Investigating the effect of farm milk consumption on childhood asthma and allergies in the context of farming, early life nutrition and innate immunity

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät der

Universität Basel

von

Georg Loss

aus Villach und Bregenz (Österreich)

Basel, 2012

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. med. Charlotte Braun-Fahrländer

Prof. Dr. med. David Strachan

Basel, den 26. Juni 2012

Prof. Dr. Martin Spiess
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

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SUMMARY

Background

The prevalence of childhood asthma and allergic diseases has increased in the past few decades in developed countries. So far, there are no preventive measures to protect from developing these outcomes in early life. Atopic diseases are strongly determined by genes but the fast increase of prevalence seems to be based on environmental exposures. It was argued that an exposure to certain factors such as air pollutants or a lack of protective environmental exposures may have increased the risk for the onset of atopic disease.

Two decades ago, a study showed that the risk for these outcomes was increased for children who lacked early life infections (the "hygiene hypothesis"). Initially, contact to other children in early life was identified as source of such infections but the hypothesis was soon extended to other sources rich in microbial exposure. In subsequent investigations in affluent countries it was repeatedly found that an exposure to farming environments was associated with lowered risks for atopic disease in childhood. The presence and strength of these associations varied with timing of exposure including *in utero* exposure, farm specific factors and different atopic outcomes. Furthermore, there is limited evidence that the susceptibility for protective associations depends on the individual genetic disposition.

Consumption of cow's milk from farms that was not commercially processed was consistently identified as one of these protective farm related factors. The associations with atopic outcomes were stronger when the farm milk was raw. Microorganisms, fatty acids and proteins in milk were speculated to possibly underlie this inverse association of farm milk consumption and decreased risk for atopic disease in childhood. Consumption of fish, fruits and vegetables were other dietary factors in early life which were related to decreased risks for asthma and allergies.

The pathways mediating these inverse associations between farm related and nutritional exposures in early life and *in utero* with atopic disease remain unclear. Recent research findings indicated an involvement of the innate immune system which acts as the pivotal defense system against invading microorganisms. First investigations showed that the gene expression of innate immunity receptors was associated with farm related exposures. Whether these gene expressions are in turn associated with atopic outcomes and whether there is a causal relationship of farm or dietary exposure with development of atopic disease in early life mediated by innate immunity remains to be elucidated.

Objectives

To elucidate the epidemiologically observed inverse associations of farm milk consumption with childhood asthma and allergic disease by identifying milk components underlying these associations, by putting these results in the context of similar associations of farm related and dietary exposures and by assessing the association of farm milk consumption in early life with the development of the innate immune system.

Methods

The cross-sectional GABRIEL study (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community) was conducted in rural areas of Germany, Switzerland, Austria and Poland to determine farming related factors which are fundamental to protecting against asthma and atopic disease in childhood. The initial study population comprised 103'219 6-12 year old children and participants for extensive assessments were selected by disproportionate stratified random samples in multiple sampling phases. Atopic health outcomes and farming and lifestyle exposures were assessed by comprehensive questionnaires. Cow's milk was collected as it was consumed at the participants' homes from about 800 children.

The prospective birth cohort study PASTURE (Protection against allergy- study in rural environments) was conducted in rural areas of Germany, Austria, Switzerland and Finland. Initially, 1'133 pregnant women were recruited in the third trimester. Environmental exposures and self reports about atopic disease were assessed by extensive questionnaires during pregnancy and yearly up to age 6. Atopic diseases at various ages were also measured objectively. A detailed food frequency diary during year 1 provided information on introduction of complementary foods. Blood samples were used to perform genotyping and to measure gene expression of Toll-like receptors 1-9 and CD14 at birth (N=938) and age 1 (N=752).

Results

The GABRIEL study showed that the prevalence of asthma, atopic sensitization, hay fever and atopic dermatitis was significantly lower in children living on a farm. A traditional type of farming namely with cows and cultivation was protective for childhood asthma, hay fever and atopy. The inverse association of general farm exposure with asthma could be explained by early life consumption of farm milk, contact with cows and contact with straw. The

association with atopy, hay fever and atopic dermatitis could not be fully explained by these factors.

Farm children consumed farm milk and unboiled farm milk more often. The latter showed higher levels of whey proteins, total viable bacterial counts and was associated with a higher fat content when compared with boiled farm milk or commercial milks. Reported consumption of unboiled farm milk was significantly associated with reduced risk for asthma, atopic sensitization, hay fever and atopic dermatitis. Associations were stronger when unboiled farm milk was consumed earlier in life. Whey proteins (bovine serum albumin, α-lactalbumin, β-lactoglobulin) were identified as milk constituents possibly explaining the epidemiologically observed protective farm milk association with asthma whereas reduced risk for atopic sensitization could not be associated with any investigated milk constituent. Microorganisms and fat content of milk showed no associations with allergic health outcomes.

A comparison of rapid methods which assess total viable bacterial counts in milk samples showed that a flow cytometry system and an automated most-probable number system were fast and inexpensive. The flow cytometry system, however, did not measure bacterial counts in heated milk samples correctly. The results of the automated most-probable number system were in good agreement with the gold standard method.

The PASTURE study showed that the increasing diversity of introduced complementary food items was inversely associated with the risk to develop atopic dermatitis after age 1, independently of other farming exposures. An inverse association was also found with the introduction of yogurt during the first year of life, independently of the diversity of introduced foods.

Maternal farming during pregnancy (*in utero* exposure) was associated with a general up-regulation of gene expression of innate immunity receptors at birth and with a significant up-regulation of *TLR7* and *8* expressions. *TLR* and *CD14* gene expression at birth and age 1 were not highly correlated indicating a change of the innate immune system during the first year of life. Child's farm milk consumption was the exposure during first year of life with the strongest associations with gene expression of innate immunity receptors at age 1 statistically significantly associated with up-regulation of *TLR4*, *5* and *6*. A previously described modification of the association of raw farm milk consumption with gene expression of *CD14* by the SNP *CD14/C-1721T* could not be confirmed.

Conclusions and outlook

The variations between associations of specific farming and dietary exposures in early life with specific atopic diseases suggest that different pathways may be involved in the protection against the development of these outcomes. Several factors explained the decreased risk for asthma in children living on farms but specific factors explaining decreased risks for atopy, hay fever and atopic dermatitis are yet to be identified. Our findings add to the evidence that early life exposures may have an effect on the development of the innate immune system. We could further demonstrate that relevant exposures differed between *in utero* and child's direct exposure during first year of life. Similarly, the timing of exposures as early as *in utero* was important for the inverse associations with atopic outcomes. Gene-environment interactions for the association of raw farm milk consumption with the gene expression of innate immunity receptors appeared to be of minor significance as reported in previous studies.

The associations of farm specific factors and farm milk consumption with atopic outcomes in childhood generated with cross-sectional GABRIEL data need to be confirmed in prospective studies to establish temporal relationships. Contrary to our expectations, microorganisms in milk were not related to asthma or atopy. Microbial assessment, however, was based on culture methods which did not capture the full diversity of the microorganisms. Future studies need to employ advanced methods to assess microbial diversity in environmental exposures and to investigate the association with atopic outcomes.

In the longitudinal PASTURE study dietary factors were only related to atopic dermatitis. Associations with asthma and atopy should be assessed as well in future analysis. An important research question for future investigations will be whether and how the innate immune system mediates the inverse associations of farm related and dietary exposure with the development of atopic disease in early life. So far, detailed investigations regarding associations of farming with atopic disease are limited to affluent countries. First studies in developing countries showed inconsistent results. Further studies are needed there to potentially prevent a rise of atopic disease prevalence as observed in developed countries.

Finally, a health impact assessment showed that raw milk consumption has the potential to be used as preventive measure for the development of atopic disease in early life. Pathogens in raw milk, however, pose a health risk and make an implementation unlikely. A native milk product that is safe and can still exert "protective effects" on atopic diseases could be facilitated by modern non-thermal pasteurization techniques.

ABBREVIATIONS

AD	Atopic dermatitis
CDC	Center for Disease Control
EHEC	Enterohemorrhagic *Escherichia coli*
FA	Fatty acid
FDA	Food and Drug Administration
GI	Gastrointestinal
GABRIEL-A	A multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community-Advanced Surveys
GWAS	Genome wide association studies
LCA	Latent class analysis
LPS	Lipopolysaccharides
PAMP	Pathogen-associated molecular pattern
PASTURE	Protection against allergy- study in rural environments
SCORAD	Scoring atopic dermatitis
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
UHT	Ultra-high temperature processing
WHO	World Health Organization

ACKNOWLEDGEMENTS

The GABRIEL-A study was supported by an European Union Research grant under the FP6-LifeSCIHEALTH Integrated Program LSH-2004-1.2.5-1 (contract no. 018996) and the PASTURE study was supported by European Union research grants PASTURE/EFRAIM (QRLT4-CT 2001-00250, KBBE-2-2-06) and the Christine Kühne Foundation (CK-Care). My work was supported by the ProDoc Doctoral Program of the Swiss National Science Foundation (PDFMP3-123115/1).

I want to thank my supervisor Charlotte Braun-Fahrländer for accepting me as her PhD student and giving me an excellent support during my studies. She always found the time for extensive and lively discussions with me. I want to thank her for integrating me in the GABRIEL-A and PASTURE study groups and giving me the opportunity to take part in many productive meetings all over Europe.

I am thankful to all the members of the international GABRIEL-A and PASTURE study groups who did a tremendous job designing and conducting these studies and who provide the data for my analyses. I want to thank them for their ideas and input at our numerous meetings and their support during my PhD studies. Many thanks go to: Sondhja Bitter, Martin Depner, Remo Frei, Jon Genuneit, Sabina Illi, Roger Lauener, Caroline Roduit, Nikolaos Sitaridis and Erika von Mutius. I want to specially thank my colleagues Silvia Apprich and Wolfgang Kneifel who I had the pleasure to work with while conducting microbial measurements at their laboratory in Vienna.

I want to thank the Environmental Exposure and Health Unit and other colleagues at the Swiss Tropical and Public Health Institute in Basel as well as colleagues from my former affiliation the Institute for Social and Preventive Medicine in Basel for supporting my work. Special thanks go to: Christian Bieli, Christian Schindler and Marco Waser. I want to further thank the PhD community at the Swiss TPH for scientific and motivational support.

I want to thank David Strachan from the St George's, University of London for his support as co-referent as well as Wolfgang Kneifel from the University of Natural Resources and Life Sciences (BOKU) in Vienna and Jakob Zinsstag from the Swiss Tropical and Public Health Institute in Basel for their inputs as experts.

Finally, I want to thank Helene, Stefanie and Hans for their support during all stages of my education. A special thanks goes to Vân for her moral support during my PhD studies.

1 INTRODUCTION AND BACKGROUND

Asthma and allergic disease

In the second half of the 20th a steep rise of childhood asthma and allergic disease prevalence was observed in affluent countries.[1-3] A few years ago it was reported that the prevalence of atopic disease (used as collective term for asthma and allergies) might be leveling off but results between different affluent countries were conflicting (Figure 1). [1,4-7] It was argued that observations of plateauing asthma numbers were based on increased quality of care and that it can be expected to see an increase of atopic disease on a global scale along with westernization of emerging and developing countries.[8] Asthma is now the most common chronic disease among children and causes together with allergic diseases a high burden on an individual and a public health level.[9] There are no primary preventive measures for these health outcomes at the moment and treatment of symptoms is often of limited success. Therefore, measures to prevent atopic disease in early life are sought after in order to reverse the observed increase of prevalence in the developed world on a population level and to possibly prevent it in emerging countries. To find such preventive measures we must understand what is causing atopic disease.

Figure 1-1: World map showing direction of change in prevalence of asthma symptoms for 6–7 year age-group
Each symbol represents a center. ▽= prevalence reduced (by >=1 SE per year). □=little change (<1 SE). △=prevalence increased (by >=1 SE per year). *Source: ISAAC study, Asher et al., The Lancet, 2006.*[10]

Causes of atopic disease: genes or environment

Although the pathogenesis contains a strong genetic component,[11,12] the sudden increase of atopic disease within only a few decades appears to be caused by environmental rather than genetic factors.[13-17] Considering the fast lifestyle changes in Western countries in the last century,[18] it seems plausible that they could be associated with increased numbers of atopic diseases and other diseases of civilization. Comparatively slow changes in genetic disposition on the other hand cannot account for this drastic increase of asthma and atopy.[13,14] It was argued, however, that genetic variance modifies the influence of environmental exposure, that is, shifts in environmental influence act on pre-existing genetic susceptibility.[12] Whereas there are many speculations about environmental factors causing asthma and atopy there are basically two potential routes of causation which might in fact occur simultaneously. The first route is characterized by an increased presence of environmental factors such as tobacco smoke, air pollution or allergens which have an adverse effect on the human body. Evidence that these factors cause atopic disease is inconsistent but they were shown to exacerbate symptoms in asthmatic children.[19-22] Associations of traffic related air pollution with asthma were found when exposure was assessed at individual level but not when it was assessed at community level.[23,24] The other route is based on the lack of formerly present environmental factors conferring protection from developing asthma and allergies on the human body. Whereas both routes might contribute to the development of atopic disease in their own right, the latter was subject to numerous investigations since the formal definition of the hygiene hypothesis and is the focus from here on out.

The hygiene hypothesis

In 1989, a publication introduced a new concept how environmental factors might influence the development of atopic disease in childhood.[25] Therein, Strachan reported an inverse association of the number of older siblings with the risk for hay fever in children. It was speculated that “unhygienic contact with older siblings” increases early life infections which are responsible for a decreased risk for this disease. Indeed, subsequent studies showed comparable results with children having lower prevalence of atopic disease when exposed to other children at day care at earlier ages or growing up with older siblings.[26,27] Research in epidemiology and immunology focused on this new concept and soon identified other “unhygienic” exposures associated with allergic disease. It was repeatedly shown that infections with viruses and bacteria were associated with human immune responses and with decreased risk for atopic disease although this was not consistent. Interestingly, similar

associations were found with non-invasive microbial exposure. The exact nature of these associations and immunological pathways, however, remain to be elucidated. von Mutius described the hygiene hypothesis as multidimensional concept with complex interactions between the following dimensions: type of environmental exposures, different atopic diseases and distinct phenotypes, timing of exposure and genetic susceptibility to react on respective exposures.[28] Therefore, a truly unifying concept to explain the hygiene hypothesis is still missing. Over two decades after the hygiene hypothesis was formulated, one specific source of protective environmental exposures for childhood atopic disease has been reported repeatedly, namely the farming environment.[29]

Farming environment

A first report of a lower hay fever prevalence found among people being employed in agriculture was published at the end of the 19th century.[30] During the 20th century agricultural reforms and urbanization lead to a decrease of the farming population in affluent countries such as Switzerland where the farming population dropped from 25% to 3% from 1920 to 2000.[31,32] In the same time the hay fever prevalence in Switzerland increased over 10-fold to 14%.[33] It is not clear to what extend and how these two events were related. On a population level, it seems reasonable that the increasing prevalence of asthma and allergies in developed countries in the past few decades could be based on a loss of traditional lifestyle closely tied to agriculture and an associated loss of environmental exposures due to industrialization and urbanization.[34] This idea was supported by a recent ecological study showing an inverse association of proportion of current rural population and prevalence of childhood wheeze in 22 European countries.[35] There are no assessments of how much of the increase of atopic diseases in a given country can be attributed to decreased farming populations and ramifications like increased cleanliness, eradication of infections and modern diet[36]. As other factors such as increased self reported disease because of public awareness due to improved diagnose and treatment[37,38] (e.g. introduction of histamine antagonists), genetic susceptibility or other environmental factors may have contributed to these disease trends they should be considered in such calculations and their interpretation.

First detailed investigations with children in a Swiss study starting in the early 1980s showed that non farmer children in rural areas showed higher rates of allergic disease than their peers living on farms.[39] But only after the hygiene hypothesis was formulated, a series of investigations regarding farming environment and atopic disease began. Since then, over 30

independent population-based studies in various affluent countries repeatedly found protective associations of farming exposure with atopic diseases in children.[29]

When investigating the farming environment and its influence on allergic diseases in early life it has to be acknowledged that there is a wide array of specific exposures to consider. After a general association of farmer vs. non farmer children with these outcomes was established and tagged the "farm effect", it was tried to ascribe this "effect" to distinctive exposures found in these farming environments. It has to be noted that non farmer comparison groups generally comprised pregnant mothers or children from rural regions who were not directly exposed to farming areas and activities rather than participants from urban regions. Farm related factors that have been speculated to underlie the "farm effect" on allergic health outcomes in early life (including pregnancy) were contact to animals,[40,41] endotoxin levels in house dust which represent gram-negative bacteria,[42] diversity of microbial exposure[43] or farm milk consumption[44] with protection being stronger when exposed earlier in life. Whereas some of these exposures were identified as protective factors more consistently than others by independent studies it was always a problem to disentangle the distinctive associations of individual exposures. Whereas a combination of the mentioned farm exposures might be necessary to fully explain the general "farm effect" on atopic disease, farm milk consumption repeatedly stood out among the associations of distinctive farming exposures with asthma and allergic health outcomes.

Farm milk and commercial milk

Farm milk is defined in the context of this thesis as cow's milk produced on one's own farm or purchased at a neighboring farm that has not undergone commercial milk processing. The term "farm milk" does not only refer to cow's milk in its native raw state (Table 1) but may also refer to home processed milk. This includes heating or boiling processes to increase product life or to facilitate consumption for children and skimming of milk to reduce the fat content. Consumption of milk from animals other than cows is rare in early life and is thus generally not covered in publications regarding health effects of farm milk consumption and will also not be the topic of this thesis. Commercial milk processing comprises more stringent treatments than home processing and induces major milk changes along the typical processing chain which was summarized by Michalski et al.[45] Homogenization facilitates a stable milk emulsion with increased shelf life due to fat globule disruption and dispersion of casein micelles. This decreases the cream separation rate caused by a density difference between milk fat and the aqueous phase and prevents coalescence. Heat treatment is used for

destruction and reduction of microorganisms and enzymes in milk to increase product safety and shelf life. Low heating regimes like pasteurization (minimum of 72° C for 15 seconds) are a minimum statutory requirement for heat processing in the European Union[46] and many other countries to provide safe dairy products for commercial distribution. Relatively low temperatures during pasteurization already lead to a substantial denaturation of whey proteins, decrease of milk activity of milk indigenous enzymes and milk's micro flora and destruction of vitamins.[45,47] Ultra high temperature processing (UHT) is comparable to a sterilization of milk leading to a strong increase of shelf life but it is also accompanied by a much stronger destructive impact on milk constituents.

Table 1-1: Composition of native cow's milk in %

Water	**87.5**		
Carbohydrates	**4.8**		
Total fat	**<4.2**		
Saturated FAs		69.4	
Mono-unsaturated FAs		25.0	
Poly-unsaturated FAs		2.3	
Trans FAs		2.7	
Conjugated linoleic acid			0.4
Proteins	**3.5**		
Caseins		80.0	
Whey proteins		20.0	
β-lactoglobulin			35.0
α-lactalbumin			12.0
Glucomacropeptide			12.0
Proteose peptone			12.0
Immunoglobulins			8.0
Serum albumin			5.0
Lactoferrin			1.0
Lactoperoxidase			0.5
Minor proteins			15.0

FA = Fatty acid, *Source*[48-50]

Milk with its high nutrient content is a popular food all around the world but this rich composition is also an ideal medium for microbes.[51-53] Therefore, pasteurization was introduced in the 20th century to decrease human illness through foodborne pathogens.[54] Nowadays, low-fat UHT milk is starting to prevail over pasteurized milk in westernized countries with a neglectable proportion of raw farm milk consumption and the concern for milkborne diseases is relatively low.[52,53,55] In other regions of the world, disease prevalence associated with unpasteurized milk consumption, however, leads to increased demands for

more stringent laws.[56,57] Pathogens commonly associated with raw milk are *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp. and enterohemorrhagic *Escherichia coli* (EHEC).[56,58-60] Brucellosis, which eluded eradication in most developed countries, and zoonotic tuberculosis can be transmitted through infected, unpasteurized animal milk products and remain major health concerns in developing countries and to some extend in industrialized countries.[57,61,62]

The protective "farm milk effect"

As mentioned before, consumption of farm milk was the most consistent farm related factor to show inverse associations with childhood asthma and allergies.[44,63,64] This was tagged the "farm milk effect" although a causal relationship has not yet been established. These associations were independent of other farming exposures and other potential confounders and were stronger when farm milk was consumed earlier in life (including pregnancy)[44] and when the farm milk was raw[63]. The mechanism of action and components of milk that underlie this observed association remain unclear. Proteins, fat composition and micro flora that can be found in the rich composition of farm milk but to a lower extend in commercial shop milk [47,58,65] have been speculated to affect the human immune system and to mediate the "farm milk effect" on atopic disease. Proteins of the whey fraction are important for host defense against infection and excessive inflammation.[66,67] Lactoferrin is an immunostimulator and an immunoregulator and the family of TGF-beta, also found in human breast milk, consists of multifunctional cytokines that were associated with less allergic outcomes in children.[67,68] Interestingly, the whey proteins α-lactalbumin and β-lactoglobulin are also the major allergens for milk allergy.[69] Consumption of products containing milk-fat were associated with a reduced risk for asthma and allergy[70] but the role of dietary fat and fatty acids in the onset of atopic outcomes remains unresolved.[71,72] Also, the micro flora of farm milk might contribute to the "farm milk effect" considering dietary effects on the human gut flora and its ties to the immune system[73] (further described in *Chapter 1: Immune system and potential pathways*).

Early life nutrition

Farm milk consumption is not only a farm specific exposure but also a nutrition specific exposure. Introduction of complementary foods in the first year of life pose a major environmental exposure for the human body. Until recently, food allergen avoidance during pregnancy and early life was recommended to decrease the child's risk to develop allergic

disease but new evidence showed that the associations on which these recommendations were based were inconclusive.[74-77] Diets in general were reported to be associated with atopic disease but information on associations of maternal diet during pregnancy and nutrition in early life with childhood allergies is limited.[78-80] There is some evidence that fish consumed before age 1, early consumption of cow's milk and intake of fruits and vegetables are associated with lowered risk for allergic disease.[12,69,81-85] Interestingly, high amounts of these foods (fresh fish, fruits and vegetables) in traditional diets were replaced by processed and synthetic foods in modern diets.[86]

The immune system and potential pathways

The immune system is protecting the human body from microorganisms which is facilitated by an innate and an adaptive component. Immunological pathways of how farming and nutrition related exposures might affect the development of atopic disease are unclear but recent evidence directed attention to the innate immune system causing a shift of the established Th1-Th2 paradigm.[12,41,87] Therein, perturbations of the balance between type 1 and type 2 T helper cells (Th1 and Th2) and their produced cytokines result in pathogenesis of allergies. This imbalance might be caused by environmental factors such as lack of microbial infection or exposure. Further arguments were made for an increased Th1 and Th2 response based on reduced immune suppression by regulatory T cells due to lack of microbial agents.[88] Whereas the adaptive immunity provides antigen specific protection by memorizing previously encountered antigens, the innate immunity serves as the pivotal system against intruding pathogens. Contrary to outdated notions, the "innate immunity is not merely a vestige of ancient antimicrobial systems that has been made redundant by the evolution of acquired immunity" but actually "dictates the conduct of the acquired immune response".[89]

The innate immunity's main components are a limited number of transmembranous and intracellular receptors, Toll-like receptors 1-13 (TLR) and CD14, which recognize pathogen-associated molecular patterns (PAMPs) and set off complex and variable downstream signaling. Furthermore, each TLR is associated with the recognition of certain groups of pathogens (e.g. TLR4 recognizes patterns of lipopolysaccharides (LPS) originating from gram-negative bacteria).[11,90,91] All these qualities allow PAMP-specific immune responses and activation of pro-inflammatory genes. The innate immune system has only been studied since the end of the 20th century and there are still many open questions.[90] In the last decade, however, farming environments and farm related exposures rich in microbes were shown to be associated with the gene expression of innate immunity receptors[92,93] and it is noteworthy

that there is evidence in humans and animal models suggesting modulation of the immune system already occurs *in utero*.[41,94,95] Interestingly, these were the same exposures that were associated with lower risk for atopic disease in childhood.[34] A new study showed that the gene expression of innate immunity receptors in turn appears to be associated with atopic dermatitis in early life.[96] Recently, it was also shown that non-microbial agents (house dust mite allergen MD-2 or Ni^{2+}) can trigger TLR responses.[97,98]

The development of local and mucosal immune response and homeostasis of the mammalian immune system depend on the colonization of the gastrointestinal tract (GI) which is highly susceptible to early life environmental exposures including breast feeding and diet.[99-105] The gut microbiota and its dysbiosis characterized by a lack of beneficial microbes (e.g. Lacobacilli and/ or Bifidobacteria[106]) or increase of pathogens in the gut were speculated to increase risk for atopic disease.[107-109] Furthermore, parasite infections were observed to lower the risk for atopic disease.[110-114] Exposures changing the gut microbiota balance might potentially influence the onset and course of atopic outcomes. However, individual susceptibility determined by genetic factors can induce protective or pathogenic response from the same commensal bacteria[109] adding to the complexity of current research.

These findings provide a promising basis to elucidate development of allergic diseases and to explain immunological pathways of protective “farm and nutrition related effects”.

Gene-environment interactions

There is limited evidence that genetic variance modifies the association of farming related exposures with asthma and atopy.[115-117] Different individual susceptibilities to “protective effects” on atopic disease due to variance in genes was also demonstrated for the association of raw farm milk consumption in early life and the development of asthma.[118] The presence of the association depended on the genotype of a single nucleotide polymorphism (SNP) in *CD14*, a gene encoding a protein which is a component of the innate immune system. Reports about such gene-environment interactions, however, are inconsistent and a recent genome wide association study (GWAS) concluded that common SNPs might be of small significance in the asthma-protective “effects” of farm exposures.[119]

1.1 Methods

The research questions of this thesis were addressed in the framework of two large European studies.

Embedded in the cross-sectional GABRIEL study (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community), the GABRIEL Advanced Surveys were conducted in rural areas of southern Germany (Bavaria and Baden-Württemberg), Switzerland, Austria and Poland to determine farming related factors which are fundamental to protecting against asthma and atopic disease in childhood.[120] The study population comprised 6-12 year old children and participants for extensive assessments were selected by disproportionate stratified random samples in multiple sampling phases to increase power. Phase 1 was a comprehensive population-based survey to assess the baseline prevalence of exposure to farming environments (see excerpt in *Appendix*: *Excerpts from GABRIEL-A questionnaires*) and of asthma and atopic diseases (N=103'219). Children eligible for phase 2 (parents' written informed consent for further sampling) were selected randomly from the following 3 exposure strata of phase 1 participants i) farm children, ii) exposed non farmer children and iii) non exposed non farmer children to ascertain detailed exposure to farming environments and to collect biomaterial and environmental samples (N = 15'255). For phase 3, a further stratified random sample (mutually exclusive disease strata were defined within each exposure stratum: i) asthma, ii) atopy but no asthma and iii) no asthma and no atopy) was taken from participants from southern Germany (Bavaria), aiming at an in-depth assessment of respiratory disease and exposure including two collections (in winter and summer) of cow's milk consumed at participants' homes (N=895).

PASTURE (Protection against allergy- study in rural environments) is a prospective birth cohort study conducted in rural areas of Germany (Upper Bavaria), Austria (Salzburg area), Eastern Switzerland and Central Finland (Kuopio).[96,121] Initially, 1'133 pregnant women were recruited in the third trimester. Environmental exposures and self reports about atopic disease were assessed by extensive questionnaires during pregnancy and at age of 2, 12, 24, 36, 54 and 72 months (see excerpt in *Appendix: Excerpt from PASTURE questionnaire: age 1*). Atopic diseases at various ages were also measured objectively such as atopy by specific IgE measurements in blood samples. A detailed food frequency diary during year 1 provided information on introduction of complementary foods. 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 at birth (N=938) and age 1 (N=752).

1.2 Goals and objectives of this thesis

The following research questions were addressed using data from the cross sectional GABRIEL-A study:

Disentangling the protective associations of a child's distinct farm exposures

To see whether specific farming characteristics had a greater impact on allergic health outcomes in children than farm exposure in general, we used a latent class analysis to disentangle the protective associations of a child's distinct farm exposures. The following questions were addressed:

1.) *Can the previously reported "farm effect" on childhood asthma and allergies be attributed to specific types of farms?*

2.) *Which distinct farm-related exposures are responsible for the association of farming environments with childhood asthma, hay fever and atopic dermatitis?*

Findings are presented in *Chapter 2: Protection from childhood asthma and allergy in Alpine farm environments – The GABRIEL advanced studies.*

Association of farm milk consumption with childhood asthma and allergy

Questionnaire based milk consumption in early life and objectively measured constituents and microorganisms in cow milk samples collected at the participant's homes were related to asthma, atopic sensitization, hay fever and atopic dermatitis. Outcomes were determined by questionnaires or measured in blood samples. Research questions were:

3.) *What is the prevalence of allergic health outcomes and cow milk consumption in the GABRIELA study sample?*

4.) *How does the composition of commercial and farm milk relate to commercial milk processing (homogenization, fat standardization, pasteurization or ultra-high temperature processing), home processing of milk (skimming, boiling), farm characteristics (farm size, cow's fodder) and milk storage (location, duration)?*

5.) *Is unprocessed cow's milk consumed in early life associated with asthma and allergic health outcomes in childhood and which specific constituents or microorganisms in milk are responsible?*

Results are presented in *Chapter 3: The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study*.

During statistical analyses with the GABRIELA dataset to answer research questions 3 - 5 it was discovered that the rapid method at first used to assess total viable bacterial counts in the GABRIELA milk samples (flow cytometry) did not yield reliable results for heat processed milk. The milk samples were measured again by a second rapid method based on a modified culture technique (automated most-probable number method). The following research questions regarding the microbial exposure assessment were addressed:

6.) *Which rapid method, a flow cytometry system or an automated most-probable number system, measures total viable bacterial counts in raw and processed cow's milk more reliably when compared with standard plate count method while keeping time and costs low?*

Findings are presented in *Chapter 4: Appropriate and alternative methods to determine viable bacterial counts in cow milk samples*.

The following research questions were addressed using data from the longitudinal PASTURE study:

Association of early life nutrition with development of atopic dermatitis

Potential health effects of raw farm milk consumption investigated in *Chapter 3* can be viewed in the context of health effects of farming as presented in *Chapter 2*. It is important to also acknowledge the farm milks role as food and to view it in the context of the full spectrum of early life nutrition and how this is associated with the development of allergic disease. With comprehensive food frequency data collected during the first year of the participating children's lives, it was possible to investigate associations of early life nutrition with atopic dermatitis.

7.) *Which complementary foods or combinations thereof do mothers introduce within the first year of their children's lives and how are they associated with the development of atopic dermatitis?*

Results are presented in *Chapter 5: The development of atopic dermatitis according to age of onset and the association with early life exposures*.

Farm related exposures in early life and associations with innate immunity

The PASTURE cohort study was also used to further elucidate potential pathways underlying the observed associations of farming related exposures with allergic health outcomes, specifically the association of farm milk exposure during pregnancy and first year of life with the development of a child's innate immune system. Information on pregnancy and early life exposures including farm, cow milk and dietary exposures and on gene expression of innate immunity receptors at birth and at age of 1 allowed to address the following research questions:

8.) *Which environmental especially farm related exposures during pregnancy are associated with a child's gene expression of innate immunity receptors (TLRs 1-9 and CD14) at birth?*

9.) *How does the innate immune system change from birth to age 1 and which farm-related and nutritional exposures during the first year of life are associated with a child's gene expression of innate immunity receptors at age 1?*

10.) *Can gene-environment interactions of associations of farm milk consumption with the gene expression of innate immunity receptors found in previous cross-sectional studies be confirmed?*

Findings are presented in *Chapter 6: Prenatal and early life exposures alter expression of innate immunity genes: The PASTURE cohort study*.

2 PROTECTION FROM CHILDHOOD ASTHMA AND ALLERGY IN ALPINE FARM ENVIRONMENTS – THE GABRIEL ADVANCED STUDIES

This paper has been published:

Illi S, Depner M, Genuneit J, Horak E, **Loss G**, Strunz-Lehner C, Büchele G, Boznanski A, Danielewicz H, Cullinan P, Heederik D, Braun-Fahrländer C, von Mutius E, the GABRIELA study group. Protection from childhood asthma and allergy in Alpine farm environments – the GABRIEL Advanced Studies. *J Allergy Clin Immunol* 2012; 129(6):1470-7.e6.

Protection from childhood asthma and allergy in Alpine farm environments—the GABRIEL Advanced Studies

Sabina Illi, PhD,[a]* Martin Depner, PhD,[a]* Jon Genuneit, MD,[b] Elisabeth Horak, MD,[c] Georg Loss, MSc,[d,e] Christine Strunz-Lehner, MPH,[a] Gisela Büchele, PhD,[b] Andrzej Boznanski, MD, PhD,[f] Hanna Danielewicz, MD,[f] Paul Cullinan, MD,[g] Dick Heederik, PhD,[h] Charlotte Braun-Fahrländer, MD,[d,e] Erika von Mutius, MD, MSc,[a] and the GABRIELA Study Group‡ *Munich and Ulm, Germany, Innsbruck, Austria, Basel, Switzerland, Wroclaw, Poland, London, United Kingdom, and Utrecht, The Netherlands*

Background: Studies on the association of farm environments with asthma and atopy have repeatedly observed a protective effect of farming. However, no single specific farm-related exposure explaining this protective farm effect has consistently been identified.
Objective: We sought to determine distinct farm exposures that account for the protective effect of farming on asthma and atopy.
Methods: In rural regions of Austria, Germany, and Switzerland, 79,888 school-aged children answered a recruiting questionnaire (phase I). In phase II a stratified random subsample of 8,419 children answered a detailed questionnaire on farming environment. Blood samples and specific IgE levels were available for 7,682 of these children. A broad asthma definition was used, comprising symptoms, diagnosis, or treatment ever.
Results: Children living on a farm were at significantly reduced risk of asthma (adjusted odds ratio [aOR], 0.68; 95% CI, 0.59-0.78; $P < .001$), hay fever (aOR, 0.43; 95% CI, 0.36-0.52; $P < .001$), atopic dermatitis (aOR, 0.80; 95% CI, 0.69-0.93; $P = .004$), and atopic sensitization (aOR, 0.54; 95% CI, 0.48-0.61; $P < .001$) compared with nonfarm children. Whereas this overall farm effect could be explained by specific exposures to cows, straw, and farm milk for asthma and exposure to fodder storage rooms and manure for atopic dermatitis, the farm effect on hay fever and atopic sensitization could not be completely explained by the questionnaire items themselves or their diversity.
Conclusion: A specific type of farm typical for traditional farming (ie, with cows and cultivation) was protective against asthma, hay fever, and atopy. However, whereas the farm effect on asthma could be explained by specific farm characteristics, there is a link still missing for hay fever and atopy. (J Allergy Clin Immunol 2012;129:1470-7.)

Key words: *Asthma, hay fever, atopic dermatitis, atopic sensitization, childhood, farming, farm milk, early life*

Discuss this article on the JACI Journal Club blog: www.jaci-online.blogspot.com.

Asthma and allergies constitute complex diseases; their cause involves both genetic and environmental determinants. Moreover, both diseases frequently have their onset in childhood and thus appear to comanifest. However, recent results from the GABRIEL Surveys contradict this concept of interdependent phenotypes. The GABRIEL Surveys were designed to identify key factors in the development of asthma using the latest research across a variety of disciplines, including genetics, epidemiology, and immunology (see Table E1 in this article's Online Repository at www.jacionline.org).[1-6] A genome-wide association study within the GABRIEL Surveys found no overlap in genes associated with asthma and total IgE levels.[1] Furthermore, within the GABRIEL Surveys, discrepant results were also observed for the protective role of microbial diversity within a farming environment.[2] Whereas the protective farm effect on childhood asthma could be explained by the overall diversity of bacteria and fungi from dust of farm and nonfarm children, this did not hold for atopy.

Previous studies on the protective effect of growing up on a typical Central European farm were fairly consistent with respect to hay fever and atopy. In contrast, results for asthma were quite heterogeneous. This potentially indicates that not all farms are the same and that specific farm characteristics are possibly of greater effect than farm exposure in general.[7-10] These previous studies mainly used questionnaires assessing the farm's characteristics but not the child's exposure. The aim of the current epidemiologic GABRIEL Advanced Studies was an in-depth analysis of the protective exposures within a farming environment both on asthma

From [a]the Asthma and Allergy Research Group, University Children's Hospital, Munich; [b]the Institute of Epidemiology and Medical Biometry, University of Ulm; [c]the Department of Paediatrics and Adolescents, Innsbruck Medical University; [d]the Swiss Tropical and Public Health Institute, Basel; [e]the University of Basel; [f]the Department of Paediatrics, Allergology and Cardiology, Wroclaw Medical University; [g]Imperial College London, National Heart and Lung Institute, London; and [h]the Division of Environmental Epidemiology, Institute for Risk Assessment Sciences, Utrecht University.
*These authors contributed equally to this work.
‡For an alphabetical listing of the members of the GABRIELA study group, see this article's acknowledgments section.
Supported by the European Commission as part of GABRIEL (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community), contract no. 018996 under the Integrated Program LSH-2004-1.2.5-1.
Disclosure of potential conflict of interest: M. Depner has received research support from the European Commission/European Research Council. A. Boznanski, H. Danielewicz, and D. Heederik have received research support from the European Commission. E. von Mutius has consulted for Novartis, GlaxoSmithKline, ALK-Abelló, and Protectimmune; has received a speaker's fee from InfectoPharm; has received research support from Airsonnett AB; and has served as a member of the Expert Panel for the UK Research Excellence Framework. The rest of the authors declare that they have no relevant conflicts of interest.
Received for publication August 31, 2011; revised March 15, 2012; accepted for publication March 16, 2012.
Available online April 24, 2012.
Corresponding author: Sabina Illi, PhD, University Children's Hospital, Lindwurmstrasse 4, 80337 Munich, Germany. E-mail: sabina.illi@med.uni-muenchen.de.
0091-6749/$36.00

doi:10.1016/j.jaci.2012.03.013

Abbreviations used
aOR: Adjusted odds ratio
LCA: Latent class analysis

and atopy. This was based on a newly designed questionnaire aiming at disentangling the protective effect of a child's distinct farm exposures.

METHODS

Study design and population

The GABRIEL Advanced Surveys were conducted by 5 study centers in rural areas of southern Germany (Bavaria and Baden-Württemberg), Switzerland (9 German-speaking cantons), Austria (Tyrol), and Poland (Silesia) from winter 2006 to spring 2008.[5] Because of differences in study design, the Polish data will be reported separately. In the population-based phase I study a short recruiting questionnaire was distributed to parents of all schoolchildren through their elementary schools. In phase II stratified random samples of all children whose parents had given written informed consent to blood sampling, genetic analyses, and dust sampling were studied. Three strata were defined: (1) farm children (ie, children living on a farm run by the family); (2) exposed nonfarm children (ie, children not living on a farm but regularly exposed to stables, barns, or cow's milk produced on a farm); and (3) unexposed nonfarm children.

In all centers the ethics committees of the respective universities and the data protection authorities approved the study.

Questionnaires

The recruitment questionnaire in phase I assessed the prevalence of respiratory and allergic symptoms and diagnoses, socioeconomic status, family history of atopy, maternal smoking, and farm characteristics comprising types of animal breeding, cultivation, and animal feeding. A comprehensive questionnaire was handed out to parents in phase II assessing characteristics of asthma and detailed information on the child's farm-related exposures. All farm-related exposures were assessed for 5 time periods (pregnancy; first, second to third, and fourth to fifth years of life; and past 12 months) and 5 frequency categories per time period (never/almost never, about once a month, about once a week, about once a day up to 15 minutes, and about once a day longer than 15 minutes). The following exposures were assessed: contact with animals (cats, dogs, cows, pigs, poultry, sheep, and horses), stay in animal sheds (cow, pig, and poultry), contact with animal feed (straw, hay, grain, corn, grass, silage, pellet feed, and sugar beet), presence during parental farming activities (harvesting/kibbling/ensiling corn, harvesting/handling hay, ensiling grass, harvesting/threshing/kibbling grain, fieldwork, manuring, and spraying pesticides), stay in barn or fodder storage room, and consumption of cow's milk produced on the farm.

Asthma and other allergic illnesses

Asthma was defined as either current wheeze (parental reporting of wheeze in the past 12 months), a positive answer to the question "Did your child ever use an asthma spray?," or a doctor's diagnosis of asthma at least once or of wheezy bronchitis more than once. Atopic and nonatopic current wheeze was defined as current wheeze with or without atopic sensitization (see the definition below), respectively, by using the children without current wheeze as a common reference group. Severe wheeze was defined as wheeze in the past 12 months with multiple triggers and asthma inhaler use ever.

Hay fever was defined as either nasal symptoms with itchy or watery eyes in the past 12 months or a doctor's diagnosis of hay fever ever. Atopic dermatitis was defined as a doctor's diagnosis ever.

All questionnaire-based outcomes were reported in phase I except for severe wheeze, which was assessed in phase II, and atopic and nonatopic current wheeze because atopic sensitization was also only assessed in phase II.

Atopic sensitization

Blood samples were collected, and serum IgE antibodies against inhalant (*Dermatophagoides pteronyssinus*, cat, grass mix [sweet vernal grass, rye grass, timothy grass, cultivated rye, and velvet grass], birch, and mugwort) and food (egg white, cow's milk, fish, wheat, peanut, and soybean) allergens were measured in one central laboratory at the Robert-Koch-Institute, Berlin, Germany, by using the UNICAP 1000 (Phadia AB, Uppsala, Sweden). Atopic sensitization was defined as specific IgE antibodies of at least 0.7 kU/L against *D pteronyssinus*, cat, or birch or a positive reaction (0.35 kU/L) to the grass mix.

Statistical analyses

For further information on statistical analyses, see the Methods section in this article's Online Repository at www.jacionline.org.

For phase I, categorical variables are presented as relative frequencies; *P* values are based on the Pearson χ^2 test. A latent class analysis (LCA) was used to derive different types of farming, the association of which with outcomes was then analyzed by using logistic regression analysis. For phase II, all questionnaire-based farm-related exposures were dichotomized into presence or absence of the exposure based on an exposure frequency of at least once a week in a specific time period. Early-life exposure was then defined as the presence of the exposure in pregnancy or the first 3 years of life. Correlation between these farm-related exposure variables was assessed by using the Kendall tau-b correlation coefficient. Diversity of farm exposures was defined by summing up all dichotomous farm exposures and division into quartiles based on the weighted distribution in the study sample. Categorical variables are presented as weighted relative frequencies and compared over categories by using the Rao-Scott χ^2 test. Weighted logistic regression models were used to calculate associations between outcomes and farm-related exposures. Stepwise logistic regression analyses were calculated to assess final models containing the most relevant exposures. Combined effects of all dichotomized farm-related exposure variables defined as 4-level categorical variables were included in this process. All models were adjusted for farming, center, and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education). Statistical analysis was performed with SAS 9.2 software (SAS Institute, Inc, Cary, NC), and a *P* value of .05 was considered significant. Because of the exploratory character of the analysis, corrections for multiple testing were not performed.

RESULTS

In phase I, 132,518 recruitment questionnaires were distributed, of which 79,888 (60.3%) were returned. Of those, 34,491 (43.2%) parents provided written informed consent for blood sampling, genetic testing, and dust sampling. Their children were eligible for phase II (Fig 1); mean age was 8.7 ± 1.4 years. Of these, 9,668 were randomly selected for phase II by exposure stratum (ie, farm children, exposed nonfarm children, and unexposed nonfarm children), and 8,419 (87%) returned the detailed phase II questionnaire. Of these participants, 7,682 (91%) provided blood samples for measurements of specific IgE levels. Families participating in phase II were of higher education and had more allergic illnesses in the family, as also observed in other studies.[11]

A lower prevalence of asthma, hay fever, atopic dermatitis, and atopic sensitization was found among farm children compared with nonfarm children in phases I and II (Table I), with the exposed nonfarm children having intermediate prevalences. After adjusting for confounding variables, the adjusted odds ratios (aORs) for asthma, hay fever, and atopic sensitization with farming status (farm vs nonfarm) were as follows: 0.68 (95% CI, 0.59-0.78; $P < .001$), 0.43 (95% CI, 0.36-0.52; $P < .001$), and 0.54 (95% CI, 0.48-0.61; $P < .001$), respectively. For atopic dermatitis, the farm effect only amounted to an aOR of 0.80 (95% CI,

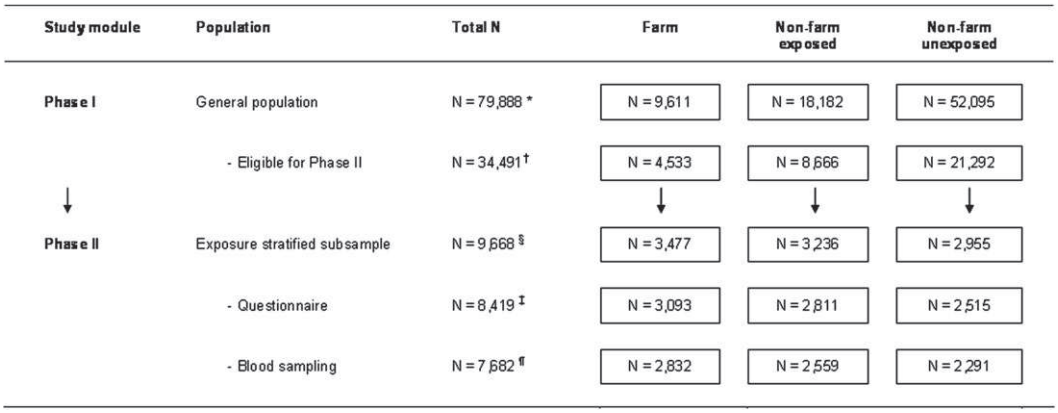

FIG 1. Study population and design. *Completed phase I recruiting questionnaire. †Completed phase I recruiting questionnaire and signed a consent form for analyses and all additional investigations in phase II. §Random selection stratified for exposure. ‡Completed phase II questionnaire. ¶Completed phase II questionnaire and participated in blood sampling and analysis of specific IgE levels.

TABLE I. Prevalence of asthma, other allergic illnesses, and atopic sensitization, as well as specific farm exposures among farm children compared with exposed and unexposed nonfarm children

		Nonfarm children		
	Farm children	Exposed	Unexposed	
Phase I*				
Atopic dermatitis	10.6%	14.4%	14.5%	†
Hay fever	4.8%	10.5%	14.7%	†
Asthma	11.4%	15.8%	18.3%	†
Current wheeze	6.7%	9.7%	11.7%	†
Phase II‡				
Atopic dermatitis	12.8%	17.3%	18.0%	‖
Hay fever	6.4%	11.6%	18.2%	‖
Atopic sensitization§	24.5%	35.5%	43.1%	‖
Asthma	14.1%	20.0%	22.2%	‖
Current wheeze	8.8%	12.6%	15.0%	‖
Atopic§	4.7%	7.5%	8.7%	‖
Nonatopic§	3.5%	5.2%	6.3%	‖
Severe wheeze	1.7%	2.9%	3.6%	‖
Phase II‡¶				
Contact with cows	70.7%	31.0%	5.2%	‖
Stay in cow shed	67.6%	24.3%	2.7%	‖
Contact with straw	64.9%	24.1%	3.8%	‖
Stay in barn	73.4%	28.5%	4.0%	‖
Stay in storage room	30.6%	7.0%	0.8%	‖
Consumption of farm milk	70.9%	51.1%	5.3%	‖

*Phase I population: n = 79,888.
†$P < .001$ of the Pearson χ^2 test for farm versus nonfarm children.
‡Phase II population: n = 8,419; analyses weighted to eligible subjects for phase II (n = 34,491).
§Reduced phase II population: n = 7,682 because of reduced sample size for blood sampling; analyses weighted to eligible subjects for phase II (n = 34,491).
‖$P < .001$ of the Rao-Scott χ^2 test for farm versus nonfarm children.
¶Farm exposures in pregnancy and the first 3 years of life assessed in the phase II questionnaire.

0.69-0.93; $P = .004$). The protective farm effect was seen for all asthma phenotypes: asthma, current wheeze, current atopic wheeze, current nonatopic wheeze, and severe wheeze.

In phase I farm characteristics with respect to animal breeding, cultivation, and animal feeding were assessed within the group of farm children. By using LCA, 3 types of farms were identified (Fig 2). The first type comprised farms without dairy cows or cattle breeding. These farms typically kept other animals, such as pigs, poultry, or horses, combined with cultivation of grain and feeding of grain shred. The second type of farming comprised farms with dairy cows and cattle breeding but nearly no cultivation. In contrast, the third farm type typically comprised those that kept dairy cows and bred cattle combined with cultivation, mostly of grain and corn. Farmers of the latter group also typically fed corn silage and grain shred to their animals. When assessing the association of the 3 types of farming with asthma, hay fever, atopic dermatitis, and atopic sensitization within the group of farm children, a protective effect of the third type of farming on asthma, hay fever, and atopic dermatitis was observed (Table II). For atopic sensitization, only a nonsignificant protective trend was observed, potentially because of the reduced sample size in phase II.

In contrast to phase I assessing farm characteristics irrespective of whether the child itself was actually exposed, in phase II the child's exposure to specific farm characteristics was assessed. First contact with these farm exposures typically occurred early in life, especially in pregnancy and the second to third year of life (Fig 3). Therefore in all subsequent analyses the timing of farm exposures relates to the period from pregnancy to the third year of life. Many of these exposures, such as contact with cows, other farm animals, or animal fodder, the consumption of cow's milk produced on a farm, and the child's presence in stables, barns, or fodder storage rooms, were inversely related to asthma, hay fever, atopic dermatitis, and atopic sensitization, even when adjusting for farming (Table III). Children were often exposed to several factors, although correlations between different factors were only moderate, with somewhat higher correlations for exposure to grass, hay, and straw (tau-b correlation coefficient, ≥0.7; data not shown). Still, many of the assessed exposures showed a strong overlap (eg, 75% of the children that "were present while the parents are manuring" also had contact with both cows and straw), requiring multivariate selection procedures to identify relevant exposures.

Therefore a stepwise variable selection process was performed. In the resulting final multivariate models, only few farm exposures remained inversely related to asthma, hay fever, atopic

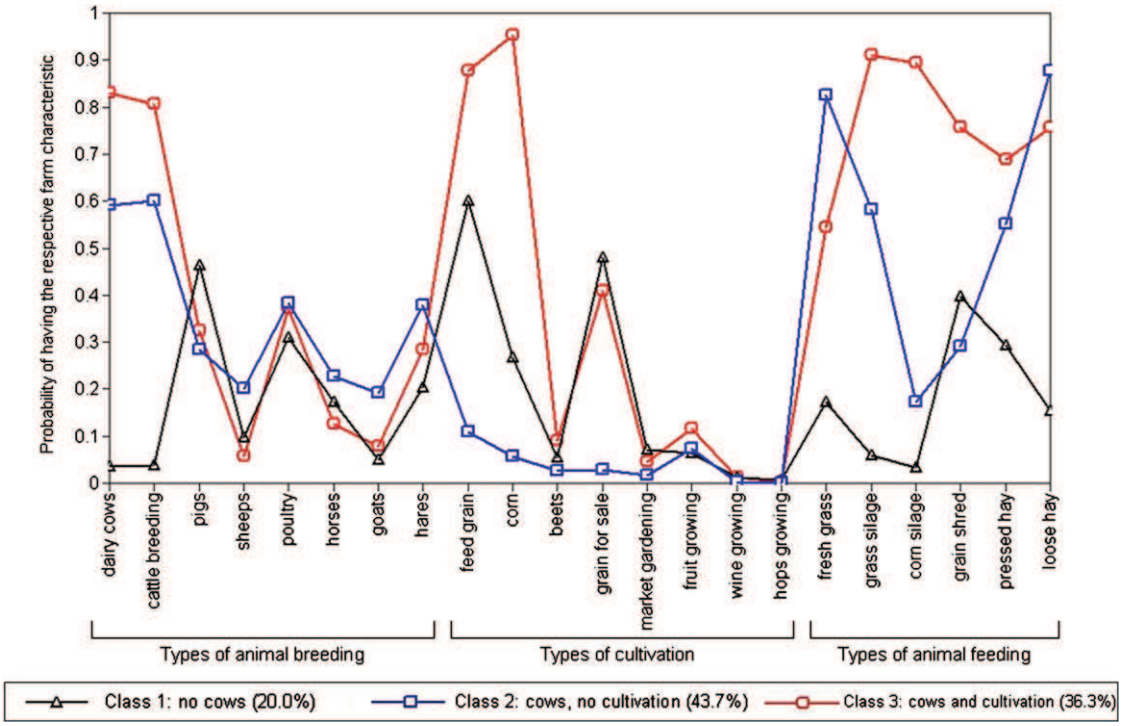

FIG 2. Types of farms based on farm characteristics. Results of LCA with 3-class solution are shown. Farm characteristics assessed in the phase I recruitment questionnaire are shown (n = 9611 farm children).

TABLE II. Types of farms and risk of asthma, hay fever, atopic dermatitis, and atopic sensitization

	Asthma*			Hay fever*			Atopic dermatitis*			Atopic sensitization†		
Farm type	**aOR‡**	**95% CI**	***P* value**	**aOR‡**	**95% CI**	***P* value**	**aOR‡**	**95% CI**	***P* value**	**aOR‡**	**95% CI**	***P* value**
No cows	1.00	—	—	1.00	—	—	1.00	—	—	1.00	—	—
Cows, no cultivation	0.84	0.69-1.03	.09	0.94	0.70-1.26	.68	0.78	0.64-0.96	.02	1.01	0.78-1.32	.94
Cows and cultivation	0.79	0.65-0.95	.01	0.70	0.53-0.94	.02	0.75	0.62-0.91	.004	0.82	0.64-1.06	.13

*Outcomes assessed in phase I recruitment questionnaire (n = 9611 farm children).
†Outcome assessed in phase II blood sampling (n = 2832 farm children).
‡Adjusted for center and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking, and parental education).

dermatitis, and atopic sensitization (Fig 4; data are shown in Table E2 in this article's Online Repository at www.jacionline.org). Concurrent contact with cows and straw and the consumption of cow's milk produced on the farm were independent protective factors for asthma. The farm effect aORs increased from 0.68 (95% CI, 0.59-0.78) to 0.89 (95% CI, 0.75-1.06) after inclusion of the relevant farm exposures, suggesting that they accounted for most of the farm effect. When stratifying the analysis into atopic and nonatopic children, the variables selected into the final model remained unchanged in the group of nonatopic subjects, whereas only farm milk remained in the model as a significant protective factor for asthma among atopic children. "Being present while the parents are manuring" showed the lowest odds ratios for all outcomes except atopic sensitization. However, in the multivariate model, when including contact with cows and with straw, manuring was no longer significant.

Similarly to asthma, protective farm exposures remaining in the final multivariate model for hay fever were contact with cows and consumption of farm milk. However, in contrast to asthma, contact with straw was no longer significant in the final model, even in combination with concurrent contact with cows. The farm effect aOR increased from 0.43 (95% CI, 0.36-0.52) to only 0.68 (95% CI, 0.55-0.84), indicating the presence of additional undetected protective exposures in the farming environment.

For atopic sensitization, contact with straw and the consumption of cow's milk produced on the farm were significant independent protective determinants in the final model (Fig 4). Similarly to hay fever, the aOR for farming only increased from 0.54 (95% CI, 0.48-0.61) to 0.74 (95% CI, 0.64-0.86). Exposure to poultry and dogs early in life additionally contributed to the model when defining atopic sensitization at a higher cutoff (≥3.5 kU/L).

With respect to atopic dermatitis, only very few distinct questionnaire-based farm exposures were significantly protective after adjusting for farming and potential confounders. Of these, only staying in a fodder storage room remained in the final model, with the effect of farming being no longer significant. In contrast to the other phenotypes, onset of atopic dermatitis typically occurs in infancy, with an increased potential role of exposures in pregnancy. We thus repeated all analyses for exposures in pregnancy only. The maternal exposures inducing the greatest change in the effect of farming on atopic dermatitis were staying in a cow shed and manuring during pregnancy. In contrast, when

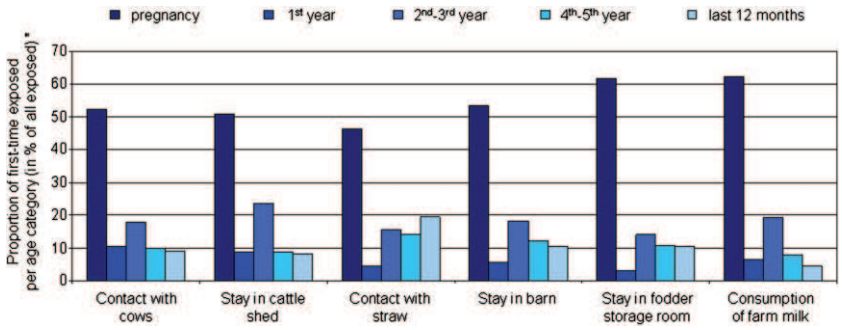

FIG 3. Timing of the first exposure to farm characteristics. Most children experienced their first exposure through their mothers in pregnancy. *Computations are based on 5 groups of children ever exposed to cows, cow sheds, straw, barns, fodder storage rooms, or farm milk. The *bars* show the proportion of children with first-time contact with the respective farm exposure per age category. Proportions of the 5 age categories add up to 100%.

including only maternal exposures during pregnancy for asthma, hay fever, and atopic sensitization, factors remaining in the final models were unchanged. Atopic dermatitis was merely defined as a doctor's diagnosis because this was assessed in phase I, whereas corresponding symptoms were only assessed in phase II. When using an outcome variable combining diagnosis and symptoms, the final multivariate models remained unchanged, except that manuring additionally remained in the model.

For the assessment of the diversity of exposures, a score was generated by summing up all dichotomous farm exposures. This diversity score was significantly associated with atopic sensitization: aORs of 0.79 (95% CI, 0.65-0.97; $P = .03$) for 2 to 4 exposures (third quartile) and 0.65 (95% CI, 0.52-0.80; $P < .001$) for 5 to 23 exposures (fourth quartile) versus no exposure (first quartile). However, when adjusting the final models for diversity, it was no longer significant, and the association of contact with straw and consumption of farm milk with the outcome remained basically unchanged (see Table E3 in this article's Online Repository at www.jacionline.org).

Sensitivity analyses investigating the individual contribution of prenatal and postnatal exposure for asthma, hay fever, and atopic sensitization showed some differences between factors but in general suggested that both periods were of importance, showing similar effects for exposure in pregnancy and in the first 3 years of life (data not shown). Furthermore, a dose-response relationship was seen (ie, stronger protection with increased frequency of exposures; see Fig E1 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Children growing up on farms in Germany, Austria, and Switzerland are protected against asthma, hay fever, and atopic sensitization. Only 3 distinct farm exposures assessed by means of questionnaire (ie, the pregnant mother's and subsequently the toddler's exposure to cows and straw and the consumption of cow's milk produced on the farm) accounted for the farm effect on asthma and partially on hay fever and atopic sensitization.

The protective effect of a farm environment on atopic dermatitis was much less pronounced than for the other outcomes. This discrepancy has already been observed in previous studies on farming and is in line with results from the German International Study of Asthma and Allergies in Childhood, in which atopic dermatitis showed no strong associations with environmental factors, indicating that the hygiene hypothesis might not hold for atopic dermatitis as much as for respiratory allergic diseases.[10,12]

The definition of asthma for population-based studies has been vividly debated. We used a broader asthma definition, including diagnosis, symptoms, and treatment, to also include milder and nonatopic phenotypes, as well as more specific definitions of current, atopic and nonatopic, and severe wheeze. Farm children were at lower risk of any of these phenotypes compared with nonfarm children, potentially indicating antiviral properties of the protective exposures. When using the International Study of Asthma and Allergies in Childhood's definition (doctor's diagnosis of asthma or recurrent wheezy bronchitis), as in previous farm studies, the farm effect remained unchanged (6.5%, 9.0%, and 10.5% in farmers and exposed and unexposed nonfarmers, respectively; $P < .001$) and was of similar magnitude as in the previous farm studies ALEX (Allergy and Endotoxin) and PARSIFAL (Prevention of Allergy–Risk Factors for Sensitization In Children Related to Farming and Anthroposophic Lifestyle) (see Table E4 in this article's Online Repository at www.jacionline.org).[8,13,14]

In cooperation with farmers and field workers coming from a farm environment, we developed an extensive questionnaire to assess the large spectrum of potential exposures that a child might encounter on a farm over the first years of life. The most relevant farm exposures were then selected into a final multivariate model through a stepwise statistical procedure based on the change in estimate of farming: the closer to the null effect the overall farming effect became when a specific farm exposure was additionally included in the model, the more likely it was to account for this farm effect. This method was very robust with respect to the selection of the final set of exposure variables. The standard stepwise variable selection procedure that merely uses the P value as an inclusion criterion resulted in the same final models, irrespective of whether farming and potentially confounding variables were forced into the model in the selection process.

In this newly developed comprehensive questionnaire, the child's contact with all types of animal feeding was assessed. The strongest protective effect on all outcomes except atopic dermatitis was seen for contact with straw. Straw is an agricultural byproduct of cereal plants (ie, the dry stalks after the grain has been removed) and is mostly used as bedding material for animals

TABLE III. Farm exposures (pregnancy to age 3 years) associated with decreased risk of asthma, hay fever, atopic dermatitis, and atopic sensitization†

	Asthma‡				Hay fever‡				Atopic dermatitis‡				Atopic sensitization§			
	aOR‖	95% CI	*P* value		aOR‖	95% CI	*P* value		aOR‖	95% CI	*P* value		aOR‖	95% CI	*P* value	
Contact with animals																
Cat	0.90	0.77-1.04	.16	#	0.92	0.77-1.10	.37	#	0.85	0.72-1.01	.06	¶	**0.81**	**0.71-0.93**	**.003**	#
Dog	0.99	0.84-1.15	.86		0.90	0.74-1.10	.31	#	0.88	0.74-1.05	.15		**0.85**	**0.74-0.97**	**.02**	#
Cow	**0.74**	**0.62-0.89**	**.002**	*,¶,#	**0.52**	**0.41-0.66**	**<.001**	*,¶,#	0.87	0.71-1.06	0.17	¶,#	**0.75**	**0.65-0.88**	**<.001**	*,¶,#
Pig	0.89	0.70-1.14	.36	¶	0.76	0.53-1.07	.12	¶,#	0.98	0.77-1.26	.89		0.87	0.70-1.07	.18	
Poultry	0.95	0.77-1.17	.63		**0.72**	**0.54-0.95**	**.02**	*,¶,#	0.95	0.76-1.17	.61		**0.76**	**0.64-0.91**	**.003**	#
Sheep	0.79	0.62-1.02	.07		0.74	0.52-1.05	.09	#	0.91	0.69-1.21	.53		0.84	0.68-1.04	.12	
Horse	1.13	0.89-1.43	.30		0.95	0.69-1.29	.73		**1.33**	**1.05-1.70**	**.02**		**0.79**	**0.63-0.99**	**.04**	
Stay in animal sheds																
Cow	**0.79**	**0.65-0.95**	**.01**	*,¶	**0.66**	**0.52-0.85**	**.001**	*,¶,#	0.82	0.67-1.01	.06	¶,#	**0.78**	**0.67-0.92**	**.003**	*,¶,#
Pig	1.04	0.81-1.33	.78		0.72	0.51-1.02	.06	¶,#	0.99	0.76-1.29	.92		0.85	0.68-1.06	.14	
Poultry	0.92	0.74-1.15	.48	¶	0.89	0.66-1.20	.44	#	0.91	0.72-1.15	.42	¶	0.84	0.69-1.01	.06	#
Contact with animal feed																
Straw	**0.79**	**0.66-0.95**	**.01**	*,¶	**0.61**	**0.47-0.80**	**<.001**	*,¶,#	0.83	0.67-1.02	.07	¶,#	**0.61**	**0.52-0.72**	**<.001**	*,¶,#
Hay**	0.87	0.73-1.04	.14	¶,#	**0.78**	**0.63-0.98**	**.03**	*,¶,#	0.91	0.76-1.10	.35	¶,#	**0.74**	**0.63-0.86**	**<.001**	*,¶,#
Grain**	0.93	0.76-1.14	.49		0.91	0.68-1.21	.52	#	0.91	0.73-1.14	.43	¶,#	**0.72**	**0.61-0.86**	**<.001**	*,¶,#
Corn**	0.86	0.67-1.09	.21	¶,#	0.88	0.64-1.20	.41	#	0.84	0.66-1.06	.14	¶	**0.78**	**0.64-0.95**	**.01**	#
Corn silage**	0.81	0.61-1.07	.14	¶,#	0.72	0.47-1.09	.12	¶,#	**0.70**	**0.54-0.93**	**.01**	*,¶,#	0.84	0.67-1.04	.11	#
Grass	0.95	0.79-1.13	.56		0.82	0.65-1.03	.09	¶,#	0.86	0.70-1.04	.11	¶,#	**0.82**	**0.70-0.96**	**.01**	
Grass silage**	0.96	0.76-1.21	.72	#	0.73	0.52-1.02	.07	¶,#	0.79	0.62-1.01	.06	¶,#	**0.79**	**0.65-0.95**	**.01**	*,¶,#
Pellet feed	0.84	0.68-1.05	.13		0.77	0.54-1.09	.13	#	0.98	0.77-1.25	.85		**0.75**	**0.62-0.92**	**.005**	#
Sugar beet	1.19	0.82-1.73	.36		0.97	0.52-1.81	.92	#	0.95	0.62-1.46	.82		0.76	0.53-1.09	.14	
Stay in —																
Barn	0.87	0.72-1.04	.13		**0.62**	**0.48-0.80**	**<.001**	*,¶,#	0.86	0.70-1.05	.14	¶,#	**0.70**	**0.59-0.82**	**<.001**	*,¶,#
Fodder storage room	0.94	0.72-1.23	.65	#	0.72	0.49-1.07	.10	¶,#	**0.72**	**0.55-0.93**	**.01**	*,¶	**0.79**	**0.64-0.98**	**.03**	
Present while parents are —																
Doing field work	1.09	0.82-1.44	.57		0.91	0.59-1.41	.68		0.85	0.62-1.19	.35	¶	0.83	0.65-1.06	.14	
Manuring	**0.65**	**0.47-0.90**	**.01**	#	**0.51**	**0.33-0.80**	**.003**	*,¶	**0.66**	**0.45-0.96**	**.03**	*,¶,#	0.85	0.65-1.11	.23	
Spraying pesticides	1.22	0.32-4.62	.77		1.00	0.12-8.14	1.00		0.74	0.27-2.07	.57		1.45	0.52-4.02	.48	
Consumption of —																
Farm milk	**0.77**	**0.66-0.90**	**.001**	*,¶,#	**0.64**	**0.53-0.77**	**<.001**	*,¶,#	0.89	0.76-1.06	.18	¶	**0.73**	**0.64-0.84**	**<.001**	*,¶,#

Significant results are shown in boldface.
*Variable included in subsequent stepwise analyses for the respective outcome (criteria: significant results and ≥10% change in aOR of farming toward the null effect).
†Farm exposures assessed in phase II questionnaire.
‡Outcomes assessed in phase II questionnaire: n = 8,419; analyses weighted to eligible subjects for phase II (n = 34,491).
§Outcome assessed in phase II blood sampling: n = 7,682; analyses weighted to eligible subjects for phase II (n = 34,491).
‖Adjusted for center, farming, and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education).
¶Ten percent or greater change in aOR of farming toward the null effect.
#Significant aOR within strata of farmer's children.
**Combination of several questionnaire items: *Hay*, contact with forage hay or present while parents were harvesting or handling hay; *Grain*, contact with forage grain or present while parents were harvesting, threshing, or kibbling grain; *Corn*, contact with forage corn or present while parents were harvesting or kibbling corn; *Corn silage*, contact with forage corn silage or present while parents are ensiling corn; *Grass silage*, contact with forage grass silage or present while parents are ensiling grass.

in the study areas. Children are exposed either in barns or in the stable when litter is placed and aerosolized or removed. However, children exposed to straw were often also exposed to hay, grass, and manure. Therefore individual effects of grass, hay, manure, and straw could not be disentangled with certainty. Recent experimental studies have shown that the oligosaccharide arabinogalactan from grass and hay protects mice against allergic asthma.[15] Cereal, the source material of straw, also contains arabinogalactan, suggesting that exposure to this plant-derived oligosaccharide might protect children against asthma and atopy.[16] Alternatively or additionally, thus far unidentified microbial exposures associated with hay and straw might explain the effect. Straw has been shown to be contaminated with a high variety of fungi and bacteria.[17]

Consumption of cow's milk produced on the farm also showed a consistently strong inverse relation with 3 of the outcomes: asthma, hay fever, and atopy. This corroborates previous findings.[8,18,19] Refined analyses on the handling of milk samples by parents (boiling or skimming) and content of microbes, fat, protein, and various enzymes have been reported separately.[6] It is important to note that the effect of consumption of cow's milk produced on the farm was independent of the protective effect of contact with cows, potentially indicating different pathways: whereas milk exerts its effect through the gut, contact with cows might potentially be an inhaled exposure affecting the airway mucosa.

This notion of different pathways is supported by the fact that contact with cows only exerted a strong effect on outcomes involving the airways (ie, on asthma, including nonatopic asthma [data not shown] and on hay fever). No such effect was observed for atopy in the final model. The effect of contact with cows on hay fever was independent of other protective exposures, such as

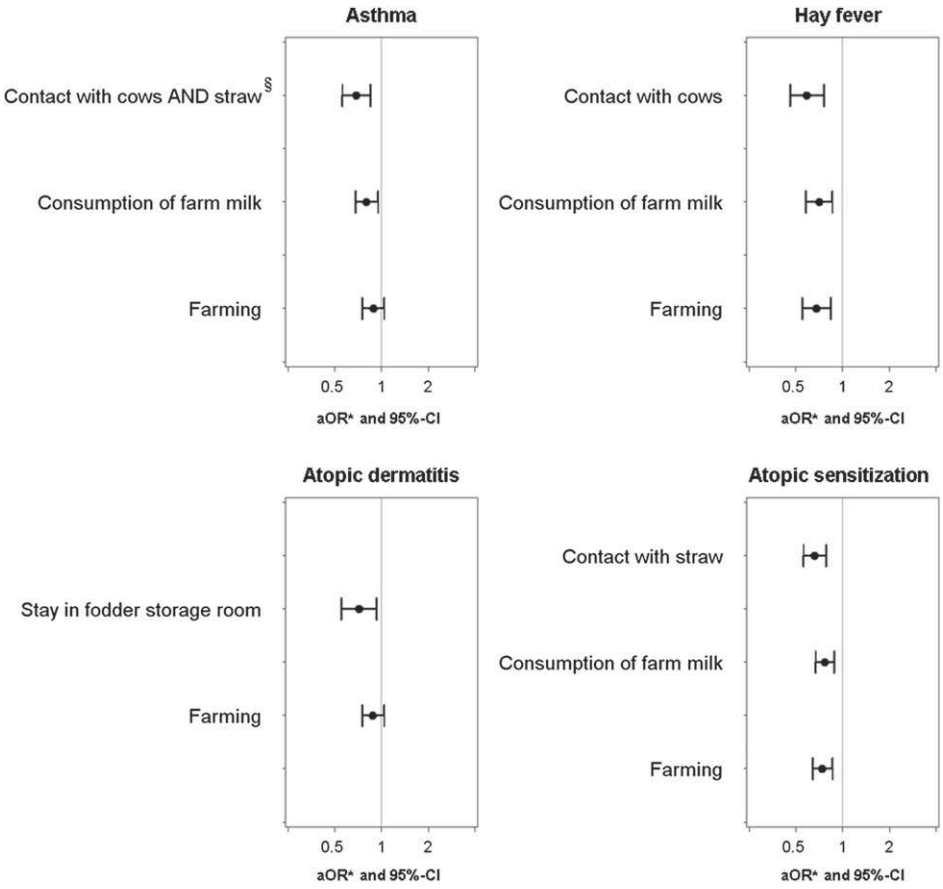

FIG 4. Specific farm exposures that best explain the overall effect of farming. Results of multivariate stepwise weighted regression models. *Mutually adjusted and additionally adjusted for center and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education). §Compared with the reference group (neither contact with cows nor straw). Odds ratios for intermediate categories (contact with cows or straw) are shown in Table E2.

contact with straw. This was in contrast to the effect on asthma: contact with cows was only inversely associated with asthma if the child also had contact with straw, potentially reflecting a specific type of farming accounting for this combined protective effect. No such interaction was observed for hay fever or atopy. This might explain why previous surveys on children from dairy farms have come to similar and homogeneous results for hay fever and atopy but have shown conflicting results with respect to protection against asthma[10,20]: perhaps specific combinations of exposures not investigated in previous studies are essential to exert a protective effect on asthma. Interestingly, similar effects were observed irrespective of whether characteristics of the farm were assessed or farm exposures of the child: both approaches resulted in a combination of cows and products of cultivation (eg, straw) as factors best explaining the overall farm effect. These results are not only observed in Alpine but also in other European areas, eg, in the Polish arm of the GABRIEL Advanced Studies (results will be reported separately). This points toward a protective effect of the traditional way of farming as it has been pursued for centuries, comprising cows, their products (eg, milk), and cultivation of grains both for alimentation and bedding material. From an evolutionary perspective, mankind has been exposed to these since settling down. Immune responses adapted to this prevailing environment might thus induce tolerance. Therefore it is not surprising that some of the farm effects observed in Central Europe are not seen in the United States because the type of farming differs greatly between continents.

The detailed questionnaire not only assessed the type of exposure but also both its time period and frequency. For most exposures, first contact in the child's life most frequently occurred during pregnancy and the second to third years of life, indicating mothers working on a farm. Furthermore, when analyzing the association of timing and outcomes, the effects of exposures early in life (ie, from pregnancy up to age 3 years, as shown in this article) showed much stronger effects than current exposure at the time of outcome assessment (data not shown). This correlates with findings from other studies that observed an effect of farm exposure in pregnancy on specific IgE levels and cytokine responses in cord blood, indicating a protective farm effect as early as *in utero*.[21-23]

Our results show that protective mechanisms differ for asthma and atopy. The exhaustive questionnaire assessed the child's farm exposures in as detailed a manner as possible. In contrast to asthma, the farm effect on atopy, although about half of it was explained by the questionnaire items, was not completely accounted for by these or their diversity, indicating a link was still missing. This is in line with previous results from the GABRIEL study group observing differing genes involved in the cause of asthma and atopy and discrepant results for the role of microbial diversity: whereas the diversity of bacteria and fungi from dust of farm and nonfarm children accounted for the protective farm effect

on asthma, this did not hold for atopy, indicating a potential role of an unknown, ubiquitous protective exposure on farms.[1,2] This unexpected finding is as puzzling as the very consistent protective effect of sibship size on atopy, which has not yet been completely explained by the hygiene hypothesis either.

The GABRIEL study group consisted of the following members listed in alphabetical order with affiliations:

Silvia Apprich, PhD,[g] Andrzej Boznanski, MD, PhD,[j] Charlotte Braun-Fahrländer, MD,[d,e] Gisela Büchele, PhD,[c] William Cookson, MD, DPhil,[a] Paul Cullinan, MD,[a] Hanna Danielewicz, MD,[j] Anna Dębińska,[j] Martin Depner, PhD,[b] Markus Ege, MD,[b] Urs Frey, MD, PhD,[q] Oliver Fuchs, MD,[k] Jon Genuneit, MD,[c] Dick Heederik, PhD,[f] Elisabeth Horak, MD,[l] Anne Hyvärinen, PhD,[h] Sabina Illi, PhD,[b] Michael Kabesch, MD,[m] Katalin Kovacs,[l] Aleksandra Kosmęda, PhD,[j] Wolfgang Kneifel, PhD,[g] Philipp Latzin, MD, PhD,[k] Roger Lauener, MD,[o] Georg Loss, MSc,[d,e] Stephanie MacNeill, MSc,[a] Bernhard Morass, MD,[l] Anne-Cécile Normand, PhD,[p] Ilka Noss, PhD,[f] Renaud Piarroux, MD, PhD,[p] Helena Rintala, PhD,[h] Mascha K. Rochat, MD,[b] Nikolaos Sitaridis,[c] Barbara Sozanska, MD,[j] David Strachan, MD,[n] Christine Strunz-Lehner, MPH,[b] Bertrand Sudre, MD, PhD,[i] Erika von Mutius, MD, MSc,[b] Marco Waser, PhD,[d,e] Juliane Weber, MD,[b] and Inge Wouters, PhD.[f]

From [a]Imperial College London, National Heart and Lung Institute, South Kensington Campus, London, United Kingdom; [b]LMU Munich, University Children's Hospital, Munich, Germany; [c]Ulm University, Institute of Epidemiology and Medical Biometry, Ulm, Germany; [d]the Swiss Tropical and Public Health Institute, Basel, Switzerland; [e]the University of Basel, Basel, Switzerland; [f]Utrecht University, Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht, The Netherlands; [g]BOKU Vienna, University of Natural Resources and Life Sciences, Department of Food Science and Technology, Vienna, Austria; [h]THL Kuopio, National Institute for Health and Welfare, Kuopio, Finland; [i]Université de Franche-Comté, UMR 6249 Chrono-Environnement, Département de Parasitologie/Mycologie, UFR SMP, Besançon, France; [j]Wroclaw Medical University, 1st Department of Paediatrics, Allergology and Cardiology, Wroclaw, Poland; [k]the Division of Pulmonology, Department of Paediatrics, Bern University Hospital, Bern, Switzerland; [l]the Department of Pediatrics and Adolescents, Division of Cardiology and Pulmonology, Innsbruck Medical University, Innsbruck, Austria; [m]Hannover Medical School, Clinic for Paediatric Pneumology and Neonatology, Hannover, Germany; [n]St George's, University of London, London, United Kingdom; [o]High Mountain Hospital Davos, Davos-Wolfgang, Switzerland; [p]the Department of Parasitology and Mycology, Hôpital de la Timone, Assistance Publique-Hôpitaux de Marseille, Marseille, France; and [q]the University Children's Hospital (UKBB), University of Basel, Basel, Switzerland.

Key messages

- **Specific types of farms with cows and cultivation exerted a protective effect on asthma, hay fever, and atopic sensitization.**
- **This protective farm effect on asthma, hay fever, and atopic sensitization was determined by 3 specific early-life exposures of the child, namely contact with cows and straw and consumption of farm milk, thereby narrowing down the farm effect.**
- **Whereas the farm effect on asthma could be explained by contact with cows, straw, and farm milk, this was not the case for hay fever and atopic sensitization, indicating differing underlying protective mechanisms.**

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METHODS

Statistical analyses

For the analysis of the farm effect, exposed and unexposed nonfarm children were combined as nonfarm children and compared with farm children.

All questionnaire-based farm-related exposures were assessed for 5 time periods and 5 frequency categories per time period. For statistical analysis, these data were dichotomized into the presence or absence of the exposure based on an exposure frequency of at least once a week in a specific time period. Early-life exposure was then defined as the presence of the exposure in pregnancy or the first 3 years of life. The correlation between the dichotomized farm-related exposure variables was assessed by using the Kendall tau-b correlation coefficient. For assessment of the diversity of exposures, a score was generated by summing up all dichotomous farm exposures depicted in Table III and dividing the sum into quartiles based on the weighted distribution in the study sample.

Data from phase II were analyzed by using weighted statistical methods, taking the specific stratified sampling design into account. Fixed *a priori* weights were calculated as the inverse of the ratio of selected to eligible children per center and strata. All analyses were weighted to the total *n* value of the study population of phase I eligible for phase II. Missing values in selected children led to slightly diminished numbers per analysis. For the final logistic regression models, a sensitivity analysis was performed by using weights additionally adjusted for missing values in the variables included in the respective model, thus truly weighting the assessed data to the total *n* value. However, results remained unchanged (data not shown).

For phase I, categorical variables are presented as relative frequencies; *P* values are based on the Pearson χ^2 test. For phase II, categorical variables are presented as weighted relative frequencies and compared over categories by using the Rao-Scott χ^2 test, which applies a design effect correction to the Pearson χ^2 statistic computed from the weighted frequencies.

In phase I LCA was used to derive different types of farming.[E1] LCA is a statistical method for finding subtypes of related subjects (latent classes) from multivariable categorical data. Farmers of our study population were clustered into a number of discrete latent classes based on the pattern of response to various questions on farm characteristics (types of animal breeding, cultivation, and animal feeding), as assessed in the phase I questionnaire. The posterior probability of each subject belonging to a particular class was estimated, and from these data, logistic regression was used to estimate associations of the respective classes or "farm types" with asthma, hay fever, atopic dermatitis, and atopic sensitization.

In phase II, weighted logistic regression models using the Taylor series method to estimate variances were used to calculate associations between dichotomous outcomes and farm-related exposures. All models were adjusted for farming, center, and potential confounders differing between farm and nonfarm children (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education). Stepwise logistic regression analyses were calculated to assess final models containing the most relevant exposures to detect specific exposure variables underlying the overall farm effect. The aim of this procedure was to explain the farm effect, and thus all exposure variables that were significant and that induced a change of at least 10% in the effect of farming toward the null-effect in farm- and confounder-adjusted analysis were included in this process. At each forward step of this model-building procedure, the exposure inducing the largest change in estimate for farming was additionally included in the multivariate model if significant. In a backward step variables were removed from the model if no longer significant. The model building ended if no additional exposure was significant if included in the model. Combined effects of all dichotomized farm related exposure variables were defined as 4-level categorical variables to detect exposures that only exert an effect if occurring concurrently with another exposure: (−−), both variables negative (reference category for statistical analysis); (+−)/(−+), 1 variable positive; and (++), both variables positive. If these combined exposures induced a change of 10% or greater in the farm effect and if only the (++) category was significant in farm- and confounder-adjusted analysis, as well as the overall type III *P* value, this categorical variable was included in the stepwise procedure based on the type III *P* value and the change in farm effect. For phase I and phase II analyses within the group of farm children, unweighted center- and confounder-adjusted logistic regression models using the same method to estimate variances as for weighted analyses were applied. aORs and 95% CIs are reported.

Statistical analysis was performed with SAS 9.2 software (SAS Institute, Inc); a *P* value of .05 was considered significant. Because of the exploratory character of the analysis, corrections for multiple testing were not performed.

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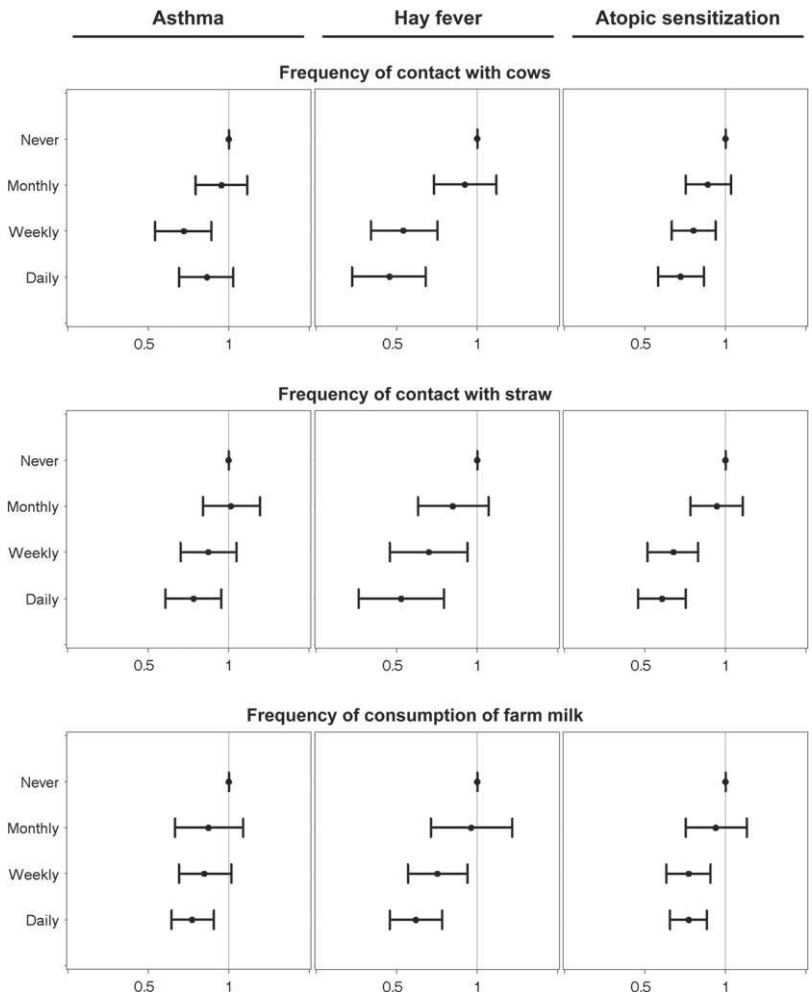

FIG E1. Frequency of exposure and risk of asthma, hay fever, and atopic sensitization. Frequency of exposure is defined as the maximum exposure of the 3 time periods (pregnancy, first year of life, and second to third year of life). aORs and 95% CIs are adjusted for farming and potential confounders.

TABLE E1. Previous publications from the GABRIEL Study Consortium

Publication	Title/key message
Moffatt et al,[E2] N Engl J Med 2010	A large-scale, consortium-based, genome-wide association study of asthma
	→ This genome-wide association study found little overlap between the principal loci that confer susceptibility to asthma and those that regulate total serum IgE levels. This suggests that an increase in IgE level is probably an inconstant secondary effect of asthma rather than its cause.
Ege et al,[E3] N Engl J Med 2011	Exposure to environmental microorganisms and childhood asthma
	→ Children living on farms were exposed to a wider range of microbes than were children in the reference group. This exposure explained a substantial fraction of the inverse relation between asthma and growing up on a farm. In contrast, atopy was only weakly associated with the diversity of microbes.
Ege et al,[E4] J Allergy Clin Immunol 2011	Gene-environment interaction for childhood asthma and exposure to farming in Central Europe
	→ A genome-wide interaction analysis revealed several novel interaction candidate genes for asthma and atopy in a farming environment. In turn, the top single nucleotide polymorphisms of a meta-analysis for childhood asthma did not interact with farming. Previously published interactions with farming-related exposures for asthma and atopy were not replicated.
Normand et al,[E5] Occup Environ Med 2011	Airborne cultivable microflora and microbial transfer in farm buildings and rural dwellings
	→ Microorganisms are transported from animal sheds and barns into farm dwellings. Therefore children living in these environments are exposed when indoors and when visiting animal sheds and barns. Indoor exposure might also contribute to the protective effect of the farm environment.
Genuneit et al,[E6] Paediatr Perinat Epidemiol 2011	The GABRIEL Advanced Surveys: study design, participation, and evaluation of bias
	→ The GABRIEL Advanced Surveys are one of the largest studies to shed light on the protective "farm effect" on asthma and atopic disease. Bias with regard to the main study question was able to be ruled out by representativeness and high participation rates in phases 2 and 3. The GABRIEL Advanced Surveys have created extensive collections of questionnaire data, biomaterial, and environmental samples, promising new insights into this area of research.
Loss et al,[E7] J Allergy Clin Immunol 2011	The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study
	→ Questionnaire-reported consumption of unboiled but not boiled farm milk was inversely associated with asthma, hay fever, and atopy. Higher levels of the whey proteins BSA, α-lactalbumin, and β-lactoglobulin in milk samples were associated with a reduced risk of asthma but not atopy. Neither total viable bacterial counts nor total fat content of milk were related to asthma or atopy.
MacNeill et al, Allergy 2011, submitted	Asthma and allergies: Is the farming environment (still) protective in Poland? The GABRIEL Advanced Studies
	→ This cross-sectional survey of schoolchildren in rural Poland showed that living on certain types of farms is significantly protective against atopic sensitization. Early-life exposure to grain might explain part of this effect.
Fuchs et al, J Allergy Clin Immunol 2011, in revision	Farming environments and childhood atopy, wheeze, lung function, and exhaled nitric oxide
	→ The protective farm effect on wheeze prevalence is independent of atopy and not attributable to improved airway size and lung mechanics. Underlying protective mechanisms include alterations of immune response and susceptibility to likely viral triggers of childhood airway disease also affecting airway inflammation.
Illi et al, J Allergy Clin Immunol 2012	Protection against childhood asthma and allergy in Alpine farm environments—the GABRIEL Advanced Studies
	→ Specific types of farms with cows and cultivation exerted a protective effect on asthma, hay fever, and atopic sensitization. This protective farm effect on asthma, hay fever, and atopic sensitization was determined by 3 specific early-life exposures of the child, namely by contact with cows and straw and consumption of farm milk, thereby narrowing down the farm effect. However, whereas the farm effect on asthma could be completely explained by these, this was not the case for hay fever and atopic sensitization, indicating differing underlying mechanisms in spite of comanifestation of these outcomes.

TABLE E2. Specific farm exposures that best explain the overall effect of farming on asthma, hay fever, atopic dermatitis, and atopic sensitization, as identified in multivariate stepwise regression models*

	aOR§	95% CI	*P* value
Asthma†			
− Contact with cows, − contact with straw	1.00	—	—
− Contact with cows, + contact with straw	1.00	0.76-1.32	1.00
+ Contact with cows, − contact with straw	0.94	0.73-1.21	.63
+ Contact with cows, + contact with straw	0.68	0.54-0.85	<.001
Consumption of farm milk	0.81	0.68-0.96	.02
Farming	0.89	0.75-1.06	.20
Hay fever†			
Contact with cows	0.59	0.46-0.76	<.001
Consumption of farm milk	0.71	0.58-0.86	<.001
Farming	0.68	0.55-0.84	<.001
Atopic dermatitis†			
Stay in fodder storage room	0.72	0.55-0.93	.01
Farming	0.88	0.75-1.04	.12
Atopic sensitization‡			
Contact with straw	0.66	0.56-0.78	<.001
Consumption of farm milk	0.77	0.67-0.88	<.001
Farming	0.74	0.64-0.86	<.001

*Weighted logistic regression models with stepwise variable selection for asthma, hay fever, atopic dermatitis, and atopic sensitization based on the largest change in estimate for farming after adjusting for confounding variables. All significant exposure variables (pregnancy to age 3 years) from previous farm- and confounder-adjusted analyses that induced a change in estimate of farming of 10% or greater toward the null effect were included in the selection process.
†Outcomes assessed in phase II questionnaire: n = 8,419; analyses weighted to eligible subjects for phase II (n = 34,491).
‡Outcome assessed in phase II blood sampling: n = 7,682; analyses weighted to eligible subjects for phase II (n = 34,491).
§Mutually adjusted and additionally adjusted for center and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education).

TABLE E3. Final multivariate models for asthma, hay fever, atopic dermatitis, and atopic sensitization adjusted for diversity score*

	aOR§	95% CI	*P* value
Asthma†			
− Contact with cows, − contact with straw	1.00	—	.08
− Contact with cows, + contact with straw	0.90	0.60-1.37	.64
+ Contact with cows, − contact with straw	0.90	0.64-1.26	.53
+ Contact with cows, + contact with straw	0.66	0.46-0.96	.03
Consumption of farm milk	0.72	0.59-0.89	.002
Farming	0.97	0.80-1.18	.76
Diversity score*			
0	1.00	—	.66
1	1.09	0.86-1.37	.47
2-4	1.17	0.91-1.51	.21
≥5	1.13	0.77-1.66	.53
Hay fever†			
Contact with cows	0.57	0.42-0.77	<.001
Consumption of farm milk	0.62	0.50-0.78	<.001
Farming	0.71	0.55-0.92	.009
Diversity score*			
0	1.00	—	.42
1	1.11	0.85-1.44	.45
2-4	1.24	0.94-1.64	.13
≥5	1.28	0.90-1.82	.16
Atopic dermatitis†			
Stay in fodder storage room	0.72	0.52-0.98	.04
Farming	0.98	0.79-1.21	.84
Diversity score*			
0	1.00	—	.29
1	0.89	0.69-1.14	.35
2-4	1.02	0.80-1.31	.87
≥5	0.82	0.63-1.06	.14
Atopic sensitization‡			
Contact with straw	0.66	0.53-0.83	<.001
Consumption of farm milk	0.77	0.65-0.91	.002
Farming	0.79	0.67-0.94	.009
Diversity score*			
0	1.00	—	.57
1	0.96	0.78-1.18	.69
2-4	0.86	0.69-1.06	.17
≥5	0.92	0.70-1.20	.53

*The diversity score is defined as the number of exposures divided into quartiles based on the weighted distribution in the study sample.
†Outcomes assessed in phase II questionnaire: n = 8,419; analyses weighted to eligible subjects for phase II (n = 34,491).
‡Outcome assessed in phase II blood sampling: n = 7,682; analyses weighted to eligible subjects for phase II (n = 34,491).
§Mutually adjusted and additionally adjusted for center and potential confounders (family atopy, ≥2 siblings, sex, maternal smoking in pregnancy, and parental education).

TABLE E4. Prevalence of asthma, hay fever, and atopic dermatitis diagnoses and atopic sensitization in farm studies

	ALEX*		PARSIFAL†		GABRIEL phase I		GABRIEL phase II‖	
	Farm children‡	Nonfarm children	Farm children§	Nonfarm children	Farm children§	Nonfarm children	Farm children§	Nonfarm children
Asthma diagnosis ever	5.4%	11.8%	6.3%	9.1%	6.5%	10.1%	8.3%	12.1%
Hay fever diagnosis ever	5.9%	15.9%	1.3%	4.4%	3.0%	9.5%	3.9%	10.6%
Atopic dermatitis diagnosis ever	—	—	7.1%	9.9%	10.6%	14.5%	12.8%	17.8%
Atopic sensitization¶	17.9%	32.9%	22.7%	34.7%	—	—	24.7%	40.8%

*Riedler et al.[E8]

†Alfven et al.[E9]

‡Farm children were defined as children with contact with farm milk or stables ever.

§Farm children were defined as children currently living on a farm run by the child's family.

‖Weighted prevalences (weighted to GABRIEL phase I).

¶ALEX: IgE ≥3.5 kU/L for house dust/storage mites, cat, grass, birch, and cow; PARSIFAL: IgE ≥0.35 kU/L in Phadiatop or mix of common food allergens (fx5); GABRIEL: IgE ≥0.70 kU/L for house dust mite, cat, and birch or IgE ≥0.35 kU/L for grass mix.

3 THE PROTECTIVE EFFECT OF FARM MILK CONSUMPTION ON CHILDHOOD ASTHMA AND ATOPY: THE GABRIELA STUDY

This paper has been published:

Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Büchele G, Weber J, Sozanska B, Danielewicz H, Horak E, van Neerven RJJ, Heederik D, Lorenzen PC, von Mutius E, Braun-Fahrländer C, the GABRIELA study group. The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study. *J Allergy Clin Immunol* 2011; 128(4):766-73.e4.

The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study

Georg Loss, MSc,[a,b] Silvia Apprich, PhD,[c] Marco Waser, PhD,[a,b] Wolfgang Kneifel, PhD,[c] Jon Genuneit, MD,[d] Gisela Büchele, PhD,[d] Juliane Weber, MD,[e] Barbara Sozanska, MD,[f] Hanna Danielewicz, MD,[f] Elisabeth Horak, MD,[g] R. J. Joost van Neerven, PhD,[h] Dick Heederik, PhD,[i] Peter C. Lorenzen, PhD,[j] Erika von Mutius, MD,[e] Charlotte Braun-Fahrländer, MD,[a,b] and the GABRIELA study group* *Basel, Switzerland, Vienna and Innsbruck, Austria, Ulm, Munich, and Kiel, Germany, Wroclaw, Poland, and Deventer and Utrecht, The Netherlands*

Background: Farm milk consumption has been identified as an exposure that might contribute to the protective effect of farm life on childhood asthma and allergies. The mechanism of action and the role of particular constituents of farm milk, however, are not yet clear.

Objective: We sought to investigate the farm milk effect and determine responsible milk constituents.

Methods: In rural regions of Germany, Austria, and Switzerland, a comprehensive questionnaire about farm milk consumption and other farm-related exposures was completed by parents of 8334 school-aged children, and 7606 of them provided serum samples to assess specific IgE levels. In 800 cow's milk samples collected at the participants' homes, viable bacterial counts, whey protein levels, and total fat content were analyzed. Asthma, atopy, and hay fever were associated to reported milk consumption and for the first time to objectively measured milk constituents by using multiple regression analyses.

Results: Reported raw milk consumption was inversely associated to asthma (adjusted odds ratio [aOR], 0.59; 95% CI, 0.46-0.74), atopy (aOR, 0.74; 95% CI, 0.61-0.90), and hay fever (aOR, 0.51; 95% CI, 0.37-0.69) independent of other farm exposures. Boiled farm milk did not show a protective effect. Total viable bacterial counts and total fat content of milk were not significantly related to asthma or atopy. Increased levels of the whey proteins BSA (aOR for highest vs lowest levels and asthma, 0.53; 95% CI, 0.30-0.97), α-lactalbumin (aOR for interquartile range and asthma, 0.71; 95% CI, 0.52-0.97), and β-lactoglobulin (aOR for interquartile range and asthma, 0.62; 95% CI, 0.39-0.97), however, were inversely associated with asthma but not with atopy.

Conclusions: The findings suggest that the protective effect of raw milk consumption on asthma might be associated with the whey protein fraction of milk. (J Allergy Clin Immunol 2011;128:766-73.)

Key words: *Allergic diseases, asthma, atopy, children, farming, hay fever, microorganism, farm milk, risk, whey protein*

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Childhood asthma and allergies remain a major health problem in industrialized countries and increasingly in developing countries.[1] Study populations with a similar genetic background but striking differences in environmental exposures have been especially informative to clarify environmental causes for the onset of asthma and atopy. Studies focusing on differences between rural farming and nonfarming communities have consistently shown that children growing up on a farm are at significantly lower risk of asthma, hay fever, and atopic sensitization than children living in the same rural area but not on a farm.[2]

Environmental factors that have been hypothesized to explain this protective effect of farm life are contact with animals,[3,4] the diversity of microbial exposure,[5] endotoxin levels in house dust,[6] and farm milk consumption.[7-9] Exposure to farm milk in early life[8] and consumption of raw farm milk[7] have been associated with a reduced asthma and atopy risk, and it has been suggested that this protection might be mediated through receptors of the innate immune system.[10]

All previous studies on the effect of farm milk consumption have been questionnaire based and lacked objective measurements of milk components. Hence determination of the biological components associated with a protective farm milk effect is warranted.

The GABRIEL (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community) Advanced studies program,[11] comprising a large population of European children, was established to investigate the environmental

From [a]the Swiss Tropical and Public Health Institute, Basel; [b]the University of Basel; [c]BOKU Vienna, Department of Food Science and Technology, University of Natural Resources and Life Sciences, Vienna; [d]the Institute of Epidemiology and Medical Biometry, Ulm University; [e]LMU Munich, University Children's Hospital, Munich; [f]the 1st Department of Paediatrics, Allergology and Cardiology, Wroclaw Medical University; [g]the Department of Pediatrics and Adolescents, Division of Cardiology and Pulmonology, Innsbruck Medical University; [h]Friesland Campina Research, Deventer; [i]the Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht University; and [j]Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Milk and Fish Products, Kiel.

*The members of the GABRIELA study group are shown in Appendix 1.

Supported by a European Union Research grant under the FP6-LifeSCIHEALTH Integrated Program LSH-2004-1.2.5-1 (contract no. 018996).

Disclosure of potential conflict of interest: J. Weber, B. Sozanska, H. Danielewicz, A Boznanski, A. Dębińska, M. Depner, A. Kosmęda, and C. Strunz-Lehner have received research support from the European Commission. D. Heederik and I. M. Wouters have received research support from the European Union. E. von Mutius is a consultant for Novartis, GlaxoSmithKline, ALK-Abelló, and Protectimmun; has received a speaker's fee from InfectoPharm; has received research support from Airsonett AB; is a member of the Expert Panel for UK Research Excellence Framework; and is an Associate Editor for the *Journal of Allergy and Clinical Immunology*. M. Ege has received research support from the European Commission and the Deutsche Forschungsgemeinschaft (DFG). M. Kabesch has financial interests in Roxall, GlaxoSmithKline, Novartis, Sanofi-Aventis, Allergopharma, and AstraZeneca GmbH and has received research support from DFG, BMBF, and the European Union. The rest of the authors have declared that they have no conflict of interest.

Received for publication February 2, 2011; revised July 14, 2011; accepted for publication July 16, 2011.

Available online August 27, 2011.

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

doi:10.1016/j.jaci.2011.07.048

Abbreviations used
ALP: Alkaline phosphatase
aOR: Adjusted odds ratio

causes of asthma and atopy and includes data on analytically determined milk constituents. The aim of the present analysis was to find biological components of cow's milk that might explain the protective effect of farm milk on childhood asthma and atopy.

METHODS

Study population and study design

The GABRIEL Advanced studies were conducted in 5 rural areas of southern Germany, Switzerland, Austria, and Poland. Because of differences in study design, the Polish data will be reported separately. In phase I a short recruitment questionnaire was distributed through elementary schools to parents of all 6- to 12-year-old school children in the selected study areas. Three strata were defined as follows: (1) farm children (ie, children living on a farm run by the family); (2) exposed nonfarm children (ie, children not living on a farm but regularly exposed to stables, barns, or cow's milk produced on a farm); and (3) nonexposed nonfarm children. For phase II analyses, a stratified random sample of 9,668 was taken from 34,491 eligible participants. Children whose parents had provided written informed consent for blood sampling, genetic analyses, and dust sampling were eligible (Table I). A comprehensive questionnaire (n = 8,334) provided information about the participants' farm-related exposures, and 7,606 also gave blood samples for IgE measurements.

For more extensive environmental sampling, the study population was restricted to 1 center (Bavaria). Three exclusive disease strata were defined within each exposure stratum: (1) asthma, (2) atopy but no asthma, and (3) no asthma and no atopy. Of the 1903 eligible Bavarian children, 895 were selected by applying disproportionate stratified random sampling to create equally sized samples within each of the 9 strata (the study design is described in more detail elsewhere[11]). Milk samples of 800 subjects were analyzed. The ethics committees of the respective universities and the data protection authorities approved the study.

Atopy

Serum IgE levels against inhalant and food allergens were measured by using a fluorescence immunoassay. Atopy was defined as positive test results for specific IgE antibodies against *Dermatophagoides pteronyssinus*, cat, or birch (cutoff, 0.7 kU/L) or against a grass mix (cutoff, 0.35 kU/L). Food allergy was defined as a positive fx5 test (fish, cow's milk, hen's egg, peanut, soybean, and wheat flour).

Clinical outcomes

Health outcomes were assessed according to International Study of Asthma and Allergies in Childhood standards.[12] Childhood asthma was defined as either wheeze in the past 12 months, asthma inhaler use ever, or a doctor's diagnosis of asthma at least once or wheezy bronchitis more than once. Current asthma was defined as childhood asthma and wheeze in the past 12 months. Hay fever required occurrence of nasal symptoms with itchy or watery eyes in the past 12 months or a doctor's diagnosis of hay fever ever. Atopic dermatitis was defined as a doctor's diagnosis ever.

Milk exposure assessed by means of questionnaire

The phase II comprehensive questionnaire provided information about the child's farm-related exposures. Cow's milk consumption was determined by asking whether the child consumed milk purchased at a shop (shop milk) or directly from a farm (farm milk) and whether farm milk was boiled or skimmed. The heating status of shop milk was not assessed. The parents had to indicate the life period of milk exposure from pregnancy to school age and the corresponding amounts of milk consumption.

Children were grouped into the following categories: (1) exclusive shop milk exposure, (2) mixed milk exposure (exposure to both shop and farm milk), and (3) exclusive farm milk exposure. The information on milk boiling was used to subdivide the farm milk exposure into "only boiled farm milk drinkers" and "any unboiled farm milk drinkers." The latter included children consuming exclusively unboiled farm milk, as well as those consuming both unboiled and boiled farm milk. The "any unboiled farm milk" group was further subdivided by frequency of consumption (daily unboiled farm milk vs less than daily unboiled farm milk) and timing of first unboiled milk exposure (first exposure to unboiled farm milk in the first year of life or during pregnancy vs after 1 year of age).

Milk sample collection and analyses

In phase III trained field workers collected cow's milk that was consumed at the participants' homes on the day of the field visit. Parents were instructed to prepare the milk as they usually did and filled out standardized milk documentation sheets. All samples were analyzed by laboratory staff blinded to the milk type and the health and exposure status.

The heating status of milk samples was defined by the residual activity of the milk indigenous enzymes alkaline phosphatase (ALP) and lactoperoxidase, according to European Commission Council Directive 92/46/EC. Low levels of ALP (<80 mU/L) correspond to milk having been heated to greater than 72°C for at least 15 seconds (minimum for pasteurized milk), and low levels of lactoperoxidase (<20,000 mU/L) correspond to milk having been heated to greater than 85°C for at least 5 seconds (minimum for high heat–treated milk). The measurements and the milk type allowed to categorize the samples as (1) high heat–treated shop milk (≥85°C), (2) pasteurized shop milk (not heated to >85°C), (3) heated farm milk (≥72°C), and (4) raw farm milk (not heated to >72°C). Because 85% of the heat-treated farm milk samples were heated to greater than 85°C, all heated farm milk samples were combined for analysis. The total fat content and whey protein levels were determined for all available phase III samples. For detailed methods, see the Methods section in this article's Online Repository at www.jacionline.org.

Microbiological analyses

The total viable bacterial count was assessed in all 800 milk samples, and 222 samples were selected for advanced microbiological analyses by using stratified random sampling (strata based on milk type, heating status, and fat content). The following microbiological groups were determined by using selective plate count methods: pseudomonades, Enterobacteriaceae, micrococci plus staphylococci, lactobacilli, yeast plus mold, bacilli plus endospores, psychrotropic bacteria, and human pathogens. For detailed methods, see the Methods section in this article's Online Repository.

Statistical analyses

All statistical analyses were performed with STATA/SE 10.1 software for Windows (StataCorp, College Station, Tex). The stratification of the study sample was taken into account by using fixed weights (weighted up to the 34,491 participants eligible for phase II) and the linearized Taylor series method for variance estimation. First, associations between milk exposure and health outcomes were determined in phase II participants by using weighted multivariate logistic regression models adjusting for age, sex, farming status (farmers vs nonfarmers), number of siblings, familial history of asthma or hay fever, study center, and breast-feeding. In sensitivity analyses all final models were adjusted for food allergens (fx5), asthma models were adjusted for atopy, and atopy and hay fever models were adjusted for asthma. An additional adjustment for contact with farm animals or contact with stables and barns was performed to avoid confounding by concomitant farm exposures.

The phase III data were used to explore associations between the objectively assessed heating status of milk or measured milk components and asthma and atopy. These regression models were adjusted for the same set of confounders as the phase II data. Milk type and heating status were categorized into 4 groups, with highly heated shop milk as the reference category. To take into account the distribution of milk constituents with high proportions of nondetectable values (total viable bacterial count, lactoferrin,

TABLE I. GABRIEL study population and design

Study module	Study area	Study population	Total no.	Farmer	Exposed nonfarmer	Nonexposed nonfarmer
Phase I	Four centers*	General population	34,491†	n = 4,533	n = 8,666	n = 21,292
↓				↓	↓	↓
Phase II	Four centers*	Subsample stratified by farm exposure	9,668‡	n = 3,477	n = 3,236	n = 2,955
		Parental questionnaires with milk exposure information available	8,334	n = 3,067	n = 2,796	n = 2,471
		IgE measurements and milk exposure information available	7,606§	n = 2,806	n = 2,544	n = 2,256
↓				↓	↓	↓
Phase III	Bavaria	Subsample stratified by exposure and outcome	895‖	n = 298	n = 300	n = 297
		Milk samples available	800¶	n = 274	n = 263	n = 263

*Germany (Bavaria and Baden-Wuerttemberg), Austria (Tyrol), and Switzerland (9 cantons).
†Eligible for phase II: Complete questionnaire plus written informed consent to further analyses were available (Bavaria: n = 11,183: 1,797/2,708/6,678).
‡Selected for phase II: Random selection of stratified (by farm exposure) eligible subjects for phase II (Bavaria: n = 2,573: 1,014/814/745).
§Blood samples with IgE measurements and parental questionnaires with milk exposure information available.
‖Selected for phase III environmental studies: Random selection of stratified (by farm exposure and health outcome) phase III eligible subjects (2,573 Bavarian children).
¶Milk samples and standardized milk documentation sheets available.

total IgG, and BSA), samples within the detection range were split at the median representing low and high levels, whereas nondetects were used as the reference group. Milk constituents that were measurable in all samples (α-lactalbumin, β-lactoglobulin, TGF-β2, and fat content) were divided into tertiles, with the lowest tertile as a reference group to test for linearity of the association with health outcomes. α-Lactalbumin and β-lactoglobulin were subsequently entered as continuous variables into the regression models. A factor analysis with continuous variables and varimax rotation (extraction of eigenvalues of ≥1.5) was used to evaluate whether the different milk constituents could be separated into different factors. Results from weighted logistic regression models were expressed as adjusted odds ratios (aORs) with corresponding 95% CIs. For full methods, see the Methods section in this article's Online Repository.

RESULTS

The distribution of milk consumption stratified by farm and nonfarm children is shown in Table II (the prevalence of health outcomes is shown in Table E1 in this article's Online Repository at www.jacionline.org). Among nonfarm children, 71.2% reported exclusive shop milk consumption, whereas 45.0% of the farm children indicated exclusive farm milk consumption. Consumption of both farm and shop milk (mixed milk exposure) was more or less comparable between farm and nonfarm children, respectively. The majority of farm milk consumers drank unboiled farm milk, and many were exposed to unboiled farm milk already during pregnancy, during the first year of life, or both. Phase II questionnaire reports of milk consumption showed high agreement with the analytically determined heating status of milk samples in phase III, which were collected at the participants' homes (see Table E2 in this article's Online Repository at www.jacionline.org).

Children exclusively drinking farm milk as reported in the phase II questionnaire had significantly lower odds ratios for asthma, current asthma, atopy, and hay fever compared with children exclusively drinking shop milk (Table III). The association with atopic dermatitis was of borderline significance. Mixed milk consumption (consumption of both shop and farm milk) was protective for hay fever and atopy. Consumption of any unboiled farm milk was consistently inversely associated with asthma, hay fever, and atopy in both exclusive and mixed farm milk drinkers. Early exposure and daily consumption of farm milk showed a stronger inverse association with health outcomes in mixed milk

TABLE II. Milk exposure of farmers and nonfarmers in phases II and III

	Total no.	Farmer (%)§	Nonfarmer (%)§
Reported milk exposure in phase II (n = 8334)			
Exclusively shop milk	3670	22.3	71.2†
Mixed milk	3010	32.7	26.4†
Only boiled farm milk	597	14.3	26.1†
Any unboiled farm milk	2413	85.7	73.9†
First unboiled farm milk <1 y	1628	68.2	42.3†
First unboiled farm milk >1 y	785	17.5	31.7†
Daily unboiled farm milk	1153	49.6	27.0†
Less than daily unboiled farm milk	1857	50.4	73.0†
Exclusively farm milk	1654	45.0	2.4†
Only boiled farm milk	174	10.7	10.6
Any unboiled farm milk	1480	89.3	89.4
First unboiled farm milk <1 y	1307	89.0	83.6*
First unboiled farm milk >1 y	173	11.0	16.4*
Daily unboiled farm milk	1051	71.6	69.3
Less than daily unboiled farm milk	429	28.4	30.7
Collected milk samples in phase III (n = 800)			
Shop milk: high heat treated§	531	42.3	78.9†
Shop milk: pasteurized‖	52	4.0	7.8†
Farm milk: heated¶	60	13.5	4.4†
Farm milk: raw#	157	40.2	8.9†

P values of the Pearson χ^2 test for farmer versus nonfarmer: *.01 ≤ *P* < .05 and †*P* < .001.
‡Percentages weighted to phase I: Differences in numbers occur because of varying proportions of missing values.
§Shop milk heated to at least 85°C.
‖Shop milk heated to at least 72°C and not more than 85°C.
¶Farm milk heated to at least 72°C (9 samples were 72°-85°C and 51 samples were >85°C).
#Farm milk not heated to greater than 72°C.

TABLE III. Adjusted association§ of reported milk exposure and asthma, atopy, hay fever, and atopic dermatitis (phase II, n = 8334)

Milk exposure reported in phase II	Asthma, aOR (95% CI)		Current asthma, aOR (95% CI)		Atopy,‖ aOR (95% CI)		Hay fever, aOR (95% CI)		Atopic dermatitis, aOR (95% CI)	
Exclusively shop milk	1.00		1.00		1.00		1.00		1.00	
Mixed milk	0.91	0.78-1.06	0.86	0.71-1.04	0.77	0.67-0.88‡	0.72	0.60-0.87‡	0.97	0.82-1.14
Only boiled farm milk	1.11	0.86-1.44	1.08	0.78-1.50	0.85	0.67-1.08	0.99	0.72-1.36	1.24	0.94-1.64
Any unboiled farm milk	0.84	0.71-1.00*	0.79	0.64-0.97*	0.74	0.64-0.86‡	0.64	0.52-0.78‡	0.88	0.74-1.05
First unboiled farm milk <1 y	0.69	0.57-0.84‡	0.66	0.52-0.84†	0.72	0.61-0.85‡	0.63	0.50-0.79‡	0.71	0.58-0.86†
First unboiled farm milk >1 y	1.08	0.85-1.37	0.98	0.73-1.30	0.78	0.63-0.97*	0.66	0.49-0.88†	1.16	0.90-1.48
Daily unboiled farm milk	0.76	0.61-0.96*	0.69	0.52-0.92*	0.68	0.57-0.82‡	0.60	0.45-0.79‡	0.81	0.65-1.02
Less than daily unboiled farm milk	0.97	0.82-1.15	0.93	0.75-1.14	0.81	0.69-0.94†	0.77	0.63-0.95*	1.03	0.86-1.24
Exclusively farm milk	0.65	0.52-0.81‡	0.64	0.48-0.84†	0.76	0.63-0.92†	0.58	0.44-0.77‡	0.78	0.61-1.00
Only boiled farm milk	1.24	0.82-1.87	1.59	0.98-2.58	0.90	0.60-1.35	1.17	0.68-1.99	1.04	0.54-2.01
Any unboiled farm milk	0.59	0.46-0.74‡	0.55	0.40-0.74‡	0.74	0.61-0.90†	0.51	0.37-0.69‡	0.75	0.59-0.96*
First unboiled farm milk <1 y	0.55	0.43-0.70‡	0.54	0.39-0.73‡	0.74	0.60-0.91†	0.51	0.37-0.71‡	0.72	0.56-0.94*
First unboiled farm milk >1 y	0.61	0.34-1.07	0.42	0.18-0.99*	0.67	0.43-1.07	0.46	0.21-1.01	0.65	0.37-1.12
Daily unboiled farm milk	0.56	0.43-0.73‡	0.51	0.36-0.72‡	0.76	0.61-0.94*	0.53	0.37-0.76‡	0.72	0.55-0.96*
Less than daily unboiled farm milk	0.61	0.43-0.86†	0.59	0.37-0.94*	0.68	0.50-0.92*	0.46	0.29-0.74†	0.77	0.53-1.11

*$P < .05$, †$P < .01$, and ‡$P < .001$.
§aORs with 95% CIs calculated by using weighted logistic regression models adjusted for age, sex, farming status, 2 or more siblings, familial history of asthma or hay fever, breast-feeding, and study center. All models weighted to phase I: n = 34,491.
‖n = 7,606.

drinkers. Because most exclusive farm milk drinkers were exposed to farm milk early in life with daily consumption, the power to detect the influence of frequency and age of first farm milk exposure was limited. Consumption of only boiled farm milk was not associated with any health outcome.

Consumption of farm milk was also inversely related to food allergen sensitization (fx5). Compared with exclusive shop milk drinking, the association between a positive fx5 test result and mixed milk consumption and exclusive farm milk drinking was an aOR of 0.85 (95% CI, 0.73-0.99) and 0.84 (95% CI, 0.69-1.03), respectively. The associations of milk consumption and asthma were robust to adjustment for atopy and food allergen sensitization.

In Table IV total fat content, total viable bacterial count, and whey protein levels are depicted and stratified by milk type and milk heating status. Highly heated shop milk showed much lower levels of all parameters compared with raw farm milk. Heated farm milk samples had a similar fat content as raw samples but significantly lower total viable bacterial counts and lower whey protein levels (not significant for α-lactalbumin). Pasteurized shop milk showed higher whey protein levels than highly heated shop or heated farm milk.

Fig 1 shows the results of the advanced microbiological analyses. Microorganisms could be detected in few shop milk and heated farm milk samples (<15% for all groups except micrococci and staphylococci [25%]). In many raw farm milk samples, micrococci and staphylococci (85.2%), lactobacilli (94.1%), bacilli and bacterial endospores (63.4%), and psychrotrophic bacteria (58.4%) could be detected. Pathogenic *Listeria innocua* and *Listeria ivanovii* strains were found in only 3 unboiled farm milk samples.

Analyses of the phase III samples (Table V) showed consumption of objectively assessed raw farm milk to be inversely associated with asthma ($P = .04$) and current asthma ($P = .03$) but not with atopy when compared with high heat–treated shop milk. A similar risk reduction, although not significant, was observed for consumption of pasteurized shop milk and asthma. Heated farm milk was not associated with asthma outcomes. Total fat content and total viable bacterial counts had no clear association with any of the analyzed health outcomes. No association was further found between these health outcomes and total protein content, somatic cell count, lactose levels, or microbiological subgroups (analyses not shown). Yet increased levels of the whey proteins tended to be inversely associated with asthma but not with atopy. Statistically significant inverse associations with asthma and current asthma were found for α-lactalbumin (asthma, $P = .03$; current asthma, $P = .03$), β-lactoglobulin (asthma, $P = .03$), and high levels of BSA (asthma, $P = .04$; current asthma, $P = .04$). Lactoferrin and total IgG levels showed a nonsignificant inverse association with asthma indicative of a dose-response relation. TGF-β2 was not significantly associated with asthma or atopy, although the highest tertile compared with the lowest tertile tended to be associated with a reduced asthma risk. In 2 exposure models including total viable bacterial counts or total fat content and individual whey proteins, the results were essentially unchanged. Applying factor analysis, the different whey proteins could not be separated from each other or from milk heating status because all were loading on the same factor.

DISCUSSION

The results of this large epidemiologic study add to the increasing body of evidence identifying consumption of farm milk (early in life) to be associated with a reduced risk of childhood asthma and allergies independently of concomitant farm exposures.[7-10] The results indicate that the effect is due to the consumption of unheated farm milk. For the first time, associations between objectively measured milk constituents and asthma and atopy could be demonstrated. Neither total viable bacterial counts nor the total fat content of the milk were related to asthma or atopy. However, some whey proteins (BSA, α-lactalbumin, and β-lactoglobulin) were associated with a significantly reduced risk of asthma but not with atopy. Prospective analyses need to confirm the results of this cross-sectional study, and further analyses are needed to determine the specific compounds underlying the

TABLE IV. Levels and percentage of detectable values of all milk constituents stratified by milk type and milk heating status

	Shop milk: high heat–treated		Shop milk: pasteurized	
Milk parameter*	**Observations (% detectable)**	**Geometric mean (95% CI)**	**Observations (% detectable)**	**Geometric mean (95% CI)**
Fat content (%)	529 (100.0)	2.01 (1.94-2.09)	52 (100.0)	2.66 (2.37-2.97)
Total viable bacteria (CFU/mL)	509 (38.3)	4.55 (3.60-5.76)	51 (94.1)	70.35 (31.89-155.24)
TGF-β2 (ng/mL)	519 (99.6)	2.97 (2.81-3.14)	47 (100.0)	8.63 (7.58-9.83)
Lactoferrin (μg/mL)	530 (14.5)	0.010 (0.008-0.012)	52 (100.0)	58.89 (45.44-76.32)
Total IgG (μg/mL)	496 (1.2)	0.016 (0.014-0.019)	52 (100.0)	29.31 (13.93-61.66)
BSA (μg/mL)	479 (13.6)	0.019 (0.015-0.024)	52 (100.0)	54.56 (42.07-70.75)
α-Lactalbumin (μg/mL)	475 (97.3)	353.92 (305.83-409.58)	52 (100.0)	1111.24 (1054.16-1171.42)
β-Lactoglobulin (μg/mL)	484 (100.0)	257.03 (242.17-272.82)	52 (100.0)	3704.11 (3524.64-3892.73)

CFU, Colony-forming unit.
*Levels are expressed as geometric means with 95% CIs. Values of less than the detection limit were set to the value of the detection limit.

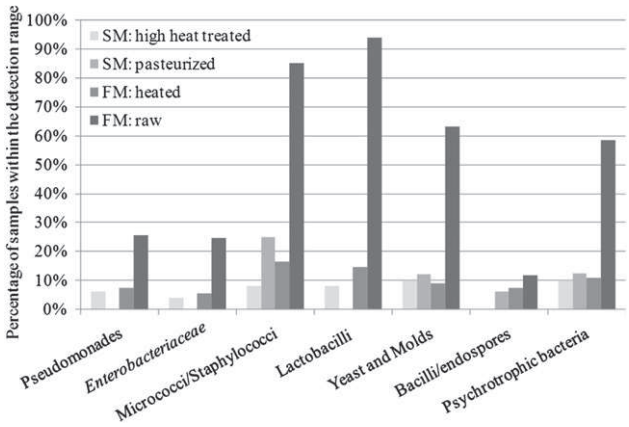

FIG 1. Proportion of samples greater than the detection limit in the advanced microbiological analyses (n = 222) shown for each microbiological group stratified by milk type and milk heating status (SM: high heat treated [n = 50], SM: pasteurized [n = 16], FM: heated [n = 55], and FM: raw [n = 101]). *FM,* Farm milk; *SM,* shop milk.

epidemiologically observed inverse association of farm milk consumption with atopy and hay fever.

The study allowed validation of parental reports of raw milk consumption against objective measurements of milk heating status and showed very good agreement. Obviously, parental reports of the raw status of the milk are reliable and not biased by social desirability, as previously speculated.[9] Under the hygiene hypothesis and given the role of microbial diversity in house dust to explain farm-related reduction of asthma risk,[5] one might assume that a higher microbial load of unboiled farm milk might be responsible for the protective farm milk effect. Milk is an excellent growth medium, allowing rapid proliferation of microbes. Indeed, the present results showed much higher counts of viable microbes in raw farm milk samples compared with heated farm milk and pasteurized and highly heated shop milk samples, as has been reported by others.[13,14] Contrary to our expectations, we did not observe an association between total viable bacterial counts in milk and investigated health outcomes. Given the cross-sectional design of the study and the restriction to viable microbe determination, the results need to be interpreted with caution. We cannot determine how representative current levels of microbes are for the long-term exposure of children, and we cannot preclude that repeated consumption of raw milk since infancy might influence the developing gut flora and interact with the immune system of the host.[15] Microbiological subgroups were measured in only 222 samples, with a high number of samples at less than the detection limit. Individual subgroups were not associated with asthma or atopy, but given the small sample size, inferences are limited. For future (prospective) analyses, new culture-independent methods to better characterize the microbial diversity of milk samples are warranted. We recently reported that the exposure to a wider range of microbes measured in house dust explained a substantial fraction of the inverse relation between asthma and growing up on a farm.[5] The association between farm milk consumption and asthma presented here was independent of and adjusted for farming and only partially attenuated the farming effect on asthma, as previously observed.[9]

Certain whey proteins were the only assessed milk components inversely associated with asthma, but the effect could not be ascribed to a single whey protein because of their high intercorrelation. Milk processing, such as heating, does not affect heat-stable caseins, whereas whey proteins, accounting for 18% of the total protein in cow's milk, are more sensitive to heat treatment[16] and might influence the bioavailability of the proteins. Bovine whey contains proteins secreted by the mammary gland, such as β-lactoglobulin, α-lactalbumin, and lactoferrin, and from serum, such as IgG, serum albumin, and TGF-β.[17] Whey proteins from bovine milk seem to play an important role in host defense against infection and excessive inflammation, yet the mechanism of action remains poorly understood.[17,18] Recent reviews have shown that lactoferrin has marked effects on immune cells in culture, being an immunostimulator and immunoregulator,[18] and that TGF-β, a multifunctional cytokine, inhibits the immunopathology to self without compromising immune responses to pathogens.[19] Higher levels of TGF-β were found in unpasteurized farm milk[20] and in human breast milk of mothers exposed to a farming environment.[21] Furthermore, TGF-β in human breast milk has been associated with reduced allergy-related outcomes in infancy and early childhood.[22] In the present study TGF-β2 was not significantly associated with asthma. Whey also contains the major milk allergens β-lactoglobulin and α-lactalbumin, and it remains perplexing that early consumption of raw cow's milk decreases the risk of asthma. Immunomodulatory effects have been ascribed to α-lactalbumin[23] and conjugates of β-lactoglobulin.[24] In addition, one might speculate that milk processing, such as homogenization, might alter the context in which potentially allergenic structures are presented to the immune system.

TABLE IV. *(Continued)*

Farm milk: heated		Farm milk: raw	
Observations (% detectable)	Geometric mean (95% CI)	Observations (% detectable)	Geometric mean (95% CI)
59 (100.0)	3.39 (3.11-3.70)	154 (100.0)	3.87 (3.66-4.11)
58 (89.7)	114.47 (58.54-223.84)	153 (98.0)	9533.94 (6206.20-14645.99)
48 (83.3)	1.52 (1.06-2.19)	125 (100.0)	5.71 (5.23-6.25)
60 (31.7)	0.028 (0.011-0.068)	157 (98.1)	80.26 (60.33-106.79)
56 (21.7)	0.095 (0.034-0.265)	154 (100.0)	224.96 (208.90-242.25)
55 (40.0)	0.15 (0.05-0.43)	154 (100.0)	84.94 (77.69-92.86)
23 (87.0)	307.59 (85.45-1107.24)	154 (100.0)	1113.08 (1075.33-1152.15)
23 (100.0)	663.96 (340.02-1296.52)	154 (100.0)	4025.40 (3892.53-4162.81)

TABLE V. Adjusted association† of asthma or atopy and milk heating status, total fat content, total viable bacterial count, or whey protein levels (phase III)

Milk parameter	No.	Asthma, aOR (95% CI)		Current asthma, aOR (95% CI)		Atopy, aOR (95% CI)	
Milk type and heating status							
Shop milk: high heat–treated	531	1.00		1.00		1.00	
Shop milk: pasteurized	52	0.50	(0.22-1.12)	0.49	(0.19-1.28)	1.28	(0.59-2.75)
Farm milk: heated	60	0.97	(0.49-1.91)	0.90	(0.38-2.16)	0.74	(0.38-1.44)
Farm milk: raw	157	0.58	(0.34-0.99)*	0.45	(0.22-0.93)*	0.90	(0.56-1.45)
Fat content (%)‡							
Lowest tertile	267	1.00		1.00		1.00	
Medium tertile	269	1.13	(0.73-1.75)	1.37	(0.83-2.26)	0.88	(0.57-1.36)
Highest tertile	258	0.98	(0.60-1.59)	0.92	(0.51-1.65)	1.39	(0.88-2.19)
Total viable bacteria (CFU/mL)							
Less than detection limit	326	1.00		1.00		1.00	
Low levels	223	0.94	(0.60-1.48)	0.88	(0.52-1.50)	0.85	(0.55-1.31)
High levels	222	1.02	(0.62-1.69)	0.85	(0.46-1.60)	0.94	(0.58-1.53)
TGF-β2 (ng/mL)‡							
Lowest tertile	247	1.00		1.00		1.00	
Medium tertile	246	1.36	(0.86-2.15)	1.23	(0.72-2.11)	1.07	(0.68-1.67)
Highest tertile	246	0.75	(0.46-1.22)	0.75	(0.42-1.32)	0.98	(0.62-1.55)
Lactoferrin (μg/mL)							
Less than detection limit	497	1.00		1.00		1.00	
Low levels	151	0.83	(0.50-1.37)	0.83	(0.46-1.52)	1.26	(0.78-2.03)
High levels	151	0.72	(0.41-1.26)	0.64	(0.31-1.32)	1.01	(0.62-1.65)
Total IgG (μg/mL)							
Less than detection limit	449	1.00		1.00		1.00	
Low levels	155	0.85	(0.52-1.40)	0.77	(0.42-1.40)	1.08	(0.68-1.73)
High levels	154	0.61	(0.34-1.07)	0.71	(0.35-1.45)	1.32	(0.81-2.17)
BSA (μg/mL)							
Less than detection limit	447	1.00		1.00		1.00	
Low levels	147	0.76	(0.46-1.26)	0.77	(0.42-1.41)	0.95	(0.58-1.55)
High levels	146	0.53	(0.30-0.97)*	0.45	(0.21-0.98)*	0.90	(0.54-1.51)
α-Lactalbumin (μg/mL)§	704	0.71	(0.52-0.97)*	0.67	(0.47-0.97)*	1.07	(0.78-1.48)
β-Lactoglobulin (μg/mL)§	713	0.62	(0.39-0.97)*	0.62	(0.39-1.06)	1.12	(0.74-1.68)

CFU, Colony-forming unit.
*$P < .05$.
†Weighted logistic regression models adjusted for age, sex, farming status, 2 or more siblings, and familial history of asthma or hay fever.
‡Divided into tertiles because requirements of linearity were not met.
§aORs for interquartile range.

Presentation of the allergenic epitopes might also be influenced by complexing the allergen with immunoglobulins, as recently proposed in an animal model.[25]

Phase III analyses allowed us to differentiate shop milk samples according to heat treatment and found pasteurized shop milk consumption to be associated with less asthma and to have higher whey protein levels than high heat–treated shop milk. Yet the association between pasteurized milk consumption and asthma was not statistically significant and needs to be confirmed in larger studies.

In this study BSA, α-lactalbumin, and β-lactoglobulin levels were found to be inversely associated with asthma but not with

atopy. It is thus conceivable that milk components not measured in the present study underlie the epidemiologically observed inverse association between farm milk consumption and atopy. The fatty acid composition of farm milk might be one such factor, which has been hypothesized before.[26,27] In the present analysis the total fat content of the milk samples was not associated with asthma or atopy, which is in contrast to other epidemiologic studies reporting a reduced risk of asthma associated with consumption of milk fat–containing products, such as full cream milk and butter,[28] or modulation of cytokine production in cord blood associated with farm-produced butter consumed by the pregnant mother.[29]

The main strength of the present study is the objective determination of several milk compounds and the enzymatic classification of the heat treatment of a comparatively large number of milk samples consumed by study participants, thus expanding questionnaire-based analysis. The cross-sectional design of the study, the lack of fatty acid measurements, and the limitations of the microbial analyses represent the main limitations of the present study.

The long-term solution to the asthma epidemic is thought to be prevention and not treatment of established disease,[30] and nutritional interventions might represent an interesting avenue. However, on the basis of current knowledge, raw milk consumption cannot be recommended because it might contain pathogens. Once the mechanisms underlying the protective farm milk effect are better understood, ways of processing and preserving a safe and preventive milk can be developed.

We thank Dr Wulf Thierfelder and Michael Thamm from the Robert-Koch-Institute, Berlin, Germany, for their cooperation and the measurement of total and specific IgE levels that was used in the definition of atopic subjects. Furthermore we thank the participating children, their families, and the fieldworkers of the GABRIEL Advanced studies.

Key messages

- **Questionnaire-reported consumption of unboiled but not boiled farm milk was inversely associated with asthma, hay fever, and atopy.**
- **Higher levels of the whey proteins BSA, α-lactalbumin, and β-lactoglobulin in milk samples were associated with a reduced risk of asthma but not atopy.**
- **Neither total viable bacterial counts nor the total fat content of the milk were related to asthma or atopy.**

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APPENDIX 1

The members of the GABRIELA study group are listed in alphabetical order: Silvia Apprich, PhD,[g] Andrzej Boznanski MD, PhD,[k] Charlotte Braun-Fahrländer, MD,[d,e] Gisela Büchele, PhD,[c] William Cookson, MD, DPhil,[a] Paul Cullinan, MD,[a] Hanna Danielewicz, MD,[k] Anna Dębińska,[k] Martin Depner, PhD,[b] Markus Ege, MD,[b] Urs Frey, MD, PhD,[l] Oliver Fuchs, MD,[l] Jon Genuneit MD,[c] Dick Heederik, PhD,[f] Elisabeth Horak, MD,[m] Anne Hyvärinen, PhD,[h] Sabina Illi, PhD,[b] Michael Kabesch, MD,[n] Katalin Kovacs,[m] Aleksandra Kosmęda, PhD,[k] Wolfgang Kneifel, PhD,[g] Philipp Latzin, MD, PhD,[l] Roger Lauener, MD,[p] Georg Loss, MSc,[d,e] Stephanie MacNeill, MSc,[a] Bernhard Morass, MD,[m] Anne-Cécile Normand, PhD,[q] Renaud Piarroux, MD, PhD,[q] Helena Rintala, PhD,[h] Mascha K. Rochat, MD,[b] Nikolaos Sitaridis,[c] Barbara Sozanska, MD,[k] David Strachan, MD,[o] Christine Strunz-Lehner, MPH,[b] Bertrand Sudre, MD, PhD,[i] Erika von Mutius, MD, MSc,[b] Marco Waser, PhD,[d,e] Juliane Weber, MD,[b] and Inge M. Wouters, PhD.[f]

From [a]Imperial College London, National Heart and Lung Institute, South Kensington Campus, London, United Kingdom; [b]LMU Munich, University Children's Hospital, Munich, Germany; [c]Ulm University, Institute of Epidemiology and Medical Biometry, Ulm, Germany; [d]Swiss Tropical and Public Health Institute, Basel, Switzerland; [e]the University of Basel, Basel, Switzerland; [f]Utrecht University, Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht, The Netherlands; [g]BOKU Vienna, University of Natural Resources and Life Sciences, Department of Food Science and Technology, Vienna, Austria; [h]THL Kuopio, National Institute for Health and Welfare, Kuopio, Finland; [i]Université de Franche-Comté, UMR 6249 Chrono-Environnement, Département de Parasitologie/Mycologie, Besançon, France; [k]Wroclaw Medical University, 1st Department of Paediatrics, Allergology and Cardiology, Wroclaw, Poland; [l]the Division of Pulmonology, Department of Paediatrics, Bern University Hospital, Bern, Switzerland; [m]the Department of Pediatrics and Adolescents, Division of Cardiology and Pulmonology, Innsbruck, Austria; [n]Hannover Medical School, Clinic for Paediatric Pneumology and Neonatology, Hannover, Germany; [o]St George's, University of London, Cranmer Terrace, London, United Kingdom; [p]High Mountain Hospital Davos, Herman-Davos-Wolfgang, Switzerland; and [q]the Department of Parasitology and Mycology, Hôpital de la Timone, Assistance Publique-Hôpitaux de Marseille, Marseille, France.

METHODS

Atopy

Serum IgE levels against inhalant (birch, cat, *D pteronyssinus*, and grass mix) and food (fx5 test: fish, cow's milk, hen's egg, peanut, soybean, and wheat flour) allergens were measured at a central laboratory (Robert-Koch Institute, Berlin, Germany) by means of fluorescence immunoassay (UNICAP 1000; Phadia AB, Uppsala, Sweden). Atopy was defined as positive test results for specific IgE antibodies against *D pteronyssinus*, cat, or birch (cutoff, 0.7 kU/L) or against a grass mix (cutoff, 0.35 kU/L). Food allergy was defined as a positive fx5 test result.

Milk sample collection and analyses

In phase III trained field workers collected 9 aliquots of milk (total of 300 mL) that were consumed at the participants' homes on the day of the field visit. Parents were instructed to prepare the milk as they usually do. Samples were transported on ice and frozen at −18°C immediately after arriving at the laboratory. During the field visit, standardized documentation sheets were filled in, including information about milk type (shop or farm purchased), storage conditions, and preparation of the milk before consumption. All milk analyses refer only to cow's milk. All samples were analyzed by laboratory staff blinded to milk type and health and exposure status.

Heating status of all milk samples was defined by residual activity of the milk-indigenous enzymes ALP and lactoperoxidase according to European Commission Council Directive 92/46/EC. ALP (fluorimetric method according to EN ISO 11816-1 [2000]; lower detection limit, 10 mU/L) and lactoperoxidase (Reflectoquant; MERCK KGaA, Darmstadt, Germany; lower detection limit, 5000 mU/L) levels were measured at the Max Rubner Institute, Kiel, Germany.

Low levels of ALP (<80 mU/L) correspond to milk having been heated to greater than 72°C for at least 15 seconds (minimum for pasteurized drinking milk [shop milk]), and low levels of lactoperoxidase (<20,000 mU/L) correspond to milk having been heated to greater than 85°C for at least 5 seconds (minimum for highly pasteurized drinking milk [shop milk]). The measurements allowed us to categorize the samples as (1) high heat–treated shop milk (≥85°C), (2) pasteurized shop milk (not heated to >85°C), (3) heated farm milk (≥72°C), and (4) raw farm milk (not heated to >72°C). The majority (85%) of heated farm milk samples were heated to greater than 85°C. The total fat content and all whey protein levels were determined for all available phase III samples.

The total fat content, total protein content, and lactose levels (infrared method), as well as the somatic cell count (flow cytometry with Fossomatic; FOSS, Hillerød, Denmark), were determined for all 800 milk samples at the Qualitaetslabor Lower Austria, Gmuend, Austria.

TGF-β2 levels (ELISA) were measured by Friesland CAMPINA Research, Deventer, The Netherlands, and all other whey proteins were measured at the University of Natural Resources and Life Sciences, Vienna, Austria. The following whey proteins were measured in all available phase III milk samples: lactoferrin (Bovine lactoferrin ELISA quantitation kit E10-126, Bethyl Laboratories, Montgomery, Tex; detection limit, 4 ng/mL), TGF-β2, total IgG (Bovine IgG ELISA quantitation kit E10-118, Bethyl; lower detection limit, 7.8 ng/mL), BSA (Bovine albumin ELISA quantitation kit E10-113, Bethyl; lower detection limit, 6.25 ng/mL), α-lactalbumin (Bovine α-La ELISA quantitation kit E10-128, Bethyl; lower detection limit, 0.78 ng/mL), and β-lactoglobulin (bovine β-Lg E10-125, Bethyl; lower detection limit, 1.95 ng/mL).

Microbiological analyses

The total viable bacterial count was assessed in all 800 milk samples, and 222 samples were selected for advanced microbiological analyses by using stratified random sampling (strata based on milk type, heating status, and fat content). Their total viable bacterial count was determined by using the standard plate count method according to Koch[E1] with a standard method agar (PCA; MERCK KGaA, Darmstadt, Germany; detection limit, 10 colony-forming units/mL). Colony-forming units of the following microbiological groups were determined by using selective plate count methods (detection limit, 10 colony-forming units/mL): pseudomonades, Enterobacteriaceae, micrococci plus staphylococci, lactobacilli, yeast plus molds, bacilli plus endospores, psychrotropic bacteria, and human pathogens. The total viable bacterial count of the remaining milk samples was assessed by using the automated most-probable-number method (TEMPO; bioMérieux, Marcy l'Etoile, France; detection limit, 1 colony-forming unit/mL) with corresponding total viable count broth. All microbiological measurements were performed at the University of Natural Resources and Life Sciences, Vienna, Austria.

For validation of TEMPO results, the viable count of every tenth milk sample was also assessed by using the standard plate count method according to Koch with standard methods agar (Plate Count Agar; Merck KGaA, Darmstadt, Germany). For 37 samples, both measurements were available and showed high agreement (Spearman rho = 0.81).

Samples selected for advanced microbiological analyses were thawed at room temperature, diluted, and analyzed with plate count methods according to Koch. The total bacterial count was determined with a standard agar method (Plate count agar, MERCK KGaA). The following microbiological groups were assessed by using the respective media and recommended incubation duration and temperature: pseudomonades (LAB 108: *Pseudomonas* Agar plus X107 C.N. selective supplement; LAB M Ltd, Bury, United Kingdom), Enterobacteriaceae (110275 Violet Red Bile Dextrose Agar according to Mossel, MERCK KGaA), micrococci and staphylococci (LAB 285: Baird Parker Media plus X085 egg yolk tellurit-supplement, LAB M Ltd), lactobacilli (110660 MRS Agar according to de Man, Rogosa and Sharpe, MERCK KGaA), yeast and molds (LAB 200: Yeast & Mould Agar, LAB M Ltd), bacilli and endospores (107324 Tryptic Soy Agar plus Polysorbate 80 und Lecithin, MERCK KGaA), and psychrotropic bacteria (1.10878 Plate count agar sugar free FIL-IDF, MERCK KGaA). The detection limit for all analyses was 10 colony-forming units/mL. Furthermore, human pathogenic bacteria (*Salmonella* species and *Listeria* species) were determined and identified.

Statistical analyses

All statistical analyses were performed with STATA/SE 10.1 software for Windows. The stratification of the study sample was taken into account by using fixed weights (weighted up to the participants eligible for phase II [34491]) and the linearized Taylor series method for variance estimation. First, the association of milk exposure and health outcomes was determined based on the phase II dataset by using weighted multivariate logistic regression models. Point estimate changes of at least 10% in bivariate models were a criterion for a covariate to be added to the final regression models. All models were adjusted for age, sex, farming status (exposed nonfarmers and nonexposed nonfarmers were combined for analyses), number of siblings, familial history of asthma or hay fever, study center, and breast-feeding. Other factors that were tested but not included in the final models were body mass index, milk avoidance caused by allergies, parental smoking, parental education, and milk storage time and location. In sensitivity analyses all final models were adjusted for food allergens (fx5), asthma models were adjusted for atopy, and atopy and hay fever models were adjusted for asthma. An additional adjustment for contact with farm animals or contact with stables and barns was performed to avoid confounding by concomitant farm exposures.

The phase III data were used to explore associations between the objectively assessed heating status of milk or measured milk components and asthma and atopy. These regression models were adjusted for the same set of confounders as the phase II data. Milk type and heating status were categorized into 4 groups, with highly heated shop milk as the reference category. To take into account the distribution of milk constituents with high proportions of nondetects (total viable bacterial count, lactoferrin, total IgG, and BSA), samples within the detection range were split at the median representing low and high levels, whereas nondetects were used as the reference group. Milk constituents that were measurable in all samples (α-lactalbumin, β-lactoglobulin, TGF-β2, and fat content) were divided into tertiles, with the lowest tertile as a reference group to test for linearity of the association with health outcomes. α-Lactalbumin and β-lactoglobulin were subsequently entered as continuous variables in the regression

models. A factor analysis with continuous variables and varimax rotation (extraction of eigenvalues of ≥1.5) was used to evaluate whether the different milk constituents could be separated into different factors. Results from weighted logistic regression models were expressed as aORs with corresponding 95% CIs.

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TABLE E1. Weighted prevalence of childhood asthma, atopy, hay fever, and atopic dermatitis by farming status (phases II and III)

	Phase II		Phase III	
Prevalence (%)§	**Farmer**	**Nonfarmer**	**Farmer**	**Nonfarmer**
Asthma	14.0	21.1‡	12.9	18.3†
Current asthma	9.2	15.2‡	8.7	12.8*
Atopic	4.5	8.1‡	5.2	8.6*
Nonatopic	3.5	5.7‡	3.8	5.0
Atopy	24.7	40.8‡	24.1	40.3‡
Hay fever	6.2	16.3‡	7.4	13.5†
Atopic dermatitis	12.9	17.8‡	11.2	18.0*

Differences in numbers occur because of varying proportions of missing values.
*P value of the Pearson χ^2 test for farmer versus nonfarmer < .05.
†P value of the Pearson χ^2 test for farmer versus nonfarmer < .01.
‡P value of the Pearson χ^2 test for farmer versus nonfarmer < .001.
§Weighted number in phase II = 34,491; weighted number in phase III = 11,183.

TABLE E2. Agreement of the reported milk consumption in phase II and the milk samples collected in phase III at the participants' homes (agreement tested for n = 796)

	Reported milk consumption in phase II		
Milk samples collected in phase III	**Exclusively shop milk (n = 419)***	**Shop and farm milk (n = 257)**	**Exclusively farm milk (n = 120)***
Shop milk	98.3%	64.2%	2.0%
Farm milk	1.7%	35.8%	98.0%
	Exclusively farm milk (n = 120)		
	Any unboiled farm milk (n = 102)	Only boiled farm milk (n = 18)	
Shop milk	2.0%	0.0%	
Farm milk >72°C	12.7%	66.7%	
Farm milk <72°C	85.3%	33.3%	

*κ Value of exclusive shop/farm milk consumption in phase II and collected shop/farm milk in phase III = 0.95 (95% CI, 0.92-0.98).

4 APPROPRIATE AND ALTERNATIVE METHODS TO DETERMINE VIABLE BACTERIAL COUNTS IN COW MILK SAMPLES

This paper has been published:

Loss G, Apprich S, Kneifel W, Von Mutius E, Genuneit J, Braun-Fahrländer C, on behalf of the GABRIELA study group. Appropriate and alternative methods to determine viable bacterial counts in cow milk samples. *Journal of Dairy Science* 2012; 95(6):2916-8.

J. Dairy Sci. 95:2916–2918
http://dx.doi.org/10.3168/jds.2011-4897

Short communication: Appropriate and alternative methods to determine viable bacterial counts in cow milk samples

G. Loss,*†[1] S. Apprich,‡ W. Kneifel,‡ E. von Mutius,§ J. Genuneit,# and C. Braun-Fahrländer,*† on behalf of the GABRIEL study group
*Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, 4002 Basel, Switzerland
†University of Basel, Petersplatz 1, 4003 Basel, Switzerland
‡BOKU Vienna, University of Natural Resources and Life Sciences, Department of Food Science and Technology, Muthgasse 18, A-1190 Vienna, Austria
§LMU Munich, University Children's Hospital, Lindwurmstrasse 4, D-80337, Munich, Germany
#Ulm University, Institute of Epidemiology and Medical Biometry, Helmholtzstraße 22, D-89081 Ulm, Germany

ABSTRACT

Farm milk consumption is reported to be inversely related to the development of asthma and atopy in children and it has been hypothesized that microorganisms in milk might contribute to this protective effect. The GABRIEL study was designed to investigate this hypothesis in a large population of European children, calling for a rapid alternative to classical culture techniques to determine bacteriological properties of milk samples. One objective was to evaluate 2 different rapid methods to determine bacteriological properties in a large number of cow milk samples collected under field conditions. BactoScan (Foss Analytical, Hillerød, Denmark), an automated standard flow cytometric method utilized for routine testing of milk quality, and TEMPO (bioMérieux, Marcy l'Etoile, France), an automated most-probable-number method, were used to assess the total viable bacterial count in farm and commercial milk samples. Both methods were compared with standard plate count method and each other. Measurements based on the TEMPO method were in good agreement with the standard plate count method and showed reliable results, whereas BactoScan results did not correlate with standard plate count measurements and yielded higher bacteria counts in heat-treated milk samples compared with raw milk samples. Most likely, these discrepant results were due to inferences with staining reactions and detection of bacteria in heat-treated milk samples. We conclude that, in contrast to the routinely used BactoScan method, the TEMPO method is an inexpensive and rapid alternative to standard culture methods suitable to assess total bacterial counts in processed and raw milk samples.

Key words: microorganism, total viable count, milk, childhood asthma

Short Communication

Previous epidemiological studies have shown that consumption of farm milk is associated with less childhood asthma and atopy (Riedler et al., 2001; Waser et al., 2007; Loss et al., 2011). The hygiene-hypothesis states that infections or microbial exposure in early childhood decrease the risk for allergy development (Strachan, 1989). The microbial load of unpasteurized milk thus offers a possible explanation for the protective effect of farm milk (Perkin and Strachan, 2006). To establish valid associations of environmental exposures and health outcomes, large numbers of samples are required. The standardized classical culture method is regarded as the gold standard to determine microbiological properties of foods. However, a serious drawback is that it is laborious and time consuming to perform. During recent decades, several rapid methods have been developed, reducing the time, and thus cost, to obtain a microbiological test result. However, it remains a challenge to choose the ideal method for the user's practical context. Although molecular, immunological, and microscopic methods are automatable, rapid, less labor-intensive, and show better reproducibility compared with conventional methods, they require high investment for equipment and materials (Jasson et al., 2010).

The GABRIEL study (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community) used 2 different rapid methods to analyze the total viable bacterial count (**TVC**) of milk samples that were collected at participants' homes during a field visit. BactoScan (Foss Analytical, Hillerød, Denmark) is a rapid flow cytometry system that is utilized in routine testing of bacteriological quality of milk where bacteria are

Received September 2, 2011.
Accepted January 29, 2012.
[1]Corresponding author: georg.loss@unibas.ch

stained with ethidium bromide and their optical characteristics are quantitatively measured by a focused light beam (Suhren and Walte, 1998; Jasson et al., 2010). The ready-to-use TEMPO system (bioMérieux, Marcy l'Etoile, France) is a modified culture method that makes use of vials with culture medium specific to the test and cards simulating the most-probable-number method. It is based on an automated system that reduces workload and number of manipulations without the necessity of a full laboratory infrastructure (Torlak et al., 2008; Jasson et al., 2010). The aim of this communication is to compare the flow cytometry and the automated most-probable-number method to standard plate count measurements and to each other, and to suggest the most appropriate rapid method to measure the TVC in this epidemiological setting.

The cross-sectional GABRIEL study was conducted in rural areas in Germany, Switzerland, Austria, and Poland. Environmental samples were taken from a stratified random subsample of 895 Bavarian participants, and milk samples were collected in 2 periods of the year [October to December (n = 543) and April to August (n = 744); Genuneit et al., 2011]. During a field visit at the participants' homes, trained field workers collected 9 aliquots of cow's milk (6 × 45 mL and 3 × 20 mL per visit) as it was consumed by the child. Children consumed either boiled or unboiled milk directly from a farm or pasteurized or UHT commercial milk. Samples were transported on ice and frozen at −18°C immediately after arriving at the laboratory. The TVC was assessed in 1,287 milk samples by flow cytometry (BactoScan) and by the TEMPO method (detection limit = 1 cfu/mL) using TEMPO TVC (total viable count). For validation of the rapid methods, the viable count of every tenth milk sample was also assessed by SPC method (IDF Standard 100B; International Dairy Federation, 1991). All microbiological measurements were performed at the University of Natural Resources and Life Sciences (Vienna, Austria).

The heating status of all milk samples was defined by residual activity of the milk intrinsic enzymes alkaline phosphatase [fluorimetric method according to EN ISO 11816–1 (ISO, 2000); lower detection limit = 10 mU/L) and lactoperoxidase (Reflectoquant, Merck KGaA, Darmstadt, Germany; lower detection limit = 5,000 mU/L) at the Max Rubner Institut (Kiel, Germany) according to the EC council directive 92/46/EC. The measurements allowed the samples to be categorized as (1) commercial milk heated to at least 85°C (n = 986), (2) commercial milk heated to below 85°C (pasteurized commercial milk, n = 72), (3) farm milk heated to at least 72°C (n = 49), and (4) raw farm milk or farm milk heated to below 72°C (n = 180). For all analyses, staff was blinded to all milk properties.

Statistical analyses were performed with Stata/SE 10.1 for Windows (Stata Corp., College Station, TX), and TVC were expressed as geometric means with 95% confidence intervals.

Measurements using the TEMPO method were in good agreement with bacterial counts based on standard culture technique (Spearman rho = 0.81), whereas BactoScan measurement did not correlate at all (Spearman rho = −0.29). Figure 1A illustrates the geometric mean levels of TVC measured with BactoScan and TEMPO in all 1,287 milk samples according to milk type and milk heating status. Figure 1B shows bacterial levels measured with BactoScan, TEMPO, and SPC method in a subsample of 95 samples that were measured by all 3 methods. Total viable bacteria counts were high in raw farm milks when measured by both the BactoScan and TEMPO methods and became decreasingly lower in heated farm milk, pasteurized commercial milk, and high-heated commercial milk samples when measured by SPC or the TEMPO method. Conversely, BactoScan measurements showed significantly higher levels of total viable bacteria in heated farm and commercial milk samples compared with raw milk levels.

The 2 rapid methods to measure TVC in milk samples collected at study participants' homes showed contrasting results when compared with each other and to the standard culture technique. Only the TEMPO method yielded valid and appropriate results when both raw and heat-treated milk samples were analyzed. BactoScan was developed as an automated instrument to assess the bacteriological quality of raw milk (Suhren and Walte, 1998) and is widely used for routine quality control of milk (Jasson et al., 2010). In contrast to routine raw milk quality control, the present study aimed at analyzing milk samples as they were usually consumed by the participating children and their families. Thus, processed milk samples such as boiled farm milk as well as pasteurized and UHT milk purchased on the retail market were tested. The discrepant results obtained by the BactoScan flow cytometry method are most likely explained by the presence of proteins and lipid globules in heated milk that have been reported to bind nonspecifically to fluorescent stains and interfere with staining and detection of bacteria unless milk-clearing treatments are applied before measurement (Gunasekera et al., 2000). Future epidemiological studies aiming at detecting microorganisms in raw and heat-treated milk need to account for this methodological aspect.

We conclude that the automated most-probable-number TEMPO method is an inexpensive and rapid alternative to standard culture that is suitable for assessing total bacterial counts in processed and raw milk samples.

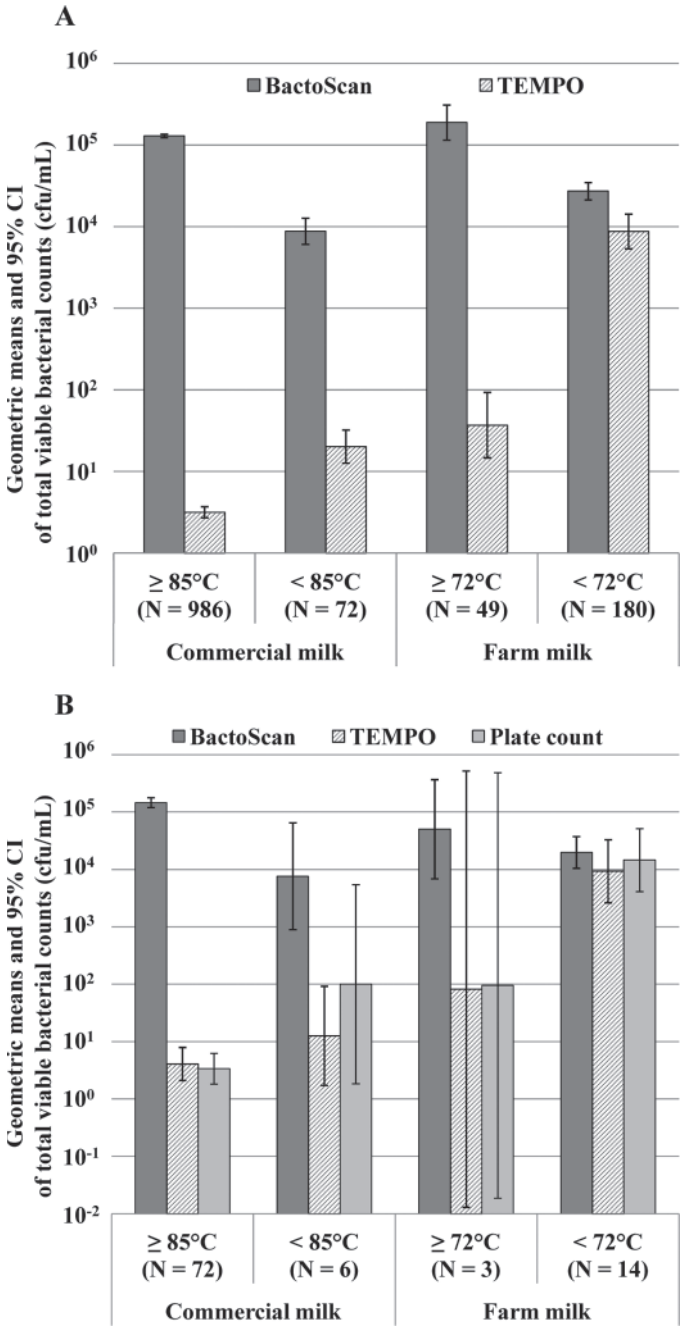

Figure 1. Geometric means and 95% confidence intervals of total viable bacterial count measured with (A) BactoScan (Foss Analytical, Hillerød, Denmark) and TEMPO method (bioMérieux, Marcy l'Etoile, France) in all 1,287 commercial and farm milk samples and (B) BactoScan, TEMPO, and plate count method in 95 commercial and farm milk samples stratified by milk's heating status.

ACKNOWLEDGMENTS

This work was supported by a European Union Research grant under the FP6-LifeSCIHEALTH Integrated Program LSH-2004-1.2.5-1 (contract number 018996). We thank Peter C. Lorenzen from the Max Rubner Institut (Kiel, Germany) for the measurement of milk intrinsic enzymes to define milk's heating status. We also thank all members of the GABRIEL study team.

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5 THE DEVELOPMENT OF ATOPIC DERMATITIS ACCORDING TO AGE OF ONSET AND THE ASSOCIATION WITH EARLY-LIFE EXPOSURES

This paper has been published:

Roduit C, Frei R, **Loss G**, Büchele G, Weber J, Depner M, Loeliger S, Dalphin ML, Roponen M, Hyvarinen A, Riedler J, Dalphin JC, Pekkanen J, von Mutius E, Braun-Fahrländer C, Lauener R, the PASTURE study group. Development of atopic dermatitis according to age of onset and association with early-life exposures. *J Allergy Clin Immunol* 2012; 130(1):130-136.e5.

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.

Supported by European Union research grants PASTURE/EFRAIM (QRLT4-CT 2001-00250, KBBE-2-2-06) and the Kühne Foundation.

Disclosure of potential conflict of interest: M. Depner receives research support from the European Commission/European Research Council. M. Roponen receives research support from the European Union FP7 and the Academy of Finland. A. Hyvärinen receives research support from the European Union FP7. J. Pekkanen receives research support from the European Union FP7, the Academy of Finland, EVO, and Farmers' Social Insurance Institution. E. von Mutius receives consultation fees from GlaxoSmithKline, Protectimmun, and Novartis; receives lecture fees from InfectoPharm and Nestlé Research; receives research support from the European Commission; has provided legal consultation/expert witness testimony for the UK Research Excellence Framework; and is Associate Editor of the *Journal of Allergy and Clinical Immunology*. R. Lauener receives research support from the Kühne Foundation and the European Union. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication November 8, 2011; revised February 28, 2012; accepted for publication February 29, 2012.

Available online April 21, 2012.

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

0091-6749/$36.00

doi:10.1016/j.jaci.2012.02.043

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.

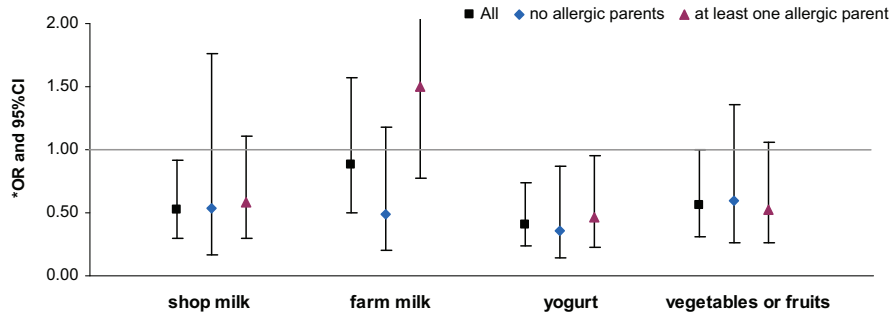

FIG 1. Association between food item introduction in the first year of life and atopic dermatitis with onset after the first year of life stratified by parental allergies. *Adjusted for farmer, center, breast-feeding, and parental history of allergies (ever eczema, hay fever, or asthma). For dairy products: 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.

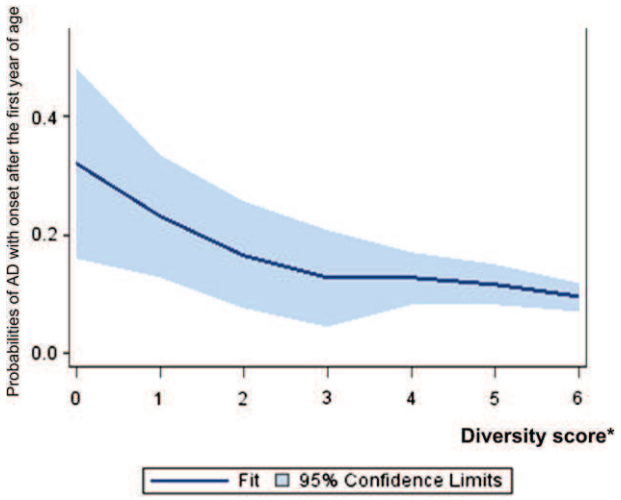

FIG 2. Association between increasing numbers of different major food items (n = 6) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Diversity score with major food items: vegetables or fruits, any cereals, meat, bread, cake, and yogurt.

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.

We thank all the fieldworkers and other PASTURE team members. We also thank Leticia Grize and Christian Schindler from the University of Basel, Switzerland, for help in statistics.

The PASTURE study group: Anne Karvonen, Maija-Riitta Hirvonen, Pekka Tittanen, Sami Remes (Finland); Vincent Kaulek (France); Jon Genuneit, Michael Kabesch, Markus Ege, Bianca Schaub, Petra Pfefferle, Harald Renz (Germany); Gert Doekes (The Netherlands); and Sondhja Bitter (Switzerland).

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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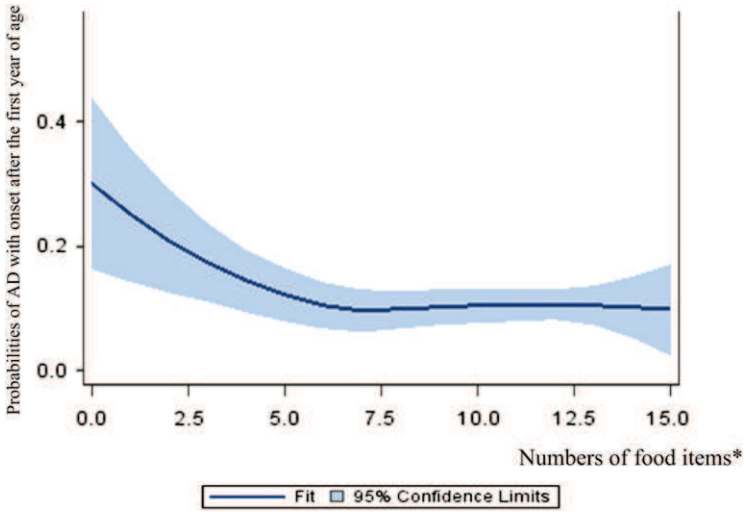

FIG E1. Association between increasing numbers of all different food items (n = 15) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Food items (n = 15): any cow's milk, yogurt, other milk products, eggs, nuts, vegetables or fruits, cereals,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

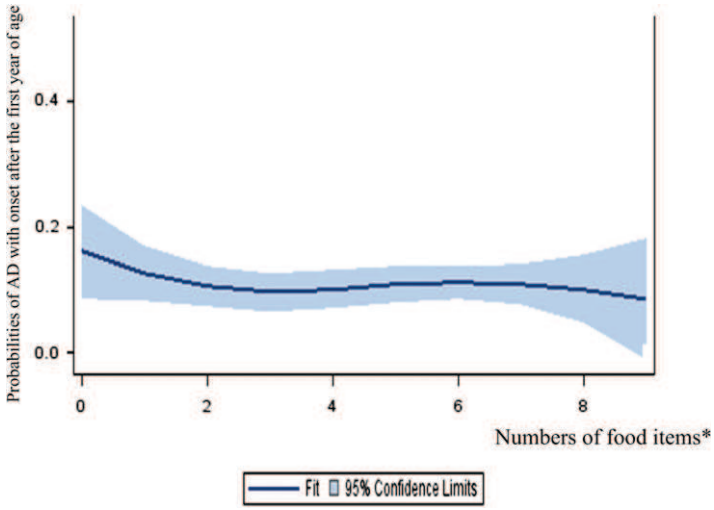

FIG E2. Association between increasing numbers of all different food items excluding major foods (n = 9) introduced in the first year of life and atopic dermatitis *(AD)* with onset after the first year of life. *Food items (n = 9): any cow's milk, other milk products, eggs, nuts, fish, soy, margarine, 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 ($P = .08$).

6 PRENATAL AND EARLY-LIFE EXPOSURES ALTER EXPRESSION OF INNATE IMMUNITY GENES: THE PASTURE COHORT STUDY

This paper has been published:

Loss G*, Bitter S*, Wohlgensinger J, Frei R, Roduit C, Genuneit J, Pekkanen J, Roponen M, Hirvonen MR, Dalphin JC, Dalphin ML, Riedler J, von Mutius E, Weber J, Kabesch M, Michel S, Braun-Fahrländer C, Lauener R, the PASTURE study group. Prenatal and early life exposures alter expression of innate immunity genes: The PASTURE cohort study. *J Allergy Clin Immunol* 2012; 130(2):523-530.e9.

* both authors contributed equally to the article

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.

Supported by European Union research grants PASTURE/EFRAIM (QRLT4-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

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

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 ($\Delta\Delta$ cycle threshold [$\Delta\Delta$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 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

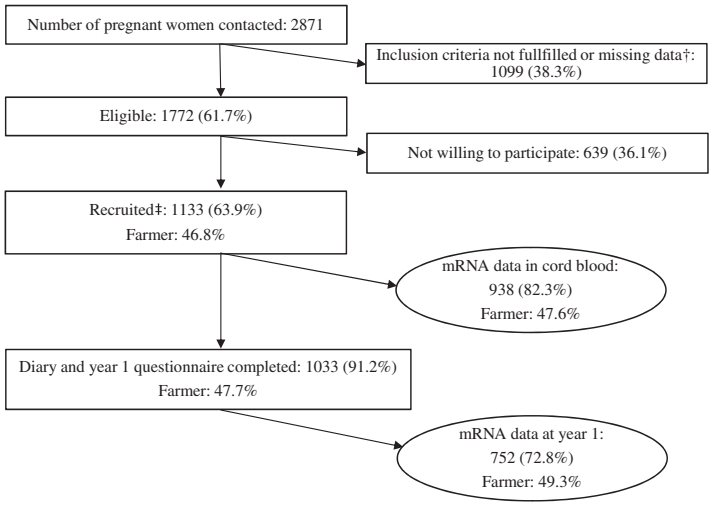

FIG 1. Selection of PASTURE study population and participants for mRNA analyses. †Inclusion criteria: living on a farm with livestock, maternal age greater than 18 years, term delivery, no genetic disease in offspring, telephone connection, and sufficient knowledge of the country's language. ‡Selection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

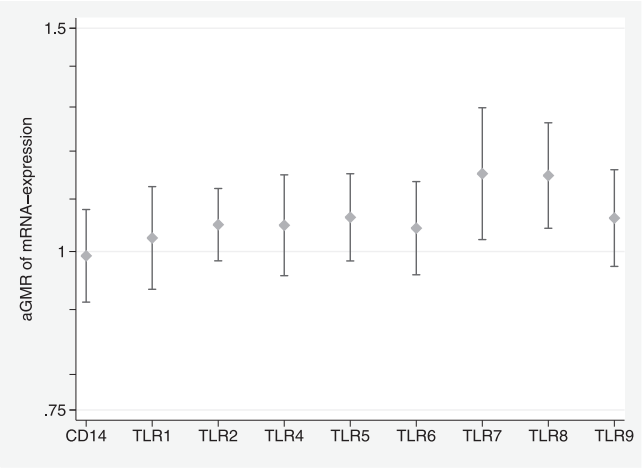

FIG 2. Adjusted association of farming/nonfarming during pregnancy and mRNA expression at birth expressed as geometric mean ratios and 95% CIs adjusted for maternal history of asthma or hay fever, sex, center (random), and maternal smoking during pregnancy. Fully adjusted multifactorial MANOVA of mRNA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

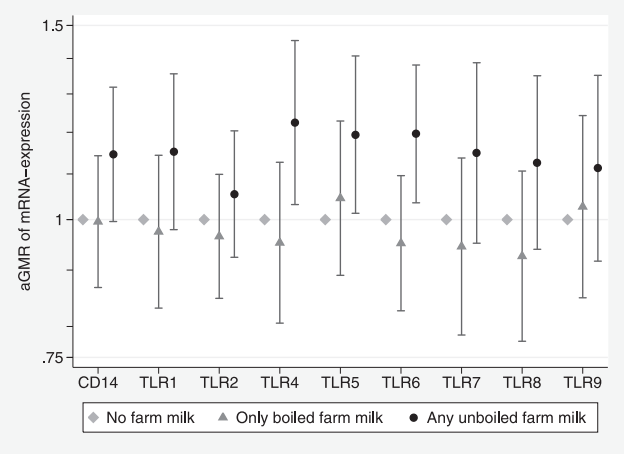

FIG 3. Adjusted association of child's farm milk consumption during first year of life and mRNA expression at year 1 expressed as geometric mean ratios and 95% CIs adjusted for farming, maternal history of asthma or hay fever, sex, center, maternal smoking during breast-feeding, solid food score, education,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

7 GENERAL DISCUSSION AND CONCLUSIONS

Findings presented in *Chapters 2* to *6* will be summarized and discussed in this chapter. After the summary and interpretation of main findings more general aspects of this thesis will be discussed.

7.1 Summary of main findings

The goals and objectives of this thesis were defined by research questions in *Chapter 1: Goals and objectives of this thesis* which will be answered here in short. More detailed results and discussions are given in the chapters covering the respective topic.

Disentangling the protective associations of a child's distinct farm exposures

1.) *Can the previously reported "farm effect" on childhood asthma and allergies be attributed to specific types of farms?*

2.) *Which distinct farm-related exposures are responsible for the association of farming environments with childhood asthma, hay fever and atopic dermatitis?*

A traditional type of farming namely with cows and cultivation was protective for childhood asthma, hay fever and atopy. The inverse association of general farm exposure with asthma could be explained by three specific early life exposures i) consumption of farm milk, ii) contact with cows and iii) contact with straw. The association with atopy and hay fever could not be fully explained by these factors indicating different underlying protective mechanisms for asthma, atopy and hay fever (detailed findings see *Chapter 2: Protection from childhood asthma and allergy in Alpine farm environments – The GABRIEL advanced studies*).

Association of farm milk consumption with childhood asthma and allergy

3.) *What is the prevalence of allergic health outcomes and cow milk consumption in the GABRIELA study sample?*

4.) *How does the composition of commercial and farm milk relate to commercial milk processing (homogenization, fat standardization, pasteurization or ultra-high temperature processing), home processing of milk (skimming, boiling), farm characteristics (farm size, cow's fodder) and milk storage (location, duration)?*

The prevalence of asthma, atopic sensitization, hay fever and atopic dermatitis was significantly lower in children living on a farm who also consumed more farm milk and unboiled farm milk. The latter showed higher levels of whey proteins, bacterial counts and was associated with a higher fat content when compared with boiled farm milk or grouped commercial milks (1 category comprising pasteurized and UHT milks). Pasteurized shop milk, however, showed higher levels of heat-sensitive milk constituents compared to UHT and boiled farm milk. The influence of skimming, milk storage and farm characteristics was tested but was not of importance in these analyses.

5.) *Is unprocessed cow's milk consumed in early life associated with asthma and allergic health outcomes in childhood and which specific constituents or microorganisms in milk are responsible?*

Reported consumption of unboiled farm milk was significantly associated with reduced risk for asthma, atopic sensitization, hay fever and atopic dermatitis. The association was stronger for children who consumed it before age 1 including maternal consumption during pregnancy. Whey proteins (bovine serum albumin, α-lactalbumin, β-lactoglobulin) were identified as milk constituents possibly explaining the epidemiologically observed protective farm milk association with asthma whereas reduced risk for atopic sensitization could not be associated with any investigated milk constituent. Microorganisms (total counts or counts of sub-groups) and fat content of milk showed no associations with allergic health outcomes (detailed findings see *Chapter 3: The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study*).

6.) *Which rapid method, a flow cytometry system or an automated most-probable number system, measures total viable bacterial counts in raw and processed cow's milk more reliably when compared with standard plate count method while keeping time and costs low?*

Both the flow cytometry system and the automated most-probable number system were fast and inexpensive, however, the flow cytometry system did not measure total viable bacterial counts in milk samples correctly. Results of the automated most-probable number system were in good agreement with the gold standard method (standard plate count method) and it should be favored for microbial exposure assessment in future epidemiological studies (detailed findings in *Chapter 4: Appropriate and alternative methods to determine viable bacterial counts in cow milk samples*).

Association of early life nutrition with development of atopic dermatitis

7.) *Which complementary foods or combinations thereof do mothers introduce within the first year of their children's lives and how are they associated with the development of atopic dermatitis?*

Feeding practices in the first year of life were associated with development of atopic dermatitis in childhood, independently of farming exposure. The increasing diversity of introduced complementary food items was inversely associated with the risk to develop atopic dermatitis after age 1. An inverse association was also found with the introduction of yogurt during the first year of life, independently of the diversity of introduced foods (detailed findings see *Chapter 5: The development of atopic dermatitis according to age of onset and the association with early life exposures*).

Farm related exposures in early life and associations with innate immunity

8.) *Which environmental especially farm related exposures during pregnancy are associated with a child's gene expression of innate immunity receptors (TLRs 1-9 and CD14) at birth?*

Maternal farming during pregnancy showed the strongest associations with gene expression of innate immunity receptors at birth statistically significantly associated with up-regulation of *TLR7* and *8* after adjustment for potential confounders. Except raw farm milk consumption, distinctive farm related exposures failed to show associations with these gene expressions at birth.

9.) *How does the innate immune system change from birth to age 1 and which farm-related and nutritional exposures during the first year of life are associated with a child's gene expression of innate immunity receptors at age 1?*

Gene expression of innate immunity receptors at birth and age 1 were not highly correlated indicating a substantial change of the innate immune system during the first year of life. Child's farm milk consumption was the exposure during first year of life with the strongest associations with gene expression of innate immunity receptors at age 1 statistically significantly associated with up-regulation of *TLR4*, *5* and *6* after adjustment for potential confounders.

10.) Can gene-environment interactions of associations of farm milk consumption with the gene expression of innate immunity receptors found in previous cross-sectional studies be confirmed?

There were two single nucleotide polymorphisms (SNPs) in *TLR8* and *9* which modified the associations of raw farm milk consumption with gene expression of the respective genes. A previously described gene-environment interaction by the SNP *CD14/C-1721T*[118] was not found (detailed findings on see *Chapter 6: Prenatal and early life exposures alter expression of innate immunity genes: The PASTURE cohort study*).

7.2 Discussion

The findings published in the framework of this thesis gave new insights into the inverse associations of farming environments, raw farm milk consumption and early life nutrition with atopic disease in childhood and further elucidated immunological pathways how this protection might be conferred on the human organism. Besides deepening the understanding of the hygiene hypothesis, methodological improvements for the microbial exposure assessment of milk samples were achieved. In the following section, I want to discuss what these findings mean in the context of the multiple dimensions of the hygiene hypothesis concept, i.e. i) type of environmental exposures in early life, ii) childhood atopic diseases and distinct phenotypes, iii) timing of exposure and iv) genetic susceptibility to react on respective exposures.[28] I will then assess how and to what extend the inverse associations of raw milk consumption with atopic outcomes would impact on atopic disease in Switzerland and how they could be translated into a public health measure. Finally, I will discuss what should be considered for future investigations in this field of research.

7.2.1 Type of environmental exposures and specific atopic disease

The first section covers two dimensions of the hygiene hypothesis concept: i) type of environmental exposures in early life and ii) childhood atopic diseases and distinct phenotypes.

7.2.1.1 Farming

The atopic outcomes in early childhood (including pregnancy) investigated with GABRIEL data in *Chapter 2* and *3* were asthma, hay fever, atopic sensitization and atopic dermatitis (AD). Due to the study design, constituents of milk samples could only be related to asthma and atopic sensitization. We showed that a general exposure to a farming environment and the consumption of raw farm milk in alpine areas had statistically significant and independent protective associations with all of these outcomes. This met our expectations and corroborated with previous studies in Western countries.[29] The strength of association for both exposures was strongest with hay fever and weakest with atopic dermatitis which is in line with previous reports. The association with asthma was heterogeneous in numerous other farming studies but in a recently published meta-analysis including all relevant studies[122] the overall farming association with childhood asthma was of similar magnitude as the farming association presented here. In our analyses, the associations of farming furthermore held for all phenotypes of asthma. When taking a closer look at the associations of distinctive farm

exposures with a novel approach using newly designed questionnaires and different types of farm milk and its constituents, only associations with asthma could be explained. In *Chapter 2*, contact to straw had protective associations with all outcomes (to a lesser extent on atopic dermatitis) whereas contact to hay, grass and manure could not be assessed separately and might be a part of this straw association. Contact to stables and barns, where participants were most likely exposed to straw, was shown before to be protective for atopic disease but distinctive exposures were not assessed.[41,44] Whether oligosaccharides in straw which showed such protective relations in animal models[123] or a high diversity of microorganisms (or specific microorganisms) found in straw[124] underlie this association with straw needs to be investigated. Recent evidence showing a relation of haying with Toll-like receptor up-regulation[92] points to an involvement of microorganisms (see *Chapter 7: The role of innate immunity*). Contact to cows was strongly related to the respiratory outcomes asthma and hay fever. Farm animals were also identified as protective farm specific exposures in other studies but they often lacked information to disentangle associations of contact to individual animals. It was argued that the exposure to the diversity of animals is actually driving this association.[29] Ege et al. found protective associations with children exposed to certain farm characteristics (associations of farms rearing pigs with asthma which was stronger on dairy farms, farms rearing poultry with atopic sensitization, cattle farms showed no associations) but no associations of individual exposure to any animal type including cattle were found with atopic disease.[92] A completely new insight presented in *Chapter 2* was that farming contact with a combination of cows and straw explained the association of general farm exposure with asthma together with exposure to farm milk. The association of farming with atopic sensitization, hay fever and atopic dermatitis couldn't be fully explained by these or other assessed specific exposures. A need for a simultaneous exposure to certain farm factors to explain the association with asthma indicates why the associations of asthma and farm environment were rather heterogeneous in the literature whereas they were more consistent for hay fever and atopy.

7.2.1.2 Farm milk consumption and dietary factors

The protective association of farm milk consumption with all atopic outcomes described in *Chapter 2* was further investigated in *Chapter 3* where it could be explained by raw farm milk consumption as opposed to consumption of boiled farm milk which showed not associations. Raw or unpasteurized farm milk consumption in childhood was previously found to be related to lower risks for atopic disease.[63,125] For further and novel investigations presented here, objectively measured milk sample components were related to outcomes. Asthma but not

atopy was inversely associated with whey proteins (statistically significantly with bovine serum albumin, α-lactalbumin, β-lactoglobulin) which were present in raw and to a lesser extend in low heated milk. These associations were indicative of dose response relationships. Associations of individual proteins could not be disentangled with GABRIEL data and it remains open whether one specific protein or a combination of proteins is involved. It is not clear from these results whether the identified whey proteins are actually responsible for the observed protective relations because the pathways are not yet known. Although these specific whey proteins were ascribed immunomodulatory effects before [48,66-68] they may only be proxies for other substances in milk that might be destroyed, denaturated or inactivated at the same temperatures but that have not been assessed in the GABRIEL samples. Fat content and total counts of microorganisms in milk were not associated with atopic outcomes. Fatty acids patterns, diversity of microflora and individual microorganisms were not assessed and cannot be excluded to be associated with atopic disease (see *Chapter 7: Study design and methodological aspects: strengths and limitations*).

Atopic dermatitis in early life and its relation to dietary factors were investigated in *Chapter 5*. With the longitudinal design of the PASTURE study, evidence for a causal relationship was stronger. Interestingly, the diversity of complementary foods introduced in the first year of life was found to be more protective for the development of atopic dermatitis (with onset after age 1) than individual food items. This was indicative of a dose response relationship. Yogurt consumption stood out among all 15 individual tested food items and was associated with a decreased risk for AD (after age 1) independently of the diversity of other complementary foods introduced. Cow milk consumption had a protective association with AD but this finding was based on shop milk consumption whereas consumption of farm milk had no clear individual association. In contrast, with cross-sectional data in *Chapter 3* it was found that farm milk consumption in early life was inversely associated with atopic dermatitis in childhood. The strength of this association was however weaker than for other outcomes. This discrepancy of results between our studies could be based on the different definitions of AD. To avoid reverse causality in the longitudinal PASTURE study, AD was defined as a doctor's diagnosis between age 1 and 4 excluding onset before age 1. In the GABRIEL study on the other hand, participants were asked retrospectively at ages 6-12 years whether they were ever diagnosed with AD. Furthermore, results of the PASTURE study showed that family history of atopic disease affected only early onset AD but not late onset which indicated that there might be 2 different phenotypes of AD, namely early and late (after age 1) onset AD. These potential phenotypes were also not considered in GABRIEL analyses.

Potential pathways of farm milk consumption and dietary factors

The question remains how these specific nutritional factors (consumption of raw farm milk, diversity of complementary foods and yogurt during first year of life) that were identified during this thesis exert their potentially protective effects on the development of atopic disease. With the introduction of foods a flush of new antigens enter the infant's body which might directly stimulate the uneducated immune system. Immunomodulatory whey proteins in native milk described in *Chapter 3* might interact with the immune system by altering T cell response as demonstrated with isolates in animal models (reviewed by Krissansen[48]). It was further shown that lactoferrin exerted anti-microbial immunity and α-lactalbumin appeared to be effective in inhibiting associations of the pathogens with intestinal cells which might contribute to balancing the gut flora.

It is known that ingestion of these foods in one way or another has an impact on the gut microbiota which in turn is associated with the development and maintenance of immune system. The role of the gastrointestinal (GI) microbiota in development of atopic disease is an unresolved issue but received much attention lately. Noverr et al. even proposed the microflora hypothesis as an alternative interpretation of the hygiene hypothesis in relation to allergic airway disease.[126] Therein, increased incidence of these outcomes is caused by perturbations in the GI microbiota because of antibiotic use and dietary differences (dietary fat, antioxidants or bottle-feeding) in industrialized countries leading to a disruption of the normal microbiota-mediated mechanisms in immunological tolerance in the mucosa. Probiotics were argued to prevent or reverse these adverse perturbations and to reduce the risk for atopic diseases in early life but studies so far could not consistently show such a protective effect on humans by probiotics containing certain bacterial strains or by prebiotics.[127] GABRIEL analyses in *Chapter 3* showed total counts of microorganisms or counts of specific groups (e.g. lactobacilli) in milk samples were not associated with asthma or atopic sensitization in early life. The influence on the GI microbiota especially during the susceptible phase in early life, however, might be determined by how diverse the composition of the ingested microorganisms is (such data were not available in the GABRIEL study, see *Chapter 7: Study design and methodological aspects: strengths and limitations*). The inverse association of farming with childhood asthma was partially explained by the child's exposure to the diversity of microorganisms in house dust[43] and it seems natural that ingestion of a wide array of microorganisms with raw milk might exert similar effects in the gut. The influence of the diversity of dietary factors on atopic dermatitis assessed in *Chapter 5* might reflect the influence of microbial exposure that is more diverse than microbes specific to

individual foods. Indeed, a study found that lower bacterial diversity of the gut flora during infancy was associated with higher risk for atopic disease at school age.[128] This is in line with conclusions drawn in recent reviews that microbiota diversity could be a more important factor for healthy immune maturation than a specific strain or strains of colonizing bacteria.[107,129] Underlying mechanisms are unclear. Protection could be based on the diversity of microbes signaling through the innate immune system but diversity could represent the importance of the actual composition of microbes or just be a proxy for levels of pro-inflammatory markers which trigger immunological responses.[130] This diversity might also have a stabilizing effect on the gut microbiota balance thus preventing colonization with adverse or pathogen microbes causing unfavorable immune responses.

The strong protective association of yogurt consumption in early life with AD found in *Chapter 5* might be mediated by abundant lactic acid bacteria affecting the balance of the gastrointestinal microbiota but as mentioned before results of human studies with isolated strains (probiotics) were inconsistent. The consumption of yogurt might also favor the colonization with bacteria producing short-chain fatty acid which are metabolites associated with immune and inflammatory responses.[131-134] It is intriguing that the colonization of the GI tract happening in the first months and years of life potentially determines the acquisition of an optimal composition of the adult flora[105] and that farm related exposures may affect atopic outcomes in early life with a potential impact lasting into adulthood.[34]

The relevant pathways of how microorganisms affect the immune system and atopic disease remain essentially unclear. Microbial antigens are actively transported by epithelial cells and constantly sampled by dendritic cells triggering T cell responses.[135] These antigens also directly stimulate Toll-like receptors (see *Chapter 7: The Role of innate immunity*). Results in *Chapter 6* showed that up-regulation of these innate immunity receptors was associated with farming and farm milk exposure which adds to the assumption that these receptors might be relevant to explain associations of farm related factors with atopic diseases in early life.

7.2.1.3 The role of innate immunity

The innate immune system has been argued before to mediate protective effects for atopic disease by inhibiting allergic immune response.[136] In the PASTURE study (*Chapter 6*), we found that farming specific and dietary factors occuring *in utero* and during first year of life were associated with an up-regulation of innate immunity receptors. Previous studies also showed that being a farm child and contact to stables during early life were associated with gene expression of innate immunity receptors but these were cross-sectional studies and only

a limited number of receptors was assessed.[41,93] We could show that *in utero* exposure to farming was associated with gene expression at birth whereas raw farm milk consumption during first year of life was associated with expression at age 1 independent of farming status. The stimulation of the innate immunity is likely to be based on microbes from these exposures which are rich in non-pathogenic microorganisms. Increased levels of microbial markers such as endotoxin, muramic acid, extracellular polysaccharide or glucan at the interface of an organism with the environment which originate from farm related or dietary exposure lead to increased stimulation of innate immunity receptors by pathogen-associated molecular patterns. There are evolved mechanisms for modulating TLR-mediated responses exemplified by the well known "endotoxin tolerance": "Exposure to microbial components such as LPS results in a severely reduced response to a subsequent challenge by LPS".[90] Thus, innate immunity in children exposed more frequently to high amounts of these markers might simply become desensitized against antigens resulting in inhibition of inappropriate adaptive immune responses. Induction of molecules which are involved in the negative regulation of TLR signaling (e.g. SOCS-1) by exposure to microbes leading to decreased adaptive immune response may also be a relevant pathway.[137,138] Unfortunately, the microflora of our investigated exposures was not assessed in detail and it could not be further explored whether the observed associations were driven by levels of microbial markers, presence of specific microbes or actual composition of microorganisms.

We found that individual foods (e.g. meat) introduced early during the first year of life were also associated with TRL expression at age 1 albeit not as strong as raw farm milk consumption. Yogurt consumption and increasing diversity of complementary foods during first year of life were associated with an up-regulation of *TLR4* however not significantly in fully adjusted models. Interestingly, these were the same dietary factors which were associated with atopic dermatitis with onset after age 1 in *Chapter 5*. It remains unresolved whether the prospective associations in *Chapter 5* represent a causal chain of relationships from exposure over innate immunity to decreased risk for atopic disease because we did not assess how these innate immunity receptors in turn were associated with atopic disease (*Chapter 6*). The up-regulation of gene expression of innate immunity receptors might merely be a proxy for increased microbial markers and not be causally involved with health outcomes. Recent findings suggest, however, that TLR activation is directly involved in development of atopic disease.[139,140]

Furthermore, the expression of receptors was not correlated between birth and age 1 which is in line with reports about rapid changes and development of innate immunity in the early life.[141] Different associations of specific farming factors with TLR expression at different times in the infant's life might indicate varying susceptibility and involvement of different pathways *in utero* and during first year of life. Whether this also reflects associations of specific exposure with only specific atopic outcomes is unclear and needs to be investigated.

The conclusion regarding the first two dimensions of the hygiene hypothesis is that the varying associations with different atopic diseases suggest that there are specific pathways involved in the inverse associations of farming exposures and nutrition with individual outcomes. From afar, the atopic outcomes seem to co-manifest when they appear in early life but recent studies already showed that both genetic and environmental factors associated with these health outcomes differed, i.e. genes related to asthma and total IgE levels did not overlap in a recent genome-wide association study and microbial diversity in house dust was associated with decreased risk for asthma but not atopy.[43,119] The fact that protective associations of farming environment and raw farm milk consumption appear to be diseases specific adds to the complexity of the hygiene hypothesis concept. During this thesis, several factors were found to explain decreased risk for asthma but specific factors explaining atopy are yet to be identified. Whereas microbial diversity seems to be of importance for the associations with asthma, associations with atopy might be more dependent on the presence of specific microbes.

7.2.2 Timing of exposures

The third dimension that was argued to be part of the hygiene hypothesis concept is the timing of exposures. In previous investigations it was shown that associations of farm related factors with atopic disease may depend on when these exposures occur in early life.[41,42,64] Riedler et al., for instance, reported that a "substantial protection against development of asthma, hay fever, and allergic sensitization was seen only in children exposed to stables, farm milk, or both in their first year of life" with an indication that "prenatal exposures had a substantial protective effect".[44] Indeed, the findings in these thesis produced with data from two different studies confirmed these timing depended associations (also with the development of innate immunity). In general, we could show that the associations were stronger when exposures occurred earlier in life including *in utero* exposure. In cross-sectional analyses in *Chapter 2*, the protective associations of farming specific exposures with atopic diseases were strongest when they happened in the first 3 years of life plus pregnancy. Associations with current

exposure at age 6-12 years were weaker. The raw farm milk association with asthma and atopic dermatitis among mixed milk drinkers (children consumed both shop and farm milk during early life) was only observed for children which consumed the raw farm milk before age 1 including maternal consumption during pregnancy (*Chapter 3*). For atopy and hay fever and among exclusive farm milk drinkers this timing effect was much less pronounced. Admittedly, with GABRIEL data we found that for a substantial part of children who were exposed to a farming specific exposure by school age the exposure already began very early; e.g. almost 70% of children exposed to raw farm milk by age 6 were already exposed to raw milk by age 1. This was also observed in other studies.[44] Hence the power to assess detailed timing effects (e.g. by age of introduction in yearly steps or to separate *in utero* and postnatal exposure) was limited. Similarly, protective associations of raw milk consumption with atopic disease were stronger if consumed more frequently in early life.

In the prospective PASTURE study, timing effects were tested as well. In *Chapter 5*, early life nutrition was related to late onset AD (after age 1) to avoid reverse causality effects but this resulted in a lack of power to assess the effects of timing when certain complementary foods were introduced during the first year of life. We found an indication that prenatal and postnatal contact to farm animals had different effects on early and late forms of AD. Prenatal exposure tended to reduced the risk for AD with onset before age 1. This was reported in more detail in a previous PASTURE publication in which contact to the diversity of farm animals was inversely associated with the risk for atopic dermatitis up to age 2.[96] Postnatal exposure, however, tended to reduce the risk for AD with onset after first year of life. These results were however not significant because of low sample sizes in regression models.

Timing proved to be of utmost importance for the associations of environmental factors with the development of the innate immune system (*Chapter 6*). Maternal exposure to general farming environment during pregnancy (*in utero* exposure) was associated with gene expression of Toll-like receptors at birth but a continued farming exposure of the child up to age 1 was not associated with gene expression at age 1. Raw farm milk consumption during first year of life was strongly related to up-regulated mRNA expression of several TLRs independent of exposure to farming environment. The association of maternal farm milk consumption during pregnancy with innate immunity receptors on the other hand, was less pronounced. A further result not presented in *Chapter 6* was that earlier introduction of raw farm milk (introduction at age 3-9 months vs. age 10-12 months) was more strongly related to the expression of innate immunity receptors. A previous study made similar observations in

which only contact to stables during pregnancy but not at school age (current exposure during period of assessment) was associated with an up-regulation of TLR and *CD14* gene expression.[41]

Apparently different pathways are involved *in utero* and when infants are exposed directly. Potentially protective effects on fetal immunity might be mediated *in utero* by transamniotic and transplacental allergen exposure and further modification of the antigen-specific immune response could then occur postnatally through interaction with the newly acquired gut microbiota.[142] Also, maternal innate immune responses to microbial stimulation in the lung were shown to transplacentally program the fetal immune system *in utero* in a mouse model.[143] Another route could be that protection is passed on to the child through epigenetic inheritance.[144] Due to given limitations these could not be investigated further.

7.2.3 Genes and susceptibility to react on environmental exposures

The estimated risk for atopic disease attributable to genes is substantial (for asthma or hay fever: 35-80%, eczema: 72%)[13,14] but it was recently shown for childhood asthma that "both genetic and environmental components may independently contribute to distinct mechanisms underlying this condition".[119] Although associations with genetic disposition were not the focus of this thesis, the analysis had to be adjusted for this strong genetic influence. This was done by assessment of the maternal and paternal history of allergic disease (in PASTURE partially with objective measurements) and adjusting for them when calculating the associations of environmental exposures with atopic outcomes. There were, however, two interesting PASTURE sub-analyses regarding genetic disposition and its association with outcomes. It was found that family history of allergies determined the development of atopic dermatitis occurring before age 1 but not the development of AD with onset after age 1 (*Chapter 5*). With information from previous studies we suggested that these could be 2 different phenotypes of AD whereas genetic and epigenetic mechanisms act only on the early onset phenotype. The second finding showed that SNPs in several innate immunity receptor genes determined the gene expression of the respective receptors (*Chapter 6*).

Results for gene-environmental interactions are debatable. The protective associations of farming, contact to straw and cow with asthma presented in *Chapter 2* were shown to be indicative of a gene-environment interaction in a previous publication of GABRIEL. In this GWAS analysis "a cluster of 4 highly correlated genotyped SNPs and 2 imputed SNPs related to the GRM1 gene were found to interact for asthma with farming, contact with cows and

straw, or contact with straw alone".[119] Replications in other settings are however needed. Genotyping data in the PASTURE study made it possible to assess a previously described association of farm milk consumption with gene expression of the respective innate immunity receptor in early life depending on genetic variations in *CD14*.[118] Indeed, we found modifications of these associations but by SNPs in *TLR8* and *9* (*Chapter 6*). These gene-environment interactions could be spurious findings because due to their strength of statistical significance they are unlikely to be found after an adjustment for multiple testing. Previous findings of such interactions were generally not reproducible and may play a minor role in protective effects on atopic outcomes.[119] How much the dimension of genetic susceptibility actually contributes to the concept of the hygiene hypothesis is still not clear and will continue to be much-debated.

7.2.4 Study design and methodological aspects: strengths and limitations

The following segment covers more general strengths and limitations of this thesis whereas several specific advantages and limitations were already mentioned in the previous sections. I had the advantage to work on two different studies with the opportunity to address several interrelated research questions. It was possible to directly compare findings of both studies and to use results from one study to generate new ideas for analyses in the other study. Moreover, the results presented in this thesis are plausible and in agreement with findings from previous studies.

7.2.4.1 The GABRIEL study

The GABRIEL study comprised a large study population of over 100'000 children living in rural areas in several countries. A strength was the comprehensive assessment which included objective measurements of exposures (e.g. milk samples) and data on a multitude of atopic diseases in early life based on validated questionnaires. Atopic sensitization was also defined by objective measurements of specific IgE. There were 3 sampling phases with a disproportionate stratified random sampling technique to provide enough statistical power with a sufficient number of children with farming exposures and atopic disease. This allowed to relate a substantial amount of objectively analyzed milk samples (800 milk samples with a full set of measurements) to atopic health outcomes using statistical weighting methods to account for the stratified sampling and to maintain the internal validity regarding the initial study population. A detailed evaluation of the GABRIEL study concluded that "the association of exposure to farm environments with asthma or rhino conjunctivitis was not biased by participation or consenting behaviour" and that "avoidance of exposure to farming

due to asthma or allergic disease and subsequent distortion of the association does not play a major role in our study population".[120]

With the cross-sectional study design, however, came inherent limitations. The temporal relationships of exposures and outcomes were assessed more or less at the same time and there is only limited possibility for causal inference. GABRIEL findings presented in *Chapter 2* and *3* are sound hypotheses about causal relationships but need to be confirmed in prospective studies.

Generalizability of findings

The results can be applied to children at school age in alpine rural regions where the participants were recruited and areas with similar farming structures and living conditions. Generalizability to the total population of 6-12 year olds might not be possible because participants were only from rural areas (urban areas were not included). Whether the protective farming and farm milk associations can be applied to rural areas in other climate zones or regions with generally different hygienic lifestyles is in question. Results from such farming studies in developing countries are inconsistent (see *Chapter 7: Farm related exposure and atopic disease in developing countries*). The farming study model established in GABRIEL was based on family run farms excluding large industrialized farms. The conception of farming, as it was understood in the geographical areas of this research, might be different in other regions of the world and even within Europe. This has been exemplified in analyses with participants from the Polish study site of GABRIEL (which were excluded from GARBIEL analyses in *Chapter 2* and *3* and will be analyzed separately). In Poland farming structures were quite different from structures in German speaking areas making it hard to interpret and compare associations of "farms" with atopic outcomes between regions.

Limited data on heat treatment of milk samples

Objective measurements of milk samples in GABRIEL phase 3 revealed that whey protein levels in milk were inversely associated with occurrence of asthma (*Chapter 3*). Interestingly there was indication of a similar association of pasteurized shop milk with asthma which was not found with UHT shop milk. Unfortunately, shop milk drinkers mainly consumed UHT milk which limited numbers of pasteurized shop milk samples. The sample size and the necessity to weight logistic regression models limited our further investigation of the effects of pasteurized milk. On an epidemiological level (questionnaire data and a large study population in GABRIEL sampling phase 2) we found that raw milk had a strong protective association with asthma, atopic sensitization, hay fever and atopic dermatitis whereas boiled

farm milk did not show this association (*Chapter 3*). A further investigation of different heat treatments was not possible because we did not have information on participants' exact heat processing of farm milk or information to distinguish the milk type of commercial milk drinkers (pasteurized or UHT shop milk).

Methodological considerations: microbial exposure assessment

The importance of microbial exposure assessment was shown in *Chapter 4*. The consequences of a misleading assessment are not only a waste of resources especially if measurements have to be repeated but they may have a strong impact on subsequent calculations of associations of exposures with health outcomes.

During statistical analyses with the GABRIEL dataset to answer research questions regarding microorganisms in milk samples (*Chapter 3*) it was discovered that the rapid flow cytometry method first used to assess total viable bacterial counts (TVC) in the GABRIEL milk samples did not yield reliable results for heat processed milk. Apparently proteins in heated milk interfere with detection of bacterial cells so that the measured levels of bacteria are higher than the true levels.[145] This error was found because of implausible results which showed levels of viable bacteria to be higher in UHT and boiled farm milk compared to raw milk. About 1400 milk samples were measured again by a modified culture method (automated most-probable number method) and validated by measuring a random sample of milk samples with the gold standard (standard plate count method). Sub-groups of microorganisms in milk were assessed in 222 milk samples by standard plate count agar. It was the first time that objectively measured microbial properties of milk were related to atopic health outcomes but neither the sub-groups nor the TVC in milk were associated with asthma or atopy in the GABRIEL study population.

Research shows, however, that such cultivation techniques can only measure a certain proportion of all microorganisms in environmental samples. Microbial investigations based on these techniques cannot be regarded as reliable in terms of their reflection of the true microbial diversity.[146] Their shortcomings can be avoided by using less biased DNA-based strategies such as PCR amplification of 16s ribosomal RNA.[147] Furthermore, recent investigations of gut microbiota composition showed that there is a need for even more powerful fingerprinting techniques than DNA-based methods to get a complete map of enteric microbiota.[148] DNA-microarrays such as phylogenetic oligonucleotide microarrays[149] and other advancements might become useful to decipher the microbial flora of environmental exposures such as raw farm milk right down to the last microbe. This might be necessary to

further assess the association of the diversity of microbial exposure with atopic disease and to see whether there is an "effect" based on actual diversity or whether key organisms are driving such a "diversity effect". It will however be challenging to interpret results of high resolution techniques due to type 1 errors (multiple comparisons issues), type 2 errors (sensitivity issues) or replication of associations considering variability in exposure over time.[130]

How representative is one milk sample?

There were 2 milk samples per participant (winter and summer) but only 1 sample (winter) was analyzed extensively and could be related to atopic health outcomes. With questionnaire data it could be shown that participants were generally consuming the same type of milk but a one-point measurement has to be considered as a shortcoming in exposure assessment. Ideally milk should be sampled over certain time periods (e.g. over 2 weeks) at least twice a year to establish a milk consumption pattern and to allow assessment of continued exposure to milk constituents. The sampling periods should also cover different seasons of the year to account for variations in milk composition due to season specific fodder (mainly affects fatty acid composition of milk which is not part of this thesis).

7.2.4.2 The PASTURE study

With the longitudinal design of this study the exposures were assessed before the outcomes occurred which ensured the establishment of temporal relationships or temporality which was described as the key component to substantiate causality.[150] Relating farm and dietary factors during pregnancy and the first year of life to gene expression of innate immunity receptors occurring after these exposures was novel (*Chapter 6*). This allowed temporal inferences thus strengthening evidence from previous cross-sectional studies.

The assessment of exposures and outcomes in PASTURE was comprehensive providing the opportunity to confirm previous hypothesis of associations with atopic outcomes and innate immunity receptors prospectively but also to investigate further associations. At age 1, already 3 extensive questionnaires were administered (during pregnancy, at month 2 and at age 1) recording farming and lifestyle factors. In addition, dietary factors analyzed in *Chapter 5* and *6* were recorded with very detailed weekly and monthly food frequency questionnaires from age of 3-12 months which allowed a detailed analysis of timing and composition of complementary food introduction. Furthermore, a wide array of innate immunity receptors (measured by mRNA expression) and SNPs in their genes were assessed. In combination with

the extensive exposure assessment, limited information on innate immunity receptors or early life exposures as found in previous studies was not an issue in PASTURE analyses.

In terms of generalizability, the PASTURE study is comparable to the GABRIEL study (see previous section). There were no noticeable differences between responders and non-responders indicating that the study population was not biased. mRNA samples for measurement of expression of innate immunity receptors were available for 82.3% (at birth) and 72.8% (at age 1) subjects. Participants with and without samples did, however, not differ regarding relevant variables.

A limitation was that consumption of food items was only reported in questionnaires and no samples were taken to be analyzed objectively. Components such as microbes and whey proteins in yogurt and farm milk would have given further insight in how these exposures were associated with late onset atopic dermatitis or the gene expression of innate immunity receptors. AD was defined not only by doctors' diagnosis but included also the standardized SCORAD score and was stratified in early and late onset to avoid reverse causality effects. This stratification also meant a reduction of power and different associations of prenatal and postnatal exposures could not be assessed.

7.2.5 How to translate these research findings

In depth analyses and new insights like presented in this thesis are much needed but it is not straightforward to comprehend what risk measures mean in the context of public health issues. In other words, what would be the potential health impacts of the findings in this thesis and could they be implemented by public health initiatives? I want to give an example to illustrate how effect measures of raw farm milk consumption produced during this thesis might impact on childhood asthma in Switzerland.

7.2.5.1 Raw milk: medicine or health hazard, the perspective counts

There are several ways to look at milk consumption. From a nutritional point of view, milk is an excellent source. For thousands of years, humans employed milk beyond infant nursing with breast milk by using milk from other animals. But from a health perspective, milk and especially raw milk are an optimal medium for microbial growth including human pathogens. Milk was described as "one of the most dangerous articles in our dietary" (Sir Graham Wilson quoted by Leedom[59]) for a good reason considering milkborne pathogens. With the emergence of the germ theory and the development of pasteurization techniques at the end of the 19th century the raw milk health hazard could be circumvented. Thermal processing

combined with improved hygienic standards removed pathogens from milk and it became a safe product. Now, at the beginning of the 21st century our study results and similar recent research findings offer yet another way to look at the consumption of raw milk. It is also from a health perspective but this time it is something promoting, namely the "protective raw milk effect" on atopic disease in childhood.

To assess the translatability of these protective associations with raw farm milk consumption, we will explore the potential impact on childhood asthma in Switzerland. I will address the two following questions: i) how many cases of asthma were prevented in the year 2000 among 6-12 year old Swiss children consuming raw farm milk? and the more hypothetical question ii) how many cases of asthma could have been prevented in the year 2000 among 6-12 year old Swiss children if 20% of exclusive shop milk drinkers had consumed raw farm milk instead of shop milk?

7.2.5.2 The risk for childhood asthma attributable to shop milk consumption

For the purpose of this calculation we shall use health outcomes of children aged 6-12 years assessed in the GABRIEL study (see *Chapter 3*) limited to the Swiss study population. Therein, a broad asthma definition was used including diagnosis, symptoms and treatment. The asthma prevalence for Swiss farmer and non farmer children was 14.6% and 21.9% respectively. The exposure to shop and raw farm milk from ages 0-6 included maternal exposure during pregnancy and was taken from the GABRIEL study where the milk consumption could be stratified by farming status. Mixed milk drinkers (consumption of both farm and shop milk) were excluded to simplify calculations. The following data restricted to Switzerland are needed for the impact assessment; Swiss population in 2000: 7'288'010 with 2.8% farming population (Table 1);[31] proportion of 6-12 year olds: 7.02%;[151] exclusive shop milk consumer: farmer: 7.4%, non farmer: 63.9%; exclusive farm milk consumers (with any raw farm milk): farmer 60.0% (69.8%); non farmer: 3.0% (57.7%). Shop milk consumption is regarded as exposure in this example. We can calculate the prevalence of milk consumption for farmers and non farmers and find that 95.6% of our example population was exclusively exposed to shop milk and 4.4% to raw farm milk (Table 2).

Table 7-1: Swiss population in 2000[31,151] (rounded values)

	All ages		Proportion of 6-12 year olds		Ages 6-12
Farmer (all in household)	203'369	x	0.0702	=	14'279
Non farmer	7'084'641	x	0.0702	=	497'422

Table 7-2: Milk consumption among 6-12 year olds in Switzerland (data from GABRIEL study) (rounded values)

	Farmer (ages 6-12)				
	Proportion		Farmer in Switzerland		Total
Exclusive shop milk consumption	0.074	x	14'279	=	1'057
Exclusive farm milk consumption	0.600	x	14'279	= 8'567	
Any raw farm milk (% of excl. FM drinkers)	0.698	x	8'567	=	5'980
total					7'037
	Non farmer (ages 6-12)				
	Proportion		Non farmer in Switzerland		Total
Exclusive shop milk consumption	0.639	x	497'422	=	317'853
Exclusive farm milk consumption	0.030	x	497'422	= 14'923	
Any raw farm milk (% of excl. FM drinkers)	0.577	x	14'923	=	8'610
Total					326'463
	Swiss-wide (ages 6-12)				
	Farmer		Non farmer		Total
Exclusive shop milk consumption	1'057	+	317'853	=	318'910
Exclusive farm milk consumption	8'567	+	14'923	= 23'490	
Any raw farm milk (% of excl. FM drinkers)	5'980	+	8'610	=	14'590
Total					333'500
Proportion of excl. shop milk drinkers	318'910	/	333'500	=	0.956

The adjusted association of raw farm milk consumption vs. exclusive shop milk consumption between ages 0-6 years with childhood asthma was: 0.59, 95% confidence interval: 0.46-0.74 (from GABRIEL study in alpine areas in 6-12 year old children, see *Chapter 3*). The reciprocal value of this odds ratio (1.695) corresponds to the adjusted association of shop milk consumption vs. raw farm milk consumption.

$$\text{Population attributable fraction} = \frac{proportion\ of\ pop.\ with\ exposure \times (risk\ measure - 1)}{proportion\ of\ pop.\ with\ exposure \times (risk\ measure - 1) + 1} \qquad (7.1)$$

Using Levin's formula[152] (7.1) we get the population attributable fraction (PAF) which is the fraction of the risk for asthma which is attributable to the exposure "exclusive shop milk consumption" for 6-12 year old Swiss children in 2000 (7.1.1) (rounded values).

$$PAF = \frac{0.956 \times (1.695 - 1)}{0.956 \times (1.695 - 1) + 1} = 0.399 \qquad (7.1.1)$$

7.2.5.3 Impact of farm milk consumption on childhood asthma

Now the two question put forward at the beginning of this section can be answer.

i) Asthma cases in early life prevented by raw farm milk consumption

First we calculate the total observed cases of asthma among children consuming raw farm milk (y) according to prevalence data from the GABRIEL study in *Chapter 3* (Table 3).

Table 7-3: Cases of asthma among 6-12 year old raw farm milk drinkers in Switzerland (rounded values)

	Population	Prevalence		
Farmer	5'980 *	0.146	=	874
Non Farmer	8'610 *	0.219	=	1'883
Observed cases (y)				2'757

To determine how many asthma cases were prevented by raw farm milk consumption (x) we use equation (7.2) solved for x, the number of expected asthma cases if all children had consumed exclusively shop milk instead of raw farm milk (y+x) and the asthma risk attributable to exclusive shop milk consumption (PAF) calculated in (7.1.1) (Table 4).

$$Total\ cases * PAF = Attributable\ cases \qquad (7.2)$$

Table 7-4: Prevented cases of asthma among 6-12 year olds in Switzerland due to raw farm milk consumption (rounded values)

	Expected cases *	PAF	=	Prevented cases
	(y+x) *	PAF	=	x
Prevented cases (x)	(y*PAF)/(1-PAF)		=	1'832

We found that 1’832 cases of asthma were prevented in the year 2000 among 6-12 year old Swiss children consuming raw farm milk between ages 0-6 years instead of consuming exclusively shop milk.

This calculation can be repeated with risk measures and respective prevalence for other atopic disease presented in *Chapter 3* to find prevented cases of hay fever: 1’840, atopic sensitization: 1’718, atopic dermatitis: 652.

ii) Asthma cases in early life potentially preventable by raw farm milk consumption

For the second question we assumed that 20% of exclusive shop milk drinkers were consuming raw farm milk between ages 0-6. First we calculate the total observed cases of asthma for our study population according to prevalence data from the GABRIEL study (Table 5).

Table 7-5: Cases of asthma among all 6-12 year olds in Switzerland (rounded values)

	Population	**Prevalence**		**Asthma cases**
Farmer	7'037 *	0.146	=	1'028
Non farmer	326'463 *	0.219	=	71'398
Observed cases				72'426

Then we calculate the total number of cases of asthma in our population attributable to exclusive shop milk consumption with equation (7.2) (Table 6), i.e. how many cases of asthma would have been prevented if all (100%) exclusive shop milk drinkers had consumed raw farm milk (N=28’914). Finally, to answer the second question we take 20% of all preventable cases.

Table 7-6: Preventable cases of asthma among 6-12 year olds in Switzerland (rounded values)

	Cases	PAF		
All preventable cases	72'426 *	0.399	=	28'914
Preventable cases if 20% of exclusive shop milk drinkers had consumed raw farm milk				* 0.2 5'783

Calculations showed that 5'783 cases of asthma could have been prevented in the year 2000 among 6-12 year old Swiss children if 20% of shop milk drinkers had consumed raw farm milk instead of shop milk between ages 0-6.

Repeating this calculation with numbers for other outcomes (*Chapter 3*) gives cases preventable by raw farm milk consumption: hay fever: 5'848, atopic sensitization: 6'969, atopic dermatitis: 2'789.

For the interpretation of these results it should be kept in mind that the group of non farmer children included rural non famers but no urban population. The prevalence of health outcomes and milk consumption especially raw milk consumption may differ between rural and urban population. The data used for this example did not allow to assess the impact of this potential difference.

7.2.5.4 Practicability of raw milk as preventive measure for atopic disease

These calculations for Switzerland show that there lies a great potential in raw farm milk consumption (or components in it) to be used as preventive measure for atopic disease. Similarly high prevalence of these diseases in other industrialized countries and reports of protective associations with farm milk consumption suggest that similar impacts would be found there. On an economical level which is beyond the scope of this thesis, such a preventive measure could have a great impact as well considering the estimated annual costs of €17.7 billion for asthma care in the EU (EU-15 plus Switzerland and Norway).[153] A Swiss-wide implementation of raw farm milk consumption let alone an international implementation would be challenging. One big issue would be the logistic effort for producers and distributors to provide a high quality raw milk product which has to be consumed within a narrow time frame due to very short shelf life of a few days. Compared to pasteurized and UHT milk where shelf life is a matter of weeks or months this would surely be less profitable. For the calculations I assumed that 20% of the Swiss children would consume raw farm milk instead of only shop milk. It is unclear whether producers are capable or would want to satisfy a

market of 20% raw milk consumers (not counting adults) given considerations of profitability and potentially high dispose of quickly expired product.

Safety issues and health concerns

The main problem associated with raw farm milk consumption is the potential health hazard due to pathogen microorganisms and this makes an implementation as health promoting food debatable. Health concerns towards raw milk are reasonable in any part of the world although it is difficult to estimate the actual number of milkborne disease. Sources of noticeable foodborne disease outbreaks are generally recorded but individual cases often remain unreported or pathogen sources are not specified. A study combining records of 6 European countries and the U.S.A. concluded that 1-5% of foodborne diseases were based on consumption of dairy products.[55] About 50% of these were ascribed to raw or unpasteurized milk products but this number might be higher because for a substantial amount of these products heat treatment was not specified. Between 2002 and 2011 the average numbers of reported cases per year of various pathogens commonly associated with food in Switzerland were Campylobacteriosis: 6'311, Salmonellosis: 1'801, EHEC: 47, Listeriosis: 45 (compare Brucellosis: 7 cases per year).[154] Assuming all of these cases were foodborne, 0.5-2.5% can be estimated to be based on raw milk consumption.

Disease caused by consumption of raw milk products is not a major health concern in German speaking regions because of an (practical) absence of brucellosis, zoonotic tuberculosis and adherence to highest quality standards. In the U.S.A. which is similarly developed milkborne pathogens remain a real problem. This discrepancy becomes evident when comparing isolation rates of *Listeria monocytogenes* in U.S. bulk tank milk from 1987-2004 which were as high as 12.6%[155] with results of the representative GARBIEL samples where in less than 2% of raw milk samples only non-pathogen listeria strains were found. Furthermore, the Swiss national dairy product monitoring system did not find any *Listeria monocytogenes* in their most recent publications about annual assessments in 2009 and 2010 which included raw milk products and it was concluded that the quality of Swiss dairy products was "good".[156,157]

In developing countries (e.g. Mali[158,159]) hygienic and quality standards are generally lower and risks for milkborne infections or exposure to antibiotic residues in milk surely outweigh potentially protective effects on atopic disease. The WHO estimated that in Sahelian countries up to 70% of diarrhea in infants may be milkborne.[160] Considering that this is a major cause of child mortality in under 5 year olds,[161] consumption of milk products and especially raw milk is a major health hazard.

Raw milk: an informed and emotional discussion

Informed and lay discussions and reports regarding the relation of raw milk consumption and health are dominated by milkborne human pathogens posing health risks. Recommendations regarding native milk consumption are inconsistent between but also within countries. There are institutions on a governmental, specifically in North America and the U.K., and on a supranational level pointing out safety issues of raw milk and products thereof for the general public and advocating that milk be pasteurized (e.g. U.S. FDA, U.S. CDC, the American Medical Association, International Association for Food Protection and the WHO).[56,58,162] For specific groups of people public health services give special recommendations to avoid raw milk consumption, e.g. the Swiss department of health issued leaflets for pregnant women informing them to avoid raw milk because of *Listeria monocytogenes*.[163] In the U.S.A. main concerns are not restricted to pathogens associated with raw milk but stretch to potential contamination of pasteurized milk and its distribution via milk pooling in dairy processing.[101] An unrelated concern is milks natural fat content. Public health campaigns encourage the consumption of fat-free or low-fat milk and milk products also early in life to reduce calorie intake and risks for obesity and cardiovascular disease.[52,53]

In contrast to these negative effects ascribed to milk in its native state, availability and promotion of organic, local and healthy foods and arguments for enhanced nutritional qualities and health benefits increase the demand for minimally processed foods like unprocessed cow's milk.[56,58,59,164] These different perspectives make raw milk a controversial topic especially in North America where laws are much more rigid than in Europe. Limited sale of raw milk is only allowed in about half of the U.S.A. and selling it across state lines violates federal law. Canada prohibits unpasteurized products nationwide with strict law enforcement.[162,165] A consequence are pro- raw milk lobbies exchanging polemic arguments with policy makers and raw milk advocates circumvent these laws with cow leasing programs which allow "drinking cow milk from their own cow".[56,166] In Europe specifically in alpine areas on the other hand, reasons for fewer restrictions regarding raw milk products might be continuing traditions of raw milk cheese production and high hygienic standards resulting in a generally low prevalence of milkborne disease.

Potential for implementation of raw milk consumption

So how should we weigh the benefits and risks of raw milk consumption? The protective association with atopic disease has only been shown for a certain age group (early life including pregnancy, see *Chapter 3*) although a prevention of atopic disease in early life

might continue into a disease free adulthood[34]. Also, so far it has only been shown in specific industrialized regions. The potential health hazard, however, is real for all age groups and in all regions of the world, in some more in some less. Ironically, the health hazard is especially high for individuals (pregnant women with developing fetuses and newborns)[167,168] who were found to be protected from atopic disease by raw milk consumption. In countries with low hygienic standards, raw milk consumption would pose a too high risk for these individuals but also in highly developed countries a commercially available raw milk product would require enormous efforts to ensure health safety. This was exemplified by an observation made in Northern Italy where raw milk has been allowed to be sold in vending machines since 2004. The raw milk was tested for pathogens and did not meet criteria fixed by the law in terms of safety for hygienic quality.[164] Giacometti et al. listed core necessities for the management and control of a commercial raw milk chain: i) authorization to produce and sell raw milk, ii) implementation of good dairy farming practices and iii) appropriate handling procedures and control of the cold chain at the farm, during transport and in vending machine.[164] A further necessity would be rigorous surveillance programs of producers and product quality.

During the course of this discussion it became evident that safety concerns and logistics do not allow a straightforward Swiss-wide (or elsewhere) implementation of raw milk consumption. A way to circumvent these limitations could be the production of a safe product which still exerts potentially protective effects of raw farm milk. To meet demands of a low processed yet pathogen-free milk product, alternatives to traditional thermal processing such as pulsed electric fields, high hydrostatic pressure, irradiation and filtration techniques are already under investigation[51](a patent application based on the findings in *Chapter 3* has been filed by members of the GABRIEL study group and is currently pending[169]). Such a native milk product could then be distributed like pasteurized milk and would also be ideal to elucidate the protective “raw milk effect” in future studies (see *Chapter 7: Outlook*). Another option would be to use isolates or preparations of protective components in milk for clinical applications. Effects and applicability of whey protein isolates, fatty acids and microoranisms (probiotics) are under investigation.

7.2.6 Outlook

Ideas and necessities for future investigations and implementation of findings can be derived from arguments made throughout this discussion. Considering the results from the cross-sectional GABRIEL study, a confirmation of these results with longitudinal data is needed. As a matter of fact, milk consumed by the participating children was also collected during the PASTURE cohort study at multiple time points during early life. Due to financial constraints these samples were kept in storage and have not been analyzed yet (for a nested case controls study fatty acid patterns were measured in only about 100 samples). An extensive objective assessment of proteins, fat and microorganisms as performed in the GABRIEL study would make it possible to relate these milk components to atopic health outcomes. With the prospective design, the components ingested over time in early life could be related to outcomes occurring after the repeated exposures or to the development of the outcome over time. With these analyses the inverse associations of whey proteins with asthma found with GABRIEL data in *Chapter 3* (which were only based on a one-point assessment of exposure) could be tested and the influence of timing and impact of continued exposure could be further explored. Raw farm milk consumption was inversely associated with asthma, atopy, hay fever and atopic dermatitis on an epidemiological level (GABRIEL phase 2) but due to the study design it was only possible to relate milk constituents to asthma and atopy. Information on all these outcomes is now available up to age 6 in the PASTURE study which would allow a comprehensive analysis of how raw milk consumption and its components are associated with a multitude of different atopic health outcomes.

Compared with asthma, atopy was not associated with whey proteins and other milk components might be relevant to explain the protective “farm milk effect” on atopy. The same may or may not be true for hay fever and atopic dermatitis. To find this out, however, microbial assessment of milk samples must be more advanced than in the GABRIEL study. As mentioned before (see *Chapter 7: Methodological considerations: microbial exposure assessment*), DNA-based or even newer methods are needed to capture the diversity and potential key organisms which may remain undetected with culture methods. A drawback of observational studies is that we can only “observe” what kind of milk children are exposed to and these types of milks can only be categorized into relatively rough heating categories (e.g. raw or boiled farm milk, pasteurized or UHT commercial milk). As demonstrated with GABRIEL data (*Chapter 3*), whey proteins are very heat sensitive and only small changes in heat treatment lead to a substantial inactivation of one protein whereas other proteins may

remain relatively unharmed. With these rough categories we cannot assess these fine differences in heating steps and are thus not able to separate the effects of individual proteins. To avoid this and other problems of observational studies we need to control all aspects of our exposure. Envisaging approaches in the more distant future, the epidemiological gold standard of an intervention study may become possible. Ideally, all the milk for the study should come from the same source (e.g. specific cow breed, same fodder, with the same processing methods) considering the season of milking (to account for seasonal fodder changes and impact on milks fatty acid composition) as opposed to commercial cow milk which is pooled milk from different farms. The intervention would then be administering milk processed at specific temperatures (e.g. raw and specific temperatures at which relevant proteins are inactivated/ denatured). An example would be to administer this milk at a certain frequency during pregnancy or early life and to assess gene expression of innate immunity receptors and atopic health outcomes in early life. Nowadays such a study would not be possible due to ethical constraints. As discussed before, raw milk consumption always comes with a certain health risk due to pathogens. The plan for the more distant future would be to produce pathogen-free native milk by novel non-thermal processing techniques and to use this milk for the trial described above. This might not allow to assess potentially protective "microbial effects" because microorganisms will be removed from all milk types.

In PASTURE, the association of dietary factors in early life was only assessed for atopic dermatitis and these analyses should be continued with asthma, hay fever and atopic sensitization. Future investigations should include objective measurements of complementary foods (e.g. microorganisms, fatty acids) and have bigger sample sizes to assess associations with early and late forms of atopic dermatitis. We established that farm and dietary exposures which were associated with lowered risk for atopic disease in childhood were also associated with gene expression of innate immunity receptors in early life. How these receptors in turn are associated with atopic disease and whether this is a causal chain from exposure over innate immunity to lowered risk for atopic disease is completely unresolved. With data from the PASTURE study it will be possible in upcoming analyses to relate the gene expression of innate immunity receptors between birth and school age to atopic outcomes and get an idea whether there are such causal relationships. Varying susceptibilities to "effects" of farming and raw milk exposure on innate immunity or atopic disease due to genetic variations appear to be of minor importance. The importance of gene-environment interactions is still not clear and should be further assessed in future studies investigating these associations.

Farm related exposure and atopic disease in developing countries

Further studies are needed to investigate associations of farm and dietary factors and related lifestyle with atopic disease in childhood in developing countries. Studies in non-Western countries are scarce and their findings are inconsistent. For example, investigations in Belarus, Chile and Nepal found that contact to farm animals in childhood was associated with lower risk for asthma, wheeze or eczema[170-172] whereas in China, Mexico and Vietnam contact to farm animals or farming was associated with an increased risk for asthma.[173-175] A recent analysis of the ISAAC study with 6-7 year olds concluded that exposure to farm animals during pregnancy and first year of life was positively associated with atopic disease at school age on a global level.[176] This global association was based on positive associations in non-affluent countries whereas in affluent countries, no protective associations with farm animals were found. This is in contrast to results from many studies in affluent countries (see *Chapter 1: Farming environment*).[29] Studies in developing countries so far lacked the inclusion of rural population, objective assessment of outcomes and exposures or consideration of parasitic infections. Exposure to parasites may in fact be important to understand these heterogeneous results between developed and developing countries as well as inconsistent findings between developing countries. Parasitic infections were generally associated with lower risk for asthma and atopy[177] but infections with certain helminths (*Ascaris, Toxocara*) from animal sources were reported to contribute to increased occurrence of atopic disease in childhood, diminished lung function or exacerbation of asthmatic symptoms.[178-182]

Findings in developing countries might in the end not be directly comparable to protective associations found in Europe because of different meanings and definitions of "farming" and "farm environment" but it is necessary to understand whether the hygiene hypothesis also applies to developing countries. If protective agents and habits could be identified and respective measures could be implemented in developing countries in a timely fashion, the expected rise of atopic disease associated with westernization might be avoidable. This research would further be "unique in having the potential simultaneously to help understand two extremely common diseases, one being one of the most common diseases in developing countries [parasitic disease] and the other one of the most common diseases in developed countries [atopic disease]"[183].

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CURRICULUM VITAE

Georg Johannes Loss was born on 11 November 1982 in Bregenz, Austria, grew up in Villach, Austria and studied in Vienna, Austria, Hanoi, Vietnam, Basel, Switzerland and London, England.

Education

1989 - 1993 Elementary school: Kevenhüller Volksschule in Villach, Austria.

1993 - 2001 Secondary school: Perau-Gymnasium in Villach, Austria. Graduated with distinction.

2001 - 2002 Military service in Klagenfurt, Austria.

2002 - 2006 **Bachelor program in Food- and Biotechnology** at the University of Natural Resources and Life Sciences, Vienna, Austria

2006 - 2008 **Master program in Food Science and Technology** at the University of Natural Resources and Life Sciences, Vienna, Austria. Graduated with distinction.

2008 Master thesis at the Institute for Biological- Food Technology at the Hanoi University of Technology in Hanoi, Vietnam.

2009 - 2011 **Postgraduate Diploma in Epidemiology** at the London School of Hygiene and Tropical Medicine, University of London, England.

2009 - 2012 **PhD student in Epidemiology** at the Swiss Tropical and Public Health Institute, Basel, Switzerland.

Work experience

1999/2000 Busboy, Hotel-Restaurant Südrast in Arnoldstein, Austria

2002 Fine food salesperson, SPAR in Velden am Wörthersee, Austria.

2003/2004 German teacher, „ActiLingua Language School für Deutschkurse“ at Theresianum, Vienna, Austria

2005/2006 Internship: Department for Microbiology, Gerot Pharmaceuticals, Vienna, Austria

2005/2006 Internship: Clinical Laboratory “Blutlabor Dr. Holzweber”, Villach, Austria

2008 English teacher, „Hello Kids English School for Children“ in Hanoi, Vietnam

Teaching and Training

2005 - 2007 Tutor for practical courses “Process engineering” and “Mechanical and Thermal Process Technology“ at the Institute for Chemical and Energy Engineering at the University of Natural Resources and Life Sciences, Vienna, Austria

2011 Training of medical doctoral student

Awards and scholarships

2007 “Wissenschaftliches Förderstipendium” (scholarship) from University of Natural Resources and Life Sciences, Vienna, Austria.

2007/2008 “Leistungsstipendium” (scholarship) from University of Natural Resources and Life Sciences, Vienna, Austria.

2009 Invited to „Profil- High Potential Day for promising graduates 2009“, Vienna, Austria.

2009 Pediatric Respiratory Epidemiology Abstract Award, European Respiratory Society

2012 Young Scientist Award (Nachwuchsforscherpreis): “science for health”, Yakult Germany GmbH.

2012 Swiss School for Public Health Award for “best published PhD article in public health” in 2011

List of publications

Böcking C, Genuneit J, Büchele G, Loss G, Ege M, Pekkanen J, Dalphin JC, Riedler J, Lauener R, von Mutius E, Renz H, Braun-Fahrländer C, Pfefferle PI on behalf of the PASTURE/Efraim-Study-group. Milk serum fatty acid composition is associated with preschool asthma but does not correspond with fatty acid pattern in serum in children from the PASTURE/EFRAIM study. J Allergy Clin Immunol; submitted 11 July 2012.

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Listed as study group author

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Congress contributions

Loss G, Waser M, Kneifel W, Apprich S, von Mutius E, Riedler J, Genuneit J, Braun-Fahrländer C, the GABRIEL study team. Can farm milk consumption prevent allergic diseases? First results from the GABRIEL advanced studies. European Respiratory Society Annual Congress; 2009; Vienna, Austria; 2009.

Abstract and oral presentation

Loss G, Waser M, Kneifel W, Apprich S, von Mutius E, Riedler J, Genuneit J, Braun-Fahrländer C, the GABRIEL study team. Can farm milk consumption prevent allergic diseases? First results from the GABRIEL advanced studies. Milk Conference; 2009; Vienna, Austria; 2009.

Abstract and oral presentation

Loss G, Braun-Fahrländer C, the GABRIEL study team. Can farm milk consumption prevent allergic diseases? Milk Seminar at the Swiss Tropical and Public Health Institute; 2010; Basel, Switzerland; 2010.

Oral presentation

Loss G, Apprich S, Waser M, Kneifel W, von Mutius E, Genuneit J, Büchele G, Weber J, Sozanska B, Danielewicz H, Horak E, van Neerven RJJ, Heederik D, Lorenzen PC, Braun-Fahrländer C, the GABRIELA study group. The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study. Organic Food Quality and Health Research Conference; 2011; Prague, Czech Republic; 2011.

Abstract and poster presentation

Loss G, Apprich S, Waser M, Kneifel W, von Mutius E, Genuneit J, Büchele G, Weber J, Sozanska B, Danielewicz H, Horak E, van Neerven RJJ, Heederik D, Lorenzen PC, Braun-Fahrländer C, the GABRIELA study group. The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study. European Academy of Allergy and Clinical Immunology Congress; 2011; Istanbul, Turkey; 2011.

Abstract and oral presentation

Loss G, Apprich S, Waser M, Kneifel W, Braun-Fahrländer C, the GABRIELA study group. The effect of farm milk on childhood asthma and allergies: The GABRIELA study. Swiss Public Health Conference; 2011; Basel, Switzerland; 2011.

Abstract, oral presentation and session chair

Loss G, Apprich S, Waser M, Kneifel W, von Mutius E, Genuneit J, Büchele G, Weber J, Sozanska B, Danielewicz H, Horak E, van Neerven RJJ, Heederik D, Lorenzen PC, Braun-Fahrländer C, the GABRIELA study group. Effect of farm milk on development of asthma and allergies. International Society for Environmental Epidemiology Conference; 2011; Barcelona, Spain; 2011.

Abstract and invited oral presentation

Loss G, Bitter S, Frei R, Roduit C, Lauener R, Genuneit J, Pekkanen J, Dalphin ML, Riedler J, von Mutius E, Braun-Fahrländer C, the PASTURE study group. Prenatal and early life exposures alter the expression of innate immunity genes: The PASTURE cohort

study. Global Risk Forum- One Health Summit; 2012; Davos, Switzerland; 2012.
Abstract and oral presentation

Loss G, Bitter S, Frei R, Roduit C, Lauener R, Genuneit J, Pekkanen J, Dalphin JC, Riedler J, von Mutius E, Braun-Fahrländer C, the PASTURE study group. Gene expression of innate immunity receptors is associated with prenatal and early life exposures: The PASTURE cohort study. European Academy of Allergy and Clinical Immunology Congress; 2011; Geneva, Switzerland; 2012.
Abstract and oral presentation

Loss G. Der protektive Effekt von Rohmilch auf Asthma und allergische Erkrankungen bei Kindern. Yakult Kolloquium „Probiotika in Praxis und Forschung"; 2012; Bonn, Germany; 2012.
Abstract and oral presentation

GABRIEL and PASTURE/EFRAIM study group meetings

Oral presentations and discussions in Vienna (Austria), Frankfurt, Günzburg, Munich, Tutzing, Ulm (Germany), Davos, Basel, Zürich (Switzerland), Amsterdam, Utrecht (The Netherlands).

Peer reviewer for

Pedriatic Allergy and Immunology, 2010

Patents based on this thesis

von Mutius E, Braun-Fahrländer C, Kneifel W, inventors; Prävention von Asthma bronchiale, allergischen und Autoimmun-Erkrankungen mittels aus Rohmilch gewonnenen bioaktiven Komponenten; European patent application number EP 11 005 880.7. applied 18 July 2011.

Course list

Course Title	Date	ECTS	Location
Various			
BOMS- Basics of medical statistics. Internetlehrgang der Universität Basel	Feb.-Mar. 2009	1.5	Basel
BEPI- Basic Epidemiology, Internetbasierter Lehrgang des ISPM	Feb.-Mar. 2009		Basel
ERS Postgraduate Course: "Asthma: Pathology and treatment"	12.09.2009		ERS Congress, Vienna
Swiss Tropical Institute: Research Seminar	HS 2010	1	Swiss TPH Basel
Master of Public Health Courses			
Einführung in die Statistiksoftware STATA	27.04-29.04	1.5	ISPM Bern
Umwelt und Gesundheit	03.-05.06. & 25.-26.06.2009	2	ISPM Basel
Statistische Methoden zum Umgang mit Confounding und Interaktionen in epidemiologischen Studien	05.08.-07.08.2009	1.5	ISPM Basel
Swiss School of Public Health + Courses			
Multilevel Modeling: Analysis of clustered data	04.11.-06.11.2009	1.5	ISPM Basel
Writing a journal article and getting it published	13.01.-15.01.2010	1	ISPM Bern
Media Training Workshop: Communication Skills	06.07.2010	0.25	ISPM Bern
How to pass your research protocol through an ethics review committee	29.11.2010	0.25	University of Zurich
Analysis of data with non-detects	14.02.-16.02.2011	1	Swiss TPH Basel
Systematic reviews and meta-analysis: A practical approach	10.03.-11.03.2011	0.75	ISPM Bern
Postgraduate Diploma in Epidemiology at London School of Hygiene and Tropical Medicine			
Fundamentals of Epidemiology	Oct. 2009- Jun. 2010	5	LSHTM DL
Statistics with computing (STATA)	Oct. 2009- Jun. 2010	5	LSHTM DL
Practical Epidemiology	Oct. 2010- Jun. 2011	5	LSHTM DL
How to write and review a paper	Oct. 2010- Jun. 2011	5	LSHTM DL
London School of Hygiene and Tropical Medicine			
Introduction to Genetic Epidemiology in the GWAS era	06.09-09.09.2011	1	LSHTM

APPENDIX

Excerpts from GABRIEL-A questionnaires

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Milk

The following questions concern the milk that your child drinks. We distinguish between milk that comes directly from the farm and milk that was bought in a store.

Milk directly from the farm

We mean the milk that was produced or acquired directly on a farm (for example from barnyard sales, milk stations). We are interested to know if you boil or skim the milk before you drink it.

m75 **75) Did your child, or the mother during the pregnancy with the child, regularly (meaning at least once a month over half a year) drink milk directly from a farm?**

☐ Yes 1 ☐ No 0 ➔ If no, please continue with the question **81**

76) How often did your child drink milk directly from the farm that was neither boiled nor skimmed?

		At least once a day	1 – 6 times a week	Less than once a week	Never
m76_1 , m76_1x - _1xx	During pregnancy	☐ 3	☐ 2	☐ 1	☐ 0
m76_2 , m76_2x - _2xx	In the first year of life	☐ 3	☐ 2	☐ 1	☐ 0
m76_3 , m76_3x - _3xx	In the 2. and 3. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m76_4 , m76_4x - _4xx	In the 4. and 5. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m76_5 , m76_5x - _5xx	In the last 12 months	☐ 3	☐ 2	☐ 1	☐ 0

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study_id

77) How often did your child drink milk directly from the farm that was boiled but not skimmed?

		At least once a day	1 – 6 times a week	Less than once a week	Never
m77_1 , m77_1x - _1xx	During pregnancy	☐ 3	☐ 2	☐ 1	☐ 0
m77_2 , m77_2x - _2xx	In the first year of life	☐ 3	☐ 2	☐ 1	☐ 0
m77_3 , m77_3x - _3xx	In the 2. and 3. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m77_4 , m77_4x - _4xx	In the 4. and 5. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m77_5 , m77_5x - _5xx	In the last 12 months	☐ 3	☐ 2	☐ 1	☐ 0

78) How often did your child drink milk directly from the farm that was skimmed but not boiled?

		At least once a day	1 – 6 times a week	Less than once a week	Never
m78_1 , m78_1x - _1xx	During pregnancy	☐ 3	☐ 2	☐ 1	☐ 0
m78_2 , m78_2x - _2xx	In the first year of life	☐ 3	☐ 2	☐ 1	☐ 0
m78_3 , m78_3x - _3xx	In the 2. and 3. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m78_4 , m78_4x - _4xx	In the 4. and 5. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m78_5 , m78_5x - _5xx	In the last 12 months	☐ 3	☐ 2	☐ 1	☐ 0

79) How often did your child drink milk directly from the farm that was boiled and skimmed?

		At least once a day	1 – 6 times a week	Less than once a week	Never
m79_1 , m79_1x - _1xx	During pregnancy	☐ 3	☐ 2	☐ 1	☐ 0
m79_2 , m79_2x - _2xx	In the first year of life	☐ 3	☐ 2	☐ 1	☐ 0
m79_3 , m79_3x - _3xx	In the 2. and 3. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m79_4 , m79_4x - _4xx	In the 4. and 5. year of life	☐ 3	☐ 2	☐ 1	☐ 0
m79_5 , m79_5x - _5xx	In the last 12 months	☐ 3	☐ 2	☐ 1	☐ 0

mom17_1 mom17_2 mom17_3
-> mom17x

study_id

Short questionnaire concerning the collection of cow milk

Please refer to the cow milk sample you filled in the test tube when answering the following questions!

1. When did you fill the cow milk sample into the test tube?

DD mom1_1 | MM mom1_2 | YY mom1_3 -> mom1x | HH mom1_4 : MM

2. Temperature of the cow milk at the time of the sample drawing:

□□.□ °C
mom2_1

3. What sort of cow milk is it?

mom3

Cow milk directly coming from one's own or another farm — 1 ○ ⇨ please go on with question 4!

Cow milk from the supermarket or shop — 2 ○ ⇨ please go on with question 7!

Please do only answer questions 4 – 6, if the cow milk you filled in the test tube was coming directly from a farm (your own or another one)!

4. Where and how long was the cow milk being stored ...

a. ...from the time of the milking until the last preparation for the child?

-> mom4ax
mom4a | mom4a1_1 | mom4a1_2 | mom4a1_3 (mom4a1_23x)

In the fridge — 1 ○ at ca. □□ °C ca. □□ Days □□ Hours

In the pantry — 2 ○ at ca. □□ °C ca. □□ Days □□ Hours
mom4a2_1 | mom4a2_2 | mom4a2_3 (mom4a2_23x)

Other depository: — 3 ○ □□□□□□□□□□□□□□□ (mom4a3_4) at ca. □□ °C ca. □□ Days □□ Hours
mom4a3_1 | mom4a3_2 | mom4a3_3 (mom4a3_23x)

Excerpt from PASTURE questionnaire: age 1

35. Hat die Mutter <u>während der Stillzeit</u> eines oder mehrere der folgenden Medikamente eingenommen?

	ja	nein	
Asthmamedikamente (Tabletten, Sprays, Inhalationen)	❒ 1	❒ 2	E35_01
Heuschnupfenmedikamente	❒ 1	❒ 2	E35_02

36. Hat die Mutter <u>während der Stillzeit</u> irgendwelche Antibiotika eingenommen?

E36

Ja ❒ 1

Nein ❒ 2 ⇨ weiter mit Frage 38

37. Hat die Mutter in dieser Zeit, während sie Antibiotika einnahm,

	ja	nein	
weiter gestillt?	❒ 1	❒ 2	E37_01
die Milch abgepumpt und verworfen?	❒ 1	❒ 2	E37_02

38. Hat Ihr Kind seit unserem letzten Hausbesuch Kuhmilch oder Ziegenmilch (verdünnt oder unverdünnt) getrunken?
Bitte beachten Sie, dass hier <u>nicht</u> Säuglingsmilchprodukte gemeint sind.

	ja	nein	
Kuhmilch direkt vom Bauernhof, ohne Abkochen	❒ 1	❒ 2	E38_01
Kuhmilch direkt vom Bauernhof, abgekocht	❒ 1	❒ 2	E38_02
Vorzugsmilch	❒ 1	❒ 2	E38_03
Schweiz: Vorzugsmilch (z.B. Demeter), Rohmilch			
Österreich: keine Antwortkategorie Vorzugsmilch!			
Pasteurisierte Frischmilch (Kuhmilch)	❒ 1	❒ 2	E38_04
H-Milch (Kuhmilch)	❒ 1	❒ 2	E38_05
Schweiz: UHT-Milch, H-Milch			
Zubereitungen aus Milchpulver (Kuhmilch)	❒ 1	❒ 2	E38_06
Nicht pasteurisierte Ziegenmilch	❒ 1	❒ 2	E38_07

39. Wie alt war Ihr Kind, als es <u>zum ersten Mal</u> Kuhmilch direkt vom Bauernhof getrunken hat?

Mein Kind hat noch keine Kuhmilch direkt vom Bauernhof getrunken ..... ❒ 1 [not ticked = 2] [E39_01]

Kuhmilch direkt vom Bauernhof, ohne Abkochen ...Alter: ____ Monate [E39_02]

Kuhmilch direkt vom Bauernhof, abgekocht .....Alter: ____ Monate [E39_03]

40. Hat Ihr Kind jemals Käse gegessen?

[E40]

Ja..... ❒ 1

Nein ..... ❒ 2⇨ weiter mit Frage 42

41. Welche Käsesorte hat Ihr Kind <u>überwiegend</u> gegessen? Bitte geben Sie die Sorte und den Markennamen an

Sorte: [E41_01] Marke: [E41_02]

K1. Welche Art von Käse haben Sie Ihrem Kind gefüttert?

Ausschließlich Käse aus pasteurisierter Milch.....❒

Ausschließlich Rohmilchkäse ..... ❒

Sowohl Käse aus pasteurisierter Milch, als auch Rohmilchkäse..... ❒

Käse aus Milch vom eigenen Bauernhof ..... ❒

Diese Frage nur in Frankreich!