidance within the first year of life because of allergies. **C,** Diversity score with all different food items for the entire study population. The *solid line* represents the predicted value of asthma as a function of the score, and *dashed lines* represent the CI. The *y-axis* is the linear logit of food allergy, and the values are centered on 0 (50/50 odds) and extended to both positive and negative values. All models are adjusted for farmer, center, duration of breast-feeding, parents with allergy, maternal education, sex, and siblings.

TABLE III. Association between diversity food score within the first year of life and gene expression for marker of IgE antibody isotype switching (Cε germline transcript) measured at 6 years

	Cε germline transcript (6 y)								
	All			No allergic parents			≥1 Allergic parent		
Diversity score, major food items within 1st y	No.	GMR*	95% CI	No.	GMR*	95% CI	No.	GMR*	95% CI
0-3 items	22	**1.81**	**1.21-2.70**	7	1.81	0.84-3.88	15	**1.78**	**1.13-2.79**
4-5 items	161	1.18	0.99-1.40	61	1.26	0.94-1.69	100	1.14	0.93-1.39
6 items, reference	336	1		156	1		180	1	

Boldface values are significant ($P > .05$).
*Geometric mean ratios are adjusted for center, farmer, parents with allergy, sex, breast-feeding, maternal education, and cord blood value for the respective gene.

children with a higher score had a lower risk of asthma compared with those with a lower score. Moreover, the analysis with mutual adjustment for the score within the first 6 months and the score within the first year of life showed a persistent significant protective effect of the first year's score, meaning that exposure to food proteins in the time period between 6 and 12 months of age might be an important time window for protection against the development of later allergic diseases. The new guidelines on early nutrition and allergy prevention recommend that complementary foods should not be introduced before 4 months of age but should be introduced for all infants by 6 months of age.[5,22] Even though more evidence is required, because our

TABLE IV. Association between diversity food score within the first year of life and gene expression for T-cell markers measured at 6 years

Diversity score, major food items within 1st year	All			No allergic parents			≥1 Allergic parent		
	No.	GMR*	95% CI	No.	GMR*	95% CI	No.	GMR*	95% CI
T-bet									
0-3 items	22	0.94	0.71-1.25	7	1.13	0.61-2.12	15	0.90	0.70-1.16
4-5 items	163	0.96	0.85-1.09	63	0.98	0.77-1.24	100	0.95	0.85-1.07
6 items, reference	339	1		157	1		182	1	
Gata-3									
0-3 items	22	0.93	0.74-1.18	7	0.93	0.56-1.56	15	0.98	0.79-1.21
4-5 items	163	1.06	0.96-1.17	63	1.10	0.91-1.34	100	1.03	0.94-1.14
6 items, reference	339	1		157	1		182	1	
Foxp3									
0-3 items	22	**0.70**	**0.51-0.96**	7	0.78	0.41-1.51	15	**0.69**	**0.50-0.96**
4-5 items	163	0.99	0.87-1.14	63	0.92	0.72-1.18	100	1.03	0.89-1.20
6 items, reference	339	1		157	1		182	1	

Boldface values are significant ($P > .05$).

*Geometric mean ratios are adjusted for center, farmer, parents with allergy, sex, breast-feeding, maternal education, and cord blood value for the respective gene.

findings form the basis for a new hypothesis, an increased diversity of food in the second part of the first year of life might be an interesting strategy to prevent allergic diseases.

The strengths of this study are the prospective design and collection of data on the introduction of complementary food within the first year of life, which avoid recall bias. One major concern with the association between feeding practices and atopic diseases is the potential bias caused by reverse causality. Among children with early symptoms of the disease, those with allergic parents, or both, introduction of certain complementary foods, especially allergenic foods, tends to be delayed. With analyses performed in a subgroup of children, excluding those with food allergy, respiratory disorders, or both within the first year of life, significant results remained with asthma, and there was a similar tendency, although weaker, with food allergy and food sensitization. Moreover, all multivariate models were additionally adjusted for atopic dermatitis, and the negative association between food diversity and asthma seems to be independent of the effect on atopic dermatitis. Sensitivity analysis with models adjusted for parental allergic status using different definitions based on atopic sensitization and/or history of allergies among the mother, father, or both showed similar results, as did stratified analysis (data not shown). Our results provide strong support to conduct a randomized clinical trial, which is essential to completely exclude the reverse causality effect. Selection bias is unlikely in this study because the excluded and included children did not differ significantly. The definition of the health outcomes based on a doctor's diagnosis might lead to an underestimation of the prevalence and, despite this, could lead to an underestimation of the association by a dilution effect; however, we still found a significant association. Information on asthma medications was not included in the definition and might be considered a limitation. Moreover, a child might be defined as having asthma if the reported doctor's diagnosis was made between 3 and 4 years of age and not later and therefore related to wheeze that resolves by school age. In any case, the strongest negative association between food diversity and wheeze phenotypes was with late-onset and persistent wheeze, which both were shown to be related to clinical phenotype of asthma.[18] Another limitation of this study is the selected study population, from rural areas in Europe, so that our findings might not be applicable to other populations and also the lack of information on lactose or other food intolerance and on conditions that could affect infant feeding or could represent subclinical manifestations of food allergy, such as colic and gastroesophageal reflux. The latter could induce a reverse causality effect and is a major limitation in this study. Therefore reverse causality cannot be completely excluded. Another reason that reverse causality cannot be excluded is that reasons for the variability in food introduction in the first year of life are not well understood.

Our findings highlight the role for diversity of environmental exposures on the development of allergic diseases. We have shown previously that the diversity of exposures might play a role in the development of allergic disease, even during pregnancy, with contact with different farm animal species.[16] Similarly, recent findings showed that the increased diversity of microbial exposures had a protective effect on asthma.[23] Our results support the hypothesis that exposure in early life to diverse food antigens, such as food proteins, could increase the maturation of the mucosal immune system and induce tolerance networks.[24] Moreover, it is believed that regulatory T cells are involved in tolerance acquisition. In this study we showed decreased expression of a marker for regulatory T cells using the transcription factor Foxp3 at the age of 6 years among children with a low food diversity scores within the first year of life. However, this transcription factor does not assess the functional activity of regulatory T cells. The protective effect of exposure to an increased diversity of food in early life might be associated with the induction of regulatory T cells rather than a shift in the T_H1/T_H2 balance. One of the mechanisms that might be involved in the inhibition of allergy development by regulatory T cells is the inhibition of isotype switching to IgE[25]; interestingly, our results showing a decrease in Cε germline transcript with an increased food diversity are in agreement with this hypothesis.

The negative association was observed between increased food diversity and asthma and food sensitization. However, no significant association was found with sensitization to inhalant allergens and allergic rhinitis, even though this is difficult to evaluate because of the low numbers. One explanation might be that early food sensitization is a better predictor for allergic diseases and asthma than inhalant sensitization because this was already shown in previous studies.[26,27] Moreover, the same

tendency toward a negative association with an increased food diversity was observed with both atopic and nonatopic asthma (even though it is not significant, this might be due to small numbers). Even though the underlying mechanism for the protective effect on asthma remains unclear, it might involve an induction of regulatory T cells, which are known to play a role in constraining inflammation.[28] Moreover, some dietary components, such as short-chain or long-chain fatty acids, as well as prebiotics, are known to have immunoregulatory properties.[3] A potential mechanism for the protective effect of an increased diversity in the infant's diet on allergic diseases might involve the gut microbiota, its metabolites, or both. It has been shown that diet, gut microbiota, and immune responses are connected.[2,29] Human studies examining the gut microbiota and allergies have shown conflicting results; however, an inverse association between the bacterial diversity of the gut microbiota in the first months of life and the development of eczema in early life was previously reported.[30]

In conclusion, this is the first study showing that infants exposed to an increased diversity of food items within the first year of life have a reduced risk of asthma, food allergy, and sensitization to food allergens up to age 6 years.

We thank all the fieldworkers and other PASTURE/EFRAIM team members.

Clinical implications: An increased diversity of complementary foods introduced within the first year of life might have a protective effect on the development of allergic diseases, such as asthma, in children.

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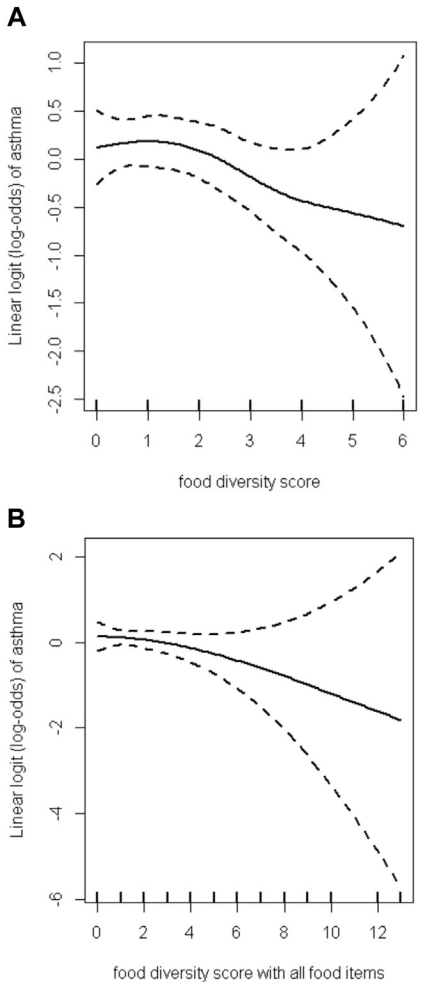

FIG E1. Association between increasing diversity of food introduced within the first 6 months of age and asthma. **A,** Diversity score with major food introduced within the first 6 months of life for the entire study population. **B,** Diversity score with all food items introduced within the first 6 months of life for the entire study population. The *solid line* represents the predicted value of asthma as a function of the score, and *dashed lines* represent the CI. The *y-axis* is the linear logit of asthma, and the values are centered on 0 (50/50 odds) and extended to both positive and negative values. All models are adjusted for farmer, center, duration of breast-feeding, parents with allergy, maternal education, sex, and siblings.

TABLE E1. Association between diversity score within the first year of life and allergic diseases and atopic sensitization, with additional adjustment for atopic dermatitis

	No.	OR*	95% CI
Asthma			
Food diversity score within 1st year			
0-3 items	7/35	**3.36**	**1.28-8.84**
4-5 items	32/243	**2.15**	**1.25-3.70**
6 items, reference	30/521	1	
Diversity score, continuous	69/799	**0.73**	**0.60-0.88**
Allergic rhinitis			
Diversity score major food within 1st year			
0-3 items	4/35	1.83	0.56-5.94
4-5 items	22/242	1.23	0.68-2.23
6 items, reference	34/518	1	
Diversity score, continuous	60/795	0.81	0.64-1.02
Doctor-diagnosed food allergy			
Food diversity score within 1st year			
0-3 items	7/32	**4.64**	**1.63-13.24**
4-5 items	22/239	1.68	0.90-3.14
6 items, reference	27/513	1	
Diversity score, continuous	56/784	**0.71**	**0.57-0.89**
Sensitization to food allergens at 4.5 or 6 y			
Food diversity score within 1st year			
0-3 items	5/24	**3.67**	**1.15-11.68**
4-5 items	21/163	1.44	0.77-2.71
6 items, reference	31/355	1	
Diversity score, continuous	57/542	**0.77**	**0.60-0.99**
Sensitization to inhalant allergens at 4.5 or 6 y			
Food diversity score within 1st year			
0-3 items	8/24	1.65	0.64-4.21
4-5 items	36/169	0.84	0.52-1.35
6 items, reference	78/362	1	
Diversity score, continuous	122/555	1.01	0.82-1.25

Boldface values are significant ($P > .05$).

*Adjusted for center, farmer, parents with allergy, sex, breast-feeding, siblings, maternal education, and atopic dermatitis up to 6 years.

TABLE E2. Association between diversity score within the first year of life and doctor's diagnosis of asthma*

	Doctor's diagnosis of asthma					
	Model 1		Model 2		Model 3	
	OR	95% CI	OR	95% CI	OR	95% CI
Food diversity score within 1st year						
0-3 items	**5.80**	**1.77-19.06**	**5.78**	**1.64-20.35**	**6.57**	**1.52-28.35**
4-5 items	**3.78**	**1.82-7.87**	**3.83**	**1.81-8.12**	2.55	0.95-6.86
6 items, reference	1		1		1	
Diversity score, continuous	**0.68**	**0.60-0.88**	**0.69**	**0.54-0.85**	**0.68**	**0.51-0.91**

Diversity scores with major food items are shown. Boldface values are significant ($P < .05$).

Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, sex, breast-feeding, siblings, and maternal education; *Model 3*, model 2 plus exclusion of food allergy at 1 year (n = 17) and only with asthma: exclusion of at least 1 episode of obstructive bronchitis and/or asthma, both doctor diagnosed and reported at 1 year (n = 102).

*Definition of asthma based only on doctor's diagnosis of asthma between 3 and 6 years of age (n = 36).

TABLE E3. Association between diversity score within the first year of life and allergic diseases and atopic sensitization: analysis among a subgroup with exclusion of children with low diversity scores (n = 819)

		Model 1		Model 2			Model 3	
	No.	OR	95% CI	OR	95% CI	No.	OR	95% CI
Asthma								
Diversity score, continuous	67/819	**0.58**	**0.41-0.82**	**0.61**	**0.43-0.87**	37/702	0.82	0.49-1.37
Allergic rhinitis								
Diversity score, continuous	60/811	0.69	0.47-1.00	0.72	0.48-1.06	56/799	0.85	0.55-1.31
Doctor-diagnosed food allergy								
Diversity score, continuous	53/777	**0.58**	**0.40-0.86**	**0.58**	**0.39-0.86**	39/763	0.82	0.50-1.36
Sensitization to food allergens at 4.5 or 6 y								
Diversity score, continuous	55/554	0.82	0.54-1.24	0.86	0.55-1.34	53/543	0.84	0.53-1.32
Sensitization to inhalant allergens at 4.5 or 6 y								
Diversity score, continuous	122/568	1.05	0.76-1.44	1.14	0.81-1.60	119/556	1.21	0.85-1.73

Diversity scores with major food items are shown. Boldface values are significant ($P < .05$).

Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, sex, breast-feeding, siblings, and maternal education; *Model 3*, model 2 plus exclusion of food allergy at 1 year (n = 17) and only with asthma: exclusion of at least 1 episode of obstructive bronchitis and/or asthma , both doctor diagnosed and reported at 1 year (n = 102).

TABLE E4. Association between introduction of single food items in the first year of life and asthma

	Asthma		
	Model 1	Model 2	Model 3
	OR (95% CI)	OR (95% CI)	OR (95% CI)
Cow's milk			
3-12 mo	0.64 (0.40-1.04)	0.67 (0.40-1.14)	0.96 (0.49-1.90)
>12 mo, reference	1	1	1
Shop milk			
3-12 mo	0.69 (0.37-1.29)	0.55 (0.28-1.08)	0.96 (0.42-2.20)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk			
3-12 mo	0.61 (0.34-1.08)	0.82 (0.40-1.69)	0.89 (0.35-2.26)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk, unboiled			
3-12 mo	0.67 (0.28-1.59)	0.95 (0.37-2.40)	1.61 (0.50-5.16)
>12 mo, reference	1	1	1
Yogurt			
3-12 mo	**0.42 (0.25-0.70)**	**0.47 (0.26-0.84)**	0.55 (0.26-1.18)
>12 mo, reference	1	1	1
Other milk products			
3-12 mo	**0.40 (0.24-0.64)**	**0.37 (0.22-0.64)**	0.65 (0.32-1.33)
>12 mo, reference	1	1	1
Vegetables or fruits			
<6 mo	0.95 (0.59-1.54)	0.98 (0.53-1.82)	0.93 (0.41-2.11)
≥6 mo, reference	1	1	1
Fish			
3-12 mo	0.72 (0.44-1.15)	0.76 (0.44-1.31)	0.80 (0.39-1.63)
>12 mo, reference	1	1	1
Nuts			
3-12 mo	0.80 (0.45-1.42)	0.69 (0.36-1.34)	0.80 (0.36-1.80)
>12 mo, reference	1	1	1
Eggs			
3-12 mo	**0.46 (0.28-0.74)**	**0.47 (0.28-0.80)**	0.65 (0.33-1.31)
>12 mo, reference	1	1	1
Meat			
<9 mo	1.28 (0.75-2.18)	1.47 (0.80-2.70)	1.44 (0.67-3.12)
≥9 mo, reference	1	1	1
Cereals			
<9 mo	1.34 (0.78-2.28)	1.13 (0.62-2.05)	1.05 (0.64-2.17)
≥9 mo, reference	1	1	1
Bread			
<9 mo	1.10 (0.67-1.80)	1.06 (0.62-1.80)	1.17 (0.58-2.35)
≥9 mo, reference	1	1	1
Soja			
3-12 mo	1.42 (0.54-3.72)	1.27 (0.46-3.46)	1.45 (0.40-5.21)
>12 mo, reference	1	1	1
Margarine			
3-12 mo	1.44 (0.86-2.41)	1.16 (0.65-2.09)	1.17 (0.55-2.48)
>12 mo, reference	1	1	1
Butter			
3-12 mo	**0.49 (0.30-0.79)**	**0.45 (0.26-0.77)**	**0.43 (0.21-0.86)**
>12 mo, reference	1	1	1
Cake			
<9 mo	0.72 (0.44-1.19)	0.71 (0.40-1.26)	0.64 (0.30-1.34)
≥9 mo, reference	1	1	1
Chocolate			
3-12 mo	**0.47 (0.28-0.78)**	**0.45 (0.25-0.79)**	**0.41 (0.19-0.88)**
>12 mo, reference	1	1	1

Boldface values are significant ($P < .05$).

Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, maternal education, sex, breast-feeding, siblings, atopic dermatitis up to 6 years, and maternal education; *Model 3*, model 2 plus exclusion of food allergy within the first year (n = 17) and of asthma within the first year (n = 102).

TABLE E5. Association between introduction of single food items in the first year of life and food allergy

	Food allergy		
	Model 1	**Model 2**	**Model 3**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**
Cow's milk			
3-12 mo	0.71 (0.42-1.21)	0.64 (0.35-1.16)	0.98 (0.49-1.96)
>12 mo, reference	1	1	1
Shop milk			
3-12 mo	0.71 (0.36-1.42)	0.56 (0.26-1.20)	1.01 (0.44-2.31)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk			
3-12 mo	0.71 (0.38-1.33)	0.82 (0.35-1.91)	1.16 (0.44-3.08)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk, unboiled			
3-12 mo	1.46 (0.69-3.07)	1.30 (0.52-3.26)	1.46 (0.50-4.24)
>12 mo, reference	1	1	1
Yogurt			
3-12 mo	**0.39 (0.23-0.69)**	**0.39 (0.20-0.75)**	0.69 (0.31-1.60)
>12 mo, reference	1	1	1
Other milk products			
3-12 mo	**0.46 (0.27-0.79)**	**0.36 (0.19-0.67)**	0.54 (0.26-1.14)
>12 mo, reference	1	1	1
Vegetables or fruits			
<6 mo	1.39 (0.82-2.36)	1.08 (0.53-2.21)	1.01 (0.45-2.29)
≥6 mo, reference	1	1	1
Fish			
3-12 mo	**0.54 (0.32-0.91)**	**0.34 (0.18-0.66)**	**0.41 (0.19-0.88)**
>12 mo, reference	1	1	1
Nuts			
3-12 mo	0.55 (0.27-1.10)	0.55 (0.24-1.25)	0.71 (0.30-1.66)
>12 mo, reference	1	1	1
Eggs			
3-12 mo	**0.55 (0.32-0.-93)**	**0.50 (0.28-0.91)**	0.82 (0.40-1.69)
>12 mo, reference	1	1	1
Meat			
<9 mo	0.80 (0.46-1.38)	0.53 (0.27-1.05)	**0.43 (0.20-0.93)**
≥9 mo, reference	1	1	1
Cereals			
<9 mo	1.41 (0.78-2.55)	1.03 (0.51-2.10)	1.11 (0.50-2.46)
≥9 mo, reference	1	1	1
Bread			
<9 mo	0.69 (0.41-1.17)	0.77 (0.43-1.40)	0.70 (0.35-1.40)
≥9 mo, reference	1	1	1
Soja			
3-12 mo	2.27 (0.92-5.63)	1.80 (0.66-4.87)	1.92 (0.60-6.13)
>12 mo, reference	1	1	1
Margarine			
3-12 mo	1.09 (0.63-1.88)	0.78 (0.41-1.50)	0.87 (0.41-1.82)
>12 mo, reference	1	1	1
Butter			
3-12 mo	**0.51 (0.30-0.87)**	0.60 (0.32-1.11)	0.83 (0.39-1.75)
>12 mo, reference	1	1	1
Cake			
<9 mo	0.62 (0.35-1.08)	0.77 (0.40-1.49)	0.89 (0.43-1.85)
≥9 mo, reference	1	1	1
Chocolate			
3-12 mo	**0.56 (0.33-0.98)**	0.60 (0.32-1.12)	0.69 (0.34-1.42)
>12 mo, reference	1	1	1

Boldface values are significant ($P < .05$).

Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, maternal education, sex, breast-feeding, siblings, and atopic dermatitis up to 6 years; *Model 3*, model 2 plus exclusion of doctor's diagnosis of food allergy within the first year (n = 17).

TABLE E6. Association between introduction of single food items in the first year of life and sensitization to food allergens

	Sensitization to food allergens		
	Model 1	Model 2	Model 3
	OR (95% CI)	OR (95% CI)	OR (95% CI)
Cow's milk			
3-12 mo	0.77 (0.46-1.31)	0.83 (0.46-1.50)	0.90 (0.49-1.67)
>12 mo, reference	1	1	1
Shop milk			
3-12 mo	0.73 (0.35-1.51)	0.73 (0.32-1.64)	0.88 (0.38-2.02)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk			
3-12 mo	0.80 (0.44-1.45)	0.80 (0.38-1.66)	0.83 (0.39-1.77)
>12 mo, reference (reference = no cow's milk)	1	1	1
Farm milk, unboiled			
3-12 mo	0.60 (0.23-1.55)	0.53 (0.17-1.64)	0.55 (0.18-1.71)
>12 mo, reference	1	1	1
Yogurt			
3-12 mo	**0.47 (0.26-0.84)**	0.69 (0.35-1.39)	0.72 (0.35-1.50)
>12 mo, reference	1	1	1
Other milk products			
3-12 mo	**0.50 (0.29-0.88)**	0.67 (0.36-1.27)	0.71 (0.36-1.37)
>12 mo, reference	1	1	1
Vegetables or fruits			
<6 mo	0.94 (0.56-1.61)	1.03 (0.52-2.02)	1.04 (0.52-2.06)
≥6 mo, reference	1	1	1
Fish			
3-12 mo	**0.49 (0.29-0.84)**	0.69 (0.38-1.26)	0.71 (0.38-1.34)
>12 mo, reference	1	1	1
Nuts			
3-12 mo	0.70 (0.35-1.39)	0.48 (0.23-1.01)	0.48 (0.23-1.02)
>12 mo, reference	1	1	1
Eggs			
3-12 mo	0.68 (0.40-1.17)	0.70 (0.38-1.29)	0.70 (0.37-1.32)
>12 mo, reference	1	1	1
Meat			
<9 mo	0.72 (0.41-1.25)	0.97 (0.51-1.84)	1.04 (0.54-2.01)
≥9 mo, reference	1	1	1
Cereals			
<9 mo	0.79 (0.45-1.36)	0.71 (0.38-1.33)	0.68 (0.36-1.29)
≥9 mo, reference	1	1	1
Bread			
<9 mo	1.08 (0.62-1.85)	0.89 (0.48-1.65)	0.93 (0.49-1.77)
≥9 mo, reference	1	1	1
Soja			
3-12 mo	1.05 (0.31-3.58)	1.32 (0.36-4.84)	1.47 (0.39-5.50)
>12 mo, reference	1	1	1
Margarine			
3-12 mo	0.83 (0.49-1.41)	0.71 (0.38-1.32)	0.68 (0.36-1.29)
>12 mo, reference	1	1	1
Butter			
3-12 mo	0.83 (0.48-1.46)	0.73 (0.39-1.39)	0.85 (0.43-1.66)
>12 mo, reference	1	1	1
Cake			
<9 mo	**0.46 (0.26-0.84)**	**0.30 (0.15-0.62)**	**0.31 (0.15-0.64)**
≥9 mo, reference	1	1	1
Chocolate			
3-12 mo	**0.37 (0.21-0.67)**	**0.39 (0.21-0.75)**	**0.36 (0.19-0.70)**
>12 mo, reference	1	1	1

Boldface values are significant ($P < .05$).

Model 1, Crude; *Model 2*, model 1 plus adjusted for center, farmer, parents with allergy, maternal education, sex, breast-feeding, siblings, and atopic dermatitis up to 6 years; *Model 3*, model 2 plus exclusion of doctor's diagnosis of food allergy within the first year (n = 17).

Chapter 7

7. Discussion:

The results of this work could identify important factors that might have a protective effect on the development of atopic dermatitis in childhood. Using data from the PASUTRE/EFRAIM birth cohort study, we could also identify that prenatal and early life are important time periods in which these exposures are effective.

The main findings of this thesis are:

First, environmental factors which could be identified to have a protective effect are the contact to farm animals and to cats. Those factors were shown to have a protective effect already when the exposure occurs during pregnancy and were especially effective on atopic dermatitis with early onset (onset within the first year of life). We also showed a tendency of gene-environment interaction between these exposures and a polymorphism of a receptor of innate immunity on atopic dermatitis.

Second, we could show that infant's feeding practices in the first year of life play an important role on the development of atopic dermatitis in childhood. Milk products, especially yogurt, and an increased food diversity introduced in the first year of life were strongly negatively associated with atopic dermatitis with onset after the first year of age. Moreover, regarding breastfeeding, the level of sIgA in breast milk was inversely associated with atopic dermatitis. Furthermore, we could replicate the negative association between an increased food diversity and other allergic diseases, such as asthma, food allergy and sensitization.

In addition, we could show that the innate immunity might play a role in the development of atopic dermatitis, and thus already at birth.

Those findings could lead to the development of new strategies for primary prevention in atopic dermatitis and allergic diseases, and the pre- and postnatal environment may represent a critical window of opportunity for those strategies.

7.1 Farming environment

Several epidemiological studies have shown that environmental factors rich in microbial compounds such as the farming environment have a protective effect on allergic disease.[88] Studies of farm environment and allergies have identified as the most protective exposures: contact to livestock and consumption of farm milk.[25, 26, 29, 37] This protective "farm effect" was mainly observed with asthma, allergic rhinitis and allergic sensitization, although the data with atopic dermatitis are inconsistently reported.[25, 26, 46]

In our birth cohort study we observed a protective effect on atopic dermatitis up to two years of age in children when the mother was working on a farm during pregnancy (chapter 2). Among the different farm-related exposures, we found that the maternal contact to farm animals during pregnancy was negatively associated with atopic dermatitis. Interestingly, we also observed a dose-response effect with an increasing number of different farm animal species the mother had contact to during pregnancy, resulting in the reduction of the risk of developing atopic dermatitis. These results from longitudinal analyses support the previous cross-sectional findings of a protective effect of prenatal exposure to farm environment on atopic sensitization and on atopic dermatitis, asthma and hay fever.[9, 10]

We also could show that the timing of exposure plays an important role and might interact differently with atopic dermatitis depending on the onset of the disease. The prenatal contact to farm animals seems to affect primarily early onset atopic dermatitis (with onset of the disease within the first year of life), whereas those exposures during the first year of life rather influence the development of atopic dermatitis with onset after the first year of life (chapter 4). One explanation could be the absence of a close contact to farm animals during the first year of life. Furthermore, the family history of atopic diseases was shown to be significantly

associated with atopic dermatitis with onset within the first year of life, but not when the onset of the disease occurred after the first year of life. Those findings indicate that there might be different phenotypes of atopic dermatitis depending on its onset and could potentially explain inconsistencies of the results between the different studies on farm lifestyle and allergic diseases. A Dutch birth cohort supports our results, by reporting that filaggrin gene mutations were associated with atopic dermatitis with onset within the first year of life but not after.[13]

Previous studies could also show that farming exposures have a strong effect on allergic diseases when occurring in utero.[47, 62] Moreover, it was shown among children from the PASTURE/EFRAIM study that prenatal exposure to farming activities, especially contact to different farm animal species and farm dairy products, could increase cord blood cytokine production, such as IFN-γ, and result in a Th1-skewed cytokine pattern at birth.[89] A number of previous studies had reported that decreased levels of IFN-γ at birth predicted the onset of allergies later in life.[90, 91] In chapter 3, we describe a positive influence of farm exposures on the expression of gene of innate immunity.

Regarding farm exposures, consumption of unpasteurized farm milk during the first two years of life was reported as a protective factor against atopic dermatitis development.[38, 92] Our findings, presented in chapter 4, show that the consumption of farm milk in the first year of life has a tendency to decrease the risk of having atopic dermatitis but only among children with no allergic parents.

7.2 Exposures to pets

Like farm animals, contact with pets has also been extensively studied and has been suggested as a potential protective factor against atopic dermatitis.

In our birth cohort study, a significant reduced risk of developing atopic dermatitis in the first two years of life was observed among children with mothers having contact with cats during pregnancy (chapter 2). A same tendency, even though not

significant, was observed for prenatal dog exposure. Those results were independent of maternal atopy or farming status.

A meta-analysis reported some strong evidence of a protective effect of dog exposure, especially when occurred in early life, with an almost uniform effect. This meta-analysis showed also significant negative association between previous cat exposure and atopic dermatitis (pooled OR for all cohort studies: 0.76; 95% CI, 0.62-0.92).[93] In the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study among children at high-risk for atopy, a tendency of a negative association between the presence of a cat in the household and atopic dermatitis was reported.[94] However parents were asked at a 3-month visit whether allergy was the reason for not having a cat and after exclusion of participants who positively answered, the protective effect disappeared. In our birth cohort study, we could not evaluate this potential reverse causality effect, as this question was not included in our questionnaire during pregnancy. However the stratified analysis among parents with or without history of allergy showed the same negative association between prenatal cat exposure and atopic dermatitis.

It is interesting to note that cat exposure was also shown as having potential effect on secondary prevention of atopic dermatitis. It was reported that cat exposures among children with atopic dermatitis had a strong protective effect on the risk of developing asthma.[95]

7.3 The role of innate immunity

It was suggested, as a result of the hygiene hypothesis, that a reduced microbial stimulation of receptors of the innate immune system in early life leads to a shift towards Th-2 responses against allergens, and therefore could induce the development of allergic diseases.[52, 53]

Analyses in school-age children enrolled in the allergy and endotoxin (ALEX) study showed that farm children expressed significantly higher levels of gene expression of innate immunity receptors (CD14 and TLR2) than non-farm children.[61]

As already mentioned above, we found that an increase of gene expression of innate immunity receptors at birth was associated with farm-related exposures, such as maternal farm work and consumption of unboiled farm milk (chapter 3). An increased gene expression of those receptors measured at one year was most strongly associated with child's consumption of raw milk. Moreover, we could show that children with an increased gene expression of TLR5 and TLR9 at birth have a decreased risk of developing atopic dermatitis in the two first years of life (chapter 2). To our knowledge, this is the first study to show an association between gene expression of receptors of innate immunity and atopic dermatitis. Immunological responses in allergic diseases are driven by a Th2-mediated immune response with insufficient Th1 stimulation. Interestingly a previous study showed that TLR5 activation and TLR9 could induce maturation of antigen-presenting cells to Th1-biased immune response.[96, 97]

Those findings are consistent with the hypothesis that the innate immune system might play a role in mediating the protective effect of exposures on the development of atopic dermatitis in childhood, along the line of data obtained in mouse models.[98]

7.4 Gene-environment interaction

Even though allergic diseases have a strong genetic background, it is now well accepted that the pathogenesis of those diseases involves an interaction between genetic and environmental factors.[99] Polymorphisms in innate immunity genes might modulate the protective effect observed among farming environment exposures. Single-nucleotide polymorphisms (SNPs) in receptors of innate immunity were studied and showed to play a role in gene-environment interactions relevant to asthma and atopy.[55, 100] It was shown that SNPs in *CD14*, as well was in *TLR2* and in *TLR4* modified associations between country living and asthma.[60, 101]

Our results support that gene-environment interactions might play as well a role in the development on atopic dermatitis in children with genes of the innate immunity genes, as we could show that the protective effect of prenatal exposures to farm

animal and cats was only observed in children with a specific genotype in a SNP of *TLR2* (chapter 2).
Moreover the relationship between farm milk consumption in the first year of life and atopic dermatitis tended to be modified by parental history of allergies (chapter 4). In previous studies looking at the association between farm milk consumption and allergic diseases, this effect modification was not described.[36, 102] These results could be explained by a gene-environment interaction, in line with a cross-sectional study showing that a polymorphism in *CD14* modified the effect of farm milk on allergic diseases.[101]

7.5 Early life nutrition

It is now well accepted that food avoidance during pregnancy or infancy has provided no consistent evidence for allergy prevention[103, 104] and is therefore no longer recommended.[67] However, studies on first introduction of complementary food in infant's diet and its association with atopic dermatitis and allergic diseases have shown inconsistent results. One remaining major concern in those analyses is the potential bias caused by the reverse causality effect.

In chapter 4, we investigate the association between food introduction in the first year of life and atopic dermatitis. A major strength of this work is the longitudinal design of the PASTURE/EFRAIM study with the prospective collection of the data, as temporality (exposure occurring before the first symptoms of the disease) can be ascertained. This design allows us to carefully evaluate the potential reverse causality effect. Therefore, and in order to take into account the reverse causality, we mainly restricted our analyses to atopic dermatitis with first occurrence after the first year of life and additionally performed stratified analyses by parental history of allergy.

We observed that an increased diversity of food introduced within the first year of life has a protective effect on atopic dermatitis, with indication of dose-response relationship, and this independently of farming status and parental history of allergy. The association between food diversity and other allergic diseases is further

investigated in chapter 6. A reduced risk of asthma, food allergy and sensitization to food allergens could also be found with an increased food diversity introduced in the first year of life, with a clear dose-response pattern.

To our knowledge this is the first study that shows a negative association between increased food diversity in first year of life and atopic dermatitis. Most of the previous studies did not find an association.[104-106] One explanation could be that those studies focused on introduction of food in the first 4-6 months of life. The time window between 6 and 12 months of age might be an important time window for potential strategies in primary prevention against atopic dermatitis, based on nutrition. Results from a very recent study support our findings and the explanation above, as it was shown that less food diversity by 12 months of age was associated with increased risk of asthma and by 6 months of age, only a tendency was observed.[107]

The results from the analyses between single food items and atopic dermatitis showed negative association with milk products, especially with introduction of yogurt in first year of life with a 50% risk reduction of developing atopic dermatitis, independently of food diversity. Those findings support recent studies showing an inverse association between early introduction of complementary food, such as fish or cow milk products, and atopic dermatitis.[34, 70] However, in order to address this more conclusively, randomized controlled trials are needed. Currently a randomized controlled trial (RCT) is underway in the UK to test whether the introduction of allergenic foods is able to reduce the risk of developing allergic diseases (Enquiring About Tolerance Study, EAT study).

One explanation for the risk reduction of allergic diseases in association with earlier introduction of complementary food could involve the induction of oral tolerance induced by exposure to those food antigens. Animal studies have also shown that early exposure to repeated doses of food antigens (allergens) can induce oral tolerance during a critical early window of development and that avoidance strategies might increase the risk of adverse immune responses to allergens.[108]

As mentioned above and based on our findings, we hypothesized that early postnatal exposure of the infant gut to a variety of food ingredients might be essential for the development of immune tolerance. A potential mechanism for the protective effect of

increased food diversity in early childhood on allergic diseases might involve changes in the gut microbiota and/or its metabolites. Specific bacterial strains which confer protection from allergic inflammation were shown to be able to induce T regulatory cells.[109] Results from several cross-sectional epidemiologic studies indicate that atopic and nonatopic subjects differ in gut microflora composition.[110-112] Moreover, an inverse association between the bacterial diversity of the gut microbiota in the first months of life and the development of atopic dermatitis was reported.[113] A recent study could also show that a low diversity of the gut microbiota during the first month of life was associated with asthma later on in childhood.[114] Therefore perturbations in the gut microbiota, induced by food exposures or other factors, may be involved in the pathogenesis of atopic dermatitis.

Moreover, metabolites produced by intestinal microbiota, such as short-chain fatty acids (SCFAs), were shown to have anti-inflammatory properties.[65, 115-117] Recently it was shown in a mouse model, that a high-fiber diet increases circulating levels of SCFAs and protects against allergic inflammation in the lung. Moreover, it has been shown that yogurt consumption with live bacteria increases fecal and plasma concentrations of SCFAs (butyrate and propionate).[118, 119]

Studies on the association between breastfeeding and development of atopic dermatitis have shown inconsistent results. As mentioned in the introduction, one reason might be the differences in the composition of breast milk which vary greatly between individual mothers. In chapter 5, we show that levels of soluble immunoglobulin A (sIgA) in breast milk measured at two months were inversely associated with the development of atopic dermatitis up to two and four years of age. The total amount of sIgA (estimated as a product of sIgA level and breast feeding duration) showed a strong negative dose-response effect with atopic dermatitis. These results were independent of the effect of complementary food introduction on atopic dermatitis. No association between TGF-β1 levels in breast milk and atopic dermatitis was observed in our study.

sIgA plays a fundamental role in the immune response at mucosal surfaces.[120] It was suggested that the sIgA in human milk may prevent an excessive uptake of foreign antigens across the mucosa, and therefore decrease the risk of allergic sensitisation.[121] However, in our study we could not find an association between atopic sensitization and sIgA in breast milk.

7.6 Outlook

Early onset of atopic dermatitis is often the start of the atopic march. Therefore primary prevention of atopic dermatitis might not only prevent the development of atopic dermatitis, but also the subsequent development of other allergic diseases, such as asthma and allergic rhinitis. From our findings, new strategies on primary prevention of atopic dermatitis can be evaluated, for instance based on nutrition. However, interventional studies are needed to confirm those results and to test these potential new strategies in primary prevention.

Even though a lot of studies could show the interaction between environment with microbial burden and allergic diseases, the hygiene hypothesis remains, since the first time described in 1989 by D. Strachan, an hypothesis. The exact mechanism and factors deriving from the hygiene hypothesis are not yet identified. One reason could be the importance of the gene-environment interaction effect, with different environmental factors having different influences depending on the genetic background, and therefore may differ between populations. Another reason could be an important role of the diversity of environmental exposures, supported by the results of this work.

Our findings highlight the role of diversity, as shown with the prenatal protective effect of the increased number of farm animal species and the protective effect of an increased food diversity in the first year of life on atopic dermatitis and other allergic diseases. Similarly, recent findings showed that the increased diversity of microbial exposures had a protective effect on asthma.[122] Those results support the hypothesis that exposures in early life or even during pregnancy to diverse antigens, such as food proteins, could increase the maturation of the immune system and induce tolerance networks.[123]

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Abbreviations

ALEX	Allergy and Endotoxin study
AMICS	Asthma Multicenter Infants Cohort Study
EFRAIM	Early Farm-Related anti-Allergy Immune Mechanisms
FLG	Filaggrin
IFN-γ	Interferon gamma
PAMP	pathogen-associated molecular pattern
PASTURE	Protection against Allergy-Study in Rural Environments
PARSIFAL	Prevention of Allergy Risk factors for Sensitization in children Related to Farming and Anthroposophic Lifestyle
PIAMA	Prevention and Incidence of Asthma and Mite Allergy
RCT	randomized controlled trial
SCORAD	SCORing atopic dermatitis
SCFAs	short-chain fatty acids
sIgA	soluble immunoglobulin A
SNP	Single nucleotide polymorphism
TGF-β	Transforming growth facto beta
TLR	Toll-like-receptor

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