

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-Fahrlander, 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

From ^athe Swiss Tropical and Public Health Institute, Basel; ^bthe University of Basel; ^cthe University of Zurich, Children's Hospital and the Christine Kühne Center for Allergy Research and Education; ^dthe Institute of Epidemiology and Medical Biometry, Ulm University; ^ethe Department of Environmental Health, National Institute for Health and Welfare, Kuopio; ^fPublic Health and Clinical Nutrition, the University of Eastern Finland, Kuopio; ^gthe Department of Environmental Science, Inhalation Toxicology Laboratory, the University of Eastern Finland, Kuopio; ^hUniversité de Franche-Comté, Department of Respiratory Disease, University Hospital, Besancon; ⁱthe Children's Hospital Schwarzach, Schwarzach; ^jLMU Munich, University Children's Hospital, Munich; ^kthe Clinic for Paediatric Pneumology and Neonatology, Hannover Medical School; and ^lthe 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.

Supported by European Union research grants PASTURE/EFRAIM (QLRT4-CT 2001-00250, KBBE-2-2-06) and the Kühne Foundation.

Disclosure of potential conflict of interest: J. Pekkanen has received research support from the European Union (EU) FP 7, the Academy of Finland and EVO funding, and the Farmers' Social Insurance Institution (Mela). M. Roponen has received research support from the European Union (FP7). E. von Mutius has received consulting fees from GlaxoSmithKline, ProtectImmun, and Novartis; has received lecture fees from InfectoPharm and Nestlé; has received research support from the European Commission and European Research Council (ERC); and has provided legal consultation/expert witness testimony for the UK Research Excellence Framework. J. Weber has received research support from the European Commission. M. Kabesch has received research support from Roxall, GlaxoSmithKline, Novartis, Sanofi Aventis, Allergopharma, AstraZeneca, the German Research Foundation (DFG), BMBF, and the EU. R. Lauener has received research support from the Kuthe Foundation and the EU. M. Ege has received research support from the ERC and the DFG. B. Schaub has received research support from the DFG and the EU. M. Depner has received research support from the European Research Council. G. Doekes has received research support from the European Commission Framework VII program. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 10, 2012; revised April 20, 2012; accepted for publication May 23, 2012.

Corresponding author: Georg Loss, MSc, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, 4002 Basel, Switzerland. E-mail: georg.loss@unibas.ch. 0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2012.05.049>

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 genes⁷ 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

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) study¹⁷ 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 studies¹⁸⁻²¹ 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 ($\Delta\Delta$ cycle threshold [$\Delta\Delta\text{Ct}$]) 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 al²³ 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

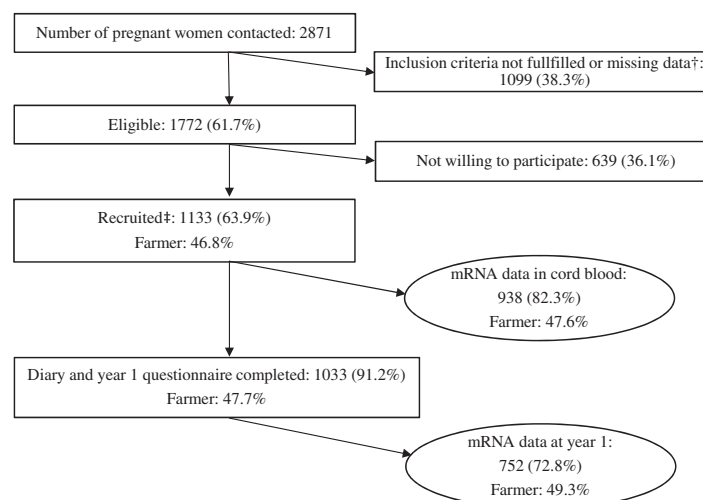


FIG 1. Selection of PASTURE study population and participants for mRNA analyses. †Inclusion criteria: living on a farm with livestock, maternal age greater than 18 years, term delivery, no genetic disease in offspring, telephone connection, and sufficient knowledge of the country's language. ‡Selection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

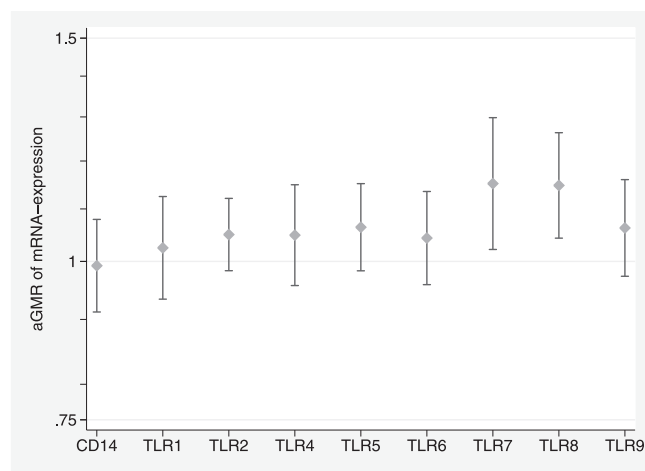
TABLE II. Crude association* of exposures during pregnancy and mRNA expression at birth (n = 938, only significant associations are shown)

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 non-farm 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 pre-natal farm animal exposure and innate immunity gene expression¹² 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										.079
No										
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										.198
>6 mo										
3-6 mo	1.15 [†] (1.02-1.29)									
≤3 mo										
Never								0.77 [†] (0.62-0.97)		
Exclusive breast-feeding										.229
>3 mo										
≤3 mo										
Never								0.75 [†] (0.60-0.94)		
No. of solid food items introduced early										.018
0										
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.

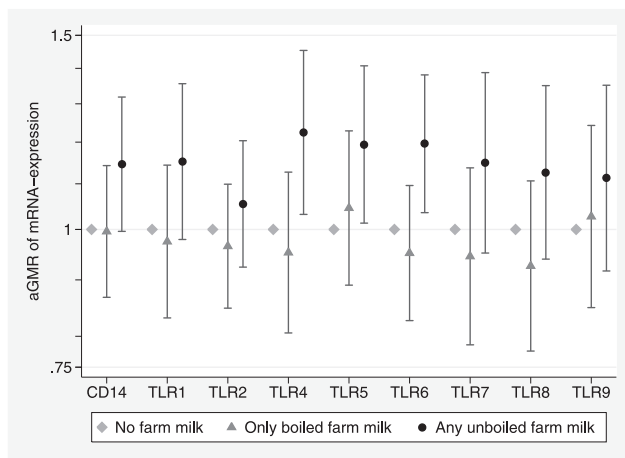


FIG 3. Adjusted association of child's farm milk consumption during first year of life and mRNA expression at year 1 expressed as geometric mean ratios and 95% CIs adjusted for farming, maternal history of asthma or hay fever, sex, center, maternal smoking during breast-feeding, solid food score, education, 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 al⁷ 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 ^athe Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland; ^bthe Department of Pediatrics, Kuopio University Hospital, Kuopio, Finland; ^cthe Department of Respiratory Disease, Université de Franche-Comté, University Hospital, Besançon, France; ^dthe Institute of Epidemiology and Medical Biometry, Ulm University, Ulm, Germany; ^eLMU Munich, University Children's Hospital, Munich, Germany; ^fthe Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany; and ^gthe 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-172T* 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, ≤ 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). 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 ($\Delta\Delta\text{Ct}$) 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 ($\Delta\Delta\text{Ct}$). To

get the numeric value, we then used the $2^{-\Delta\Delta C_t}$ 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)

Exposure during pregnancy	mRNA expression					
	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)

Exposure during first year of life	mRNA expression					
	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

Receptor/SNPS	Genotype	mRNA expression in cord blood				mRNA expression at age 1 y			
		No.	GMR	95% CI	P value	No.	GMR	95% CI	P value
<i>TLR1/C-2299T</i> (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
<i>TLR1/T-2192C</i> (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
<i>TLR1/A742G</i> (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
<i>TLR2/T1349C</i> (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			
<i>TLR2/T596C</i> (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
<i>TLR2/T-16934A</i> (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
<i>TLR4/C8851T</i> (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
<i>TLR4/A8551G</i> (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
<i>TLR4/G-2570A</i> (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
<i>TLR4/T-1607C</i> (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
<i>TLR5/A1774G</i> (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
<i>TLR5/T1845C</i> (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
<i>TLR5/C1173T</i> (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
<i>TLR6/T-1928C</i> (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			
<i>TLR6/T-2079A</i> (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
<i>TLR7/A17961T</i> (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
<i>TLR7/C12318T</i> (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
<i>TLR8/C10907A</i> (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
<i>TLR8/C9008T</i> (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
<i>TLR8/A-4824G</i> (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)

Receptor/SNPS	Genotype	mRNA expression in cord blood				mRNA expression at age 1 y			
		No.	GMR	95% CI	P value	No.	GMR	95% CI	P value
<i>TLR9/T-2622C</i> (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
<i>TLR9/T-2871C</i> (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
<i>CD14/C-1721T</i> (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

Receptor/SNPS	Genotype	mRNA expression in cord blood				mRNA expression at age 1 y			
		No.	aGMR*	95% CI	P value	No.	aGMR†	95% CI	P value
<i>TLR1/C-2299T</i> (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
<i>TLR1/T-2192C</i> (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
<i>TLR1/A742G</i> (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
<i>TLR2/T1349C</i> (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			
<i>TLR2/T596C</i> (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
<i>TLR2/T-16934A</i> (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
<i>TLR4/C8851T</i> (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
<i>TLR4/A8551G</i> (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
<i>TLR4/G-2570A</i> (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
<i>TLR4/T-1607C</i> (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
<i>TLR5/A1774G</i> (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
<i>TLR5/T1845C</i> (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
<i>TLR5/C1173T</i> (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
<i>TLR6/T-1928C</i> (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			
<i>TLR6/T-2079A</i> (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
<i>TLR7/A17961T</i> (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
<i>TLR7/C12318T</i> (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
<i>TLR8/C10907A</i> (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
<i>TLR8/C9008T</i> (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
<i>TLR8/A-4824G</i> (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)

Receptor/SNPS	Genotype	mRNA expression in cord blood				mRNA expression at age 1 y			
		No.	aGMR*	95% CI	P value	No.	aGMR†	95% CI	P value
<i>TLR9/T-2622C</i> (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
<i>TLR9/T-2871C</i> (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
<i>CD14/C-1721T</i> (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.