Hygiene hypothesis and innate immunity: Evaluating the role of environmental factors and genetic polymorphisms on the expression of receptors of the innate immune system

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Content

Summary

Background

In ‘westernised’ countries, the prevalence of childhood asthma and allergy has risen throughout the last few decades. Changes in lifestyle and environmental factors like an increase in exposure to air pollutants, environmental tobacco smoke, or indoor allergen and pet exposure have been considered as plausible explanations. However, little evidence in support of these causal risk factors for these common chronic childhood diseases has been found. Lower risk of hay fever and atopic sensitisation were reported in children with a higher number of siblings, and later also in children who attended day-care centres early in infancy. These findings were summarised in the so-called ‘hygiene hypothesis’: Limited exposure to bacterial and viral pathogens during early childhood results in a higher risk of developing allergic diseases.

A series of epidemiological studies in Europe, Canada, and Australia showed reduced prevalence of asthma and allergy among farmers’ children compared to non-farmers’ children. Stable visits early in life and consumption of raw cow’s milk were suggested as the main factors of the farming environment conferring protection against atopic diseases. These results have been seen as an extension of the ‘hygiene hypothesis’, since a farm environment provides an enormous habitat for microorganisms.

Pattern-recognition receptors (PRR) of the innate immune system, such as toll-like receptors (TLR) or CD14, recognize LPS (lipopolysaccharide), a component of the outer membrane of gram-negative bacteria, and other nonviable environmental compounds. Activation of PRR signalling pathways initiates regulatory mechanisms which in turn modulate the adaptive immune response. Interestingly, recently it has been shown that farmers’ children express higher levels of PRR than children from non-farming families suggesting that innate immune mechanisms are involved in the allergy-protective effect of the farming environment.

For various genetic loci, i.a. the CD14 gene, an association with the occurrence of atopic diseases have been described. However, studies investigating the same genetic variants in other populations often failed to reproduce the original results.

Gene-environment interactions have been found for several genetic polymorphisms in PRR genes, therefore differing environmental exposures between study populations might explain the conflicting results of genetic studies.

Objectives

To assess factors of the farming environment that influence the innate immune system with respect to gene expression of crucial proteins of innate immunity pathways and test if this association is modulated by unknown genetic factors. We expected that results might give a deeper understanding of the immunological pathways and the complex relationship between environment, genes and atopic disease.

Methods

The PARSIFAL study (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) was a cross-sectional survey in rural areas of Austria, Germany, the Netherlands, Sweden, and Switzerland. 14893 children aged 5-13 years belonging to four study groups (farmer's children, children attending Steiner schools, and their respective control groups) completed a standardised questionnaire including questions about asthma and allergy, the child's activities on farms, and characteristics of the home environment. In subpopulations, further data was available. Blood samples were obtained from selected children and tested for atopic sensitisation, especially for specific IgE antibodies to common inhalant and food allergens. Indoor dust samples were collected to determine exposure to microbial compounds (LPS and fungal extracellular polysaccharides). Furthermore, gene expression measurements and SNPs (single nucleotide polymorphisms) of PRR were available.

Results

Gene expression of CD14, TLR2, and TLR4 was higher in farmers' children compared to non-farmers' children. Mainly prenatal factors accounted for these differences. Expression of PRR was higher in children when the mother worked on the farm during pregnancy. In addition, we observed a dose-dependent increase in gene expression of CD14, TLR2 and TLR4 with the number of farm animal species the mother had contact with during pregnancy, which probably serves as proxy for an

increasing variation in microbial exposure. Prenatal factors also exert their effect on the child's health later in life. Children of mothers who worked on the farm during pregnancy were less sensitised at school age to common inhalant and food allergens than children of unexposed mothers. However, the development of clinical symptoms of atopic diseases seemed to depend on exposures that occurred postnatally.

Farm milk consumption was significantly associated with lower prevalence of atopic sensitisation and atopic health outcomes. Of particular importance is the consistency of the findings across the four study groups. The inverse association was not explained by concurrent farm activities of the child and was most pronounced in children drinking farm milk since their first year of life. Farm milk consumption and other specific farm-related exposures (pig farming, feeding silage, child's involvement in haying, and regular stay in animal sheds and barns) taken together explained the protective effect of being a farm child on asthma.

Neither of the genotyped SNPs of innate immunity genes was directly associated with differential gene expression. However, a polymorphism in the CD14 gene (*CD14/A-1721G*) significantly modified the effect of farm milk consumption on CD14 gene expression. Farm milk drinking children homozygous for the A allele expressed significantly more CD14 than non-exposed children. No association between farm milk drinking was observed in children with the GG genotype, heterozygous children showed an intermediate difference in CD14 gene expression between exposed and non-exposed children. We observed the same interaction between genotypes of *CD14/A-1721G* and farm milk exposure on asthma and related atopic health outcomes. Moreover, we were able to replicate the gene-environment interaction on atopic health outcomes in two independent populations.

We compared RNA quality in two epidemiological studies using different tubes for RNA sampling (EDTA and RNA stabilizing tubes). The amount of RNA that could be extracted from the blood samples was lower in the EDTA tubes leading to higher proportions of blood samples unavailable for measurement of gene expression. Moreover, we found decreasing agreement between samples repeatedly measured suggesting RNA degradation over time. Important recommendations for future epidemiological studies measuring gene expression could be developed.

Conclusions and Outlook

Whereas the manifestation of atopic diseases such as asthma and allergies depends on postnatal exposures, protection against atopic sensitisation is conferred by prenatal exposure to a farming environment. Immune modulation by activation of innate immune mechanisms might underlay these observations. However, cross-sectional studies are not appropriate for drawing conclusions regarding the temporal sequence of events and, therefore, our results need confirmation by longitudinal studies. The ongoing PASTURE cohort study will address these questions as longitudinal clinical and immunological data will be available.

Biological data of the composition of farm milk was not available in PARSIFAL. Thus, the allergy-preventive ingredients contained in the farm milk are still unknown. Within the ongoing cross-sectional GABRIEL study milk samples from children's homes will be sampled and components analysed regarding their allergy-protective potential.

Our results of the genetic analyses are limited due to the insufficient power of the PARSIFAL study. In future the ongoing large-scale GABRIEL study will allow adequately powered genetic analyses to be conducted. Genome-wide association studies and gene-environment-interaction studies in the GABRIEL population will result in a deeper insight into the genetics of atopic diseases.

Current scientific evidence has not developed strongly enough to provide a reliable course of action for primary prevention or therapy. Infectious diseases resulting from exposure to pathogens continue to be a serious public health problem. However, further investigation and characterisation of environmental compounds conferring protection against atopic diseases is promising and will hopefully result in future efficient preventive measures.

Zusammenfassung

Hintergrund

In westlichen Ländern wurde über die letzten drei Jahrzehnte beobachtet, dass Asthma und Allergien rasch und deutlich zugenommen haben. Mögliche Erklärungen sind Änderungen des Lebensstils und in der Exposition gegenüber Umweltfaktoren wie Luftschadstoffen, Passivrauch oder häuslichen Allergenen und Haustieren. Ein direkter kausaler Zusammenhang mit diesen Risikofaktoren konnte aber bisher nicht schlüssig gezeigt werden. Kinder mit mehreren Geschwistern und Kinder, die früh in ihrem Leben Kinderbetreuungsstätten besuchten, zeigten ein niedrigeres Risiko, an Heuschnupfen und allergischer Sensibilisierung zu erkranken. Diese Befunde wurden in der sogenannten „Hygienehypothese“ zusammengefasst: Eine eingeschränkte frühkindliche Exposition gegenüber bakteriellen und viralen Pathogenen führt zu einem erhöhten Risiko, eine allergische Krankheit zu entwickeln.

Epidemiologische Studien aus Europa, Kanada und Australien zeigten niedrigere Prävalenzen für Asthma und Allergien bei Bauernkinder gegenüber Nichtbauernkindern. Dabei gibt es deutliche Hinweise, dass vor allem regelmässige Stallbesuche und der Konsum von roher Kuhmilch vor Erkrankungen des atopischen Formenkreises schützen. Diese Resultate werden als eine Erweiterung der „Hygienehypothese“ gesehen, da ein bäuerliches Umfeld als riesiges Biotop für Mikroorganismen betrachtet werden kann.

Pattern-recognition Rezeptoren (PRR) des angeborenen Immunsystems, wie z. B. Toll-like Rezeptoren oder CD14, erkennen LPS (Lipolysaccharid), eine Komponente der äusseren Zellmembran Gram-negativer Bakterien, und andere Stoffe aus unserer Umwelt. Aktivierung von zellulären PRR-Signalwegen löst regulatorische Mechanismen aus, die wiederum das adaptive Immunsystem beeinflussen können. Interessanterweise konnte kürzlich gezeigt werden, dass Bauernkinder grössere Mengen an PRR exprimieren als Kinder aus Nichtbauernfamilien. Dies deutet darauf hin, dass das angeborene Immunsystem am Schutz der bäuerlichen Umgebung vor atopischen Erkrankungen beteiligt ist.

Für verschiedene Genloci, u. a. das CD14-Gen, konnte eine Assoziation mit dem Auftreten atopischer Erkrankungen beschrieben werden. Häufig konnten jedoch diese Resultate in anderen Populationen nicht reproduziert werden. Gen-Umwelt-Interaktionen konnten für verschieden Polymorphismen in Genen, die für PRR kodieren, gezeigt werden. Expositionsunterschiede in den verschiedenen Studienpopulationen sind deshalb eine mögliche Erklärung für die widersprüchlichen Resultate.

Ziel

Faktoren der bäuerlichen Umfelds zu identifizieren, die das angeborene Immunsystem bezüglich Genexpression von wichtigen Proteinen in dessen Signalwegen beeinflussen und überprüfen, ob diese Zusammenhänge von unbekannten genetischen Einflüssen verändert werden. Wir erwarteten, dass unsere Resultate ein tieferes Verständnis für das komplexe Zusammenspiel zwischen Umwelt, Genen und atopischen Erkrankungen ergeben.

Methoden

Die PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) Studie war eine Querschnittsstudie, die in ländlichen Gebieten von Österreich, Deutschland, Holland, Schweden und der Schweiz durchgeführt wurde. Die Eltern von 14893 Kinder im Alter zwischen 5 und 13 Jahren aus vier Studiengruppen (Bauernkinder, Kinder aus Steiner Schulen und ihre entsprechenden Kontrollgruppen) füllten einen standardisierten Fragebogen bezüglich der Gesundheit des Kindes (mit Schwerpunkt auf Asthma und Allergien), den kindlichen Akivitäten auf Bauernhöfen und des heimischen Umfelds aus. In Teilgruppen wurden weitere Daten erhoben. Blutproben zur Bestimmung der atopischen Sensibilisierung (spezifische IgE-Antikörper gegen häufige inhalative und Lebensmittelallergene) und Staubproben zur Messung der häuslichen mikrobiellen Exposition (LPS und extrazelluläre Polysaccharide von Pilzen) wurden gesammelt. Ausserdem, waren die Genotypen (SNPs, single nucleotide polymorphisms) und die Genexpression von Genen einzelner PRR verfügbar.

Resultate

Die Genexpression von CD14, TLR2 und TLR4 wär höher bei Bauernkindern als bei Nichtbauernkindern. Vor allem pränatale Faktoren waren für diesen Unterschied verantwortlich. Die Expression der PRR war höher bei Kindern, deren Mutter während der Schwangerschaft auf dem Bauernhof gearbeitet hat. Zusätzlich haben wir eine Dosis-Wirkung-Beziehung zwischen der Anzahl verschiedener Nutztiere, mit denen die Mutter während der Schwangerschaft Kontakt hatte und die wahrscheinlich ein Mass für die Variabilität der mikrobiellen Exposition darstellt, und der Geneexpression von CD14, TLR2 und TLR4. Pränatale Faktoren beeinflussten ausserdem die Gesundheit des Kindes im Schulalter: Kinder von Müttern, die während der Schwangerschaft auf dem Bauernhof gearbeitet haben, zeigten weniger häufig eine atopische Sensibilisierung als Kinder nicht exponierter Mütter. Das Auftreten von klinischen Symptomen atopischer Erkrankungen wurde jedoch vor allem von postnatalen Expositionen beeinflusst.

Der Konsum roher Kuhmilch war signifikant mit einer tieferen Prävalenz atopischer Sensibilisierung und Erkrankungen assoziiert. Hervorzuheben ist die Konsistenz der Resultate in allen vier Studiengruppe. Dieser schützende Effekt der Rohmilch konnte nicht mit kindlichen Aktivitäten auf dem Bauernhof erklärt werden und war am ausgeprägtesten bei Kindern, die seit ihrem ersten Lebensjahr Rohmilch konsumiert hatten. Der Konsum roher Kuhmilch und weitere für ein bäuerliches Umfeld typische Expositionen (Schweinehaltung, Verfütterung von Silofutter, Mithilfe des Kindes beim Heuen und regelmässige Aufenthalte in Stall oder Scheune) erklärten zusammen genommen den protektiven Bauerneffekt.

Keiner der genotypisierten SNPs von Genen des angeborenen Immunsystems war mit unterschiedlicher Geneexpression assoziiert. Ein Polymorphismus im CD14-Gen (*CD14/A-1721G*) modifizierte jedoch signifikant den Effekt von Rohmilchkonsum auf die Geneexpression von CD14. Für das A-Allel homozygote Kinder, die Rohmilch konsumierten, exprimierten mehr CD14 alles nicht exponierte Kinder. Diese Assoziation konnten wir in Kindern mit dem GG-Genotypen nicht beobachten. Ausserdem konnten wir dieselbe Interaktionen zwischen *CD14/A-1721G* und Rohmilchkonsum in Bezug auf die Entstehung atopischer Erkrankungen beobachten.

Zudem war es uns möglich die Gen-Umwelt-Interaktionen in zwei unabhängigen Populationen zu replizieren.

Wir verglichen die RNA-Qualität in zwei epidemiologischen Studien, in denen verschiedene Blutsammelsysteme (EDTA-Röhrchen und Röhrchen mit einem RNA-stabilisierenden Zusatz) verwendet wurden. Die RNA-Menge, die extrahiert werden konnte, war tiefer in den EDTA-Röhrchen und führte zu einem höheren Anteil für Genexpressionsanalysen nicht mehr verwendbare Proben. Außerdem konnten wir eine abnehmende Übereinstimmung zwischen Resultaten von RNA-Analysen, die mehrmals mit einem zeitlichen Abstand durchgeführt worden sind, feststellen. Dies kann am ehesten mit RNA-Degradataion im Verlauf der Zeit erklärt werden. Aus diesen Resultaten konnten wir wichtige Empfehlungen für kommende epidemiologische Studien, in denen Gene Expression gemessen wird, herleiten.

Schlussfolgerungen und Ausblick

Während die Manifestation atopischer Erkrankungen von postnatalen Faktoren abhängt, führt die pränatale Exposition gegenüber einem bäuerlichen Umfeld vor allem zum Schutz vor atopischer Sensibilisierung. Die Beeinflussung des Immunsystems durch Aktivierung der angeborenen Immunität könnte diesen Beobachtungen zu Grunde liegen. Querschnittsstudien sind jedoch nicht geeignet Rückschlüsse auf die zeitliche Abfolge von Ereignissen zu ziehen und entsprechende Resulate müssen durch longitudinale Studien bestätigt werden. Die laufende PASTURE-Kohortenstudie wird sich mit diesem Fragen beschäftigen können, da entsprechende longitudinale klinische und immunologische Daten gesammelt werden.

Biologische Daten bezüglich der Zusammensetzung von Rohmilch waren in der PARSIFAL-Studie nicht verfügbar. Der allergieprotektive Bestandteil der rohen Kuhmilch ist deshalb immer noch unbekannt. In der laufenden GABRIEL-Studie werden heimische Milchproben der Kinder gesammelt und bezüglich ihres allergieprotektiven Potenzials analysiert.

Die Resultate unserer genetischen Analysen sind durch die für diese Forschungsfragen geringe Studiengrösse limitiert. In Zukunft wird die größer angelegte GABRIEL-Studie adäquate genetische Analysen zu lassen. Genomweite

Assoziations- und Gen-Umwelt-Interaktions-Studien in der GABRIEL-Population werden zu einem tieferen Einblick in die Genetik atopischer Erkrankungen führen.

Die wissenschaftliche Beweislage ist noch zu wenig fortgeschritten, um zuverlässige Empfehlungen für die Primärprävention oder Therapie zu entwickeln. Infektionskrankheiten sind nach wie vor ein grosses Problem für die öffentliche Gesundheit. Intensive Forschungen und Charakterisierung von Bestandteilen in unserer Umwelt, die vor atopischen Erkrankungen schützen können, ist vielversprechend und wird in Zukunft hoffentlich zu wirksamen Präventivmassnahmen führen.

Chapter 1 General introduction and background

I Epidemiology of Asthma and Allergies

I.I Increase in Prevalence of Asthma and Allergies

Asthma and allergies (henceforth denoted as atopic diseases) are among the most common chronic diseases in childhood. Besides being the cause of extensive healthcare costs these diseases represent a physical and psychological burden for the affected children and their families. In the course of the last century atopic diseases showed a steady increase in prevalence [2]. In Switzerland the prevalence in adults raised from about 1% in 1926 to 13% in 1991 [3]. Nowadays, asthma is the third leading cause of hospitalization in U.S. children under 18 years of age, exceeded only by pneumonia and injuries [4]. This pronounced increase occurred in such a short time that experts refer to it as an “asthma epidemic”. Interestingly, this epidemic stands out through large regional differences. Whereas particularly ‘westernised’ countries have been affected by this rapid increase it occurred markedly less in developing countries. In Eastern Germany where drastic changes towards westernisation of living conditions have occurred after reunification of the Federal Republic of Germany and the German Democratic Republic an increase in the prevalence of hay fever (from 2.3 to 5.1%) and atopic sensitization (19.3 to 16.7%) has been documented in children aged between 9 and 10 years of age [5, 6]. It has to be noted, however, that in the last decade several authors have reported stabilizing prevalences of atopic diseases [7, 8].

I.II Environmental Risk Factors

This alarming trend opened up a whole area of research trying to identify the responsible factors for the ‘allergic epidemic’. De novo occurrence of genetic mutations is unlikely to account alone for the change in disease prevalence regarding the short time in which it was observed. Changes in lifestyle and environmental factors are therefore more plausible. Detrimental environmental factors like air pollution increased indoor allergen exposure (due to better house insulation, reduced indoor air ventilation and more carpeting on floors) or common pet ownership offered an explanation. However, a clear causal association between the environmental

exposure to air pollutants like particulate matter, NO2, SO2, or ozone in Europe and the development of childhood asthma and allergy has not been found [9, 10]. However, a number of studies have shown that short-term increase of exposure to air pollution and environmental tobacco smoke results in increasingly severe symptoms among asthmatic children [11-13] suggesting these factors to be a triggering co-factor rather than a factor initiating or inducing the asthmatic state. Similarly, the increase in the prevalence childhood asthma and allergy could not consistently be explained by increased indoor allergen exposure [14-16] or pet ownership [17, 18]. Several studies have even demonstrated a lower prevalence of childhood allergy prevalence in children exposed to pets early in life [19-21] – an observation that might be in favour of the idea that also a decrease in allergy-protective rather than a increase in allergy-prone environmental factors account for the increase in atopic diseases.

II The Hygiene Hypothesis

II.I Emergence of the Hygiene Hypothesis

In 1989, Strachan et al. reported a lower risk of hay fever and atopic sensitisation in children with a higher number of siblings [22]. He hypothesised that protection from allergic diseases might be acquired through infections in early childhood, transmitted by ‘unhygienic contacts with older siblings, or acquired prenatally’. Thus, more children, less improved household amenities and lower standards of personal cleanliness may be protective against developing allergic diseases. This interpretation is now known as the ‘hygiene hypothesis’. Krämer et al. infer that ‘if this hypothesis is true, early exposure to childcare outside the home would protect against atopy by promotion of cross infections’, and she could show higher prevalence of atopy among children who started to attend day nursery at an older age than in those who started to attend it at a younger age [23]. Since day-care attendance was more frequent among Eastern German children compared to Western German children the aforementioned German studies were in line with this data. Data from a longitudinal study provided further evidence showing that growing up with older siblings and also early attendance of a day-care centre were protective against the development of asthma later in childhood [24]. The hygiene hypothesis was given further support from Matricardi et al. who demonstrated in Italian military

cadets an inverse association between seropositivity of orofecal infections (hepatitis A, Toxoplasma gondii and herpes simplex virus 1), which may be regarded as a marker of poor hygiene, and atopy defined as a positive skin-prick test or increased specific IgE to common inhalant allergens [25].

II.II Extension of the Hygiene Hypothesis: Farm Studies

Until then the idea behind the hygiene hypothesis was that mainly exposure to living microbes and the number of undergone infections confer to the protection against atopic diseases. However, reports that persons working with hay rarely suffer from hay fever go back as far as the 19th century [26]. M. Gassner, a Swiss paediatrician from a rural area systematically collected serological data from 15-year-old school children in his village from 1983 and he observed that farmers' children suffer less from allergic diseases than their non-farming peers from the same village [27]. The cross-sectional Swiss SCARPOL study, a multicentre study originally designed to investigate associations between air pollution and respiratory and allergic symptoms in school children, offered to investigate this observation in a larger sample. In the 1620 SCARPOL children aged 6-15 years the prevalence of seasonal symptoms of hay fever and atopic sensitisation were significantly lower in children who were raised on a farm compared to non-farmers' children from the same rural areas [28]. The living conditions of farming families differed in this study population in many respects from living conditions of other families. Farming families had, in general, larger family sizes, higher numbers of pets, were more likely to heat with wood or coal, mothers were less likely to smoke, homes had more indoor dampness, and the families differed in dietary habits. However, none of these factors could explain the strong inverse association between atopy and growing up on a farm. The results were consistently confirmed in pediatric populations from Finland [29], Canada [30], Australia [31], Austria [32] and Germany [33]. The latter investigated school children aged 5-7 years in rural areas of Bavaria. The authors could show that contact with stable animals was inversely related to the prevalence of diagnosed hay fever, asthma and wheeze among farmers' children. Furthermore they observed a dose-response relationship of farming (non-farming, part-time farming and full-time farming) and risk for atopic disease.

II.III Which farm-related exposures protect from atopic diseases?

Box 1. Definition of farm milk.
In this thesis farm milk is of particular interest; thus clarification of the expression farm milk is needed. Herein "farm milk" denotes fresh cow's milk which has undergone no further industrial processing like pasteurization or homogenisation. However, treatment on the farm before consumption like heating or skimming is generally not known and specifically mentioned if respective data is available.

The farming environment is a 'human model' of an environment rich in opportunities for exposure against microorganisms. Based on previous findings, research teams from Germany, Austria, and Switzerland designed 1999 the cross-sectional ALEX (Allergy and Endotoxin) study to assess the role of environmental and lifestyle factors that affect the prevalence of allergy. Analyses of the 812 farmers' and non-farmers' children of the ALEX population led to novel insights into how an environment rich in microbial exposure might confer protection for atopic diseases. Riedler et al. published intriguing results showing that children who have been in stables or have been drinking farm milk during the first year of life had less asthma and hay fever than children who were exposed later in life or not at all [34]. Interestingly, the lowest prevalence of asthma and hay fever was found in children who were exposed to stables *and* farm milk in the first year of life. However, the ALEX population did not provide enough power to disentangle the individual effects of the two exposures.

Further analyses of the ALEX data by Braun-Fahrländer et al. revealed a negative association between exposure to lipopolysaccharides (LPS or endotoxin, see also section III.II), an integral part of the outer cell membrane of Gram-negative bacteria, and atopic asthma, hay fever and atopic sensitisation [35]. Intriguingly, in the same publication an impaired innate immune response determined by a down-regulation of cytokine production in children highly exposed to LPS was reported. The authors suggest that long-term, high-level environmental exposure to LPS might favour a state of tolerance, which in turn prevents the development of allergic immune responses.

II.IV The exposure has to take place early in life

Exposures of the mother during pregnancy and its influence on the fetus have been investigated in some detail for other exposures namely smoking [36], intake of

antibiotics [37] or paracetamol [38] or exposure to allergens [39]. There is also evidence from animal models that prenatal exposure to microbial compounds prevent allergic disease in the offspring [40, 41]. Similarly, the aforementioned ALEX data did, in addition to provide evidence for a protective effect of a farming environment, highlight the timing of exposure by demonstrating that the strongest effect was seen in children exposed to farm-related factors in the first year of life. Furthermore, in the same publication it was reported that children of mothers who worked daily on the farm were less likely to develop asthma and hay fever later in life compared to children of mothers who were less often or not at all active on the farm. Again the sample size of the ALEX study was too small to investigate whether prenatal or postnatal exposures had stronger effects on the child's health. The larger PARSIFAL study provided enough power to investigate the question of the timing of exposure in more detail.

III Immunology

Atopic diseases are characterised by exaggerated immune responses to common, typically harmless proteins in our environment. Atopic patients are predisposed to the clinical manifestation of chronic disorders like allergic asthma, allergic rhinoconjunctivitis and atopic eczema, but also acute allergic reactions ranging from urticaria to anaphylaxis. This part of the introduction is intended to review the function of the human immune system and describe recent findings in immunological research to cross-link biological pathways in the development of allergic disease and the observations of epidemiological studies as discussed in the previous sections.

III.I The immune system

The human immune system recognizes and eliminates invading microorganisms in two ways: The adaptive or acquired immunity, consisting of B and T cells, uses a vast set of antibodies and T cell receptors with a high specificity against any foreign pattern or peptide. The highly effective acquired immunity is responsible for elimination of infection in a late phase and for the establishment of immunological

memory. By contrast, the innate immune system constitutes the first-line defence of a host to survive the early phase of infection. It recognizes only a limited number of conserved structures of invading microorganisms through a limited number of germ-line encoded receptors and it seems to be independent of immunologic memory [42]. Furthermore, innate immunity provides co-stimulatory molecules and cytokines to direct the adaptive immune response.

III.II The innate immune system

Pattern-recognition receptors

The innate immune system has been highly conserved during evolution [43-45]. Germ-line encoded receptors recognize molecular structures not present in the host, but conserved among pathogens. Such structures are called Pathogen-Associated Molecular Patterns (PAMP) [46]. Cellular receptors recognizing such structures have been named Pattern-Recognition Receptors (PRR) [46]. The most prominent best-investigated exponent of the PRR is the toll-like receptor (TLR) family. The Toll protein was first described in Drosophila where it is a crucial regulator of the immune system. In mammalian, twelve homologue forms of the Toll receptor are described and called Toll-Like Receptors (TLR), constituting the principal family of PRR in mammals. Humans have 10 functional TLR while TLR11 is non-functional due to a stop codon in the gene. TLR12 has not been documented so far. TLR are mainly expressed on cells of the immune system such as macrophages, Dendritic Cells (DC), mast cells, B-cells and some T-cells [47]. Not all TLR are located extracellularly in the plasma membrane. TLR3, 7, 8, and 9 are found almost exclusively expressed in endosomal compartments. Based on their amino acid sequence, human TLR can be divided into five subgroups, each recognizing related structures [48, 49]. Thus, the ligand recognition concept of TLR is completely different to that of antibodies or T cell receptors generating a binding domain against almost any imaginable foreign structure (see section III.III). The expression of TLR is not static but modulated in response to pathogens, cytokines, or environmental exposure to microbial components [50].

TLR4 binds bacterial LPS and is the TLR investigated in most detail. LPS is a component of the outer cell membrane of Gram-negative bacteria. The cell wall of a single Escherichia coli contains about two million LPS molecules. They are released

upon cell death and during growth and division. In humans LPS is able to provoke the systemic inflammatory response syndrome (SIRS) potentially leading to multiple organ dysfunction. By contrast, its chronic exposure to LPS in the environment of children has been associated with protection against the development of atopic diseases [35].

Binding of LPS occurs in close interaction of TLR and CD14 [51]. CD14 is a glycoprotein with 356 amino acids, encoded on chromosome 5q 23–31 [52]. Membrane CD14 (mCD14) is expressed in mature myeloid cells and is coexpressed and forms complexes with TLR4 and also TLR2 [53]. Two soluble (sCD14) forms of CD14 are constitutively generated [54]. Beside LPS, CD14 recognizes a wide spectrum of microbial compounds [55], such as lipoteichoic acid [56], and peptidoglycan [57], but also nonmicrobial compounds, such as phospholipids [58-60]. It has been shown that expression of CD14 is correlated with airway inflammation following inhalation of bacterial LPS [61].

The TLR2 subfamily is composed of TLR1, 2, 6 and 10. This subgroup recognizes lipids of many different microorganisms. TLR1 and 6 act as co-receptors of TLR2 recognizing lipoproteins of bacteria, mycoplasma, and mycobacteria [62-64]. TLR2 alone recognizes various microbial compounds from Gram-positive bacteria and fungi, such as lipoteichoic acid, lipoarabinomannan, and glucans.

The ligands of the other TLR consist of further viral and bacterial compounds: Bacterial Tri-acyl lipopeptides (TLR 1), viral single and double stranded RNA (TLR 7/8 and TLR 3, respectively), bacterial flagellin (TLR 5) and bacterial or viral CpG DNA (TLR 9). The ligands of TLR10 are unknown so far.

TLR signalling pathway

Activation of TLR by binding to one of its ligands triggers an intracellular signalling pathway involving recruiting Myeloid Differentiation primary-response protein 88 (MyD88) and translocation of Nuclear Factor κB (NF-κB,) to the nucleus leading to transcription and production of inflammatory mediators like Interferon (IFN) γ and Tumor Necrosis Factor (TNF) α [42, 65]. Moreover, it has been shown that TLR activation induces antimicrobial effector mechanisms [66-68].

Regulators of the TLR signaling cascade

Activation of the TLR signaling cascade is absolutely necessary to establish an immune response. But the process has to be tightly regulated, because misguided activation of the innate immune system may enhance the risk to develop chronic inflammation, allergy, and autoimmunity [69]. Repeated challenge with LPS leads to a reduced responsiveness to a subsequent stimulation with LPS. This is a well-known phenomenon and is designated as endotoxin or LPS tolerance [70]. Many different molecules, e.g., the Suppressor Of Cytokine Signalling (SOCS) family, IL-1-Associated Kinase (IRAK) 2, and the Toll-Interacting Protein (Tollip) have been proposed to be involved in negative regulation of the TLR signaling cascade (reviewed in [71]). Regulation also occurs through down-regulation of transcription and translation of TLR genes or by degradation of TLR protein [72].

III.III The adaptive immune system

Interaction between innate and adaptive immunity

The components of both the innate and the adaptive immune system do not act independently. Activation of an innate immune system is a major prerequisite for activation of an adaptive immune response (reviewed in [73]). Recent studies further suggest that TLR on dendritic cells plays a crucial role in immune response by activation of T regulatory cells, which in turn may down-regulate TH2-type immune response [74]. Naïve CD4+ T helper (Th) cell activation is initiated by interaction of the T Cell Receptor (TCR) - CD3 complex with a processed antigenic peptide bound to MHC class II molecules presented on Antigen-Presenting Cells (APC). The TCR dictates the antigen specificity of the response and plays the central role in initiating T cell activation.

T cell activation and differentiation

Immature T cells migrate from bone marrow to the thymus. There T cells begin to express the TCR for later antigen recognition. The TCR is a highly variable molecule attaining its diversity by combinatorial joining of variable-region gene segments generating a large number of random gene combinations. Positive and negative selection then ensures survival of T cells able to recognize self (MHC restriction, positive selection), but elimination of cells expressing high-affinity receptors to self-

MHC molecules (negative selection) resulting in self-tolerance. These mature T cells migrate to the periphery, ready to get activated by APC. Depending on the density of the peptides presented, types of co-stimulatory molecules expressed and cytokines released by the APC, naïve T helper cells start to proliferate and differentiate into the T effector cell subsets, Th-1, Th-2, T regulatory cells (Treg), and Th-17.

TLR-induced Interleukin (IL)-12 production of APC generally induces differentiation towards Th-1 cells. TheTh-1 response is characterized by the secretion of IL-2, Transforming Growth Factor (TGF)-β, and IFN-γ induced by transcription factor T-bet [75]. IFN-γ mediates secretion of high-affinity IgG2a antibodies in B cells and activates macrophages but inhibits Th-2 cells. Dysregulation of the Th-1 response is associated with autoimmunity and inflammatory diseases.

Th-2 cell differentiation, evolved to enhance clearance of parasites, is induced by IL-4. Th-2 cells express transcription factor GATA-3 enhancing the secretion of IL-4, IL-5, and IL-13 [76]. These cytokines activate eosinophils, mast cells, and mediate IgE secretion in B cells, but inhibit Th-1 cells. A dysregulated Th-2 response is associated with atopy.

Treg cells act as suppressor for Th-1 and Th-2 cells and are therefore important regulators of the immune response. The suppression depends, at least in part, on cell-to-cell contact [77]. Treg cells are characterized through expression of the transcription factor forkhead box (FOX) P3 and secrete elevated amounts of the cytokines IL-10 and TGF-β [78, 79]. Since these cells should not interfere with induction of pathogen-specific protective immune response, TLR-mediated IL-6 production of APC blocks the suppressive activity of Treg cells [80].

Th-17 cells are induced through TGF-β and IL-6 and produce high amounts of IL-17. The development of Th-17 cells is blocked by IL-4 and IFN-γ and it seems that these cells have a function in suppressing autoimmune disease [81].

B cell activation

B cells produce antibodies, a most effective tool of the immune system to fight infections. B cells mature in the bone marrow. During maturation, the process of gene rearrangement of B cell receptors (equates to the membrane bound form of a antibody) takes place to generate antibodies with a vast diversity of binding specificities. Mechanistically, the process is very similar to the rearrangement of the

TCR genes. After maturation, B cells bearing the immunoglobulin (Ig, antibody) isotype M on the cell surface migrate to peripheral lymphoid organs, where they get activated through antigenic contact. Further signals required for B cell activation come from the T helper cell, the interaction of CD40 on the B cell with CD40 ligand on the T helper cell and cytokines secreted by T helper cells, respectively. After immunization with antigen, B cells proliferate for about a week. Then, they migrate to germinal centers of secondary lymphoid organs (lymph nodes, spleen, and mucosal lymphoid tissues), where somatic hypermutation and class-switch recombination (CSR) take place. Via somatic hypermutation, point mutations are induced in the variable region of the antibody gene to generate antibodies with enhanced specificity. B cells bearing receptors with the highest affinity to the antigen are selected by affinity maturation. CSR exchanges the constant region of the antibody, generally Cμ (IgM) by Cγ (IgG), Cα (IgA) or Cε (IgE) in order to change the antibodies' effector function. For induction of immunoglobulin class switching, two signals are required. The first CSR activation signal occurs (i) Th-dependently by cell-to-cell contact (CD40 ligand - CD40 receptor on the B cell) or (ii) Th-independently (by BAFF, B lymphocyte stimulator protein, expressed by neutrophils and macrophages). The second signal is provided by cytokines determining the isotype of the antibody (Th1 – IFN-γ – IgG2a/igG3; Th2 – IL-4 – IgE/IgG1).

A necessary prerequisite for atopy is an elevated production of IgE and hence CSR to IgE. Switching to IgE is under tight control of Th-2 cytokine IL-4 activating the transcription factor Signal Transducer And Activator of Transcription (STAT) 6. Interestingly, a STAT6 haplotype was found to be associated with higher IgE levels [82]. Furthermore, recent work has shown that the protective effect of farm exposure might be switching stage and allergen specific and confined to Th-2-dependent IgG1, IgG4, and IgE expression [83]. The authors suggested that distinct mechanisms regulate individual steps within allergen-induced class switching.

From IgE to Allergy

Antigens that are inherently harmless but nevertheless able to trigger an IgE-prone immune response are called allergens. The atopic state is characterised by an exaggerated tendency to mount IgE responses to common environmental allergens. Plasma cells of atopic patients secrete IgE instead of IgG in response to allergens

and have therefore elevated serum levels of IgE [84]. IgE binds to Fc ε receptors on mast cells and basophils. These cells have granules containing pharmacologically active mediators. Cross-linking of IgE bound to Fc ε receptors by allergens leads to degranulation of the mast cells and basophils. Inflammatory mediators like histamine, leukotrienes, prostaglandins, and cytokines like IL-4, IL-5, IL-6, and TNF are released. The mediators are responsible for clinical presentation of allergic asthma (mucosal inflammation, smooth-muscle contraction [85]), allergic rhinoconjuntivitis (conjunctival and mucosal inflammation [86, 87]) and atopic eczema (skin inflammation, pruritus [88]).

IV Genetics

The understanding that genetics play a role in allergic disease and asthma has been recognized for more than 100 years. This genetic component was suggested through observations that allergic subjects had a significantly higher incidence of family histories of disease as compared with controls [89]. Follow-up studies have shown that if one parent has allergies, a child has a 33% chance of developing allergies and if both parents are allergic that number jumps to a 70% chance. The results of twin studies suggest that approximately 50% of the risk for developing asthma is related to genetic factors with an equivalent risk associated with environment [90].

IV.I Tools for studying genetics in atopic diseases

With the introduction of new powerful genetic tools, the heritable component of atopic disease, in particular of asthma, has gained increasing attention over the last few years. With recent technological advances, the identification of alterations in the sequence of the base pairs of our DNA may help to understand better the underlying biology and lead to the discovery of so far unknown processes resulting in atopic diseases.

Genome-wide linkage studies rely on families with individuals affected by asthma [91]. Evenly spaced genetic markers covering all chromosomes are typed in family members, and a search is made for genetic regions containing a higher than expected number of shared alleles among affected individuals within a family. The identification of such a region signals that somewhere within this genomic interval, a

disease-predisposing allele is to be found. The genes within this region are further examined by positional cloning - that is, by typing denser and denser collections of genetic variants, until the underlying disease-associated gene(s) are found.

Candidate-gene association studies focus on a selected number of genes that have been implicated as having a role in disease pathogenesis [91]. Association studies between variants in candidate genes and relevant phenotypes are mostly conducted by comparing allele or genotype frequencies between groups of unrelated cases and unrelated controls. Association analysis is expected to be more powerful for the detection of common disease alleles that confer modest disease risk in sample sizes that are comparable with those used in linkage studies [92]. Moreover, recruiting large numbers of unrelated affected individuals in association studies is easier than to collect large numbers of families, and there is increased statistical power in studying the equivalent number of individuals in a case-control association study than in family-based linkage studies.

Box 2. The nomenclature of single nucleotide polymorphisms.

The nomenclature of genetic variants is still not standardized completely and is therefore occasionally confusing. For example, single nucleotide polymorphisms (SNPs), the variants most frequently assayed in genetic studies, are ideally defined by their position within the gene and the two alleles found at that position. However, the same SNP often receives different identifiers. For SNPs in regulatory regions, some groups refer to the translation start site for numbering, whereas other groups count from the transcription start site. Thus, the same replacement of a C with a T in the CD14 promoter may be identified as CD14/C-260T or CD14/C-159T. A solution to this problem may be found through concerted efforts such as the one led by the SNP database at the National Center for Biotechnology Information, which acts as a public-domain archive for a collection of genetic polymorphisms in various organisms [1]. dbSNP maps each submitted SNP assay to the genome and assigns to each submitted SNP assay an unambiguous ID (rs number) that corresponds to the position in an idealized genome. For example, the dbSNP notations of the CD14 polymorphism mentioned above is rs2569190. The strength of this system is that submitted SNPs that map to the same location are clustered into the same RefSNP and have the same rs number. The SNPs discussed in this article are identified using traditional notation, which has the advantage of pointing readily to the gene, and by their rs number (at their first appearance in the text).

The innate immune system harbours several candidates potentially playing a role in the development of atopic disease. One of them is the CD14 cell surface protein

which is, as discussed, part of the receptor for bacterial LPS. The C allele of a single nucleotide polymorphism (SNP) in the promoter region of the CD14 gene (*CD14/C-260T* also known as *CD14/C-159T*; rs2569190; see Box 2 for more information about the nomenclature of SNPs) has been linked to the presence of atopy in some, but not all [93-95] populations. Among the studies in support of a protective role of *CD14/*-260T*, Dutch adults homozygous for the C allele had a higher number of positive skin-test responses and higher total serum IgE levels (in individuals with positive skin-test responses) and subsequently more allergic symptoms [96]. Among 481 U.S. American children TT homozygotes had significantly lower levels of IgE than did carriers of the other two genotypes [97]. In this publication TT homozygotes also had significantly higher sCD14 levels than did carriers of both the CC and CT genotypes, suggesting a functional role of the polymorphism in the production of sCD14. Likewise, atopic Chinese children homozygous for the C allele in *CD14/C-260T* had the highest serum total IgE levels compared with those of subjects in the CT and TT subsets [98]. In a population-based cohort followed from primary school age, it was found that the CC genotype in *CD14/C-260T* was associated with an increased risk of early onset atopy and bronchial hyperresponsiveness [99].

Almost equally impressive is the evidence against an association between CD14/*-260T and protection from allergy. Thus the T allele of *CD14/C-260T* was associated with increased total serum IgE levels in allergic subjects who worked at the Jackson Laboratories [100]. The same allele was more often transmitted in Hutterites with positive skin-test responses [101] and more common among US patients with non-atopic asthma and food allergy than among control subjects, particularly among white subjects [102].

Thus, ignoring the studies that did not observe at all an association between *CD14/C-260T* and the development of atopic diseases, the C allele has evolved as both a risk and protective for allergic disorders. Considering that the studies discussed above have been conducted in different populations in their respective environments and that *CD14/C-260T* is only an example among many polymorphisms that have been described as both protective *and* risk factors, these conflicting results suggest that genes and environment are intertwined in complex, nonlinear relationships, such that the same genetic background might result in the expression of different phenotypes in different environments.

IV.II Inseparable friends: Genes and environment

Gene-by-environment interactions have been defined as a situation when, because of their genetic differences, two or more individuals, families or genotypic lines respond differently, or to different extents, to a change in the environment [103]. Most of the performed studies on gene-environment interactions are based on this principle. In contrast to population-based studies in which the average effect of an environmental exposure is compared between groups, the identification of susceptible individuals within populations via genotyping allows a better estimation of the true magnitude of effect of an environmental exposure for the population at risk. Conversely, the function of a genetic variant may also be amenable to modification through environmental exposures.

On the statistical level, an interaction in either direction typically refers to the effects of product terms or to heterogeneity of its main effects. In other words, the difference of a genetic effect between two strata - exposed and non-exposed individuals - is investigated. Conversely, the difference of an environmental effect between two strata of genetic make-up is also studied. To examine the interaction between genotype and environmental exposure on asthma risk, two general approaches have been used that parallel the approaches taken for identifying asthma-susceptibility genes in general. The first is a genome-wide approach, in which exposure status is incorporated into a genome-wide screen for asthma-related traits to identify loci that contribute to asthma risk in exposed cases only, and those that contribute to asthma risk in unexposed cases only. The second is a candidate gene approach in which genes are selected because of their association with asthma-related traits and with biological pathways involved in the metabolism of environmental exposures. The genome-wide approach has the potential to identify novel loci interacting with environmental exposures, whereas the candidate approach tests the contribution of biological pathways potentially interacting with the environmental exposures under the assumption that the polymorphisms significantly alter the function of the gene.

The innate immune system offers several candidate genes for investigation of gene-environment interaction in atopic diseases, since it serves, on the one hand, as a primary recognition system of environmental, particularly microbial, exposure, and, on the other hand, owns the ability to initiate immunological processes potentially

preventing atopic disease. In a sub-sample of the ALEX study population, it has been reported that a SNP in the gene encoding TLR2 (*TLR2/A–16934T*; rs4696480) significantly interacts with a farming environment [104]. Only farmers' children with a T allele were susceptible to protective factors on the farm, whereas children homozygote for the A allele had prevalences of asthma and atopy comparable with those of non-farmers' children. Among non-farmers' children, no effect of TLR2 polymorphisms was seen. In the same population children with the CC genotype of the aforementioned *CD14/C-260T* SNP had lower levels of specific IgE to common aeroallergens than the children with the CT or the TT genotype, but only when exposed to high LPS loads [105]. Likewise, being homozygotes for the CC allele was a risk factor in terms of total IgE and specific IgE to inhalant allergens in children exposed to pets, but was a beneficial factor in children having regular contact with stable animals. Other gene-environment interactions for the CD14 gene have recently been published [106-109].

IV.III Functionality of genetic polymorphisms

Regardless of the method used to discover genes that potentially modify disease susceptibility, a causative role for such genes can only be established through further functional characterization of the genes and their variants.

For example, a SNP in the promoter of the IL-13 gene (*IL13/C-1112T*; rs1800925), a key cytokine in asthmatic airway inflammation, enhanced IL-13 promoter activity in primary human and murine CD41 TH2 lymphocytes, whereas the same polymorphism had opposite transcriptional effects in non-polarized CD41 T cells [110]. The nuclear milieu may thus determine the functional outcome of a genetic variation. The nuclear milieu in turn may be affected by the extracellular environment that will eventually be modified by a subject's environmental exposures.

In the first description of *CD14/C-260T* polymorphism not only a negative association with the TT genotype and total IgE levels was shown, but the children with two T alleles also expressed higher levels of sCD14 [97]. Later work described that the relative transcriptional activities of the C and T alleles of *CD14/C-260T* differ in monocytes and hepatocytes, depending on the ratio between SP1 and SP3, which are the transcription factors that bind the polymorphic promoter region [111]. Recently, the same author described in a pediatric population an age-dependent

transcriptional activity of the genotypes of *CD14/C-260T* and *CD14A-1721G*, the latter being discussed in Chapter 5. There we show that the gene-environment interaction is not only observable for the health outcomes under study, but also on the level of the gene expression of CD14.

V Objectives of the Thesis

Overall goal

The overall goal of this thesis was to assess factors of the farming environment that influence the innate immune system with respect to gene expression of crucial proteins of innate immunity pathways and test if this association is modulated by unknown genetic factors. We expected that results might give a deeper understanding of the immunological pathways and the complex relationship between environment, genes and atopic disease.

In particular, the following research questions were addressed:

Environmental factors and expression of innate immunity genes

1. *Can the results of the ALEX study [50] be confirmed in the PARSIFAL data?*

   Reproduction of results in different populations strengthens the evidence that the measured effect is true. We address this question of reproducibility in Chapter 2.

2. *Are the levels of microbial compounds in house dust associated with the expression of genes of the innate immune system?*

3. *Is the protective effect of farm milk consumption that has been shown in the ALEX study reproducible in the PARSIFAL study and is it at least partly explained by differential gene expression of PRR?*

4. *Which other specific factors of farming lifestyle have an impact on the expression of PRR of the innate immune system?*

5. *Is there a window of age where potential beneficial factors take effect?*

In PARSIFAL detailed information about timing and frequency of farm related exposures including farm milk consumption was asked and in a subsample house dust was collected. In Chapter 2 we investigate the association between distinct farm exposures, including exposure to microbial compounds, and gene expression of PRR. We can show that prenatal exposures to farming environment influence the expression of innate immunity genes and confer protection against atopic sensitisation in the unborn child. The exploration of the beneficial effect of farm milk consumption on atopic diseases is presented in Chapter 3. But we could also show that not all farming environments protect against atopic diseases as outlined in Chapter 4.

DNA polymorphisms, gene expression of PRR and environmental factors

6. *To what extent do polymorphisms of genes encoding for PRR modify the respective expression of PRR?*

7. *Does such a relation depend on environmental factors (gene-environment interaction) most notably the factors known to be protective regarding atopic diseases?*

In a sub-sample of PARSIFAL genetical analyses were done. DNA of peripheral blood leucocytes was genotyped for SNPs of the PRR to investigate gene expression among genotypes and gene-environment interactions. We present the results of these analyses in Chapter 5 of this thesis.

Gene expression in the epidemiological study setting

8. *Are there qualitative differences of the gene expression results between the PARSIFAL and the ALEX study using different methodological approaches to some extent?*

9. *Which pitfalls have to be accounted for when doing gene expression measurements in epidemiological studies?*

Gene expression measurements are a new tool to collect biological data in epidemiological studies. Circumstances (logistics, different laboratory staff and field workers) in which blood samples are handled differ in a large extent compared to the laboratory or clinical research. In Chapter 6 we therefore investigated potential pitfalls regarding gene expression measurements in epidemiological studies and were able to formulate recommendations for further projects.

VI Methods: The PARSIFAL study

Based on the previous findings of ALEX and other farm studies research teams of five European countries designed the cross-sectional PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) study to further investigate the protective effect of a farming lifestyle on atopic diseases. In addition to the three alpine countries participating in ALEX (Austria, Germany and Switzerland) research teams from the Netherlands and Sweden joined the PARSIFAL study group. The expansion of the number of participating study centres and efforts of the respective research teams resulted in a large study population providing enough power for a closer investigation of potential protective factors of a farming lifestyle. As apparent in the acronym PARSIFAL investigation of an anthroposophic lifestyle and its influence on the development of atopic disease was a further goal of this study, since several publications reported lower prevalence of atopic diseases among children raised in anthroposophic families. However, this additional research question is beyond the scope of this thesis.

The PARSIFAL population consisted of 14893 children aged 5-13 years belonging to four study groups: farmer's children, children attending Steiner schools and their corresponding reference groups [112]. The parents completed a detailed questionnaire, which included questions on environmental exposures, lifestyle, socio-economic conditions, history of infections, diet, contact with animals, and on symptoms of bronchial asthma, rhinoconjunctivitis and atopic eczema. The questions were based on the internationally validated and translated ISAAC phase-II questions [113] and the Swedish BAMSE study [114]. The questionnaires were distributed and collected from October 2000 to May 2002.

Allergen-specific IgE

In all countries a sub-sample of the children whose parents had consented were invited to blood sampling and a clinical examination. Allergen-specific IgE was measured in 4049 children against a mix of common inhalant allergens (Dermatophagoides pteronyssinus, D. farinae, birch, timothy, mugwort, cat, dog, horse and Cladosporium herbarum) and a mix of common food allergens (hen's egg white, codfish, cow's milk, peanut, soy bean and wheat flour).

Environmental microbial exposure

LPS and fungal extracellular polysaccharide (EPS) were measured in mattress dust samples of 83.9% of children with complete gene expression data (n=270). Sampling and detection methods are described elsewhere [115]. In brief, LPS was measured with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker, Walkersville, Md) and EPS with a specific sandwich enzyme immunoassay for EPS of Aspergillus and Penicillium species [116].

Gene expression measurements

For the Swiss branch of the PARSIFAL study, RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression of innate immunity receptors. The total RNA was isolated with the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). Quantitative real-time PCR (TaqMan; Applied Biosystems, Foster City, California, USA) was performed, as described elsewhere [117]. In brief, primers and probes were designed by using the primer design software Primer Express (Applied Biosystems). Optimal concentrations for primers and probes were determined according to the manufacturer's instructions. The reactions for the target and the endogenous control were performed in separate tubes. All PCR reactions were analyzed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Experiments assessing background signals were performed for every assay by running the reaction without templates (‘‘no template controls’’). No amplification was observed in any of these ‘‘no template controls,’’ indicating that there was neither contamination nor unspecific. The data are

presented as normalized values (the amount of mRNA of the target molecule divided by the amount of mRNA of the endogenous control [18s rRNA]).

Genotyping of genes of the innate immune system

Genotyping was done in 1478 PARIFAL children. To reproduce our results in an independent sample we also analysed genetic data of 576 children of the ALEX study. Parental consent for genetic analyses was available for all these children. DNA was extracted from peripheral blood leukocytes (PBLCs) by using standard techniques. All genes were screened for single nucleotide polymorphisms (SNPs) in white subjects. Haplotype tagging SNPs closely linked with known polymorphisms ($R2 > 0.7$) and with a minor allele frequency of greater than 10% were chosen for this study by using the algorithm implemented in ldSelect [118]. This algorithm is based on linkage disequilibrium. Genotyping for both populations was done in the same laboratory by using identical methods. Genomic DNA was assayed by using the 5' exonuclease reaction (Taqman, Applied Biosystems).

Chapter 2 Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children

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Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children

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Background: There is increasing evidence that environmental exposures determining childhood illnesses operate early in life. Prenatal exposure to a farming environment through the mother might also play an important role.
Objective: We sought to investigate the role of maternal exposures to environments rich in microbial compounds for the development of atopic sensitization, asthma, and corresponding alterations in the innate immune system in offspring.
Methods: In the children of the cross-sectional Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style study, asthma and atopy were assessed by means of standardized questionnaires (n = 8263) and serum IgE measurements (n = 2086). In a subsample (n = 322) gene expression of Toll-like receptors (TLR2 and TLR4) and CD14 was assessed. Maternal exposures were defined through questionnaire information.
Results: Both atopic sensitization (adjusted odds ratio, 0.58; 95% CI, 0.39-0.86) and the gene expression of receptors of innate immunity were strongly determined by maternal exposure to stables during pregnancy, whereas current exposures had much weaker or no effects. A dose-response relation was found between the extent of upregulation of these genes and the number of different farm animal species the mother had encountered in her pregnancy. Each additional farm animal species increased the expression of TLR2, TLR4, and CD14 by a factor of 1.16 (95% CI, 1.07-1.26), 1.12 (95% CI, 1.04-1.2), and 1.10 (95% CI, 1.03-1.23), respectively.
Conclusion: Maternal exposure to an environment rich in microbial compounds might protect against the development of atopic sensitization and lead to upregulation of receptors of the innate immune system. The underlying mechanisms potentially operating through the intrauterine milieu or epigenetic inheritance await further elucidation.
Clinical implications: When assessing risk factors of allergies in an infant's medical history, attention must also be paid to environmental exposures affecting the mother. (J Allergy Clin Immunol 2006;117:817-23.)

Key words: *Asthma, allergy, atopic sensitization, gene expression, Toll-like receptors, CD14, farming, maternal exposure, microbial exposure*

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Childhood asthma and allergies manifest in the first years of life.[1,2] Environmental exposures implicated in the inception of these illnesses must therefore occur even earlier (ie, before the manifestation of the first symptoms). In recent years, a number of studies have shown that

Abbreviations used
EPS: Extracellular polysaccharide
OR: Odds ratio
PARSIFAL: Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style
TLR: Toll-like receptor

exposures in the first 12 to 24 months of life might indeed determine the development of childhood asthma and allergies.[1,3-5] The temporal sequence of events might, however, be traced back to prenatal exposures, namely exposures of the mother. Active smoking during pregnancy[6]; exposure of the pregnant mother to antibiotics,[7] paracetamol,[8] and allergens[9,10]; and the effect of such exposure on the fetus have been investigated in some detail. Still, little is known about the effects of maternal exposure to microbial compounds in the environment on the development of atopy and asthma in offspring.

A deviated humoral immune response resulting in the production of IgE rather than IgG antibodies is the common feature of atopic diseases. The regulation of the humoral response by B cells depends on T effector cells. In turn, T-cell function is regulated by mediators of the innate immune system. Although it has been shown that adaptive immune responses can be shaped *in utero*,[11] little is known about potential prenatal determinants of the innate immune response and its relation to the development of atopy and asthma.

The aim of the present study was to investigate the role of exposures of the mother and the child, respectively, to environments rich in microbial burden for the development of atopy and asthma, as well as the gene expression of receptors of the innate immune response in the offspring.

METHODS

Population and study areas

The cross-sectional Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Life Style (PARSIFAL) study aimed at studying the determinants of childhood asthma and allergies in farming and anthroposophic populations, as described previously.[12] A child who lived on a farm and whose family ran the farm was considered a *farm child*. Other children were termed *farm reference children*. The present analyses focus on 2823 farm and 5440 farm reference children aged 5 to 13 years from rural areas of Austria, Germany, the Netherlands, Sweden, and Switzerland. In Sweden farmers with children were identified from the Farming Registry at the National Bureau of Statistics, and farm reference children were randomly selected from the population registry among children living in the same area. In Austria all farm children of a given school class were chosen by teachers who had a good knowledge of the region, and reference children were randomly selected from the same schools by the local study group. In the other countries children were randomly selected from schools in areas known to have a high percentage of farmers.

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

Questionnaires

The questionnaire comprised questions on sociodemographic background, parents' atopic diseases, family, and housing characteristics. In addition, information on the child's farm activities, as well as the mother's farm exposures during pregnancy, were available. Questions related to the child's contact with different farm animals (during the first year of life or later), consumption of farm milk (during the first year of life or current), and regular stable or barn visits and helping with haying at present. Activities occurring at least weekly were defined as occurring regularly. Maternal exposure included contact to different farm animal species during pregnancy and regularly working in stables (sheep shelters, hog, cow, and chicken houses) during pregnancy and lactation. The questionnaire asked for contact with 6 different farm animal species (cows, pigs, sheep, horses, goats, and poultry), and these were summed.

Questions on health outcomes and farm exposures were derived from the internationally validated International Study of Asthma and Allergies in Childhood II[13] questionnaire and the Allergy and Endotoxin study,[14] respectively. Children were considered to have current rhinoconjunctivitis symptoms if sneezing, runny nose, stuffy nose, and itchy eyes were reported in the last 12 months without the child having a cold at the same time. Current wheezing was defined as at least one episode of wheezing during the last 12 months. Children reported to have had symptoms of seasonal rhinoconjunctivitis in the last 12 months were defined as symptomatic, whereas children with a report of a physician's diagnosis of hay fever were considered to have a physician's diagnosis of seasonal rhinoconjunctivitis. Children with a report of a physician's diagnosis of asthma or of obstructive bronchitis more than once in their lifetime were considered to have a physician's diagnosis of asthma.

Measurement of allergen-specific serum IgE levels

In Austria, the Netherlands, and Sweden all children whose parents had consented to blood sampling were invited to a physical examination with blood sampling. In Germany and Switzerland only a random sample of those who consented were invited because of the comparatively large number of children included in these countries.[12] This subsample of children with blood analysis (n = 2086) did not significantly differ from the whole sample in regard to disease frequencies or family history of atopic disease (data not shown).

Allergen-specific IgE for common inhalant (Phadiatop) and food allergens (fx5; Pharmacia CAP System; Pharmacia Diagnostics AB, Uppsala, Sweden) was measured in serum. Two cut-off values for atopic sensitization were used: IgE values of 0.35 kU/L or greater and 3.5 kU/L or greater for either inhalant or food allergens.

Expression of Toll-like receptors

For the Swiss branch of the PARSIFAL study, RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression of innate immunity receptors. Children with available RNA samples did not differ significantly from the total Swiss PARSIFAL population with respect to farm exposures and health outcomes (data not shown).

The total RNA was isolated with the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). Quantitative real-time PCR (TaqMan; Applied Biosystems, Foster City, Calif) was performed, as described elsewhere.[15] In brief, primers and probes were designed by using the primer design software Primer Express (Applied Biosystems).

Optimal concentrations for primers and probes were determined according to the manufacturer's instructions. The reactions for the target and the endogenous control were performed in separate tubes. All PCR reactions were analyzed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Experiments assessing background signals were performed for every assay by running the reaction without templates (''no template controls''). No amplification was observed in any of these ''no template controls,'' indicating that there was neither contamination nor unspecific fluorescence (see Fig E1 in the Online Repository at www.jacionline.org). The data are presented as normalized values (the amount of mRNA of the target molecule divided by the amount of mRNA of the endogenous control [18s rRNA]).

Measurement of endotoxin and extracellular polysaccharide in dust samples

Endotoxin and fungal extracellular polysaccharide (EPS) were measured in mattress dust samples of 83.9% of children with complete gene expression data (n = 270). Sampling and detection methods are described elsewhere.[16] In brief, endotoxin was measured with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker, Walkersville, Md) and EPS with a specific sandwich enzyme immunoassay for EPS of *Aspergillus* and *Penicillium* species.[17]

Statistical analysis

Statistical analysis was performed with SAS 9.1.3 (The SAS Institute, Cary, NC) and Stata/SE 8.2 (StataCorp LP, College Station, Tex) software. Odds ratios (ORs) or geometric means ratios and 95% CIs were calculated in multiple logistic regression analysis.

In preliminary logistic analyses the associations of atopic sensitization, atopic symptoms, or diseases and exposure to farm characteristics were explored and adjusted for predefined covariates (age, sex, parental education, maternal and paternal atopy, number of older siblings, exposure to pets, and study center). We included farm characteristics that best predicted being a farm child, namely the child's current exposure to a farming lifestyle (regular stable or barn visits or helping with haying), the child's exposure to farm animals (ever), predominant consumption of farm milk (ever), and regular maternal work in stables during pregnancy. In the final regression model all variables were mutually adjusted. The independent effects of maternal work in stables during pregnancy and lactation were investigated to evaluate the effect of timing of the exposure.

Because the distribution of gene expression levels of the CD14, Toll-like receptor (TLR) 2, and TLR4 genes was skewed, these variables were log-transformed, resulting in a good approximation to the normal distribution (data not shown). For farm and farm reference children, these log-transformed values were compared by using the *t* test, and crude geometric means with their 95% CIs were computed. Multiple regression models adjusting for the same covariates as mentioned above were used to explore the associations between gene expressions and farm-related exposure measures. Results were expressed as adjusted geometric means ratios. In addition, adjusted geometric means of normalized gene expression were calculated for different levels of specific exposure variables (eg, the number of farm animal species the mother was exposed to during pregnancy).

In further analyses the effect of additional exposures during intervening years (5-13 years) that might influence atopic sensitization or gene expression were tested. In turn, the following variables were included in the regression models: worm infestations ever, respiratory infections during the first 2 years of life, day-care attendance, prescription of antibiotics or antipyretics before or after the first year of life, and history of measles, rubella, mumps, pertussis, or mononucleosis.

RESULTS

Of the 11,969 invited farm and farm reference children, 8402 (70%) returned the questionnaires. A total of 139 children were excluded because of missing values for sex and age or because they did not meet the age criteria of 5 to 13 years.

The prevalence of all outcomes (atopic sensitization, rhinoconjunctivitis symptoms and physician's diagnosis of seasonal rhinoconjunctivitis, and current wheezing and physician's diagnosis of asthma) was significantly lower in farm children compared with in nonfarm children (see Fig E2 in the Online Repository at www.jacionline.org),[12] despite some heterogeneity across the countries. The most pronounced differences between farm and nonfarm children were seen in Germany.

Factors mediating the farm effect

Determinants of the protective farm effect were assessed in multiple regression models (Table I). Predominant farm milk consumption was inversely related to the symptoms ($P = .079$) and diagnosis of seasonal rhinoconjunctivitis ($P = .022$), asthma ($P = .038$), and wheezing ($P = .065$). Additionally, regular contact with farm animals contributed to the protective effect on the physician's diagnosis of seasonal rhinoconjunctivitis ($P = .049$). Rhinoconjunctivitis symptoms in the last 12 months were also inversely related to frequent stable or barn visits or help with haying ($P = .007$). The strongest protective effect, however, was found for maternal stable work during pregnancy on atopic sensitization (OR, 0.58; 95% CI, 0.39-0.86; $P = .007$). Moreover, this association remained significant after additional adjustment for being a farm child or correction for multiple testing ($P_{Bonferroni} = .035$).

Maternal stable exposure during pregnancy versus lactation

Because 20% of all mothers working in the stable during pregnancy (n = 2184) stopped working in the stable after giving birth to the child, it was possible to disentangle the contributions of pregnancy and lactation, respectively, to the maternal effect. The effect of maternal stable work during lactation tended to be less pronounced (OR, 0.66; 95% CI, 0.45-0.96). It was no longer statistically significant when adjusted for maternal exposure in pregnancy (OR, 0.92; 95% CI, 0.52-1.61). When assessing the effect of timing of first exposure to farm animals on atopic sensitization, adjusted ORs for first contact in pregnancy, first contact in first year of life, first contact between first year and current, and current first contact were 0.36 (95% CI, 0.25-0.51), 0.54 (95% CI, 0.32-0.92), 0.77 (95% CI, 0.49-1.22), and 0.71 (95% CI, 0.43-1.20), respectively.

Variation of the effect on atopic sensitization across countries

We furthermore assessed the effect of maternal stable work during pregnancy on atopic sensitization for each

TABLE I. Mutually adjusted ORs for associations of farm-related exposures with health outcomes

	Atopic sensitization (≥3.5 kU/L) (n = 285/2086)	Rhinoconjunctivitis symptoms (n = 507/8174)	Physician's diagnosis of rhinoconjunctivitis (n = 343/8130)	Wheezing (n = 552/8169)	Diagnosis of asthma (n = 656/8080)
Current farm exposure*	0.96 (0.63-1.46), P = .854	0.63 (0.45-0.88), P = .007	0.66 (0.41-1.07), P = .090	0.88 (0.65-1.19), P = .403	0.82 (0.62-1.09), P = .172
Regular contact with farm animals ever	0.76 (0.51-1.15) P = .194	0.87 (0.67-1.14), P = .321	0.69 (0.47-1.00), P = .049	0.97 (0.75-1.26), P = .822	0.94 (0.75-1.19), P = .629
Farm milk consumption ever	0.76 (0.52-1.11), P = .162	0.77 (0.58-1.03), P = .079	0.63 (0.42-0.93), P = .022	0.77 (0.58-1.02), P = .065	0.76 (0.59-0.99), P = .038
Stable exposure in pregnancy†	0.58 (0.39-0.86), P = .007	0.74 (0.50-1.09), P = .126	0.77 (0.44-1.36), P = .371	0.76 (0.54-1.07), P = .120	0.86 (0.63-1.16), P = .325

ORs are given with 95% CIs in parentheses and P values. The models are adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever, study center, and the other exposure variables presented in the table.

*Current regular exposure to stable or barn or regular participation in haying.

†Mother worked regularly in stable during pregnancy.

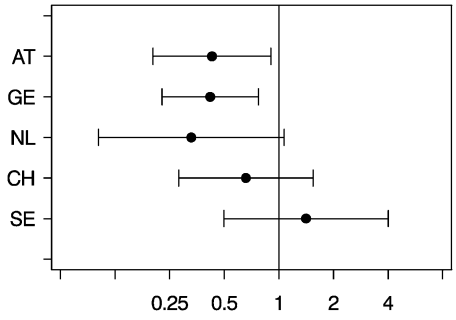

FIG 1. Adjusted ORs for maternal work in stables during pregnancy on atopic sensitization across countries. As a cut-off point for atopic sensitization, a value of 3.5 kU/L or greater was used. The ORs are given separately for the following countries: Austria *(AT)*, Germany *(GE)*, the Netherlands *(NL)*, Switzerland *(CH)*, and Sweden *(SE)*.

country separately (Fig 1). The protective maternal effect was found in all countries but Sweden (OR, 1.41; 95% CI, 0.50-4.01). We investigated different cut-off definitions in the 4 countries showing a protective maternal effect. Using the detection limit as cut-off (≥0.35 kU/L) yielded a somewhat weaker but still significant effect (OR, 0.73; (95% CI, 0.54-0.995) compared with a cut-off level of 3.5 kU/L or greater (OR, 0.53; 95% CI, 0.35-0.80).

Association of farm-related exposure with gene expression

Because environmental factors have been shown to correlate with the expression of receptors of the innate immune system, we next investigated the expression of the genes for TLR2, TLR4, and CD14 in the Swiss subpopulation. Geometric means of the expression of these genes were significantly higher among farm children than among farm reference children (TLR2: 0.74 [95% CI, 0.65-0.84] vs 0.49 [95% CI, 0.43-0.56], $P < .0001$; TLR4: 0.98 [95% CI, 0.88-1.09] vs 0.82 [95% CI, 0.72-0.93], $P = .034$; CD14: 0.48 [95% CI, 0.41-0.55] vs 0.28 [95% CI, 0.24-0.31], $P < .0001$), thereby confirming previous data from another farming population.[18] It was, however, not the current exposure to several farm characteristics but rather maternal exposure during pregnancy that was associated with increased gene expression (Table II). Likewise, current levels of endotoxin and EPS in mattress dust were not correlated with gene expression of their respective receptors (data not shown and Fig E3 in the Online Repository at www.jacionline.org). Day-care attendance, reported childhood infections, worm infestation, and use of antibiotics or antipyretics during intervening years were not associated with gene expression of TLR/CD14 and atopy and did not affect reported point estimates. However, a dose-response relation was found when relating the number of different farm animal species the mother had contact with during pregnancy to gene expression (Fig 2).

Each additional species of farm animal the mother was exposed to during pregnancy increased the expression of TLR2, TLR4, and CD14 by a factor of 1.16 (95% CI, 1.07-1.26), 1.11 (95% CI, 1.03-1.19), and 1.14 (95% CI, 1.04-1.24), respectively. Neither restricting the analysis to farm children only nor omitting the highest category of contact to farm animal species during pregnancy materially changed the results. In a model including exposure to different animal species during pregnancy and during the first year of life simultaneously, stronger effects were observed for exposure during pregnancy (TLR2, 1.11 [95% CI, 1.01-1.21]; TLR4, 1.05 [95% CI, 0.97-1.14]; CD14, 1.09 [95% CI, 0.99-1.21]) than during the first year of life (TLR2, 1.03 [95% CI, 0.94-1.12]; TLR4: 1.02 [95% CI, 0.95-1.10]; CD14: 1.00 [95% CI, 0.91-1.10]), respectively. The correlation between these 2 variables was moderate ($r = 0.69$).

DISCUSSION

The present study showed a clear inverse association of a farming environment (farm milk consumption, stable/barn visits, and contact with farm animals) with

TABLE II. Mutually adjusted geometric means ratios for associations of farm-related exposures with the expression of the TLR2, TLR4, and CD14 genes (n = 322)

	TLR2	TLR4	CD14
Current farm exposure*	1.04 (0.69-1.55), P = .851	0.93 (0.66-1.3), P = .671	1.01 (0.66-1.54), P = .964
Regular contact with farm animals ever	1.09 (0.75-1.58), P = .650	0.92 (0.67-1.25), P = .577	0.97 (0.65-1.43), P = .866
Farm milk consumption ever	1.04 (0.77-1.42), P = .813	1.06 (0.81-1.4), P = .656	1.16 (0.83-1.64), P = .385
Stable exposure in pregnancy†	1.44 (1.04-1.98), P = .027	1.4 (1.07-1.83), P = .015	1.66 (1.18-2.33), P = .003

Geometric means ratios are given with 95% CIs in parentheses and P values. The models are adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever, and the other exposure variables presented in the table.

*Current regular exposure to stable or barn or regular participation in haying.

†Mother worked regularly in stable during pregnancy.

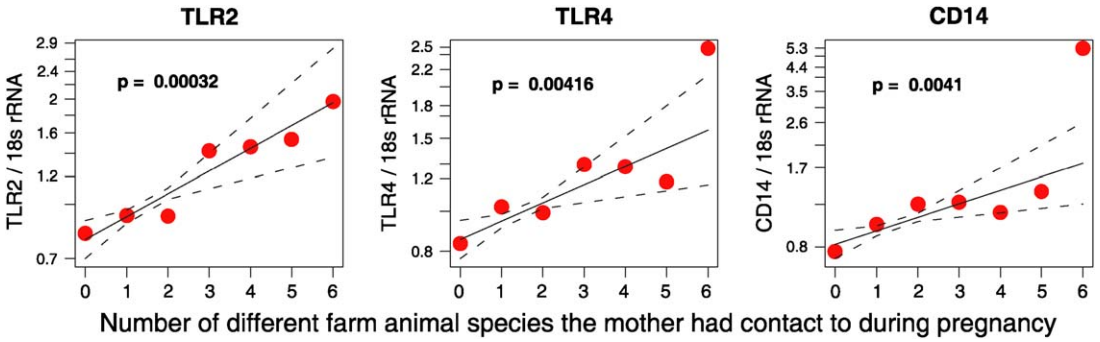

FIG 2. The x-axis shows increasing numbers of different farm animal species the mother had contact with during pregnancy (n = 322). The y-axis shows geometric means *(dots)* of gene expression of TLR2, TLR4, and CD14, respectively, which were normalized by dividing by gene expression of 18s rRNA. In addition, the linear fit of the association *(solid line)*, along with the corresponding 95% CI *(dashed line)*, is displayed. All analyses were adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever, child's current exposure to a farming lifestyle, child's exposure to farm animals, and predominant farm milk consumption of the child.

the prevalence of seasonal rhinoconjunctivitis, symptoms of seasonal rhinoconjunctivitis, and asthma. Maternal exposure to stables in pregnancy was associated with atopic sensitization and the expression of genes for receptors of the innate immune system (ie, TLR2, TLR4, and CD14). A dose-response relation was found between the extent of the upregulation of these genes and the number of different farm animal species the mother had contact with during her pregnancy. However, only longitudinal studies will eventually establish a causal relationship.

The identification of farm milk and stable animal contact as being protective for asthma and seasonal rhinoconjunctivitis is in accordance with the results of the Allergy and Endotoxin study.[14] The much larger size of the PARSIFAL population allowed, however, a more detailed investigation of the relevant time windows of exposure for each outcome. A stronger effect of prenatal (ie, maternal) exposure was seen for atopic sensitization. Moreover, this effect was observed for gene expression of innate immunity receptors. Stable exposure during pregnancy was more relevant than exposure while the mother was breast-feeding, thereby shifting the focus to prenatal exposure. The cross-sectional design of the study might have resulted in recall bias. However, this is unlikely because the outcomes (IgE levels and gene expression) were laboratory parameters not known to the mothers when answering the questionnaires. The maternal effect was independent of the mother's history of atopy and consistent over geographic areas, with the exception of Sweden. In Sweden none of the stables were connected to farmhouses in contrast to the other countries, potentially indicating a different pattern and intensity of exposure. Moreover, the levels of microbial compounds in house dust differ between Sweden and the other countries.[16]

Exposure to farm animals and stables has been found to be associated with increased exposure to various microbial products.[19,20] TLR2 is a receptor for various microbial compounds from gram-positive bacteria and fungi, such as lipopeptides, lipoarabinomannan, lipoteichoic acid, and glucans, whereas TLR4 recognizes LPSs mostly from gram-negative bacteria. CD14 in turn is involved in the recognition of LPSs and other bacterial wall components.[21] It seems therefore likely that the upregulation of these genes with an increasing number of farm animal species the mother was exposed to during pregnancy is attributable to increasing levels, diversified levels, or both of microbial exposure associated with contact with these animals.

By recognizing microbial compounds, TLRs are part of innate immunity, the first line of defense. The innate immune response communicates with the effector cells of the adaptive immune response, which includes the T helper cell populations.[22] T_H1 cells favor the production of IgG antibodies, whereas T_H2 cells promote the production of IgE antibodies and thereby the development of atopic diseases. In experimental studies it has been shown that stimulation of the innate immune response might skew the balance toward antiallergic T_H1 responses.[23,24] However, in children of farming environments with high endotoxin exposure, a decreased secretion of both T_H1 and T_H2 cytokines was observed on restimulation of leukocytes through the TLR4 ligand LPS.[25] This finding suggests that mechanisms other than a T_H1/T_H2 imbalance might be operational in these environments. T regulatory cells or mechanisms inherent to innate immune signaling, such as LPS tolerance, might play a role.[18,26,27]

In the present study current exposures to either stables and barns or to measured levels of microbial products indoors were not related to expression of these innate immunity genes. Yet other microbial exposures than the ones assessed might have been of greater importance. However, endotoxin and EPS are specific ligands for TLR4 and TLR2, respectively,[21] which makes it improbable that we have missed major environmental signals.

In contrast, maternal contact with increasing numbers of farm animal species, which is likely to parallel microbial exposures, was significantly related to a long-lasting upregulation of the expression of these innate immunity genes, which was still detectable at school age. Similarly, maternal work in stables during pregnancy was associated with decreased production of allergen-specific IgE antibodies, which was again detectable at school age. Thus prenatal environmental influences might affect the long-term development of innate and adaptive immune responses. In turn, the manifestation of organ-specific atopic conditions of the upper and lower respiratory tract might depend on the interplay between the growing and maturing child and his or her environment.

It is unknown how the maternal environment exerts its effect on the offspring. One might speculate that the fetal immune system interacts with a certain cytokine pattern prevailing in the maternal organism[28] or with the antigens themselves,[29] implying the establishment of immunologic tolerance. Another attracting explanation might consist in epigenetic inheritance. Differential patterns of gene expression arise during development *in utero* and are subsequently retained through mitosis. Stable alterations are heritable but do not involve mutations of the DNA itself.[30,31] Recent data suggest that environmental factors might alter methylation processes, a key element of epigenetic inheritance.[30] Furthermore, there is some evidence suggesting that endotoxin has a role in gene silencing because LPS is a potent inflammogen that stimulates acetylation of histones.[32]

The PARSIFAL study does not provide information on the maternal exposure before pregnancy, and therefore we cannot exclude a preconceptional effect. In other words, the mother's earlier exposure to a farming environment might have determined a configuration of her immune system, which she then passed on to her child. If indeed preconceptional exposures were important, environmental determinants of disease inception would also operate in the parental generation. Recent observations demonstrating a plateau of the prevalences of asthma, seasonal rhinoconjunctivitis, and atopic sensitization after 3 decades,[33-35] which approximates a generation, might indirectly support the notion of a generational effect. Furthermore, a transgenerational association of a grandmother's smoking with her grandchildren's risk for asthma has recently been reported.[36] Of note, in our rural population the effect of maternal work in stables during pregnancy on the child's atopy risk was independent from the mother's smoking habits. The lack of insight into the determinants of the increase in the prevalence of asthma and allergies over time might therefore in part be explained by the neglect of the potential effect of preconceptional exposures of the parents.

In conclusion, our results suggest that maternal exposures to environments rich in microbial exposures determine the priming of a child's immune response. For organ-specific manifestation of allergic diseases, additional factors, continuing exposure, or both effective later in life seem to be important. Our findings stress the importance of the identification of the appropriate time window leading to the modulation of immune responses and disease manifestation, respectively. Longitudinal studies will eventually deepen our understanding of the relevant temporal sequences.

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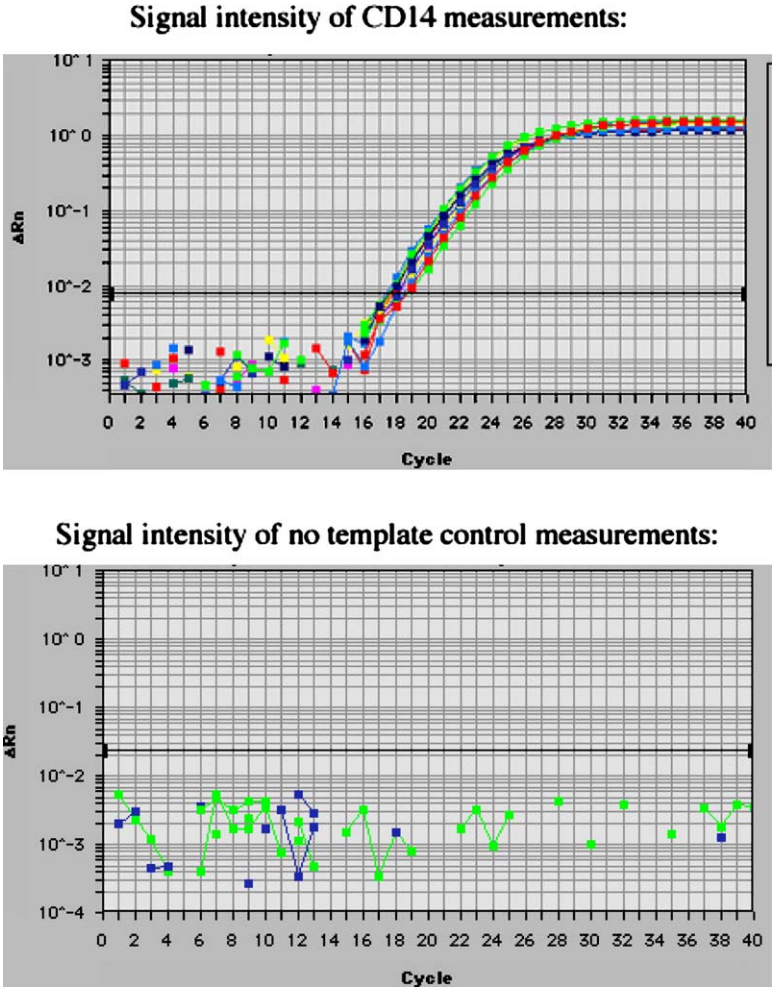

FIG E1. Signal intensity of CD14 measurements and "no template controls."ΔRn values, which indicate the magnitude of signal generated by the PCR reaction, are given for number of PCR cycles for 12 and 5 representative experiments, respectively.

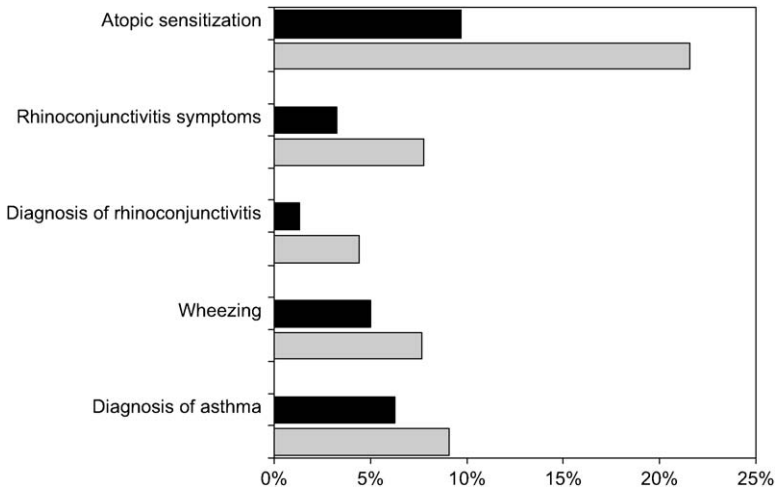

FIG E2. Prevalences of atopic sensitization, hay fever, and asthma for farm *(black bars)* and reference *(gray bars)* children.

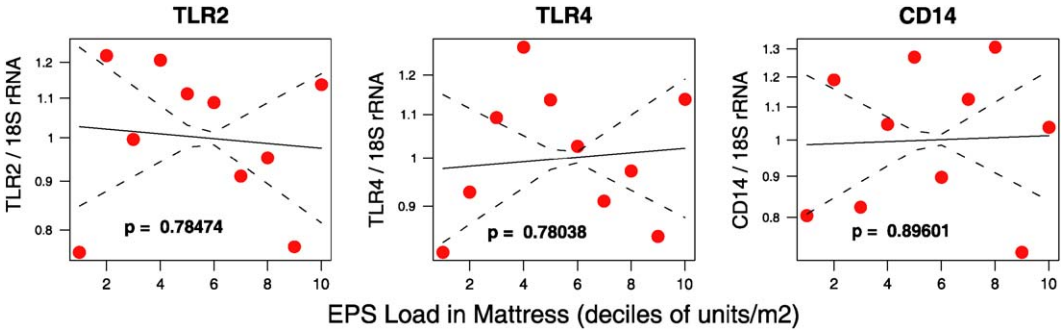

FIG E3. The x-axis shows deciles of EPS load per square meter of mattress (n = 322). The y-axis shows geometric means *(dots)* of gene expression of TLR2, TLR4, and CD14, respectively, which were normalized by dividing by gene expression of 18s rRNA. In addition, the linear fit of the association *(solid line)*, along with the corresponding 95% CI *(dashed line)*, is displayed. All analyses were adjusted for age, sex, family history of atopy, parental education, environmental tobacco smoking, maternal smoking during pregnancy, number of older siblings, contact with pets ever, child's current exposure to a farming lifestyle, child's exposure to farm animals, and predominant farm milk consumption of the child.

Chapter 3 Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe

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ORIGINAL PAPER

Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe

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Clinical and Experimental Allergy

Summary

Background Dietary interventions as a means for atopy prevention attract great interest. Some studies in rural environments claimed an inverse association between consumption of farm-produced dairy products and the prevalence of allergic diseases, but current evidence is controversial.

Objective To investigate whether consumption of farm-produced products is associated with a lower prevalence of asthma and allergy when compared with shop-purchased products.

Methods Cross sectional multi-centre study (PARSIFAL) including 14 893 children aged 5–13 years from five European countries (2823 from farm families and 4606 attending Steiner Schools as well as 5440 farm reference and 2024 Steiner reference children). A detailed questionnaire including a dietary component was completed and allergen-specific IgE was measured in serum.

Results Farm milk consumption ever in life showed a statistically significant inverse association with asthma: covariate adjusted odds ratio (aOR) 0.74 [95% confidence interval (CI) 0.61–0.88], rhinoconjunctivitis: aOR 0.56 (0.43–0.73) and sensitization to pollen and the food mix fx5 (cut-off level of $\geqslant 3.5$ kU/L): aOR 0.67 (0.47–0.96) and aOR 0.42 (0.19–0.92), respectively, and sensitization to horse dander: aOR 0.50 (95% CI 0.28–0.87). The associations were observed in all four subpopulations and independent of farm-related co-exposures. Other farm-produced products were not independently related to any allergy-related health outcome.

Conclusion Our results indicate that consumption of farm milk may offer protection against asthma and allergy. A deepened understanding of the relevant protective components of farm milk and a better insight into the biological mechanisms underlying this association are warranted as a basis for the development of a safe product for prevention.

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Introduction

The role of dietary factors in the development of asthma and atopy is still controversial. It has been postulated that the decrease in vegetable consumption and a shift from animal to vegetable fats has contributed to the increase in asthma and allergic diseases over the last decades [1, 2]. Several studies reported positive associations between elevated margarine consumption and childhood atopy risk [3, 4], while studies in rural environments reported an inverse association between consumption of farm-produced dairy products such as yogurt and farm milk and the prevalence of atopy [5–8], allergic rhinitis [5, 8, 9], asthma [5], and atopic dermatitis [8, 9]. However, in a Finnish study among farm and non-farm children, no effect of dairy product consumption and atopy was observed, but regular intake of fresh vegetables, predominantly when grown in the own garden, significantly reduced the risk of atopic sensitization [10]. Thus, current evidence of the relation between farm-produced products and the prevalence of allergic disease is controversial and mechanisms underlying the observed associations are unknown. Experiments demonstrating a beneficial effect of adding apathogenic bacteria (probiotics) to children's diet as a means of preventing atopic dermatitis have [11, 12], however, stimulated the interest in a possible role of microbes associated with the consumption of farm-produced foods.

The large European PARSIFAL study (Prevention of allergy risk factors for sensitization in children related to farming and anthroposophic lifestyle) offered the opportunity to examine specifically the relation between self- or farm-produced products and the prevalence of asthma and allergic diseases in more than 14 000 school-aged children [13]. The study included farm children, children from Rudolf Steiner schools (families with an anthroposophic lifestyle), and reference children from rural and sub-urban areas. The anthroposophic lifestyle includes factors like a restrictive use of antibiotics, antipyretics, and vaccinations, and often a biodynamic diet. An earlier study conducted in an anthroposophic community in Sweden showed a lower prevalence of childhood allergy [14].

The present analyses of the PARSIFAL study specifically addressed the question of whether, first, consumption of self- or farm-produced products, especially farm milk is associated with a lower prevalence of asthma, allergic diseases, and atopic sensitization when compared with shop-purchased products, and second, whether these associations are limited to children from rural environments or explained by other concurrent farm exposures.

Methods

Study population

Within the PARSIFAL study, children aged 5–13 years, from farm families or attending Rudolf Steiner schools, were compared with children from rural non-farming environments (reference for the farm children) and with children from (sub)urban environments attending State schools (reference for the Steiner school children) in Austria, Germany, the Netherlands, Sweden, and Switzerland. Children in Steiner schools often come from families with an anthroposophic lifestyle, which includes a holistic approach to life, education, and medicine. The details of the study design are described elsewhere [13]. In brief, a total of 15 137 (70% participation rate) parents' completed questionnaires were collected. Two hundred and forty-four questionnaires were excluded because children were outside the age limits or main information was missing, leaving 14 893 children for the analyses. In all, 8788 children gave consent for blood sampling and of these 4854 were invited for blood sampling [all children from Austria (806), the Netherlands (691), and Sweden (944) and a random sample of children from Germany (1548) and Switzerland (865) due to the comparatively large number of recruited children]. Four thousand and forty-nine (83.4%) children provided a blood sample and 3979 samples yielded a sufficient volume for allergen-specific IgE measurements. The study was approved by local research ethics committees in each country and informed consent was obtained from the parents of each child.

Parents' questionnaire

The dietary component of the PARSIFAL questionnaire included questions on the frequency of current average consumption of self-produced or directly purchased on a farm- and store-purchased foods. These foods included milk, butter, and other dairy products as well as margarine, eggs, meat, vegetables, and fruits. Response options were restricted to four categories: never, less than once per week, one to six times per week, or once a day or more.

Validity and reproducibility were assessed with 24-h recalls performed as telephone interviews of 493 randomly selected participants from all involved study groups and study areas. The validity for foods consumed with high frequency such as milk, vegetables, and fruits was the highest (positive predictive value above 60%, and negative predictive value above 80%). The reproducibility of reported consumption of milk during the first year of life was concordant among 71% of participants for store-purchased milk and 85% for farm-produced milk. No significant differences were found in the validity of responses between the five countries participating or between the four groups of children.

Besides the dietary component, the parental questionnaire included questions on socio-demographic background, parents' atopic diseases, food avoidance due to allergies in the family, breastfeeding, and the child's height and weight. In addition, information on the child's farm activities as well as the mother's farm exposures during

pregnancy was available. A child who lived on a farm and whose family ran the farm was coded as being a farm child.

The prevalence of diseases and symptoms were assessed by questions of the validated and translated International Study of Asthma and Allergies in Childhood (ISAAC) [15]. Children ever diagnosed with asthma, or obstructive bronchitis more than once, were considered to have a doctor's diagnosis of asthma. Current wheezing was defined as having wheezing at least once during the past 12 months. In a subsample of the PARSIFAL study questionnaire, responses on asthma and current wheeze have been validated against a bronchial challenge with hypertonic saline and no significant differences in validity were found between the four study groups [16].

Children diagnosed with hayfever and whoever had the symptoms of hayfever, were considered to have a doctor's diagnosis of rhinoconjunctivitis. Current rhinoconjunctivitis symptoms were defined as sneezing, runny nose, nasal block-up, and itchy eyes during the past 12 months, without having a cold at the same time.

Children with an intermittent itchy rash lasting at least 6 months and who had been diagnosed with atopic/allergic eczema were considered to have a doctor's diagnosis of atopic eczema. Current atopic eczema symptoms was considered present if the child had ever had an itchy rash intermittently for at least 6 months and, in addition, reported an itchy rash in defined locations (bend of the arm/knee, backside of thighs, neck, and around eyes/ears) at any time during the last 12 months.

Allergen-specific immunoglobulin E measurements

All samples were screened with a mix of common inhalant allergens (Phadiatop: birch, timothy, mugwort, *Dermatophagoides pteronyssinus* and *farinae*, cat, dog and hose epithelium and *Cladosporium herbarum*) and a mix of common food allergens (fx5: egg white, milk, fish, wheat, peanut, soya bean) (Pharmacia CAP System; Pharmacia Diagnostics AB, Uppsala, Sweden). Sera that were scored positive in Phadiatop were further analysed separately against *Dermatophagoides pteronyssinus* (house dust mite) and *Lepidoglyphus destructor (storage mite)*, a mix of grass pollen, a mix of tree pollen, cat, and horse epithelium. All analyses were performed centrally at the Department of Clinical Immunology (Karolinska University Hospital, Stockholm, Sweden). Atopic sensitization was defined as allergen-specific IgE $\geqslant$ 0.35 kU/L. In addition, a cut-off value of 3.5 kU/L was also considered for the analyses. Pollen sensitization was defined as positive grass pollen mix and/or positive tree pollen mix.

Statistics

χ^2 Statistics were used to evaluate differences in dietary habits between farm children and farm reference children as well as between Steiner school children and their reference group. Consumption of products from self-production or directly purchased on a farm was compared with shop-purchased products.

Logistic regression analyses were performed to calculate adjusted odds ratios (aOR) of the association between asthma or allergy outcomes and farm-produced foods. Multivariate models evaluating the effect of each food item on allergy outcomes were adjusted for predefined covariates including study group (farm children, Steiner school children, and the respective reference groups), country, sex, age, mother's and father's reported asthma and/or hayfever, parents' education, maternal smoking during pregnancy, current environmental smoking at home, older siblings, exclusive breastfeeding $>$ 4 months, BMI, and food avoidance due to familial asthma and/or allergy. In a second step, models were mutually adjusted for all food items.

We also calculated stratified estimates for the four study groups. The degree of heterogeneity of the stratum-specific ORs across study groups was evaluated using standard meta-analytic techniques [17]. In models examining the association between farm milk consumption and health outcomes, the timing of consumption (only in the first year of life, only at present, both in the first year of life, and at present), and relevant concomitant farm exposures such as frequency of the child's current visits to animal sheds (less or more than once a week) were additionally tested. In a sensitivity analysis, we also tested whether the effect of farm milk consumption varied across individual countries. Stability of effect estimates was examined by removing each country, one at a time.

To assess the possible influence of allergy-related changes in dietary habits, the respective question was included in all regression models. For the final analyses, we excluded the 469 non-milk-drinking children to avoid potential primary milk avoidance due to allergy-related symptoms at younger ages. Statistical analyses were performed using STATA (version 8.2, Stata Corp LP, College Station, TX, USA).

Results

Table 1 shows the distribution of children's consumption of selected farm-produced or shop-purchased foods across study groups. Although the consumption of farm milk and of self-produced products was most common among farm children, relevant proportions of all other study groups also ate and drank these products.

Table 2 gives the results of the multivariate analyses evaluating the effect of each individual farm-produced food on asthma and allergy adjusting for the predefined covariates (left side). A significant inverse association with a doctor's diagnosed asthma was observed for all

Table 1. Consumption of selected foods according to the study group

	Farm children (%) ($n = 2823$)	Farm reference (%) ($n = 5440$)	P-value*	RSS children (%) ($n = 4606$)	RSS reference (%) ($n = 2024$)	P-value*
Milk consumption						
Never	5.1	2.8		3.2	1.4	
Shop milk	28.1	77.3		65.7	90.5	
Farm milk (ever)	66.8	19.9	< 0.001	31.1	8.1	< 0.001
Only in the first year of life	6.1	8.3		16.8	3.7	
Only at present	8.9	5.2		6.1	2.8	
Both in the first year and at present	51.9	6.3	< 0.001	8.2	1.7	< 0.001
Butter consumption						
No margarine and no butter	3.0	4.5		3.6	5.4	
Shop-purchased butter only	52.6	75.4		80.4	78.8	
Margarine (exclusively)	5.0	6.2		4.0	7.4	
Butter from farm milk (any)	39.4	14.0	< 0.001	12.0	8.5	< 0.001
Yoghurt consumption						
No	4.6	5.6		4.0	5.5	
Shop purchased only	56.5	77.9		78.2	82.4	
Self-produced or directly purchased on a farm	38.9	16.5	< 0.001	17.8	12.1	< 0.001
Egg consumption						
No	3.7	5.7		4.0	6.1	
Shop purchased only	27.8	55.4		70.3	75.3	
Farm-produced or directly purchased on a farm	68.5	38.9	< 0.001	25.7	18.6	< 0.001
Vegetable or fruit consumption						
No vegetables and no fruits	0.6	1.1		0.7	1.0	
Shop purchased only	15.8	42.8		56.2	67.3	
Self-produced or directly purchased on a farm	83.5	56.1	< 0.001	43.1	31.7	< 0.001
Food avoidance due to allergies in the family	4.9	8.8	< 0.001	17.6	10.3	< 0.001

*P-values are given for the comparison of farm children and Rudolf Steiner School (RSS) children vs. their respective reference groups.

farm-produced products except vegetables and fruits. In addition, farm milk and egg consumption were inversely related to diagnosed rhinoconjunctivitis. When simultaneous adjustment was made for all farm-produced foods (right side), only consumption of farm milk remained significantly and inversely associated with the prevalence of diagnosed asthma, diagnosed rhinoconjunctivitis, and current rhinoconjunctivitis symptoms. None of the food items was significantly associated with atopic eczema and current eczema symptoms.

Table 3 shows the association between farm milk consumption and sensitization to aero and food allergens, adjusted for predefined covariates and all farm-produced foods. Using a cut-off level of 0.35 kU/L, a significant inverse association was found for a sensitization to horse allergen, and associations for sensitization to pollen, cat dander, and to the food mix fx5 tended to be negative, whereas the association with house dust and storage mites tended to be positive. When the more clinically relevant cut-off level of $\geqslant 3.5$ kU/L was chosen, the negative association with farm milk consumption became stronger and statistically significant for pollen sensitization and the food mix fx5.

The inverse relation between farm milk consumption and the prevalence of diagnosed asthma, rhinoconjunctivitis, and pollen sensitization was observed in all four study groups without significant heterogeneity (Fig. 1). Similarly, no significant heterogeneity of the effects across study groups was observed for fx5 (p-heterogeneity 0.610) and horse dander (p-heterogeneity 0.465).

When the use of shop-purchased butter and the consumption of butter made out of farm milk was contrasted to margarine consumption, an inverse association was seen between butter consumption and asthma and wheeze even when simultaneously adjusted for farm milk consumption. For asthma: aOR 0.80 (95% CI: 0.65–1.00), 0.62 (0.46–0.84) for shop-purchased and farm-produced butter, respectively. For wheeze aOR 0.84 (0.65–1.06), 0.79 (0.57–1.08) for shop-purchased and farm-produced butter, respectively. No significant associations were observed for other allergy-related health outcomes.

A strong and consistent inverse association was observed for the prevalence of asthma, wheeze, rhinoconjunctivitis (diagnosed and symptoms), pollen sensitization, and fx5 in children who consumed farm milk since their first year of life (Table 4). The inclusion of other relevant concomitant farm activities in the multivariate regression model somewhat attenuated the effects and widened the confidence intervals (CIs) (Table 4), but the estimated protective effect

Table 2. Associations between asthma, rhinoconjunctivitis, and atopic eczema and production type of consumed foods

	Prevalence of health outcomes[†], *n* (%) (*N* = 14 424)	Adjusted[‡] OR (95% CI) for individual farm-produced foods (consumed ever in life[†]) Reference category: shop purchased products					Models simultaneously adjusted for all farm-produced foods				
		Farm milk	Butter from farm milk	Yoghurt from self-production[§]	Eggs from farm-production[§]	Vegetables or fruits from self-production[§]	Farm milk	Butter from farm milk	Yoghurt from self-production[§]	Eggs from farm-production[§]	Vegetables or fruits from self-production[§]
Dr's diagnosis of asthma	1250 (8.9)	0.74* (0.61–0.88)	0.72* (0.58–0.90)	0.81* (0.67–0.98)	0.81* (0.69–0.95)	0.90 (0.78–1.04)	0.79* (0.65–0.95)	0.79 (0.59–1.06)	1.03 (0.80–1.33)	0.90 (0.74–1.09)	1.02 (0.86–1.20)
Current wheezing	1073 (7.6)	0.86 (0.72–1.04)	0.89 (0.71–1.12)	0.92 (0.75–1.12)	0.84* (0.71–0.99)	0.92 (0.79–1.07)	0.90 (0.74–1.10)	0.97 (0.71–1.30)	1.05 (0.80–1.37)	0.86 (0.70–1.06)	1.00 (0.84–1.20)
Dr's diagnosis of rhinoconjunctivitis	591 (4.2)	0.56* (0.43–0.73)	0.73 (0.52–1.02)	0.87 (0.66–1.15)	0.79* (0.63–0.99)	0.87 (0.71–1.06)	0.58* (0.44–0.76)	0.81 (0.53–1.25)	1.20 (0.83–1.73)	0.89 (0.67–1.19)	0.98 (0.78–1.25)
Current rhinoconjunctivitis symptoms	1037 (7.3)	0.70* (0.57–0.85)	0.98 (0.77–1.24)	0.95 (0.77–1.17)	0.86 (0.72–1.03)	0.94 (0.80–1.10)	0.70* (0.57–0.86)	1.14 (0.83–1.56)	1.04 (0.78–1.38)	0.87 (0.70–1.09)	1.02 (0.85–1.22)
Dr's diagnosis of atopic eczema	1436 (10.1)	0.89 (0.75–1.06)	0.89 (0.73–1.09)	0.98 (0.82–1.18)	0.92 (0.79–1.06)	0.98 (0.86–1.13)	0.91 (0.76–1.08)	0.87 (0.66–1.14)	1.12 (0.89–1.43)	0.92 (0.77–1.10)	1.04 (0.88–1.22)
Current atopic eczema symptoms	1517 (10.7)	0.89 (0.76–1.05)	0.94 (0.77–1.14)	0.91 (0.76–1.08)	0.98 (0.85–1.13)	0.91 (0.80–1.04)	0.91 (0.77–1.07)	1.02 (0.79–1.32)	0.92 (0.73–1.16)	1.08 (0.90–1.29)	0.91 (0.78–1.06)

*P-value < 0.05.

[†]Four hundred and sixty-nine never-milk-drinking children excluded. Models were limited to 89.2% children without missing values in any of the exposure or outcome variable.

[‡]Adjusted for study group, country, sex, age, mother's and father's reported asthma and/or hayfever, parent's education, maternal smoking during pregnancy, current environmental smoking at home, older siblings, exclusive breastfeeding > 4 months, BMI, food avoidance due to familial asthma and/or allergy.

[§]Or directly purchased on a farm.

OR, odds ratio; CI, confidence interval.

Table 3. Adjusted[†] odds ratios (95% CI) for farm milk consumption ever in life and sensitization against aero and food allergens

	Sensitization cut-off (kU/L)	Prevalence of health outcomes[‡], *n* (%) (*N* = 3818)	Farm milk consumption Reference category: shop milk consumption only
Phadiatop	⩾0.35	1,056 (28.1)	1.07 (0.86–1.32)
	⩾3.5	595 (16.0)	0.93 (0.71–1.22)
Food mix fx5	⩾0.35	430 (11.4)	0.80 (0.59–1.07)
	⩾3.5	33 (0.9)	0.42* (0.19–0.92)
Pollen	⩾0.35	702 (18.7)	0.80 (0.62–1.02)
	⩾3.5	348 (9.3)	0.67* (0.47–0.96)
House dust mite	⩾0.35	574 (15.3)	1.24 (0.94–1.62)
	⩾3.5	368 (9.8)	1.35 (0.98–1.87)
Storage mite[§]	⩾0.35	168 (4.5)	1.22 (0.78–1.92)
Cat dander[§]	⩾0.35	267 (7.1)	0.86 (0.59–1.25)
Horse dander[§]	⩾0.35	130 (3.5)	0.50* (0.28–0.87)

*P-value < 0.05.

[†]Adjusted for study group, country, sex, age, mother's and father's reported asthma and/or hayfever, parent's education, maternal smoking during pregnancy, current environmental smoking at home, older siblings, exclusive breastfeeding > 4 months, BMI, food avoidance due to familial asthma and/or allergy, and all other farm-produced foods in the table.

[‡]One hundred and sixty-one never milk drinkers were excluded from the analyses.

[§]Due to small numbers of sensitized children, only models with cut-off level 0.35 kU/L were performed.

CI, confidence interval.

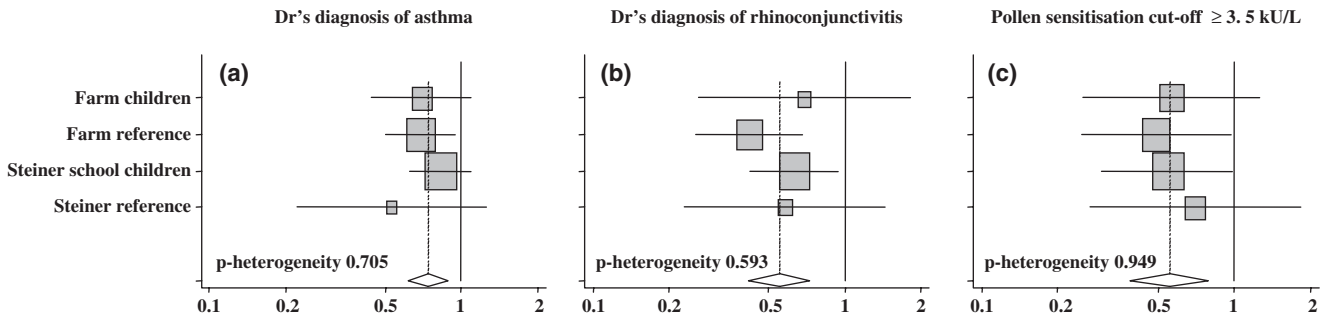

Fig. 1. (a–c) Forest plots of the association between asthma and allergy and consumption of farm milk ever for study groups using meta-analysis. The horizontal lines correspond with the 95% confidence intervals for each study group, with the corresponding box area drawn proportional to the weight for that group in the meta-analysis. The diamond represents the summary estimate. The *P*-value is given for the test of heterogeneity. Adjusted for country, sex, age, mother's and father's reported asthma and/or hayfever, parent's education, maternal smoking during pregnancy, current environmental smoking at home, older siblings, exclusive breastfeeding > 4 months, BMI, food avoidance due to familial asthma and/or allergy.

of farm milk consumption on asthma, rhinoconjunctivitis, and fx5 remained statistically significant.

The effect of being a farm child on asthma [aOR 0.73 (0.59–0.91)], rhinoconjunctivitis [0.42 (0.28–0.62)], and pollen sensitization [0.28 (0.19–0.40)] was only partially explained by farm milk consumption: farm milk adjusted effect of being a farm child on asthma [aOR 0.84 (0.67–1.06)], on rhinoconjunctivitis [aOR 0.55 (0.37–0.83)], and on pollen sensitization [aOR 0.36 (0.24–0.54)].

We also examined whether the observed effect of farm milk consumption was sensitive to the exclusion of specific countries by rerunning the analyses with each country being removed one at a time, but no relevant changes were observed.

Discussion

The analyses of the large cross-sectional study PARSIFAL give evidence of a significant inverse association between farm milk consumption and childhood asthma, rhinoconjunctivitis, sensitization to pollen, a mix of food allergens, and horse dander. Other farm-produced foods were not independently related to asthma and allergy prevalence. Of particular importance is the consistency of the findings across children from farming, rural non-farming, anthroposophic, and (sub)urban environments indicating that farm milk consumption represents a route of exposure that is independent of concomitant exposures to microbial compounds present in animal sheds and farm homes. The

Table 4. Sensitivity analyses for the association between asthma and allergy and farm milk consumption; adjusted OR (95% CI)

	n	Dr's diagnosis of asthma	Current wheezing	Dr's diagnosis of rhinoconjunctivitis	Current rhinoconjunctivitis symptoms	Pollen sensitization cut-off ⩾3.5 kU/L	fx5 cut-off ⩾3.5 kU/L
Timing of farm milk exposure							
Shop milk only (reference category)	9805	1	1	1	1	1	1
Farm milk only in the first year of life	1467	0.79 (0.61–1.01)	0.95 (0.74–1.22)	0.52* (0.35–0.75)	0.69* (0.52–0.90)	0.61* (0.37–1.00)	0.98 (0.69–1.44)
Farm milk only at present	869	0.74 (0.54–1.02)	0.85 (0.61–1.17)	0.58* (0.36–0.94)	0.85 (0.61–1.18)	0.73 (0.42–1.27)	0.86 (0.56–1.33)
Farm milk in the first year and at present	2204	0.67* (0.51–0.88)	0.77 (0.58–1.03)	0.61* (0.40–0.94)	0.60* (0.43–0.83)	0.51* (0.31–0.86)	0.61* (0.42–0.89)
Additional adjustment for the child's current visits to animal sheds							
Shop milk consumption only (Reference category)	9805	1	1	1	1	1	1
Farm milk in the first year and at present	2204	0.74* (0.56–0.98)	0.82 (0.61–1.10)	0.70 (0.45–1.07)	0.68* (0.48–0.94)	0.62 (0.37–1.04)	0.62* (0.42–0.91)

*P-value < 0.05.

†Adjusted for study group, country, sex, age, mother's and father's reported asthma and/or hayfever, parent's education, maternal smoking during pregnancy, current environmental smoking at home, older siblings, exclusive breastfeeding > 4 months, BMI, food avoidance due to familial asthma and/or allergy.

OR, odds ratio; CI, confidence interval.

inverse association was not explained by concurrent farm activities of the child or farm exposures during pregnancy and was most pronounced in children drinking farm milk since their first year of life.

The results of the PARSIFAL study thus confirm an inverse association between farm milk consumption and allergic health outcomes reported previously [5–9]. However, the specific allergic health outcomes associated with farm milk consumption differ between studies. The strong effect on asthma was only reported by the ALEX study, which has been conducted in the same three alpine countries as the PARSIFAL study, but in geographically different and independent study populations [5]. Inverse associations between farm milk consumption and allergic rhinoconjunctivitis have been reported by the ALEX study [5], a study in New Zealand [9], and a recent study in the United Kingdom [8]. The ALEX study and surveys conducted in Crete [7], Northern Germany [6], New Zealand [9], and the United Kingdom [8] reported an inverse association between farm milk consumption and atopy whereas the PARSIFAL study observed an inverse association with most tested allergens, but not mites. No association between farm milk consumption and atopic sensitization was reported by a study conducted among rural children from Finland [10].

Allergen-specific differences in response to environmental exposures have already been reported by a series of farm studies that suggested a stronger association between farm residency and pollen sensitization and no or weak relations with mite sensitization [18–21]. However, other farm studies did not confirm these findings [8, 22, 23]. To evaluate whether the allergen-specific effects associated with farm milk consumption in the PARSIFAL study represent so far unappreciated peculiarities in the biology of allergen/immune system interactions deserves replication of these findings and further investigations of potential mechanisms.

The studies conducted in the United Kingdom and New Zealand reported strong and statistically significant inverse relations between farm or raw milk consumption and atopic eczema that was not observed in the present study. These contrasting findings may in part be explained by the amount of control for food avoidance that differed between the studies. Food avoidance due to a pre-existing allergy may bias the results in cross-sectional analyses. Eleven percent of the PARSIFAL population reported avoiding certain foods due to an existing allergy in one of the family members. Adjusting all regression models for this variable only slightly attenuated the effect estimates of farm milk consumption on asthma or rhinoconjunctivitis. However, the association between farm milk consumption and diagnosed eczema became non-significant when adjustment was made for food avoidance [diagnosed eczema without adjustment for food avoidance (aOR 0.80, 95% CI 0.68–0.95), and current eczema

symptoms (aOR 0.84, 95% CI 0.71–0.98), respectively]. When the study population was restricted to those without food avoidance, no significant association between farm milk consumption and diagnosed atopic eczema (aOR 0.90, 95% CI: 0.73–1.10) or current eczema symptoms (aOR: 0.94, 95% CI 0.79–1.13) was observed. We also excluded all non-milk-drinking children because this group may have changed dietary habits due to allergy-related skin or gastrointestinal problems early in life and not report it as deliberate food avoidance. However, this restriction had no strong impact on reported atopic dermatitis or any other allergic health outcome.

At present, we can only speculate about the components of farm milk responsible for the observed protective effect. Farm milk possibly contains different levels or a different composition of pathogenic and nonpathogenic microbes compared with milk purchased in a shop. The health effects of pathogens in raw milk such as salmonella or enterohaemorrhagic *Escherichia coli* (EHEC) are well recognized, and transmission of EHEC through unpasteurized cow's milk continues to cause serious health effects [24]. It is conceivable that the microbial burden of farm milk influences the gut microflora and thus the development of oral tolerance [25, 26]. Recent animal experiments have shown that colonization of germ-free mice with polysaccharide-A-producing *Bacteroides fragilis*, a ubiquitous gut microorganism and an important Gram-negative anaerobe that colonizes the mammalian lower gastrointestinal tract, restored normal cytokine production and established a proper T-helper type 1 (Th1)/Th2 balance for the host [27]. Gut microflora may also regulate immune responses outside the gut as has been evidenced in recent animal experiments. Mice were treated with antibiotics in drinking water, followed by a single oral lavage of yeast (*C. albicans*) [28]. They developed alterations of gastrointestinal bacterial populations and increased yeast numbers in the gastrointestinal microbiota. Subsequent intranasal exposure to mould spores led to an allergic response in the airways that was not observed when exposure occurred without prior alteration of the gut microflora. These results indicate that events in distal mucosal sites may play an important role in regulating immune response in the airways. Commensal microorganisms present in farm milk might therefore be responsible for the decreased risk for respiratory allergies such as asthma and hayfever.

The present study does not allow evaluating the effect of pasteurized vs. raw milk consumption because no objective confirmation of the raw milk status of the farm milk samples was available. Parental answers to a question on consumption of boiled vs. raw farm milk are likely to be biased due to the social desirability of responses because raw milk consumption is not recommended especially for young children. About half of the parents indicated that they usually did not boil the milk before consumption but no differential effects were observed between those boiling and those not boiling the milk. This might be a result of biased parental answers or may indicate that pasteurization is not of key importance because compounds other than microbes may play a role. This interpretation is supported by an analysis of Swiss alpine farm milk from exclusively grass-fed cows showing a higher content of omega-3 fatty acids than milk from cows fed conserved grass such as silage [29]. The relative concentrations of linolenic acid (18 : 3) and eicosapentaenoic acid (20 : 5) and the ratio of eicosapentaenoic acid to arachidonic acid (20 : 4) that is critical for the formation of omega-3-derived eicosanoids were significantly higher in milk from grass-fed cows and in cheese made from this milk [29, 30]. Research into fatty acid effects on allergic diseases has focused on the intake of omega-3 fatty acids that is potentially beneficial, and of omega-6 and trans-fatty acids which might be detrimental to asthma [31]. Elevated margarine consumption, which contributes to the intake of omega-6 fatty acid and of trans-fatty acids has been reported to increase childhood atopy risk in several epidemiological studies [3, 32]. Other studies indicated that full-fat milk and butter was associated with a reduced risk of asthma in young children [33–35]. In the present study, butter compared with margarine consumption was associated with a lower risk for asthma supporting a possible role of fatty acid intake. Future analyses of the farm milk compounds responsible for the beneficial effect therefore have to include fatty acid profiles in addition to microbial compounds.

Several limitations of the study have to be taken into account. First, dietary assessment is based on a limited set of variables that do not provide a complete representation of the child's diet. However, the primary aim of the present analyses was to compare farm-produced vs. shop-purchased products and their effect on allergic diseases and not to evaluate the effect of diet per se. The validity and reproducibility of the present dietary assessment has been shown to be good especially for farm milk consumption. Second, as no measurements of biological compounds of farm milk or other farm-produced products are available, the study reports associations and cannot provide an insight into the mechanism underlying the observed association between farm milk consumption and allergic diseases. Third, as the underlying mechanism of the farm milk effect is not known, the study does not allow to elucidate why consumption of farm milk is associated with different allergic health outcomes in different study populations.

In conclusion, the results of the present study indicate that consumption of farm milk is associated with a lower risk of childhood asthma and rhinoconjunctivitis. These results might be transferred to non-farming populations as they were observed in all subpopulations of the PARSIFAL study. Dietary interventions are an attractive

means for primary prevention. However, raw milk may contain pathogens such as salmonella or EHEC, and its consumption may therefore imply serious health risks [24]. A deepened understanding of the relevant 'protective' components of farm milk and a better insight into the biological mechanisms underlying the reported epidemiological observation are warranted as a basis for the development of a safe product for prevention. At this stage, consumption of raw farm milk cannot be recommended as a preventive measure.

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Chapter 4 Not all farming environments protect against the development of asthma and wheeze in children

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Not all farming environments protect against the development of asthma and wheeze in children

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Background: In recent years, studies have shown a protective effect of being raised in a farm environment on the development of hay fever and atopic sensitization. Inconsistent data on the relation of farming to asthma and wheeze have raised some doubt about a true protective effect.
Objective: We sought to study the differential effects of farm-associated exposures on specific asthma-related health outcomes.
Methods: The cross-sectional Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle study included 8263 school-age children from rural areas in 5 European countries. Information on farm-related exposures and health outcomes was obtained by using questionnaires. In subsamples allergen-specific IgE and RNA expression of *CD14* and *Toll-like receptor* genes were measured, and dust from children's mattresses was evaluated for microbial components.
Results: Inverse relations with a diagnosis of asthma were found for pig keeping (odds ratio [OR], 0.57; 95% CI, 0.38-0.86), farm milk consumption (OR, 0.77; 95% CI, 0.60-0.99), frequent stay in animal sheds (OR, 0.71; 95% CI, 0.54-0.95), child's involvement in haying (OR, 0.56; 95% CI, 0.38-0.81), and use of silage (OR, 0.55; 95% CI, 0.31-0.98; for nonatopic asthma) and in Germany for agriculture (OR, 0.34; 95% CI, 0.22-0.53). Protective factors were related with higher expression levels of genes of the innate immunity. Potential risk factors for asthma and wheeze were also identified in the farm milieu. Levels of endotoxin and extracellular polysaccharides were related to the health outcomes independently of the farm exposures.
Conclusions: The protective effect of being raised in a farm environment was ascribed to distinct exposures.
Clinical implications: The development of atopic sensitization and atopic and nonatopic asthma is most likely determined by different environmental factors, possibly reflecting distinct pathomechanisms. (J Allergy Clin Immunol 2007;119:1140-7.)

Key words: *Asthma, wheeze, atopic sensitization, farming, microbial components*

Numerous studies have shown a protective effect of being raised in a farm environment on the development of hay fever and atopic sensitization among children.[1-11] The effects on asthma, however, are inconsistent. Whereas in some studies being raised on a farm was inversely associated with asthma or wheeze ever,[1,2,6,7,9,12,13] a substantial number of studies did not find any significant association,[4,5,10,11,14-16] and in some regions a tendency toward a positive association of farming with asthma was revealed.[2,17] These controversial results have raised some doubt about a true farm effect on asthma. However, a more differentiated look at distinct farm-related exposures and specific asthma phenotypes might be useful.

Moreover, little is known about potential specific determinants of asthma and atopy in the farm environment. As candidate protective factors, consumption of unpasteurized farm milk[7,18] and exposure to animal sheds have

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Abbreviations used
EPS: Extracellular polysaccharide
OR: Odds ratio
PARSIFAL: Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle
TLR: Toll-like receptor

been identified. Dust from animal sheds is rich in endotoxin[19] and muramic acid,[20] both bacterial components that can activate innate immunity, thereby potentially affecting the development of asthma and allergic diseases.

Farming practices and hence microbial exposures vary between farms. The global analysis of the multicenter Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study revealed a weak effect of being raised on a farm on asthma, with substantial heterogeneity across study regions.[21] The present analysis aimed at disentangling the differential effects of various farm-associated exposures on specific asthma-related health outcomes, thereby explaining the heterogeneous findings. Furthermore, the contribution of several microbial components to the identified effects was studied.

METHODS

Population and study areas

The PARSIFAL study was a cross-sectional survey on children of farmers, children attending Rudolf Steiner schools, and their respective reference groups. The study was carried out as described previously.[21] In the farming branch of the study, children aged 5 to 13 years from rural areas of Austria, Germany, The Netherlands, Sweden, and Switzerland were invited to participate. Of the 11,969 invited children, 8402 (70%) returned the questionnaires. A total of 139 children were excluded because of missing values for sex and age or because they did not, on review, meet the age criteria of 5 to 13 years. Of the included 8263 children, 88% had complete values for all farm exposure variables. The study was approved by the national ethical boards of the 5 study centers, and informed consent was obtained from the children's parents or guardians for questionnaires and blood samples.

Questionnaire

The questionnaire included questions on sociodemographic background, family history of asthma and atopy, exposure to farm environment, animals, pets, housing, nutrition, and the child's health. Questions on health outcome and farm exposure were derived from the internationally validated International Study of Asthma and Allergies in Childhood II[22] questionnaire and the Allergy and Endotoxin study.[7] Children with reported physician-diagnosed asthma once or obstructive bronchitis more than once in their lifetime were defined as having *asthma ever*. Wheezing during the past 12 months was considered *current wheeze*. A child who lived on a farm and whose family ran the farm was coded as being a *farm child* (n = 2823), whereas all other children were termed *reference children* (n = 5440). Questions concerning farm life and farm exposure were asked to both farm and reference children, covering regular stays in animal sheds or barns; regular participation in farm activities, such as harvesting of hay; consumption of farm milk; regular contact with farm animals; and maternal involvement in farm work during pregnancy. Farmers were asked whether they performed livestock farming, agriculture (cultivation of grain), or combinations of these. Data on farm characteristics, such as animal species kept on the farm (cattle, pigs, poultry, horses, sheep, goats, hares, and rabbits) or animal feed (pressed hay, loose hay, pellet feed, and silage), were also collected. The children's farm activities at present were dichotomized into rarely or regularly (at least once a week).

Measurement of allergen-specific serum IgE

Blood analysis was performed in a subsample of 2086 children, which did not differ substantially from the whole sample with respect to farm exposures. Allergen-specific IgE for common inhalant (Phadiatop) and food allergens (fx5; Pharmacia CAP System; Phadia AB, Uppsala, Sweden) was measured in the serum. Definition of atopic sensitization and distinction between atopic and nonatopic asthma or wheeze was based on IgE values of 0.35 kU/L or greater for inhalant or food allergens.

Expression of *CD14* and *Toll-like receptor*

Gene expression of *CD14* and *Toll-like receptor* (*TLR*) was assessed in 268 Swiss children, among them 56% farm children. Children with available RNA samples (95.3% of children who provided blood samples) did not differ significantly from the total Swiss PARSIFAL population with respect to farm exposures and health outcomes (data not shown). Detection methods are described in the Online Repository at www.jacionline.org.

Detection of microbial components

Levels of endotoxin, β(1→3)-glucan, and fungal extracellular polysaccharides (EPSs) were measured in mattress dust samples of 440 children. Sampling and detection methods are described elsewhere[23] (see also this article's supplementary Methods text in the Online Repository at www.jacionline.org).

Statistical analysis

Statistical analysis was performed with SAS 9.1.3 (The SAS Institute, Cary, NC). Odds ratios (ORs) from bivariate analysis or logistic regression models are given with 95% CIs. Frequencies of farm characteristics were calculated as percentages of all farms (Table I), and children's activities on farms were calculated as percentages of all children (Table II).

Parsimonious models (Fig 1) were established for every outcome separately by means of backward elimination with a significance level of .157, corresponding to Akaike's information criterion, to identify the individual effects of single farm characteristics and the children's activities. The backward elimination started with the following set of variables: family history of asthma or atopy, respectively; sex, being a farm child, and study center; agriculture; keeping pigs, poultry, horses, sheep, goats, hares, or rabbits; feeding pressed hay, loose hay, silage, or pellet feed at the farm; and predominant farm milk consumption by the child, regular stays in animal sheds, regular stays in barns, regular contact with farm animals, and helping with haying. Models for atopic sensitization additionally included maternal exposure to animal sheds during pregnancy.[24] In some study regions, being a farm child, livestock farming, and cattle keeping were collinear, and therefore the latter 2 variables were not included in backward elimination.

The specific determinants for the atopic and nonatopic phenotypes of asthma and wheeze were explored by using the same procedure starting from the same set of variables (Fig 2). In a sensitivity analysis further variables (age, parental educational level, daycare attendance,

TABLE I. Characteristics of farms in the PARSIFAL study

Farm characteristics	Percentage of farm children (n = 2813)
Farm types	
Livestock farming	82%
Agriculture	40%
Animals kept on farm	
Cattle	70%
Poultry	46%
Pigs	33%
Hares and rabbits	33%
Horses	21%
Sheep	16%
Goats	15%
Fodder used on farm	
Pellet feed	64%
Loose hay	57%
Silage	53%
Pressed hay	42%

TABLE II. Children's farm-related activities in the PARSIFAL study

Children's activities	Farm children (n = 2813)	Reference children (n = 5440)
Regular* farm milk consumption	68%	18%
Regular stays in animal sheds	75%	13%
Regular stays in barns	61%	8%
Regular involvement in haying	51%	3%
Regular contact with farm animals	93%	34%
Regular contact with cattle	77%	16%
Regular contact with pigs	38%	8%
Regular contact with sheep	21%	8%
Regular contact with poultry	47%	16%
Regular contact with horses	29%	17%
Regular contact with goats	20%	7%
Regular contact with hares/rabbits	39%	29%

*"Regular" is defined as at least once a week.

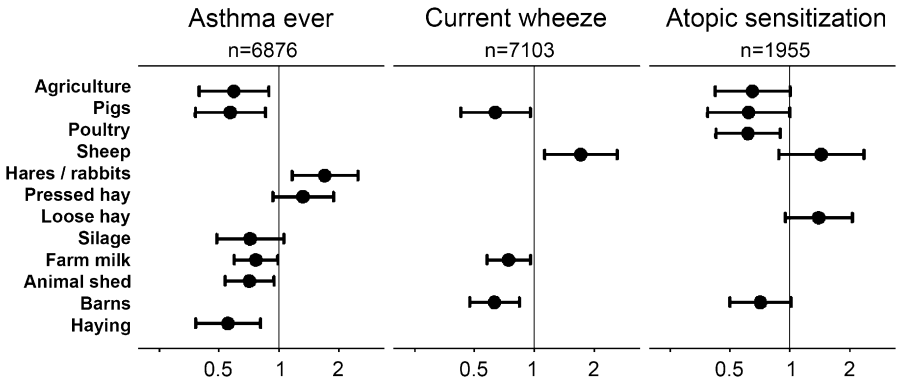

FIG 1. Farm-related specific determinants of asthma, wheeze, and atopic sensitization. Mutually adjusted ORs with 95% CIs additionally adjusted for sex, study center, group, and family history of asthma are shown. Atopic sensitization was adjusted for sex, study center, family history of atopy, and additionally for maternal exposure to animal sheds during pregnancy. The models were established by using backward elimination based on Akaike's information criterion.

duration of breast-feeding, contact with pets, reported childhood infections, worm infestation, and use of antibiotics or antipyretics) were explored but did not confound the associations.

All exposures that contributed significantly to the models in Fig 1 were used to calculate propensity scores for being a farm child (ie, the probability of being a farm child dependent on the farming exposures). The resulting propensity scores were then used as scalar variables to adjust for being a farm child in the analyses investigating the effects of microbial components on asthma, wheeze, and atopy.[25] Values for exposure levels of microbial contaminants were obtained by means of division of measured concentrations by weight of collected mattress dust followed by log transformation, resulting in normal distribution. Data on microbial components were only available for a subsample selected for wheezing and atopy status,[23] and therefore stratified weighted logistic regression was performed.[26] Each observation was weighted by the inverse probability of being selected to the subsample of dust analysis.

In a final step heterogeneity across study regions of the effect of farming on asthma was assessed, looking at farms with and without agriculture separately. ORs for farming were calculated stratified for study regions and compared by using Breslow-Day tests. In multiple regression models interaction terms for farming and study regions were explored.

RESULTS

Characteristics of farms and children's activities on farms

Information on farms is provided in Table I. The questions regarding farm characteristics were not mutually exclusive, and therefore percentages do not sum up to 100%. Because the PARSIFAL study was deliberately performed in regions of dairy farming, the predominant form of farming was livestock, in particular cattle. Yet substantial numbers of other animals were kept on the farms as well. Of the livestock farms, 37% also performed agriculture, most likely to provide fodder for their animals. Only 24% of agricultural farms did not also raise livestock. Because farm

characteristics might reflect a child's exposure only to a limited extent, children's activities on a farm were assessed also for farm and farm reference children (Table II). A third of the reference children in these rural areas were exposed to farm animals on a regular basis (ie, at least once a week). The prevalences of the lifetime diagnosis of asthma and of current wheeze in farm children were 6.3% and 5.0%, respectively, and in reference children they were 9.1% and 7.7%, respectively.

Effects of farm characteristics and children's activities on asthma, wheeze, and atopic sensitization

Fig 1 shows the mutually adjusted models for asthma, wheeze, and atopic sensitization resulting from backward elimination. Variables with nonsignificant associations with the health outcomes in these models are not listed (ie, horses kept on the farm, goats kept on the farm; pellet feed or other animal feed; and children's regular contact with specific farm animal species [cattle, pigs, sheep, poultry, horses, goats, and hares/rabbits]). The lifetime diagnosis of asthma was inversely associated with agriculture (OR, 0.60; 95% CI, 0.40-0.89), pig farming (OR, 0.57; 95% CI, 0.38-0.86), farm milk consumption (OR, 0.77; 95% CI, 0.60-0.99), frequent stay in animal sheds (OR, 0.71; 95% CI, 0.54-0.95), and child's involvement in haying (OR, 0.56; 95% CI, 0.38-0.81), and there was borderline significance with using silage as feed (OR, 0.72; 95% CI, 0.49-1.06). Keeping of hares and rabbits (OR, 1.70; 95% CI, 1.16-2.50) and the use of pressed hay (OR, 1.32; 95% CI, 0.93-1.88) were positively associated. Current wheeze was inversely related to pig farming (OR, 0.64; 95% CI, 0.43-0.95), farm milk consumption (OR, 0.74; 95% CI, 0.58-0.96), and frequent stays in barns (OR, 0.63; 95% CI, 0.47-0.84); the presence of sheep, however, was positively associated with wheezing (OR, 1.72; 95% CI, 1.13-2.61). Frequent stays in barn were also inversely associated with atopic sensitization, as well as agriculture and keeping pigs and poultry on the farm. The use of loose hay as fodder was positively associated with atopic sensitization. When adjusting for the child's activities and farm characteristics, the variable of being a farm child was not any longer inversely related with asthma, wheeze, or atopy; in other words, the variables included in the final models explained the effect of farming on asthma, wheeze, and atopy completely (data not shown).

Effects of farm characteristics and children's activities on the expression of *CD14* and *TLR* genes

Table III gives the geometric means ratios for the expression of *CD14* and *TLR* genes, depending on the various farm characteristics and children's activities that were related to asthma or wheeze from Fig 1. Interestingly, the different exposures exhibited specific effects on different genes. Because of a high correlation ($r > 0.8$) of the variables regular stays in animal sheds, regular stays in barns, and maternal exposure to stables during pregnancy with being a farm child in the Swiss subsample, independent effects of these variables could not be studied.

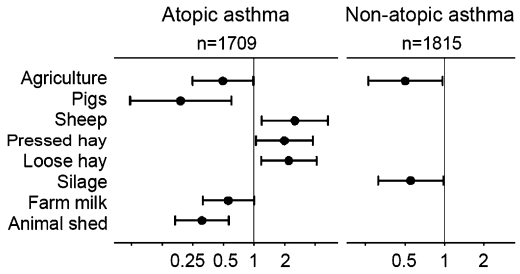

FIG 2. Farm-related specific determinants for atopic and nonatopic asthma. Mutually adjusted ORs with 95% CIs additionally adjusted for number of older siblings, sex, study center and family history of asthma, where appropriate, are shown. The models were established by using backward elimination based on Akaike's information criterion in the subsample of children selected for measurement of allergen-specific IgE levels.

Atopic and nonatopic phenotypes of asthma and wheeze

Besides common determinants for asthma and atopic sensitization, some specific determinants for either asthma or atopic sensitization were also detected. This observation prompted a differential evaluation of the asthma and wheeze phenotypes with respect to a child's atopy status. As shown in Fig 2, only agriculture exhibited an inverse relation with both phenotypes of asthma. Further effects on atopic asthma were exerted by pig farming (OR, 0.19; 95% CI, 0.06-0.60), regular consumption of farm milk (OR, 0.56; 95% CI, 95% CI, 0.31-1.01), and stays in animal sheds (OR, 0.31; 95% CI, 0.17-0.57). Sheep farming and feeding loose or pressed hay were positively associated with the atopic phenotype. Interestingly, the use of silage was protective for nonatopic asthma (OR, 0.55; 95% CI, 0.31-0.98). For wheeze, almost the same determinants were found (data not shown): pig farming, silage, and barn stays were inversely related to atopic wheeze, and sheep farming and feeding loose hay were positively related; barn stays tended to be protective for nonatopic wheeze.

Protective and risk-associated farm characteristics with respect to asthma and wheeze

The 742 of 2813 farm children who were growing up in a strongly protective farm environment (ie, exposed to 4 or 5 ''asthma-protective'' factors) had an asthma prevalence of less than 3%. On the other hand, 210 farm children were exposed to asthma risk factors (keeping hares or rabbits and feeding pressed hay), and only 1 or no protective factors. This group of children had an asthma prevalence of 17%. Children living on farms where sheep but no pigs or cows were kept had prevalences of wheeze and asthma up to 4 times higher than their peers living on farms where pigs or cows but no sheep were kept (12% vs 3% and 14% vs 3% for wheeze and asthma, respectively). Farm

TABLE III. Geometric means ratios for expression of CD14 and TLR dependent on children's farm exposures and farm characteristics

Exposure variables	*CD14*	*TLR1*	*TLR2*	*TLR3*	*TLR4*	*TLR5*	*TLR6*	*TLR7*	*TLR8.1**	*TLR8.2**	*TLR9*	*TLR10*
Being a farm child	1.67, $P < .001$	1.38, $P = .006$	1.52, $P < .001$		1.20, $P = .046$			1.26, $P = .048$	1.29, $P = .034$	1.34, $P = .032$		
Haying†				1.72, $P = .067$				1.53, $P = .021$				1.83, $P = .011$
Farm milk consumption†								1.38, $P = .040$		1.42, $P = .060$		
Keeping pigs†						1.41, $P = .054$						
Feeding silage†							1.35, $P = .027$			1.39, $P = .065$	1.32, $P = .079$	
Keeping sheep†												
Keeping hares†			1.27, $P = .072$									
Feeding pressed hay†						0.53, $P = .001$						

Only geometric means ratios with P values of less than .1 are shown. The geometric means ratios are adjusted for age, sex, and for being a farm child (†).
*Isoform 1 (TLR8.1) encodes the longer isoform, which has an extended N-terminus compared with isoform 2 (TLR8.2).

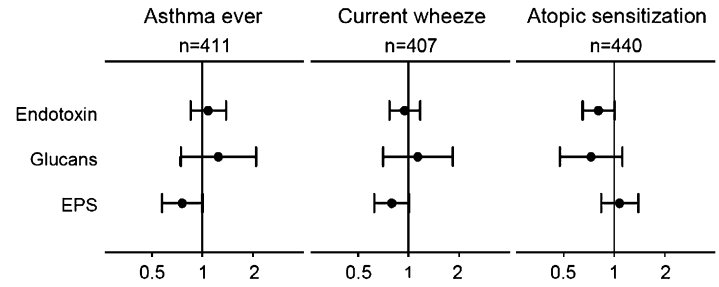

FIG 3. Effects of microbial compounds measured in mattress dust on asthma, wheeze, and atopic sensitization. Mutually adjusted ORs with 95% CIs additionally adjusted for all variables contributing to the respective models in Fig 1 are shown.

children with risk factors for atopy (sheep and loose hay) but no protective factors had a prevalence of 14% for atopic sensitization, whereas among the 109 farm children with no risk-associated factors but at least 3 protective factors, only 2 (1.8%) children were sensitized.

Effects of microbial substances on asthma and related health outcomes

Fig 3 shows the mutually adjusted effects of endotoxin, glucans, and EPS levels in mattress dust on asthma, wheeze, and atopic sensitization. Endotoxin was inversely related to atopic sensitization ($P = .0531$). Glucans were not significantly associated with any of the 3 outcomes but tended to be protective for atopic sensitization. EPSs were inversely related to asthma ($P = .0483$) and with borderline significance to wheeze ($P = .0581$). Further distinction of atopic and nonatopic phenotypes did not reveal more information, most likely because of limited sample sizes. The protective effect of being a farm child on current wheeze was explained by the levels of exposure to endotoxin, glucans, and EPSs (adjusted OR, 0.89), whereas for asthma (adjusted OR, 0.55) and atopic sensitization (adjusted OR, 0.38), the protective effect of being a farm child was not explained by the levels of these exposures. Although children from farms with pigs but without sheep had endotoxin levels (geometric mean, 41,438 EU/g; 29,551-58,107 EU/g) in mattress dust almost twice as high as those seen in children from sheep farms without pigs (26,909 EU/g; 17,192–42,118 EU/g), this difference is unlikely to reflect the underlying mechanisms of the effects of pig and sheep keeping on asthma and sensitization because the variables of keeping sheep and keeping pigs did not change the OR for endotoxin (data not shown).

Heterogeneity across countries

The crude asthma OR for being a farm child versus that seen in reference children varied substantially across countries.[21] A low OR was found in Germany (OR, 0.45; 95% CI, 0.32-0.64), whereas in the other 4 countries the OR was close to unity (OR, 0.92; 95% CI, 0.72-1.17). The effect was not heterogeneous among these 4 countries ($P = .85$), but it was heterogeneous between them and Germany ($P = .013$). Thus the 2 subsamples (children from countries without a farm effect and the German subsample) were analyzed for differences in farm characteristics. When comparing children from farms without agriculture with reference children, the effect of farming

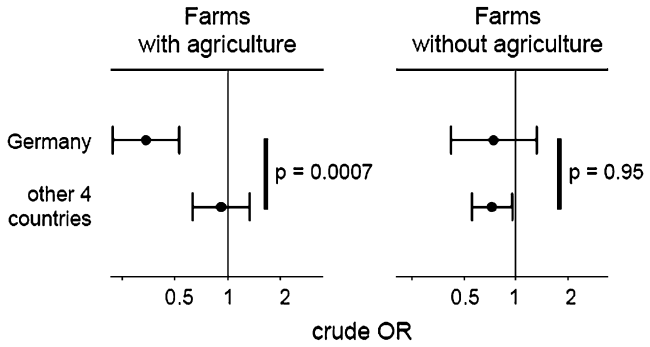

FIG 4. Effect of being a farm child on asthma in agricultural and nonagricultural farms. Crude ORs are given with 95% CIs for asthma diagnosis dependent on being a farm child separately for the German subsample and the subsample of the 4 other countries. *P* values refer to Breslow-Day tests for homogeneity of effects.

on asthma did not vary between the 2 subsamples (Fig 4). However, when comparing children from farms with agriculture with reference children, the effect was strong in Germany (OR, 0.35; 95% CI, 0.23-0.55) but marginal in the other 4 countries (OR, 0.93; 95% CI, 0.65-1.33). Heterogeneity of effects between the 2 subsamples was formally proved by using a highly significant Breslow-Day test ($P = .0007$) and a significant interaction term in multiple regression analysis ($P = .0015$).

DISCUSSION

The present study revealed distinct protective exposures for asthma and wheeze (agriculture, pig farming, silage, haying, farm milk, animal sheds, and barns). Most of these exposures were related to the atopic phenotypes, but silage exerted a protective effect only on the nonatopic phenotype. Furthermore, potential risk factors for asthma and wheeze have been identified in the farm milieu (sheep and hare keeping and using hay as feed). For atopic sensitization, agriculture, pig, and poultry farming and barns were identified as protective factors. Collectively, the identified factors explained the protective effect of being a farm child on asthma. Endotoxin and EPS levels were related to asthma, wheeze and atopic sensitization independently of the farm characteristics and the child's activities.

Potential determinants of asthma, wheeze, and atopic sensitization

The effects of frequent stays in animal sheds and farm milk consumption have been described previously.[7,24] A detailed analysis of the effects of farm milk and other dairy products is the subject of a separate article.[18]

The large sample size of the PARSIFAL study provided the opportunity to differentiate between several animal species usually kept on farms. In this context the identification of pig keeping, in our study populations mostly in combination with cattle keeping, as a potential protective factor for all 3 health outcomes is an interesting finding. An inverse relation of pig keeping with atopic sensitization has also been detected in two studies performed in Guinea-Bissau[27] and New Zealand.[16] In contrast, in an Iowa population pigs were found to be positively related to asthma.[17] In our analysis the protective effects of pigs on asthma and atopy were consistent across all countries (data not shown). The discrepancy between the American study and the other studies might be explained by different farm sizes and hence children's exposure. At least in our study population, more than 80% of pig farmers kept less than 10 pigs, and exclusive pig farming was very uncommon in the study population (n = 34). Four (12%) children from exclusive pig farms had a physician's diagnosis of asthma, whereas only 35 (4%) of 884 children from dairy farms with additional pig keeping had asthma ($P = .0514$). An exploratory analysis of the number of pigs kept on a farm did not reveal any dose-response effect. An explanation for the effects of pig keeping might be found in specific microbial exposures present in farms where pigs are kept in addition to cattle.

In contrast to pig keeping, sheep farming increased the risk for atopic asthma and wheeze. This might be a plausible finding because for sheep breeders, a higher risk of respiratory syndromes has been described, possibly caused by disinfectants, such as formaldehyde or copper sulfate.[28] Whether this explanation can be applicable to children remains to be elucidated. Nevertheless, sheep keeping might obscure a protective farm effect on asthma in populations where sheep breeding is common.[16]

Harvesting, storage, and feeding of hay in various forms is another key feature of farms. Active participation in haying, however, might be avoided by children with asthma or allergies, implying potential reverse causation. In the PARSIFAL study only 62 families (<1% of all) reported that the child avoided haying because of manifest disease or for prevention, and the effect of haying was still present when the analysis was restricted to children without sensitization to grass pollen (data not shown). One could imagine that small dust particles inhaled during haying might act as carriers of protective agents, such as microbial antigens or immunomodulatory substances (eg, mycotoxins). This notion is supported by the gene expression studies showing that active participation in haying was associated with higher gene expression of several *TLR* genes (Table III).

In turn, a positive association of pressed hay with asthma and of loose hay with atopic asthma and atopic sensitization was found. Yet this does not necessarily contradict the potential protective effect of active participation in haying because the microbial colonization of fresh hay differs substantially from that of stored hay.[29,30] Barns do not only comprise hay lofts but are also storage places for other farm products, such as crop, grain, and straw. Therefore it is conceivable that the protective effect of barns is present despite a disadvantageous effect of pressed or loose hay.

Silage is of special interest because it was only clearly protective for the nonatopic phenotypes of asthma and wheeze. Silage contains vast amounts of lactobacilli but also other microbes with potential immunomodulatory effects, such as aspergilli[31] and listeriae.[32] Small but continuously inhaled or ingested doses of microbial contaminants, such as mycophenolic acid from aspergilli, might suppress T-cell activity.[31] *Listeria monocytogenes*[32] is known for its ability to stimulate the innate immune system and to convert T_H2-dominated immune responses into T_H1-dominated responses.[33]

The exact molecular mechanisms of the farm-related exposures and their beneficial and, in some cases, also probably harmful effects on asthma remain, however, unknown. The observation that these farm-related exposures are associated with the differential expression of the *CD14* and *TLR* genes (Table III) supports the idea of distinct effects on a molecular level. Various exposures might differentially affect the innate immune system in qualitative and quantitative terms.

A striking finding of the present analysis was the identification of agriculture as having the power to explain the heterogeneous effects of farming on asthma between Germany and the other study regions. Because the effect of agriculture on asthma was only present in Germany, comparing the practices and products of agriculture of the German areas with those of the other PARSIFAL study regions might be helpful. Unfortunately, no supplemental data on agriculture were available in the data set. However, because most of the agricultural farms (>80%) of the German subsample concomitantly raised livestock (cattle/pigs), cultivation of feed grain is the most probable form of agriculture. Culture of fodder beets, oilseeds, potatoes, or cereals for human nutrition is likely to play a minor role in these areas. Contamination of feeding grain with immunomodulatory mycotoxins is a well-known phenomenon,[34-37] and one might speculate that feeding grain could contain beneficial immunomodulatory substances. In particular, pigs are fed with bruised grain, which can be contaminated with deoxynivalenol, a trichothecene mycotoxin that enhances proinflammatory gene expression in macrophages after costimulation by TLR ligands, such as endotoxin.[38]

Independent effects of endotoxin and glucans

Mattress dust samples of selected farm and reference children were examined to back up the questionnaire data with objective biologic parameters. A previous analysis of the same data set focused on atopic wheeze ever as an outcome because of the stratified sampling design.[39] After adjustment of farming, no significant associations of the outcomes with the biocontaminants under investigation were seen. The present analysis used weighted stratified logistic regression to overcome problems of the sampling design, thereby rendering exploration of further outcomes, such as lifetime diagnosis of asthma, current wheeze, and atopic sensitization, possible.

The protective effect of endotoxin on atopic sensitization is in accordance with that seen in previous studies.[19,40] $\beta(1\rightarrow3)$-glucans are cell-wall constituents of most fungi, but are also found in some bacteria and many plants.[41] The inverse association of glucans with atopic sensitization did not reach statistical significance, potentially because of limited sample size, but the estimate was stronger than for endotoxin. EPSs of *Aspergillus* and *Penicillium* species are more specific markers of indoor fungal exposure.[41] The inverse association of EPSs with asthma is a novel finding. Of all 3 microbial substances, EPSs were most closely related to being a farm child (data not shown).

The associations of the 3 biocontaminants with the asthma-related health outcomes were independent of the farm characteristics and the children's activities. Hence they might represent exposures not adequately covered by questionnaire data. On the other hand, the exposures identified through questionnaires, such as agriculture or pig farming, were not traced back to the microbial compounds under investigation, ultimately prompting exploration of further microbial substances (eg, those found in agriculture or animal feed).

Conclusion

The asthma-protective effect of being raised on a farm in the PARSIFAL study can be attributed to pig farming, feeding silage, child's involvement in haying, farm milk consumption, and regular stay in animal sheds and barns. In Germany performing agriculture contributed importantly to the asthma-protective effect. The microbial compounds investigated thus far do not explain the inverse relation of asthma prevalence with farm characteristics and children's activities but exert independent effects. The identification of highly protective farms might direct future research to investigate microbial contamination of distinct farms with their individual animal species and feeds.

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METHODS

Detection of microbial components

Atopic and nonatopic wheezers were selected, as well as healthy nonsensitized control subjects.[E1] Dust from mattresses was collected by parents of the participating children. They were sent nylon sampling socks and instructed to vacuum the whole area of the mattress. Endotoxin was measured with the kinetic chromogenic Limulus Amebocyte Lysate test (Bio Whittaker, Walkersville, Md), glucans with an inhibition enzyme immunoassay,[E2] and EPSs with a specific sandwich enzyme immunoassay for EPSs of *Aspergillus* and *Penicillium* species.[E3]

Expression of *CD14* and *TLR*

For the Swiss branch of the PARSIFAL study, RNA samples were collected from 195 farm and 127 reference children (95.3% of children who provided blood samples) to analyze gene expression of innate immunity receptors. Children with available RNA samples did not differ significantly from the total Swiss PARSIFAL population with respect to farm exposures and health outcomes (data not shown).

The total RNA was isolated by using the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). For RT of RNA, 300 ng of total RNA in a final volume of 30 μL was used, and adequate amounts of TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif) were added. Quantitative real-time PCR of *CD14*, *TLR2*, and *TLR4* was performed as previously described.[E4] The quantification of mRNA of the other *TLR* genes was performed on an ABI Prism 7900 Sequence Detection System using the TaqMan low density array system (Applied Biosystems). The determined gene expression values were normalized to the parallel measured endogenous control 18S rRNA. We analyzed the data with the comparative cycles threshold method, according to the manufacturer's instructions (Applied Biosystems).

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Chapter 5 A polymorphism in CD14 modifies the effect of farm milk consumption on allergic diseases and CD14 gene expression

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A polymorphism in *CD14* modifies the effect of farm milk consumption on allergic diseases and *CD14* gene expression

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Background: Consumption of farm milk in early life is associated with less asthma and allergies.
Objective: We hypothesized that genetic variation in the innate immunity receptor *CD14* might modify the association between farm milk consumption and asthma and atopy.
Methods: Questionnaire data, serum IgE levels, and genotypes for 4 single nucleotide polymorphisms in *CD14* were assessed in farmers' and nonfarmers' children from 2 European populations (Allergy and Endotoxin study, n = 576; Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle study, n = 1539). In a subsample (n = 222) *CD14* gene expression was measured in peripheral blood leukocytes. The effects of farm milk and *CD14* genotypes on asthma, allergies, and *CD14* expression and their interactions were investigated.
Results: We found a significant interaction between genetic variation in *CD14/−1721* and farm milk consumption. Adjusted odds ratios for the association between farm milk and asthma varied between the genotypes: AA, 0.18 (95% CI, 0.07-0.47); AG, 0.47 (95% CI, 0.26-0.86); and GG, 0.98 (95% CI, 0.46-2.08). Similar patterns were observed for symptoms of allergic rhinoconjunctivitis and pollen sensitization. *CD14/−1721* also modified the association between farm milk and *CD14* gene expression (adjusted geometric means ratios: AA, 1.61 (95% CI, 0.98-2.66); AG, 1.11 (95% CI, 0.71-1.72); and GG, 0.76 (95% CI, 0.39-1.48).
Conclusion: The protective effect of farm milk consumption on allergic diseases is stronger in children carrying the A allele in *CD14/−1721* than in children homozygous for the G allele. This might be mediated through farm milk–induced upregulated *CD14* gene expression.
Clinical implications: Our results support the hypothesis that the inverse association between farm milk consumption and allergic diseases is mediated by *CD14*-activated innate immune mechanisms. (J Allergy Clin Immunol 2007;120:1308-15.)

Key words: *Allergy, asthma,* CD14*, gene-environment interaction, gene expression, farming, epidemiology*

Several studies in rural populations have demonstrated that living on a farm provides a protective environment for the development of allergic diseases.[1] In addition to farm animal contact[2] and microbial compounds,[3] such as LPS, more recently, farm milk consumption has been identified as another characteristic of a farming lifestyle that might confer protection against allergic diseases.[2,4-7] The relevant compound or compounds responsible for the protective farm milk effect and the underlying biologic mechanisms are still unclear, although several suggestions have been put forward. Viable (lactobacilli[6]) and

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Abbreviations used
ALEX: Allergy and Endotoxin
OR: Odds ratio
PARSIFAL: Prevention of Allergy – Risk Factors for Sensitisation in Children Related to Farming and Anthroposophic Lifestyle
PBLC: Peripheral blood leukocyte
SNP: Single nucleotide polymorphism

nonviable (LPS[3]) microbial components of farm milk interacting directly or indirectly (eg, through modification of the intestinal microflora) with the immune system have been proposed as possible causal factors. Alternatively, compounds other than microbes, such as high levels of omega-3 polyunsaturated fatty acids, in farm milk might play a role.[8-10]

CD14 is a key molecule of the innate immune system serving as a receptor and carrier for microbial ligands[11] and other lipid-containing molecules.[12-14] As previously shown in genetic studies, a polymorphism in the *CD14* gene, *CD14/C-260T*, might interact with environmental factors in the development of asthma and atopic diseases.[15,16] Although the mechanisms of gene-environment interactions are largely unknown, a polymorphism in the promoter region of a gene might exert its effect by modulating gene expression. Recently published reports support this possibility and suggest that variation in gene expression is of importance in the interplay among environment, genes, and disease.[17,18]

In the present study we therefore aimed to investigate whether the negative association between allergic health outcomes and farm milk consumption is modified by polymorphisms in *CD14* and whether a genotype-specific protective effect is associated with increased expression of *CD14*.

METHODS

Population

Data from farmers' children and nonfarmers' children enrolled in 2 cross-sectional surveys, the Allergy and Endotoxin (ALEX) study and the Prevention of Allergy – Risk Factors for Sensitisation in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study, were analyzed in this study. Both surveys aimed to explore protective factors associated with farming and asthma and allergies in childhood. The methods used were similar and partly identical. Details of the selection process are described in Fig E1 in the Online Repository (available at www.jacionline.org). Ethical permission for each study was obtained from local authorities in each center.

In brief, the ALEX study[2] was a cross-sectional study conducted in 1999 with an overall participation rate of 75%. Of the families who gave consent for further investigations, all farmers' children, all nonfarmers' children with contact to farming, and a random sample of nonexposed nonfarmers' children were invited for blood sampling. Complete questionnaire data and a blood sample were available for 812 children. Of these, consent for genetic analyses was not given for 120 Swiss children, and no adequate DNA sample was available for 8 children from Germany and Austria, leaving 684 children (258 farmers' and 426 nonfarmers' children) for genotyping.

In 2001, farmers' children and nonfarmers' children were recruited as part of the PARSIFAL study in the same countries but from different geographic areas and additionally in Sweden and The Netherlands.[19] Parents of these children completed a self-administered questionnaire to assess asthma and allergies and exposures associated with farming. The overall participation rate was 70%. Participants were also asked for consent to further investigations, including a venous blood sample. Consent to genetic analyses and an adequate sample of DNA were available for 1907 children.

In case more than 1 child per family participated in the study, only the eldest sibling was included in the analyses to fulfill the assumption of independent observations. The final sample sizes for the ALEX and PARSIFAL studies were therefore reduced to 576 (209 farmers' and 367 nonfarmers' children) and 1478 (957 farmers' and 521 nonfarmers' children) children, respectively.

Phenotypes

In both study populations the same questions and thereby the same definition of phenotypes were used. Asthma was defined as a physician's diagnosis of asthma or recurrent asthmatic, spastic, or obstructive bronchitis. Children whose parents reported "wheezing or whistling in the chest in the last 12 months" were classified as having current wheeze. Current symptoms of allergic rhinoconjunctivitis were defined as a positive response to the following question: "In the last 12 months, has your child had problems with sneezing or a runny or blocked nose without a cold accompanied by itchy-watery eyes?"

The concentrations of specific IgE against common aeroallergens were measured with the ImmunoCAP assay (Phadia, Uppsala, Sweden; ImmunoCAP codes for the individual allergens are given in brackets below) in all sera and in both populations. In the ALEX study specific serum IgE levels against timothy grass pollen (g6), birch pollen (t3), house dust mite (*Dermatophagoides pteronyssinus*, d1), storage mite (*Lepidoglyphus destructor*, d71), cat dander (e1), and cow dander (e4)[2] were measured, and in the PARSIFAL study specific serum IgE levels against grass pollen mix (gx3; timothy grass, sweet vernal grass, rye grass, cultivated rye, and velvet grass), tree pollen mix (tx9; birch, grey alder, hazel, oak, and willow), house dust mite (*D pteronyssinus*, d1), storage mite (*L destructor*, d71), cat dander (e1), and horse dander (e3) were measured.[4] Two different cut-off values (0.35 and 3.5 kU/L) were used for analyses.

Exposure to farm milk in the first year of life

In both studies exposure to farm milk (cow's milk from self-production or directly purchased from a farm) in the first year of life was defined in children whose parents reported that the child began to drink farm milk at the age of 1 year or less (n = 909). Children not exposed to farm milk in the first year of life started either later (n = 257) or did not drink farm milk but rather any kind of shop-purchased cow's milk (n = 847). The latter 2 groups formed the group of children (n = 1104) who were not exposed to farm milk in the first year of life.

Non–milk-drinking children (n = 8) were excluded from the analyses to avoid potential primary milk avoidance because of allergy-related symptoms at younger ages.

Genotyping

DNA was extracted from peripheral blood leukocytes (PBLCs) by using standard techniques. *CD14* was screened for single nucleotide polymorphisms (SNPs) in white subjects (http://innateimmunity.net). Haplotype tagging SNPs closely linked with known polymorphisms ($R^2 > 0.7$) and with a minor allele frequency of greater than 10% were chosen for this study by using the algorithm implemented in ldSelect.[20] This algorithm is based on linkage disequilibrium. Statistical power to detect unassayed, disease-associated polymorphisms depends on the correlation (R^2) between the unassayed site and the

TABLE I. Frequencies of *CD14* genotypes in the 2 study populations

CD14 polymorphism	Genotype	ALEX study (n = 576) n (%)	PARSIFAL study (n = 1478) n (%)
CD14/C−4191T	CC	320 (55.6%)	816 (55.2%)
	CT	213 (37.0%)	521 (35.3%)
	TT	21 (3.6%)	85 (5.8%)
	Missing	22 (3.8%)	56 (3.8%)
CD14/C−2839T	CC	283 (49.1%)	768 (52.0%)
	CT	232 (40.3%)	537 (36.3%)
	TT	38 (6.6%)	111 (7.5%)
	Missing	23 (4.0%)	62 (4.2%)
CD14/C−1721T	AA	172 (29.9%)	494 (33.4%)
	AG	272 (47.2%)	639 (43.2%)
	GG	89 (15.5%)	254 (17.2%)
	Missing	43 (7.5%)	91 (6.2%)
CD14/C−260T	CC	133 (23.1%)	399 (27.0%)
	CT	279 (48.4%)	683 (46.2%)
	TT	121 (21.0%)	337 (22.8%)
	Missing	43 (7.5%)	59 (4.0%)

assayed site. These SNPs were as follows: *CD14/−4191* (rs574441), tagging 5 other SNPs; *CD14/–2839* (rs2569193) and *CD14/−260* (rs256190), each tagging 3 other SNPs; and *CD14/−1721* (rs2915863), a single SNP because of linkage disequilibrium with an R^2 value of 0.7 or less to other SNPs in the reference population (http://innateimmunity.net). Genotyping for both populations was done in the same laboratory by using identical methods. (Arizona Respiratory Center, Tucson, Ariz). Genomic DNA was assayed by using the 5′ exonuclease reaction (Taqman; Applied Biosystems, Foster City, Calif). Primer and probe sequences used and further details of the genotyping method are available from the author on request.

Expression of *CD14*

For the Swiss branch of the PARSIFAL study, RNA samples of PBLCs were collected from 316 children (95.3% of children who provided blood samples in Switzerland) to analyze gene expression of innate immunity receptors (see Fig E1 in the Online Repository at www.jacionline.org). After restriction to the eldest siblings, the final sample consisted of 222 children, of whom 132 were exposed to farm milk in the first year of life and 89 were not (1 missing).

Total RNA was isolated by using the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). Quantitative real-time PCR (TaqMan, Applied Biosystems) was performed as described elsewhere.[14] All PCR reactions were analyzed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Experiments assessing background signals were performed for every assay by running the reaction without templates ("no template control"). In none of these was any template control amplification observed, indicating that there was no contamination or unspecific fluorescence. The data are presented as normalized values (the amount of mRNA of the target molecule divided by the amount of mRNA of the endogenous control [18S rRNA]).

Statistical analyses

Genotype frequencies in the ALEX and PARSIFAL populations were calculated and compared with those of a population in Hardy-Weinberg equilibrium by using the χ^2 goodness-of-fit test with 1 *df*.

The prevalences of reported asthma, current wheezing, current rhinoconjunctivitis symptoms, and atopic sensitization (allergen specific serum IgE, ≥3.5 kU/L) were calculated in the genotypes of the 4 investigated *CD14* SNPs. Odds ratios (ORs) for the association between the phenotypes and being a farm child (a child who lived on a farm and whose family ran the farm), between phenotypes and contact with stable animals in the first year of life, and between phenotypes and farm milk consumption in the first year of life were estimated in the *CD14* genotypes and tested for homogeneity by using Mantel-Haenszel statistics.

The following factors were considered as potential confounders for the association between exposure and outcome and included in the multiple logistic and multiple linear regression models in case of a univariate association ($P < .150$) to the independent variable of interest: sex, age in years (5-6, 7-8, 9, 10-11, or 12-13 years), maternal education (low, medium, and high), number of elder siblings (none, 1, 2, 3, or more), passive smoking (0, 1-9, or >10 cigarettes per day), study center, being a farm child, and breast-feeding (exclusively ≥5 months and <5 months).

Associations are presented as ORs for binomial data and as exponentiated regression coefficients (geometric means ratios) for numeric data.

The coding of the genotype was done according to the best-fitting model on the basis of Akaike's information criterion[21] to test for statistical interaction: ordinal (reflecting an additive genetic model), binomial (homozygous of one allele vs heterozygous and homozygous of the other allele, reflecting a dominant or a recessive genetic model, respectively), or categorical (no genetic model). The likelihood ratio test was performed to test the null hypothesis of no interaction. Two-sided *P* values of less than .05 were considered significant.

The robustness of our results was checked by repeating the analyses in the subsamples of farmers' and nonfarmers' children.

All analyses were carried out with STATA/SE 8.0 software (StataCorp, College Station, Tex) and R 2.4.0 (R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study population

Table I shows the genotype frequencies of the 4 investigated *CD14* polymorphisms. Genotype frequencies did not differ between the ALEX and PARSIFAL studies (Table I) or between early farm milk drinkers and non–farm milk drinkers, respectively (data not shown). In both populations, no significant deviation from Hardy-Weinberg equilibrium was observed (data not shown) for all 4 polymorphisms. Residual linkage disequilibrium was found between *CD14/−1721* and *CD14/−260* in both populations (see Tables E1 and E2 in the Online Repository at www.jacionline.org).

Characteristics and the prevalence of asthma and allergies in the 2 main populations and in the PARSIFAL subpopulation are described in Table II. Because of different study designs in the ALEX and PARSIFAL surveys, which resulted in different proportions of farmers' children, the prevalence of asthma, allergic rhinoconjunctivitis, and specific serum IgE to aeroallergens differed between the study populations.

Effects of *CD14/−1721* genotypes and farm milk consumption on allergic diseases

The *CD14/−1721* SNP is characterized by a substitution of adenine by guanine in the promoter of the *CD14* gene. There was no significant association between *CD14/*

TABLE II. Description of the 2 study populations

Characteristic		ALEX study (n = 576) n (%)	PARSIFAL study (n = 1478) n (%)	PARSIFAL subpopulation (n = 222) n (%)
Farmer's child	Yes	209 (36.3%)	957 (64.7%)	124 (55.9%)
Farm milk consumption in the first year of life	Yes	288 (50.0%)	621 (42.0%)	132 (59.5%)
	Missing	0 (0.0%)	18 (1.2%)	1 (0.5%)
Sex	Girl	270 (46.9%)	713 (48.2%)	110 (49.5%)
Age groups	5-6 y	0 (0.0%)	126 (8.5%)	26 (11.7%)
	7-8 y	174 (30.2%)	399 (27.0%)	50 (22.5%)
	9 y	115 (20.0%)	259 (17.5%)	20 (9.0%)
	10-11 y	231 (40.1%)	507 (34.3%)	60 (27.0%)
	12-13 y	56 (9.7%)	187 (12.7%)	66 (29.7%)
Asthma	Yes	48 (8.3%)	125 (8.5%)	15 (6.8%)
	Missing	0 (0.0%)	23 (1.6%)	2 (0.9%)
Current wheezing	Yes	59 (10.2%)	108 (7.3%)	16 (7.2%)
	Missing	9 (1.6%)	14 (0.9%)	3 (1.4%)
Current symptoms of allergic rhinoconjunctivitis	Yes	58 (10.1%)	90 (6.1%)	10 (4.5%)
	Missing	11 (1.9%)	16 (1.1%)	1 (0.5%)
Specific serum IgE ≥3.5 kU/L				
Any allergen tested	Yes	125 (21.7%)	193 (13.1%)	35 (15.8%)
	Missing	0 (0.0%)	61 (4.1%)	0 (%)
Pollen	Yes	89 (15.5%)	101 (6.8%)	21 (9.5%)
	Missing	0 (0.0%)	60 (4.1%)	0 (0%)
House dust mite	Yes	59 (10.2%)	120 (8.1%)	17 (7.7%)
	Missing	0 (0.0%)	4 (0.3%)	0 (0%)

−1721 genotypes and allergic diseases (data not shown). Children who consumed farm milk in the first year of life were less likely to have asthma, symptoms of allergic rhinoconjunctivitis, and atopic sensitization (allergen specific serum IgE, ≥3.5 kU/L; crude ORs [95% CIs] for asthma, rhinoconjunctivitis symptoms, and atopic sensitization were as follows: 0.36 [95% CI, 0.25-0.51], 0.55 [95% CI, 0.38-0.78], and 0.60 [95% CI, 0.47-0.77], respectively), thereby confirming previously reported results.[2,4]

By using Mantel-Haenszel test statistics for homogeneity of ORs across strata, the protective effect of farm milk consumption differed between the genotypes of *CD14/−1721* in the ALEX and PARSIFAL populations (data not shown; prevalences of allergic health outcomes stratified by genotypes and main exposures are shown in Tables E3-E5 in the Online Repository at www.jacionline.org). This heterogeneity between genotypes remained after adjusting for potential confounding in multiple regression analyses (Table III).

Moreover, the effect patterns were similar in both populations for asthma, current wheeze, symptoms of allergic rhinoconjunctivitis, and pollen sensitization. Overall, in the majority of the health outcomes, children homozygous for GG in *CD14/−1721* did not show an inverse association with farm milk consumption. For asthma, current wheeze, and symptoms of allergic rhinoconjunctivitis, the inverse association with farm milk was strongest in the AA genotype, intermediate in the AG genotype, and absent in the GG genotype. No effect modification by *CD14/−1721* genotypes was observed for the association between farm milk consumption and sensitization to any allergen (≥3.5 IU/mL). However, for specific IgE against pollen (≥3.5 IU/mL), a strong protective farm milk effect was seen in the *CD14/−1721* AA and AG genotypes but not in the GG genotype (Table III). Similar associations were observed when a cut-off value of 0.35 kU/L was used to define sensitization, even though trends were more pronounced in the PARSIFAL population.

When the combined population was stratified into farmers' and nonfarmers' children, the gene-environment interaction between *CD14/−1721* and farm milk consumption in the first year of life was also seen, although the formal test for interaction was mostly not significant (Table IV).

When both study populations were combined, interactions between *CD14/−1721* genotypes and farm milk consumption were significant for asthma ($P = .003$), current symptoms of allergic rhinoconjunctivitis ($P = .04$), and specific IgE against pollen of 3.5 kU/mL or greater ($P = .027$).

To evaluate the effect of timing of farm milk consumption, we categorized early and current farm milk consumption into 4 groups (early no, current no; early yes, current no; early no, current yes; early yes, current yes). Only children who drank farm milk in the first year of life (independently of current consumption) showed heterogeneity in frequencies of allergic health outcomes when stratified by *CD14/−1721* genotypes (see Table E6 in the Online Repository at www.jacionline.org).

The genotypes of *CD14/−1721* modified neither the association between being a farm child and allergic phenotypes (asthma, current wheeze, symptoms of allergic rhinoconjunctivitis, and atopic sensitization) nor the association between stable animal contact in the first year of life and allergic phenotypes (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org).

TABLE III. Adjusted ORs for the association between farm milk consumption in the first year of life and allergic diseases in *CD14/−1721* genotypes in 2 independent rural populations

		ALEX study (n = 533)			PARSIFAL study (n = 1387)		
Response	***CD14/−1721***	**n***	**aOR (95% CI)†**	***P* value‡**	**n***	**aOR (95% CI)†**	***P* value‡**
Asthma	AA	172	0.29 (0.07-1.14)	.331[d]	488	0.11 (0.03-0.49)	.004[a]
	AG	272	0.38 (0.12-1.16)		628	0.54 (0.26-1.11)	
	GG	89	0.75 (0.18-3.1)		251	1.14 (0.46-2.86)	
Current wheezing	AA	169	0.53 (0.17-1.67)	.150[d]	488	0.69 (0.3-1.62)	.441[a]
	AG	270	0.80 (0.31-2.1)		634	0.90 (0.43-1.9)	
	GG	88	1.87 (0.53-6.6)		251	1.12 (0.4-3.19)	
Current symptoms of allergic rhinoconjunctivitis	AA	167	0.50 (0.14-1.76)	.242[d]	491	0.42 (0.15-1.22)	.074[a]
	AG	269	0.51 (0.19-1.36)		629	0.72 (0.32-1.6)	
	GG	87	1.21 (0.33-4.39)		251	1.57 (0.54-4.63)	
Specific serum IgE ≥3.5 kU/L							
Any allergen tested	AA	172	0.23 (0.09-0.57)	.119[r]	479	0.86 (0.46-1.63)	.848[r]
	AG	272	0.52 (0.27-1.02)		612	0.69 (0.38-1.27)	
	GG	89	0.50 (0.18-1.40)		241	1.14 (0.48-2.71)	
Pollen	AA	172	0.15 (0.04-0.56)	.107[a]	480	0.58 (0.25-1.36)	.049[d]
	AG	272	0.28 (0.12-0.66)		612	0.39 (0.15-0.97)	
	GG	89	0.56 (0.18-1.78)		241	1.73 (0.54-5.56)	
House dust mite	AA	172	0.34 (0.11-1.03)	.207[a]	494	0.83 (0.37-1.84)	.523[a]
	AG	272	0.69 (0.28-1.69)		637	0.93 (0.45-1.90)	
	GG	89	0.97 (0.23-4.08)		253	1.29 (0.44-3.82)	

Reference group: no farm milk consumption in the first year.
aOR, Adjusted OR.
*Sums of genotype group sizes and study population differ because of missing values in the respective phenotype variable.
†ORs are adjusted for being a farm child, age, study center, sex, environmental tobacco smoke, maternal education, and breast-feeding (exclusively ≥5 months).
‡Likelihood ratio test for interaction. *P* values are given for the best-fitting model, as represented by superscript letters: *a*, additive; *d*, dominant; *r*, recessive.

Effects of *CD14/−1721* genotypes and farm milk consumption on gene expression of *CD14*

The subpopulation (n = 222) in which gene expression of *CD14* was measured consisted of 132 children who did drink farm milk in the first year of life and 89 who did not (no information about first-year farm milk consumption was available for 1 child). Previously published results showed that farmers' children expressed higher amounts of CD14 on PBLCs than nonfarmers' children (data not shown).[17,18] In the univariate analysis PBLCs of early farm milk drinkers appeared to express more CD14 than those of non−farm milk drinkers. However, when adjusted for being a farm child, this association was no longer significant (adjusted geometric means: no early farm milk consumption, 0.35 [95% CI, 0.29-0.43]; early farm milk consumption, 0.41 [95% CI, 0.36-0.47]; $P = .258$]), thus making a direct farm milk effect unlikely.

Moreover, mean gene expression was similar in *CD14/−1721* genotypes (geometric means: AA, 0.39 [95% CI, 0.32-0.47]; AG, 0.37 [95% CI, 0.32-0.43]; GG, 0.42 [95% CI, 0.33-0.54]; $P = .666$).

However, stratification by genotype of *CD14/−1721* revealed heterogeneity of the farm milk effect on *CD14* gene expression (see Fig 1, including ORs for asthma presented in Table III to emphasize the similarity of the effect pattern). Children with a history of drinking farm milk in the first year of life and having either the AA or the AG genotype in *CD14/−1721* expressed 61% and 11%, respectively, more CD14 compared with children not drinking farm milk in the first year of life. In contrast, children with the GG genotype expressed 24% less CD14 if they had consumed farm milk in the first year of life than if they had not.

Results of the *CD14/−4191, CD14/−2839,* and *CD14/−260* polymorphisms

There was no significant association between the *CD14/−4191*, *CD14/−2839*, and *CD14/−260* polymorphisms and asthma or allergies or *CD14* gene expression. There was also no clear pattern of interaction between any of the polymorphisms and farm milk consumption on the phenotypes under study, except for symptoms of allergic rhinoconjunctivitis in the last 12 months in *CD14/−4191* (see Tables E7-E10 in the Online Repository at www.jacionline.org).

DISCUSSION

The results of our analyses suggest that a polymorphism in the *CD14* gene *(CD14/−1721)* modifies the previously described inverse association of early farm milk consumption on allergic diseases in children. This gene- environment interaction was seen in 2 independent rural populations and was independent of being a farm child.

TABLE IV. Adjusted ORs for the association between farm milk consumption in the first year of life and allergic diseases in *CD14/−1721* genotypes in nonfarmers' and in farmers' children

		Nonfarmers' children (n = 839)			Farmers' children (n = 1081)		
Response	***CD14/−1721***	**n***	**aOR (95% CI)†**	***P* value‡**	**n***	**aOR (95% CI)†**	***P* value‡**
Asthma	AA	258	0.14 (0.05-0.45)	.001[a]	402	0.18 (0.02-1.39)	.288[a]
	AG	430	0.57 (0.25-1.31)		470	0.27 (0.09-0.81)	
	GG	145	1.72 (0.50-5.96)		195	0.58 (0.15-2.20)	
Current wheezing	AA	255	0.99 (0.39-2.51)	.347[d]	402	0.11 (0.01-0.85)	.029[a]
	AG	431	0.89 (0.35-2.30)		473	0.53 (0.22-1.29)	
	GG	145	1.87 (0.48-7.33)		194	1.01 (0.31-3.24)	
Current symptoms of allergic rhinoconjunctivitis	AA	252	0.34 (0.12-1.0)	.026[a]	406	0.60 (0.16-2.22)	.165[d]
	AG	427	1.16 (0.41-3.32)		471	0.38 (0.15-0.96)	
	GG	144	1.76 (0.45-6.93)		194	1.2 (0.37-3.88)	
Specific serum IgE ≥3.5 kU/L							
Any allergen tested	AA	258	0.58 (0.28-1.19)	.283[a]	393	0.45 (0.18-1.10)	.277[d]
	AG	431	0.83 (0.40-1.73)		453	0.34 (0.17-0.69)	
	GG	144	1.13 (0.36-3.5)		186	0.69 (0.26-1.85)	
Pollen	AA	258	0.25 (0.1-0.67)	.025[a]	394	0.37 (0.12-1.13)	.038[d]
	AG	431	0.64 (0.2-2.02)		453	0.18 (0.07-0.49)	
	GG	144	1.65 (0.3-8.93)		186	0.96 (0.33-2.78)	
House dust mite	AA	259	0.89 (0.33-2.38)	.796[r]	407	0.60 (0.19-1.85)	.82[r]
	AG	433	0.62 (0.29-1.35)		476	0.62 (0.24-1.58)	
	GG	146	1.38 (0.35-5.50)		196	0.93 (0.23-3.69)	

Reference group: no farm milk consumption in the first year.
*Sums of genotype group sizes and study population differ because of missing values in the respective phenotype variable.
†ORs are adjusted for study, age, study center, sex, environmental tobacco smoke, maternal education, and breast-feeding (excluding >5 months).
‡Likelihood ratio test for interaction. *P* values are given for the best-fitting model, as represented by superscript letters: *a*, additive; *d*, dominant; *r*, recessive.

In addition, *CD14/−1721* genotypes significantly modified the association between farm milk consumption and *CD14* expression in peripheral blood cells. These results suggest a biologic mechanism underlying the gene-environment interaction found for genetic variation in the CD14 gene and farm milk consumption in early life on the development of asthma and allergies in this study.

CD14 is a pattern-recognition receptor of the innate immune system for a wide spectrum of microbial compounds,[11] such as LPS,[22] lipoteichoic acid,[23] and peptidoglycan,[24] but also for nonmicrobial compounds, such as phospholipids.[12-14] This diversity of potential CD14-binding ligands and the fact that the allergy-protective compounds of farm milk have not yet been identified allow speculation about ways in which farm milk consumption might mediate the protective effect through CD14.

Farm milk is known to contain various bacterial species,[25] and there are 2 plausible and not mutually exclusive ways as to how the microbial composition of the farm milk might interact with the immune system through CD14. First, farm milk itself might contain microbial compounds that are able to bind to CD14 after penetrating the intestinal mucosa.[26] Second, strains of probiotic bacteria contained in farm milk might have a balancing effect on the intestinal microflora, and their degradation products might interact with CD14 after absorption.[27] Because CD14 is known to be a coreceptor of Toll-like receptors 4[28] and 2,[29] complexes of CD14 and their ligands might bind to one of these pattern-recognition receptors on the cell surface of antigen-presenting cells and hence activate the immune system, resulting in subsequent production of immunomodulatory chemokines and cytokines. Yet first analyses of milk samples of an ongoing birth cohort study in rural areas of Europe indicate that endotoxin levels are similar in milk consumed by farmers' and nonfarmers' families (Gehring, submitted) and thus challenge the role of gram-negative microorganisms for the protective farm milk effect.

Alternatively, nonmicrobial molecules contained in the farm milk might be responsible for the protection against allergic diseases. There is some evidence that omega-3 dietary fatty acids, which have been found at higher levels in the milk of exclusively grass-fed cows than in the milk of cows fed with conserved grass,[30] are potentially beneficial to patients with allergic diseases.[8-10] Thus animal feeding and also milk processing (fat standardization, pasteurization, and homogenization)[31] might influence the milk's fat content or fatty acid profile. One might therefore speculate that farm milk differs from shop-purchased milk (which represents a mixture of milk produced in different regions and different types of farms) regarding its fat amount or composition. Because it is known that CD14 also acts as a receptor and carrier for phospholipids,[12-14] fatty acids offer another potential mechanism as to how farm milk consumption might mediate protection against allergic diseases through CD14. However, because the immunologic

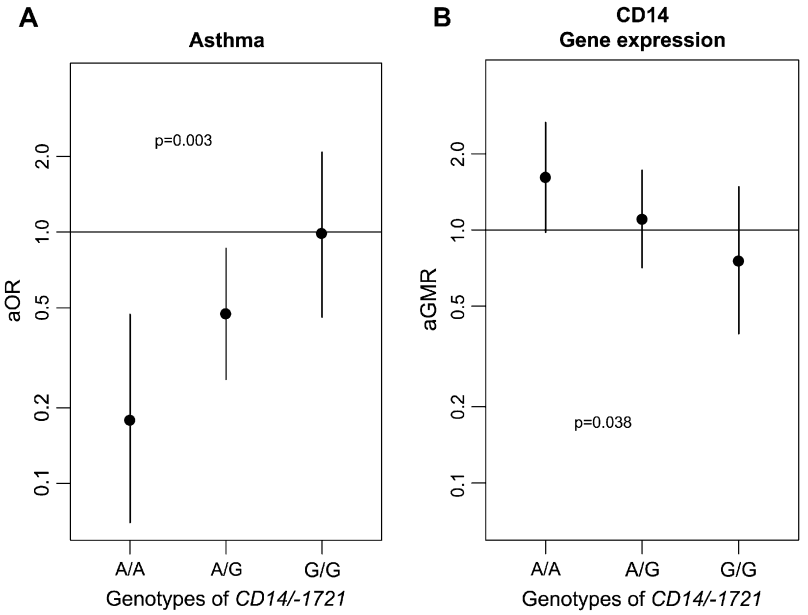

FIG 1. The effect of farm milk consumption on asthma (**A**; ALEX and PARSIFAL studies combined, n = 1920) and *CD14* gene expression (**B**; Swiss PARSIFAL subsample, n = 222) by genotypes of *CD14/−1721*. ORs and geometric means ratios are adjusted for being a farm child, age, and sex. ORs for asthma are additionally adjusted for study center, environmental tobacco smoke, maternal education, and breast-feeding (exclusively ≥5 months). *P* values are based on the test for interaction, assuming an additive effect of the A allele. *aOR*, Adjusted OR; *aGMR*, adjusted geometric means ratio.

relevant component has not been identified, the mechanisms underlying the observed association between farm milk consumption and allergic diseases remain speculative.

Variations in genotypes of *CD14/−1721* not only interacted with the effect of farm milk consumption on clinical outcomes but also on gene expression of *CD14*, suggesting that differential gene expression of *CD14* might mediate the farm milk effect on allergic diseases. Because *CD14/−1721* is located in the promoter region of the *CD14* gene, it appears plausible that a biologically relevant effect of this polymorphism would be mediated by changes in the transcriptional activity of the *CD14* gene. *CD14* gene expression was measured in a subsample of the Swiss PARSIFAL children to confirm the biologic relevance of the *CD14/−1721* polymorphism. However, neither farm milk consumption nor one of the *CD14/−1721* genotypes was directly associated with gene expression of *CD14*. An association between the *CD14/−1721* polymorphism and *CD14* gene expression became evident only on exposure to farm milk consumption. Thus the gene-environment interactions underlying the different health outcomes become evident at the level of gene expression, indicating that assessment of gene expression might be helpful when evaluating the biologic relevance of polymorphisms in promoter regions. One might speculate that exposure to farm milk activates antigen-presenting cells and induces expression of *CD14*. This might lead to an increased susceptibility to further stimulation by farm milk–associated agents.

To date, no functional data for *CD14/−1721* have been published. We can therefore not exclude the possibility that polymorphisms in linkage disequilibrium with *CD14/−1721* underlie our findings. For example, a well-characterized functional promoter polymorphism in *CD14 (CD14/−260)*[32] has been found in different populations to modify the effect of microbial exposures on the development of atopy.[33,34] *CD14/−260* is in high linkage disequilibrium with *CD14/−1721* in our population ($R^2 > 0.7$). Therefore, not surprisingly, several ORs for the associations between farm milk and the phenotypes studied were similar in both SNPs (Table III and Table E9 in the Online Repository at www.jacionline.org). The ORs were, however, less heterogeneous across the genotypes of *CD14/−260* than across *CD14/−1721* in both populations. Functional studies are needed to further evaluate the mechanisms underlying our observations and to elucidate the role of *CD14/−1721* in the development of asthma- and allergy-related phenotypes in the context of environmental exposures.

Recent studies suggest that pollen and house dust mite allergy represent 2 different phenotypes with respect to their genetic and environmental determinants.[4] Therefore this finding might represent thus far unappreciated peculiarities in the biology of allergen/immune system interactions, but experimental studies are needed for further investigation of potential mechanisms.[35]

We are aware that the sample size, particularly that of the ALEX population, was low to study gene-environment interactions. Therefore several formal tests for interaction might not have reached statistical significance. We do, however, believe that similar trends in the majority of the health outcome measures in the 2 independent study

populations strongly suggest that the results have not only emerged because of chance.

Although our study included objective measurements, such as gene expression measurement, genotyping, and measurement of allergen-specific IgE, the limitations of the cross-sectional and observational design in elucidating biologic mechanisms are well recognized. Nevertheless, our results strongly suggest that biologic effects underlie the observed inverse association between farm milk consumption and allergic diseases, thus offering attractive avenues for future preventive measures.

We thank all field workers and coworkers of the PARSIFAL and ALEX studies. We especially thank Susanne Loeliger from the University Children's Hospital Zurich for support with the RNA analyses and Susan Salomon and Lizhi Yu from the Arizona Respiratory Center (Tucson, Ariz), who did DNA processing and genotyping of the *CD14* polymorphisms. We also thank all school physicians and teachers for their support, and all children and parents for participation in this study.

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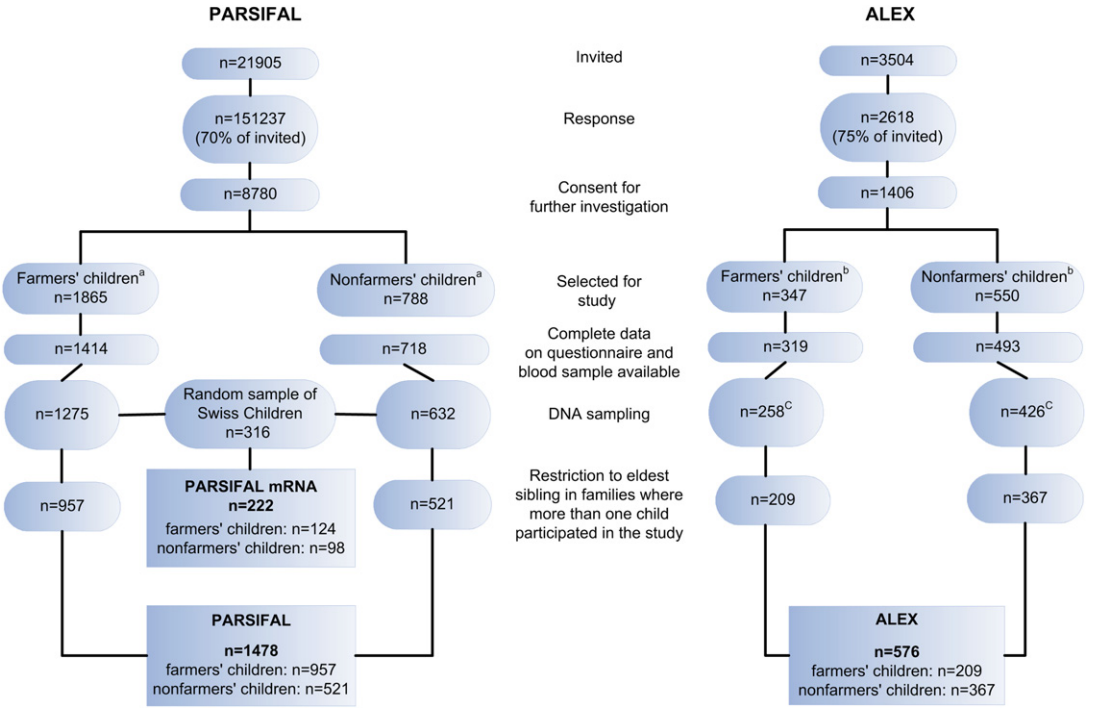

FIG E1. Selection of the 2 study populations. [a]Restriction to farmers' and nonfarmers' children (1450 children from Steiner schools and their 751 reference children excluded). [b]All farmers' children, all nonfarmers' children with contact to farming, and a random sample of nonexposed nonfarmers' children were selected. [c]No genetic testing was done for the Swiss children (n = 120).

TABLE E1. Linkage disequilibrium *(R^2)* between *CD14* SNPs in the ALEX population (n = 576)

	CD14/C−2839T	***CD14/A−1721G***	***CD14/C−260T***
CD14/C−4191T	0.115	0.218	0.278
CD14/C−2839T	—	0.282	0.369
CD14/A−1721G	—	—	0.764

The R^2 value is calculated with R (R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria; package ''genetics,'' function ''LD'').

TABLE E2. Linkage disequilibrium *(R^2)* between *CD14* SNPs in the PARSIFAL population (n = 1539)

	CD14/C−2839T	***CD14/A−1721G***	***CD14/C−260T***
CD14/C−4191T	0.117	0.226	0.294
CD14/C−2839T	—	0.258	0.335
CD14/A−1721G	—	—	0.769

The R^2 value is calculated with R (R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria; package ''genetics,'' function ''LD'').

TABLE E3. Prevalences of allergic health outcomes stratified by *CD14/−1721* genotypes and farm milk consumption in the first year of life

		Farm milk consumption in the first year of life	
		No	Yes
Phenotype	***CD14/−1721***	**n (%)**	**n (%)**
Asthma	A/A	45/374 (12.1)	6/287 (2.1)
	A/G	58/507 (11.5)	18/395 (4.6)
	G/G	20/180 (11.3)	15/160 (9.4)
Current wheezing	A/A	39/374 (10.5)	14/287 (5.0)
	A/G	45/507 (8.9)	22/395 (5.6)
	G/G	18/180 (10.1)	17/160 (10.8)
Current symptoms of allergic rhinoconjunctivitis	A/A	31/374 (8.4)	9/287 (3.2)
	A/G	46/507 (9.2)	19/395 (4.9)
	G/G	16/180 (9.1)	14/160 (8.8)
Specific serum IgE $\geq$3.5 kU/L			
Any allergen tested	A/A	72/374 (19.7)	31/287 (11.0)
	A/G	89/507 (18.1)	46/395 (12.0)
	G/G	31/180 (18.0)	23/160 (14.8)
Pollen	A/A	50/374 (13.7)	13/287 (4.6)
	A/G	64/507 (13.0)	18/395 (4.7)
	G/G	18/180 (10.5)	14/160 (9.0)
House dust mite	A/A	37/374 (9.9)	19/287 (6.6)
	A/G	43/507 (8.5)	31/395 (7.9)
	G/G	14/180 (7.8)	15/160 (9.4)

TABLE E4. Prevalences of allergic health outcomes stratified by *CD14/−1721* genotypes and being a farm child

		Farmers' child	
		No	Yes
Phenotype	***CD14/−1721***	**n (%)**	**n (%)**
Asthma	A/A	25/259 (9.7)	27/407 (6.7)
	A/G	46/433 (10.7)	30/478 (6.4)
	G/G	21/147 (14.5)	15/196 (7.7)
Current wheezing	A/A	29/259 (11.4)	25/407 (6.2)
	A/G	42/433 (9.7)	25/478 (5.3)
	G/G	21/147 (14.5)	15/196 (7.7)
Current symptoms of allergic rhinoconjunctivitis	A/A	23/259 (9.1)	17/407 (4.2)
	A/G	45/433 (10.5)	20/478 (4.2)
	G/G	17/147 (11.8)	14/196 (7.2)
Specific serum IgE $\geq$3.5 kU/L			
Any allergen tested	A/A	62/259 (24.0)	43/407 (10.9)
	A/G	91/433 (21.1)	46/478 (10.2)
	G/G	36/147 (25.0)	19/196 (10.2)
Pollen	A/A	41/259 (15.9)	23/407 (5.8)
	A/G	64/433 (14.8)	18/478 (4.0)
	G/G	22/147 (15.3)	10/196 (5.4)
House dust mite	A/A	34/259 (13.1)	24/407 (5.9)
	A/G	40/433 (9.2)	36/478 (7.6)
	G/G	17/147 (11.6)	13/196 (6.6)

TABLE E5. Prevalences of allergic health outcomes stratified by *CD14/−1721* genotypes and stable animal contact in the first year of life

		Stable animal contact in the first year of life	
		No	Yes
Phenotype	***CD14/−1721***	**n (%)**	**n (%)**
Asthma	A/A	25/262 (9.6)	15/264 (5.7)
	A/G	33/390 (8.5)	21/341 (6.3)
	G/G	17/148 (11.6)	7/129 (5.5)
Current wheezing	A/A	25/262 (9.8)	18/264 (6.8)
	A/G	30/390 (7.8)	19/341 (5.6)
	G/G	20/148 (13.7)	8/129 (6.2)
Current symptoms of allergic rhinoconjunctivitis	A/A	19/262 (7.4)	12/264 (4.6)
	A/G	37/390 (9.6)	10/341 (3.0)
	G/G	19/148 (13.1)	7/129 (5.5)
Specific serum IgE $\geq$3.5 kU/L			
Any allergen tested	A/A	53/262 (20.5)	23/264 (9.0)
	A/G	73/390 (19.0)	29/341 (8.9)
	G/G	28/148 (19.3)	11/129 (8.9)
Pollen	A/A	33/262 (12.7)	10/264 (3.9)
	A/G	47/390 (12.2)	13/341 (4.0)
	G/G	20/148 (13.8)	2/129 (1.6)
House dust mite	A/A	26/262 (9.9)	14/264 (5.3)
	A/G	35/390 (9.0)	19/341 (5.6)
	G/G	12/148 (8.1)	10/129 (7.8)

TABLE E6. Prevalence of allergic health outcomes by *CD14/−1721* genotypes and timing of farm milk consumption

		Timing of farm milk consumption			
		First no, current no	First yes, current no	First no, current yes	First yes, current yes
Phenotype	***CD14/−1721***	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Asthma	A/A	32/289 (11.1)	0/44 (0.0)	11/80 (13.8)	6/240 (2.5)
	A/G	49/378 (13.1)	4/63 (6.5)	9/114 (8.0)	14/328 (4.3)
	G/G	16/130 (12.4)	5/27 (18.5)	4/47 (8.9)	10/130 (7.7)
Current wheezing	A/A	30/289 (10.5)	3/44 (7.0)	7/80 (9.0)	11/240 (4.7)
	A/G	34/378 (9.0)	3/63 (4.8)	9/114 (8.0)	19/328 (5.9)
	G/G	16/130 (12.4)	7/27 (26.9)	2/47 (4.3)	10/130 (7.8)
Current symptoms of allergic rhinoconjunctivitis	A/A	24/289 (8.5)	1/44 (2.3)	6/80 (7.5)	8/240 (3.3)
	A/G	34/378 (9.1)	5/63 (7.9)	9/114 (8.0)	14/328 (4.4)
	G/G	12/130 (9.4)	5/27 (18.5)	4/47 (8.7)	7/130 (5.4)
Specific serum IgE $\geq$3.5 kU/L					
Any allergen tested	A/A	57/289 (20.1)	5/44 (11.9)	11/80 (14.1)	26/240 (11.0)
	A/G	67/378 (18.2)	8/63 (13.3)	20/114 (18.2)	38/328 (11.9)
	G/G	25/130 (20.2)	8/27 (30.8)	6/47 (13.0)	15/130 (11.7)
Pollen	A/A	40/289 (14.1)	2/44 (4.8)	7/80 (9.0)	11/240 (4.6)
	A/G	48/378 (13.0)	3/63 (5.0)	14/114 (12.7)	15/328 (4.7)
	G/G	16/130 (12.9)	8/27 (30.8)	2/47 (4.3)	6/130 (4.7)
House dust mite	A/A	29/289 (10.0)	6/44 (13.6)	6/80 (7.5)	13/240 (5.4)
	A/G	32/378 (8.5)	7/63 (11.1)	10/114 (8.8)	24/328 (7.3)
	G/G	10/130 (7.8)	2/27 (7.4)	4/47 (8.5)	13/130 (10.0)

TABLE E7. Adjusted ORs for the association between farm milk consumption in the first year of life and allergic diseases in *CD14/−4191* genotypes in 2 independent rural populations

		ALEX study (n = 554)			PARSIFAL study (n = 1422)		
Phenotype	***CD14/−4191***	**n***	**aOR (95% CI)†**	***P* value‡**	**n***	**aOR (95% CI)†**	***P* value‡**
Asthma	CC	320	0.29 (0.12-0.74)		810	0.77 (0.43-1.38)	.017
	CT	213	0.33 (0.11-1.01)		509	0.21 (0.07-0.61)	
	TT	21	No cases		83	No cases	
Current wheezing	CC	316	0.95 (0.46-1.96)		811	0.95 (0.50-1.79)	
	CT	209	0.53 (0.19-1.50)		513	0.71 (0.31-1.63)	
	TT	20	No cases		84	0.82 (0.18-3.84)	
Current symptoms of allergic rhinoconjunctivitis	CC	317	0.87 (0.42-1.81)	.014	810	1.04 (0.54-2.00)	.082
	CT	208	0.14 (0.03-0.63)		511	0.40 (0.14-1.11)	
	TT	19	No cases		85	No cases	
Specific serum IgE ≥3.5 kU/L							
Any allergen tested	CC	320	0.49 (0.27-0.88)		781	1.31 (0.80-2.14)	.095[r]
	CT	213	0.31 (0.15-0.66)		500	0.65 (0.34-1.23)	
	TT	21	0.13 (0.01-1.06)		84	1.28 (0.32-5.12)	
Pollen	CC	320	0.32 (0.16-0.65)		781	0.97 (0.50-1.88)	.086[r]
	CT	213	0.22 (0.09-0.54)		501	0.40 (0.16-0.99)	
	TT	21	0.11 (0.01-1.37)		84	0.66 (0.05-7.95)	
House dust mite	CC	320	1.41 (0.65-3.07)	.006[r]	813	1.49 (0.80-2.78)	
	CT	213	0.28 (0.09-0.83)		520	0.76 (0.36-1.58)	
	TT	21	0.16 (0.01-2.23)		85	2.01 (0.30-13.41)	

Reference group: children without farm milk consumption in the first year of life.

aOR, Adjusted OR.

*Sums of genotype group sizes and study population differ because of missing values in the respective phenotype variable.

†ORs are adjusted for being a farm child, age, sex, and study center.

‡Likelihood ratio test for interaction. *P* values are given for the best-fitting model: *r*, recessive.

TABLE E8. Adjusted ORs for the association between farm milk consumption in the first year of life and allergic diseases in *CD14/−2839* genotypes in 2 independent rural populations

		ALEX study (n = 553)			PARSIFAL study (n = 1416)		
Phenotype	***CD14/−2839***	**n***	**aOR (95% CI)†**	***P* value‡**	**n***	**aOR (95% CI)†**	***P* value‡**
Asthma	CC	283	0.33 (0.13-0.85)		755	0.40 (0.2-0.77)	
	CT	232	0.38 (0.13-1.10)		530	0.68 (0.33-1.39)	
	TT	38	No cases		111	No cases	
Current wheezing	CC	278	0.85 (0.40-1.83)		760	0.67 (0.35-1.26)	.045[d]
	CT	229	0.89 (0.33-2.38)		532	1.41 (0.67-2.98)	
	TT	37	0.28 (0.03-2.76)		110	0 (0-Inf)	
Current symptoms of allergic rhinoconjunctivitis	CC	275	0.55 (0.24-1.25)		758	0.64 (0.31-1.32)	
	CT	231	0.73 (0.28-1.90)		531	0.65 (0.28-1.52)	
	TT	37	0.35 (0.03-3.63)		111	2.78 (0.43-18.0)	
Specific serum IgE ≥3.5 kU/L							
Any allergen tested	CC	283	0.42 (0.23-0.77)		737	0.94 (0.56-1.57)	
	CT	232	0.35 (0.17-0.72)		513	1.10 (0.61-1.99)	
	TT	38	0.29 (0.05-1.70)		108	0.95 (0.28-3.23)	
Pollen	CC	283	0.31 (0.15-0.62)		737	0.8 (0.39-1.64)	
	CT	232	0.22 (0.09-0.55)		514	0.49 (0.21-1.14)	
	TT	38	No cases		108	0.85 (0.19-3.82)	
House dust mite	CC	283	0.75 (0.33-1.72)		766	0.98 (0.53-1.83)	
	CT	232	0.69 (0.29-1.68)		535	1.62 (0.79-3.33)	
	TT	38	1.01 (0.12-8.51)		111	0.61 (0.11-3.32)	

Reference group: children without farm milk consumption in the first year of life.

*Sums of genotype group sizes and study population differ because of missing values in the respective phenotype variable.

†ORs are adjusted for being a farm child, age, sex, and study center.

‡Likelihood ratio test for interaction with $P \leq .1$. *P* value is given for the best-fitting interaction model: *d*, dominant.

TABLE E9. Adjusted ORs for the association between farm milk consumption in the first year of life and allergic diseases in *CD14/−260* genotypes in 2 independent rural populations

		ALEX study (n = 533)			PARSIFAL study (n = 1419)		
Phenotype	***CD14/−260***	**n***	**aOR (95% CI)†**	***P* value‡**	**n***	**aOR (95% CI)†**	***P* value‡**
Asthma	CC	133	0.45 (0.12-1.70)		393	0.09 (0.01-0.67)	.018[r]
	CT	279	0.24 (0.07-0.75)		672	0.51 (0.27-0.98)	
	TT	121	0.37 (0.10-1.32)		333	0.87 (0.38-1.95)	
Current wheezing	CC	131	0.65 (0.20-2.18)		393	0.77 (0.30-1.99)	
	CT	276	0.71 (0.28-1.79)		678	0.82 (0.42-1.61)	
	TT	119	1.64 (0.54-4.94)		334	0.9 (0.37-2.19)	
Current symptoms of allergic rhinoconjunctivitis	CC	129	0.69 (0.15-3.10)		396	0.66 (0.22-1.97)	
	CT	275	0.48 (0.20-1.16)		674	0.56 (0.26-1.20)	
	TT	118	1.01 (0.32-3.21)		333	1.11 (0.44-2.78)	
Specific serum IgE ≥3.5 kU/L							
Any allergen tested	CC	133	0.30 (0.12-0.79)		386	1.00 (0.51-1.99)	
	CT	279	0.37 (0.20-0.7)		656	0.84 (0.48-1.45)	
	TT	121	0.60 (0.25-1.45)		321	1.55 (0.77-3.14)	
Pollen	CC	133	0.12 (0.02-0.56)		387	0.53 (0.21-1.37)	.033[d]
	CT	279	0.22 (0.10-0.49)		656	0.51 (0.24-1.11)	
	TT	121	0.53 (0.18-1.50)		321	1.73 (0.65-4.6)	
House dust mite	CC	133	0.41 (0.13-1.28)	0.087[a]	398	1.16 (0.5-2.71)	
	CT	279	0.75 (0.31-1.8)		682	0.99 (0.51-1.91)	
	TT	121	1.47 (0.45-4.85)		336	1.61 (0.67-3.88)	

Reference group: children without farm milk consumption in the first year of life.

*Sums of genotype group sizes and study population differ because of missing values in the respective phenotype variable.

†ORs are adjusted for being a farm child, age, sex, and study center.

‡Likelihood ratio test for interaction with $P \leq .1$. *P* values are given for the best-fitting interaction model: *d*, dominant; *r*, recessive; *a*, additive.

TABLE E10. Effect modification of *CD14* polymorphisms on the association between farm milk consumption in the first year of life and gene expression of *CD14* (n = 222)

			PARSIFAL Switzerland	
Polymorphism	**Genotype**	**n**	**aGMR (95% CI)***	***P* value†**
CD14/C−4191T	CC	121	1.29 (0.89-1.86)	.077[d]
	CT	82	1.57 (1.01-2.46)	
	TT	17	3.30 (1.25-8.76)	
CD14/C−2839T	CC	118	1.40 (0.97-2.01)	
	CT	86	1.66 (1.07-2.59)	
	TT	15	1.75 (0.61-5.05)	
CD14/C−260T	CC	64	2.10 (1.26-3.49)	.073[r]
	CT	105	1.37 (0.92-2.03)	
	TT	48	1.03 (0.58-1.82)	

aGMR, Adjusted geometric means ratio.

*Geometric means ratios are given for farm milk consumption in the first year of life. Reference group: no farm milk consumption in the first year of life. Geometric means ratios are adjusted for being a farm child, age, and sex.

†Likelihood ratio test for interaction with $P \leq .1$. *P* values are given for the best-fitting interaction model: *d*, dominant; *r*, recessive; *a*, additive.

Chapter 6 Gene expression measurements in the context of epidemiological studies

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Short communication

Gene expression measurements in the context of epidemiological studies

Background: Gene expression measurements became an attractive tool to assess biological responses in epidemiological studies. However, collection of blood samples poses various technical problems. We used gene expression data from two epidemiological studies to evaluate differences between sampling methods, comparability of two methods for measuring RNA levels and stability of RNA samples over time.
Methods: For the PARSIFAL study, PBLC of 1155 children were collected using EDTA tubes in two countries. In the PASTURE study, tubes containing RNA-stabilizing solutions (PAXgene® Blood RNA Tubes; PreAnalytiX) were used to collect cord blood leucocytes of 982 children in five countries. Real-time PCR (conventional single tube assay and high-throughput low density arrays) was used to quantify expression of various innate immunity genes. In 77 PARSIFAL samples, gene expression was measured repeatedly during prolonged storage.
Results: In PARSIFAL (EDTA tubes) the median RNA yield after extraction significantly differed between the two centres (70 and 34 ng/μl). Collecting blood into an RNA-stabilizing solution markedly reduced differences in RNA yield in PASTURE (range of medians 91–107 ng/μl). The agreement [Spearman rank correlation (r)] between repeated measurements of gene expression decreased with increasing storage time [e.g., for CD14: r (first/second measurement) = 0.35; r (first/third measurement) = 0.03]. RNA levels measured with either the conventional method or low-density arrays were comparable ($r > 0.9$).
Conclusion: Collecting blood samples into tubes containing an RNA-stabilizing solution increases RNA yield and reduces its variability. Long-term storage of samples may lead to RNA degradation, requiring special attention in longitudinal studies.

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Key words: blood sampling; epidemiology; gene expression; innate immunity; PAXgene; RNA degradation

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High-throughput genomic technologies allow to measure large-scale gene expression profiles in a time-efficient way. These methodological advances have spread the range of potential applications and allow gene expression analyses to be used in the context of epidemiological studies (1, 2).

However, in epidemiological study settings, other sources of measurement error have to be considered than in clinical or laboratory research: distance between location of blood collection and further processing, different fieldworkers involved in the studies, and, particularly in international studies, different laboratories and laboratory staff are likely to affect quality of the obtained gene expression data.

In the present study, we had the possibility to analyse gene expression data of two epidemiological studies [PARSIFAL (3) and PASTURE (4)]. We compared centre-associated variations in RNA quantities obtained using two sampling methods. Furthermore, we tested the comparability of low-density arrays, a high-throughput method, with the conventional single tube assay to assess gene expression. Finally, the PARSIFAL study allowed us to follow up RNA quality, as multiple measures of the same samples at different time points were available. The aim was to evaluate sources of measurement error in gene expression data in order to avoid these factors in future investigations.

Abbreviations: CBLC, cord blood leucocytes; EDTA, ethylene diamine tetraacetic acid; LDA, low density array; PARSIFAL, prevention of allergy risk factors for sensitization in children related to farming and anthroposophic lifestyle; PASTURE, protection against allergy: study in rural environments; PBLC, peripheral blood leucocytes; rtPCR, real-time polymerase chain reaction; STA, (conventional) single tube assay.

Methods

Population

We analysed data from farmers' and nonfarmers' children enrolled in the cross-sectional study PARSIFAL (3) (Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle) and in the longitudinal study PASTURE (4) (Protection Against Allergy: Study in Rural Environments).

In PARSIFAL, parents from five countries completed a self-administered questionnaire to assess asthma and allergies and exposures associated with farming. Participants were also asked for consent to further investigations, including venous blood sampling. In two countries (A and B), a random sample of 1155 farmers' and nonfarmers' children was drawn for gene expression analyses of innate immunity genes.

PASTURE is a longitudinal study conducted in five European countries (A, B, C, D and E). Each country built a birth cohort of about 100 farmers' and 100 nonfarmers' children. Several detailed questionnaires were completed and cord blood were collected for the measurement of the child's immunological status. Gene expression of innate immunity genes was measured in the total population of 982 children.

Sample collection and RNA extraction

For the PARSIFAL study, PBLC were collected in EDTA blood in 2002. Of the 1155 selected samples, 1140 (98.7%) were available for total RNA isolation using the QIAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). The RNA was solved in water and stored at −80°C.

For the PASTURE study cord blood leucocytes from the 982 children were collected in tubes containing an RNA-stabilizing solution (PAXgene® Blood RNA Tubes; PreAnalytiX, Qiagen, Hilden, Germany) in 2005 and isolated according to the manufacturer's instructions. The RNA was treated with DNase and solved in *Tris*-Buffer and stored at −80°C.

Reverse transcription and gene expression analyses

Reverse transcription was performed with reagents from Applied Biosystems according to the manufacturer's instructions. The RNA concentration had to reach a cut-off concentration of 21 ng/μl to be used for the reverse transcription.

Two different methods of the quantitative real-time polymerase chain reaction (rtPCR) were used to measure mRNA levels:

1. rtPCR with plates (conventional single tube assay, STA-rtPCR) were performed in a final volume of 30 μl containing the adequate amounts of cDNA, primers, probe and TaqMan Universal MasterMix (Applied Biosystems, Foster City, CA, USA) and were analyzed on an ABI Prism 7700 Sequence Detection System™ (Applied Biosystems). All oligonucleotides were synthesized by Microsynth.
2. rtPCR with low-density arrays (LDA-rtPCR) allows to measure the expression of 48 genes with a small amount of cDNA. The LDA-rtPCR was performed in a final volume of 1 μl and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems).

Gene expression of CD14, TLR2 and TLR4 was analysed in PARSIFAL samples from countries A and B in March 2002 with STA-rtPCR. The high-throughput method LDA-rtPCR was used to measure the expression of the same genes in a subsample of the PARSIFAL population from country A in November 2004. In September 2005, gene expression measurement of the genes was repeated in all PARSIFAL samples from country A. For CD14, STA-rtPCR analyses were repeated in December 2005 to test the reproducibility of the different methods.

Statistical analyses

To test for equal proportions of nominal variables in different groups, the Chi-squared (χ^2) test was used. Agreement between numeric variables (gene expression measurements) was measured by the spearman rank correlation coefficient.

All analyses were carried out using R 2.5.0 (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria).

Results

RNA amounts extracted using two methods of blood collection

Using EDTA tubes for blood sampling in the PARSIFAL study, the median of extracted RNA was twofold higher in country A than in country B (Table 1). Consequently, the proportion of samples below the cut-off value for further gene expression analyses (21 ng/μl) differed greatly between the two countries: whereas 98.8% of the samples of country A yielded sufficient RNA for gene expression analyses, this was only true for 68.9% of the samples of country B.

In the PASTURE study, blood was sampled in tubes containing RNA-stabilizing solutions; only slight differences in the amount of extracted RNA were observed between the participating countries (Table 1). The country-specific medians were in a narrow range between 91 and 107 ng/μl. The proportion of samples with sufficient RNA amount for gene expression ranged between 91.1% and 99.4%.

Reproducibility of high-throughput LDA-rtPCR measurements

We measured agreement of rtPCR results of CD14 mRNA levels between the conventional (single tube assay) and the high-throughput method (low-density arrays) by calculating rank correlation coefficients. To exclude interfering effects of the reverse transcription, we

Table 1. RNA yield after RNA extraction using two methods of blood sampling

	EDTA tubes			RNA-stabilizing tubes		
Country	Median amount (ng/μl)	Below 21 ng/μl [*n*/*N* (%)]	*P**	Median amount (ng/μl)	Below 21 ng/μl [*n*/*N* (%)]	*P**
A	70	5/417 (1.2)	<0.0001	91	17/191 (8.9)	0.006
B	34	225/723 (31.1)		100	10/214 (4.7)	
C	–			99	1/180 (0.6)	
D	–			99	10/200 (5.0)	
E	–			107	9/197 (4.6)	

*χ^2 test of equal proportions.

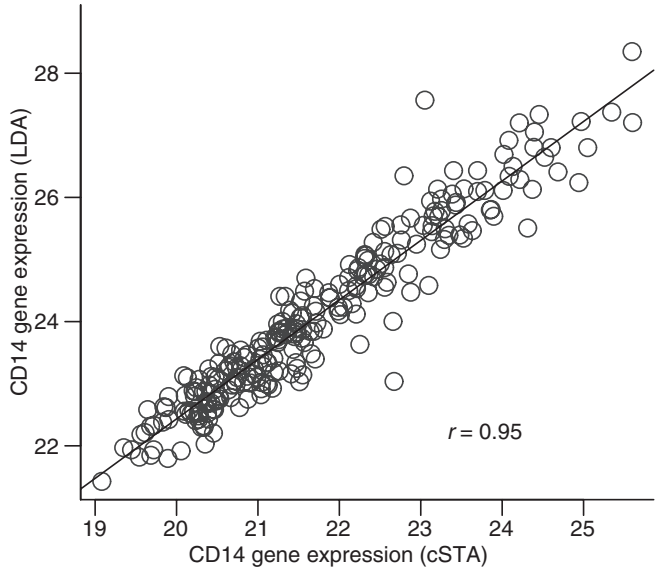

Figure 1. Correlation between CD14 gene expression values resulting from conventional single tube assay cSTA and from low-density arrays LDA using the same CD14 primers and probe set. (r, spearman rank correlation coefficient; straight line, least squares regression line).

measured the expression of the CD14 gene in the same samples either with the conventional STA or with the high-throughput LDA using the same primers and probe set. Correlation of STA-rtPCR and LDA-rtPCR results was high ($r > 0.9$, Fig. 1).

Consistency of rtPCR CD14 gene expression in repeated measurements over time

In 77 samples of the PARSIFAL population of country A gene expression of CD14, TLR2 and TLR4 were analysed repeatedly in 2002, 2004 and 2005. Figure 2 shows agreement (Spearman rank correlation) of gene expression measurements between 2002 and 2004 and between 2002 and 2005 for CD14, TLR2 and TLR4. For all three genes, the agreement of measurements between 2002 and 2004 is moderate (0.35–0.56) and low between 2002 and 2005 (0.03–0.32).

Discussion

We observed that the amount of extracted RNA depends on the method used for blood sampling and that agreement between rtPCR results decreases over storage time. These results suggest that preanalytical procedures and length of storage time are crucial factors for sample quality.

The median amounts of the total extracted RNA amount markedly differed between the two countries studied in PARSIFAL. In this study, blood was collected in EDTA tubes and subsequently transported

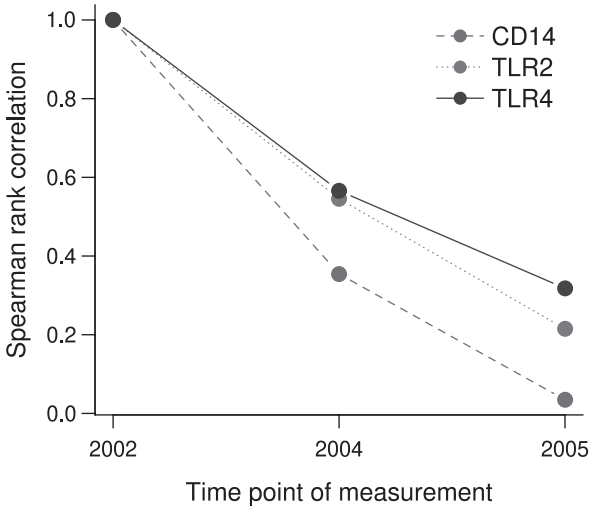

Figure 2. Agreement between repeated measurement of gene expression of CD14, TLR2 and TLR4 during long-time storage. (*y*-axis, Spearman rank correlation between CD14 gene expression measured in 2002 and subsequent measurements (2004 and 2005); *x*-axis, time points of measurements).

to labs in the two study centres, where RNA has been extracted. Although maximal attention was given to control temperature during transport, to minimize transportation time and time until RNA extraction in the labs, it seems probable that differences in these parameters translated into different RNA yields between centres. Based on this experience, we then used in the PASTURE study a blood collection method suitable to stabilize RNA directly in the field. This method resulted in considerably higher RNA yields with lower variability between different centres. It therefore helped to minimize preanalytical errors and proved to yield useful amounts of RNA in the different countries involved.

Figure 2 shows that the agreement between the results of repeated measurements of the expression of the same genes in the same samples at various time points during long-time storage decreased with each additional measurement. Total RNA of these samples was extracted in 2002, thereafter stored in water at −80°C and only thawed up for the two subsequent analyses. Water as solvent for long-term storage of RNA may not be optimal, since unbuffered solutions enhance RNA degradation. This can be avoided with pH-buffered solvents such as *Tris*-buffer, storage of the RNA in precipitated form or reverse transcription of all RNA into cDNA being more stable during long-term storage. RNA degradation caused by duration of storage most likely occurs at random, resulting in a nondifferential error in statistical analyses. Weak associations between gene expression and environmental factors may therefore not be detectable anymore in older samples.

In this study, we assessed gene expression with two different rtPCR methods, the conventional single tube

assay and the high throughput low-density arrays. We found a high comparability between these two methods.

Biological data, such as hosts' immune responses to environmental exposures gathered in the context of epidemiological studies, are most important in order to better understand the biological mechanisms triggered by gene-environment interactions. However, collection of samples and initial work-up in epidemiological studies are made in the field or nonspecialized laboratories rather than in well-controlled lab-settings. Stringent quality control mechanisms are therefore mandatory when performing biological analyses in the context of epidemiological studies.

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Chapter 7 General Discussion and Outlook

This chapter includes general aspects of the PARSIFAL study results. We begin with the discussion of allergy-protective factors of a farming environment. Next we discuss immunological and genetical issues arising from our results and close the first section by addressing major limitations of the PARSIFAL study. Next, the research questions put forward in Chapter 1 are answered in form of short summaries of the main findings. Finally, possible scientific questions arising from the results of the PARSIFAL study are outlined.

VII General Aspects of the Results of the PARSIFAL study

VII.I Protective factors of a farming environment

A key result of this thesis is that the timing of an environmental exposure is crucial to exert its effect on the development of atopic diseases. In Chapter 2 and particularly in Chapter 3 and Chapter 4 we describe factors exerting their effects during growing and maturing of the child in his or her environment. This is in contrast to the development of atopic sensitisation, which is mainly influenced by prenatal exposures.

In Chapter 2 we reported a significant association between maternal work in stables during pregnancy and decreased production of allergen-specific IgE antibodies in her child detectable at school age. Albeit the large sample size of PARSIFAL disentangling pre- and postnatal exposure was difficult because most of the mothers working in stables after birth also worked there before. When assessing the time point of the first contact to stable animals the strongest effect on occurrence of atopic diseases was observed when the first contact occurred during pregnancy and decreased when the first contact took place later. These results suggest that prenatal environmental exposures affect the long-term development of the child's immune system. How the maternal environment exerts its effect on the offspring is unknown. Certain cytokine patterns prevailing in the maternal organism [119] or diaplacental transfer and interaction with the fetal immune system of the antigen itself [120] might exert the protective effects. One might also speculate that epigenetic inheritance meaning stable alterations (e.g., DNA methylation or histone acetylation

[121], but not mutations of the DNA) is involved in the prenatal protection against atopic sensitisation. Thus, preconceptional exposure to a farming environment might imprint the parents genetic make-up by epigenetic means which in turn are passed on to the offspring. The PARSIFAL study does not provide information on the parental exposure before pregnancy, and therefore we cannot exclude these preconceptional effects. Future studies are needed to address the question of epigenetic inheritance by collecting appropriate questionnaire data and assessing epigenetic changes in the parental, as well as in the child's genome

In Chapter 4 we describe protective effects for atopic diseases of pig farming, feeding silage, child's involvement in haying, and regular stay in animal sheds and barns, and farm milk consumption in the PARSIFAL study. Taken together these factors explained the effect of being a farm child and suggest that protection is not conferred by a single environmental component, but rather several factors contribute to a different extent.

There are a few epidemiological studies indicating that consumption of raw cow's milk has a protective effect on the development of atopic diseases (see Chapter 1). PARSIFAL was the first study specifically investigating the allergy-protective effect of farm milk consumption. In Chapter 3 we report detailed analyses of the PARSIFAL dietary data and show that the protective effect of farm milk consumption is reproduced in the whole population as well as in the four study groups suggesting that the effect is not farming specific. The strongest effect was shown for children who drank farm milk during the first year of life and who were also currently consuming farm milk, thus were most likely being exposed to it their whole life. This result favours the assumption that besides the effect of farm milk consumption in early life on a child's immune system, drinking farm milk later in life reinforces protection against atopic diseases. At present we can only speculate about specific components of farm milk that might be responsible for the observed protective effects. It is conceivable that the microbial burden of farm milk influences the gut microflora and thus the development of oral tolerance [122]. Recent animal experiments have shown that colonization of germ-free mice with polysaccharide-A-producing Bacteroides fragilis, a ubiquitous gut microorganism and an important Gram-negative anaerobe that colonizes the mammalian lower gastrointestinal tract, restored normal cytokine production and established a proper Th-1/Th-2 balance for

the host [123]. Probiotical treatment for allergy prevention has recently been investigated in several clinical trials which, systematically reviewed, showed a beneficial effect on the development of atopic disease [124, 125]. However, particularly in immunodeficient patients adverse effects have been observed [126-129].

Alternatively, farm milk might exert its beneficial effect on the development of atopic diseases by containing nonviable, microbial or nonmicrobial molecules. CD14 is a pattern-recognition receptor of the innate immune system for a wide spectrum of microbial compounds and nonmicrobial compounds (see Chapter 1). Farm milk is known to contain various bacterial species [130] and microbial compounds that are able to bind to CD14 after penetrating the intestinal mucosa. Because CD14 is known to be a coreceptor of Toll-like receptors 4 and 2 complexes of CD14 and their ligands might bind to one of these pattern-recognition receptors on the cell surface of antigen-presenting cells and hence activate the immune system, resulting in subsequent production of immunomodulatory chemokines and cytokines.

Because of the diversified binding properties of CD14 also non-microbial molecules contained in the farm milk might thus be responsible for the protection against allergic diseases. There is some evidence that omega-3 dietary fatty acids are potentially beneficial to patients with allergic diseases [131, 132]. Thus animal feeding and also milk processing (fat standardization, pasteurization, and homogenization) might influence the milk's fat content or fatty acid profile [133, 134]. One might therefore speculate that farm milk differs from shop-purchased milk regarding its fat amount or composition and subsequent intake of higher amounts of omega-3 dietary fatty acids might result in impaired production of proinflammatory prostaglandins and leukotrienes [135].

When evaluating the use of farm milk as an allergy-preventive agent, health effects of pathogens in raw milk such as salmonella or enterohaemorrhagic Escherichia coli (EHEC) have to be carefully considered. Particularly, transmission of EHEC through unpasteurized cow's milk continues to cause serious health effects [136] and therefore better knowledge of the protective ingredients of raw cow's milk have to be established before using it in a clinical setting. Future analyses of farm milk compounds responsible for the beneficial effect have to focus on a better

characterisation of the consumed milk, e.g., by measuring fatty acid profiles in addition to microbial compounds.

VII.II Imprinting of the innate immune system

The availability of biological data, specifically gene expression of PRR in the PARSIFAL population allowed us to link our epidemiological observations to transcriptional activity of cells interacting with the innate immune system. In Chapter 2 we showed that prenatal exposure to a farming environment influenced gene expression of PRR at school age. Both measures of prenatal exposure, maternal stable work during pregnancy and the number of farm animals the mother had contact with during pregnancy, were significantly associated with increased gene expression of TLR2, TLR4 and CD14. These PRR serve as receptors for a wide spectrum of microbial compounds (see Chapter 1) and it seems likely that the number different animals reflects increasing levels, diversified levels, or both of microbial exposure. TLR4 and CD14 bind specifically LPS, and TLR2 binds fungal cell wall components and it is known that farm activities correlate with indoor concentration of LPS and other microbial components [137, 138]. However, we did not observe an association between indoor exposure of these ligands and their respective receptor. Thus, measuring individual components of the microbial environment might reflect a too-narrow spectrum.

Expression of PRR was not associated with any health outcomes under study. Whether upregulation of PRR primarily reflects a marker of exposure or whether higher levels of PRR take an active part in the pathogenesis of atopic diseases cannot be clarified with the PARSIFAL data. However, experimental data strongly suggest an involvement of PRR activation in the development of atopic diseases [139-141]. Until quite recently the most accepted mechanistical conception of how microbial exposure exerts its allergy-protective effects was the induction of Th1 immune response that in turn suppresses the allergy-prone Th2 response. The epidemiological observation that the increase in atopic diseases is paralleled by Th1-driven autoimmune diseases [142] and experimental data [143] challenges this simple concept of deviation towards a Th1 immune response. Recent research came up with other concepts that might explain the contradictory results. (i) The identification of Treg, a cell line secreting inhibitory cytokines such as IL-10 and TGF-

β, offered an elegant way to explain that a missing suppression of both, the Th1 and Th2 system, might account for exaggerated Th2 immune responses and thereby development of atopic diseases [144, 145]. (ii) One might also speculate that children, exposed to microbes in the environment, build up a network of molecules desensitizing the innate immune system against harmless antigens or antigens in low dose. This network would have the potential to inhibit an inappropriate adaptive immune response, as it is the case in atopic disorders. SOCS-1, a potent suppressor of the TLR signaling cascade (see Chapter 1), is induced in response to microbes and suppresses inflammatory and autoimmune responses in mice [146, 147]. Similar findings in humans were observed in a subanalysis of the ALEX study: Exposure to LPS was associated with the expression of SOCS-1 that was in turn inversely associated with the expression of cytokines of adaptive immunity such as IL-5, IFN-γ and IL-10 suggesting a state of LPS tolerance [148]. Finally, (iii) the cytokine IL-17 of the recently identified TH17 cell line was reported to be increased in the sputum, bronchoalveolar lavage fluids and sera from asthmatics [149] thereby offering another pathogenetic mechanism.

VII.III The genes have a say

In Chapter 5 we report a gene-environment interaction between a polymorphism in the CD14 gene (*CD14/A-1721G*) and farm milk consumption in the first year of life on atopic health outcomes in two independent populations. In unstratified analyses farm milk consumption had no effect on gene expression, highlighting the venture of missing relevant signals that are masked by genetic variation in epidemiological studies. In our population the well-characterized functional promoter polymorphism in *CD14/C-260T* (see Chapter 1) was in high linkage disequilibrium with *CD14/A-1721G*, therefore results for the associations between farm milk and the phenotypes studied were similar in both SNPs, although less heterogeneous across the genotypes of *CD14/A-1721G*. However, we can not exclude the possibility that polymorphisms in linkage disequilibrium with *CD14/A-1721G* underlie our findings. Moreover, the power of our study population was too limited too conduct haplotype analyses. Effects of combinations of different SNPs in the CD14 gene might be more distinct than effects of the individual SNPs.

Variations in genotypes of *CD14/A-1721G* not only interacted with the effect of farm milk consumption on clinical outcomes but also on gene expression of CD14. Several mechanisms for how the genotype could influence the phenotype are possible. Mutations in the coding region may modify the structure and thus the function of the protein. *CD14/A-1721G* is located in the promoter region of the CD14 gene and thus rather affects gene expression and, in turn, the amount of the translated protein.

However, neither farm milk consumption nor one of the *CD14/A-1721G* genotypes was directly associated with gene expression of CD14. An association between the *CD14/A-1721G* polymorphism and CD14 gene expression became evident only on exposure to farm milk consumption. We speculate that subjects with higher expression of CD14 produce higher levels of the CD14 protein and may therefore be more susceptible to further stimulation by farm milk-associated agents. However, investigation of the transcriptional activity of the genetic variations of *CD14/A-1721G* and its function are needed.

VII.IV Limitations

The results of this thesis have to be interpreted in the light of three serious limitations of the PARSIFAL study: The cross-sectional design, the lack of biological data regarding the composition of farm milk, and the limited power to for genetical analyses.

Results of a cross-sectional study do not allow conclusions to be drawn regarding the temporal sequence and therefore causal relationships between events, although multiple regression techniques may allow disentangling temporality to a certain extent. A temporal relationship can only be addressed adequately in longitudinal studies which generally are financially extensive and time-consuming. In turn, cross-sectional studies offer the possibility to study research questions with moderate expenses in time and money. Studies like PARSIFAL stand out by their hypotheses generating, rather than hypotheses proving potential. Furthermore, cross-sectional studies are prone for the occurrence recall bias, meaning that e.g. mothers from atopic children tend to recall past exposures better than mothers of healthy children. Objective measurements, such as gene expression measurement, genotyping, and

measurement of allergen-specific IgE, however, are not known to the parents and therefore the results are unlikely to be biased by recall.

Moreover, the results of this thesis are limited by the lack of biological data regarding milk composition. Therefore, the underlying mechanisms of the allergy-preventive effect of farm milk consumption remain speculative.

Finally, we are aware that the sample size was low to study gene-environment interactions. We do, however, believe that similar trends in the majority of the health outcome measures in the two independent study populations strongly suggest that the results have not only emerged because of chance.

VIII Summary of the Main Findings

Environmental factors and expression of innate immunity genes

1. *Can the results of the ALEX study [50] be confirmed in the PARSIFAL data?*

We could reproduce the observation in a small sub-sample of the ALEX population that children raised on a farm express higher amounts of PRR. In PARSIFAL gene expression of CD14, TLR2 and, in contrast to ALEX, TLR4 were higher in farmers' children compared to non-farmers' children.

2. *Are the levels of microbial compounds in house dust associated with the expression of genes of the innate immune system?*

We determined levels of LPS and EPS levels in house dust collected from the living room and from the child's mattress. Gene expression of both, the TLR4 ligand LPS and the TLR2 ligand EPS, were not correlated with gene expression of their respective receptor in neither the living room nor the mattress dust samples.

3. *Is the protective effect of farm milk consumption that has been shown in the ALEX study reproducible in the PARSIFAL study and is it at least partly explained by differential gene expression of PRR?*

We were able to reproduce the protective effect of farm milk consumption in the larger PARSIFAL study independently of potential confounders as shown in multiple

regression models. Of particular importance is the consistency of the findings across children from farming, rural non-farming, anthroposophic, and suburban environments indicating that farm milk consumption represents a route of exposure that is independent of concomitant exposures to microbial compounds present in animal sheds and farms houses. The inverse association was not explained by concurrent farm activities of the child or farm exposures during pregnancy and was most pronounced in children drinking farm milk since their first year of life.

In addition, we describe a genetic polymorphism in the CD14 gene that modifies the effect of farm milk consumption on both, the development of atopic diseases and gene expression of CD14. However, we did not observe a direct association between farm milk consumption and the gene expression of neither CD14, nor TLR2 and TLR4.

4. *Which other specific factors of farming lifestyle have an impact on the expression of PRR of the innate immune system?*

5. *Is there a window of age where potential beneficial factors take effect?*

Expression of PRR was higher in children when the mother worked on the farm during pregnancy. This association remained even after adjustment for other farm-related factors and potential confounders in multiple regression models. In addition, we observed a dose-dependent increase in gene expression of CD14, TLR2 and TLR4 with the number of farm animal species the mother had contact with during pregnancy, which probably serves as proxy for an increasing variation in microbial exposure.

Interestingly, prenatal factors also exert their effect on the child's health later in life. Children of mothers who worked on the farm during pregnancy were less sensitised at school age to common inhalant and food allergens than children of unexposed mothers. Other farm-related exposures did not confound this relationship. However, the development of clinical symptoms of atopic diseases seemed to depend on exposures that occurred postnatally. Thus, children currently exposed to a farming environment (defined as barn or stable visits or helping with haying at least on a weekly basis) had fewer symptoms of allergic rhinoconjuntivitis, whereas farm

milk consumption and regular contact with farm animals were inversely associated with the diagnosis of asthma.

DNA polymorphisms, TLR expression and environmental factors

6. *To what extent do polymorphisms of genes encoding for PRR modify the respective expression of PRR?*

7. *Does such a relation depend on environmental factors (gene-environment interaction) most notably the factors known to be protective regarding atopic diseases?*

Neither of the genotyped SNPs was directly associated with differential gene expression. However, a polymorphism in the CD14 gene (*CD14/A-1721G*) significantly modified the effect of farm milk consumption on CD14 gene expression. Farm milk-drinking children homozygous for the A allele expressed significantly more CD14 than non-exposed children. No association between farm milk drinking was observed in children with the GG genotype; heterozygous children showed an intermediate difference in CD14 gene expression between exposed and non-exposed children. In unstratified analyses farm milk consumption had no effect on gene expression. Interestingly, we observed the same interaction between genotypes of *CD14/A-1721G* and farm milk exposure on asthma and related atopic health outcomes. Moreover, we were able to replicate the gene-environment interaction on atopic health outcomes in two independent populations: the PARSIFAL and the ALEX population.

Gene expression in the epidemiological study setting

8. *Are there qualitative differences of the gene expression results between studies using different methodological approaches?*

9. *Which pitfalls have to be accounted for when doing gene expression measurements in epidemiological studies?*

In PARSIFAL as well as in the longitudinal PASTURE study gene expression was measured to investigate environmental influences on the innate immune system.

However, there were some methodological differences between the two studies: In PARSIFAL blood was collected in EDTA tubes, whereas tubes containing an RNA-stabilizing solution were used in PASTURE. Using these different tubes had a marked impact on the amount of RNA that could be extracted from the blood samples: In PASTURE larger amounts of RNA could be extracted resulting in smaller proportions of samples that could not be used for further analyses. We also observed less heterogeneity in terms of extracted RNA amounts between study centres. Using RNA stabilizing tubes is therefore preferable for maintaining the quality of blood samples, particularly in logistically extensive, epidemiological studies. Furthermore, the PARSIFAL samples have been stored with water as a solvent. We found decreasing agreement between samples repeatedly measured, suggesting RNA degradation over time. Thus, particularly in longitudinal studies, other solvents than water have to be considered for long-term storage of RNA samples.

IX What Scientific Questions Arise from these Results

IX.I Further studies: Large and longitudinal

For a better characterisation of the allergy-protective compounds contained in the farming environment, as well as identification of susceptibility genes for atopic diseases, large-scale epidemiological studies are needed that provide enough power to detect effects also in small subsets of a population. An ongoing project, the cross-sectional GABRIEL study taking place in Austria, Germany, Poland, and Switzerland, recruited about 100,000 children from farming and non-farming families for a close investigation of genetic and environmental factors and their role in the development of atopic diseases [150]. This large population with well-characterised sub-samples by means of environmental (dust samples and milk samples), clinical (measurement of bronchial hyperresponsivness) and biological (gene expression, genotyping and specific IgE) data promises novel results regarding the identification allergy-protective factors of a farming environment. A close investigation of the relevance of farm milk consumption in the development of atopic disorders will be possible as data of processing (skimming, heating) or storage time of the milk consumed by the children will be available.

As pointed out in section VII.IV the cross-sectional design of the PARSIFAL study limits the informative value of our results regarding the causal relationship between exposure and outcome. Thus, longitudinal studies help us to gather information on the temporal sequence and thereby allowing causal inference. The ongoing PASTURE cohort consists of about 1000 children from five European countries (Austria, Finland, France, Germany, and Switzerland). Each country built a birth cohort of about 100 farmers' and 100 non-farmers' children [151]. Several detailed questionnaires were completed during pregnancy and after birth of the child referring to the health of the child and the family and intensity and timing of farm-related exposures of the child and the mother. Cord blood and blood samples at one year of life were collected for measurements of specific IgE, as well as for genotyping and measurement of gene expression of innate immunity genes. Furthermore, the children will be followed up to the age of 6 years, and beyond. This data will allow the investigators to evaluate prenatal factors affecting the child's immunological and health status at birth and later in life. Furthermore, exposure data will be collected close to the time point when the child was actually exposed and before manifestation of disease, thereby limiting recall bias. It would be desirable to follow up this cohort until school age when most atopic disorders have already become manifest.

IX.II The magic of raw cow's milk

Analyses of milk samples of an ongoing birth cohort study in rural areas of Europe (PASTURE) indicate that LPS levels are similar or even lower in milk consumed by farmers' than in milk consumed by non-farmers' families [152]. There, higher levels of LPS in the milk of non-farming families could be largely explained by storage time before consumption and thereby most likely results from recontamination. This study challenges the role of gram-negative microorganisms for the protective farm milk effect. Though, since it is known that the microbial spectrum of recontaminated cow's milk is dominated by Gram-negative bacteria and essentially differs from fresh, unprocessed milk [153, 154], LPS might not be the decisive compound reflecting the microbial load conferring beneficial, immunomodulatory effects on the child's health.

In the GABRIEL study questionnaire data regarding milk processing and also storage time, as well as biological data of farm milk is available. Along with the large sample size a closer investigation of the effect of farm milk consumption on atopic

health outcomes is possible. Farm milk can be processed in multiple ways. Each processing step has different influences on the composition of the milk. Whereas skimming might affect the lipid profile and bioavailability of fatty acids, heating of the milk might change the microbial content as a function of the extent and duration of heating. These factors will be investigated in the large GABRIEL population as it provides enough power for subgroup analyses. In a sub-sample of the GABRIEL population shop and farm milk samples are collected directly from the child's home. These samples very closely represent what kind of milk the child is actually drinking and extensive analyses of these samples regarding composition, particularly fat content and profile, and microbial contamination will be conducted. An accurate characterisation of the farm milk and recognition of the differences compared to the milk bought in the shop will bring us closer to the potential components that confer protection against atopic diseases and might offer the opportunity to develop a product safe enough to investigate in clinical trials.

IX.III A closer look at genetics

The achievements in genetics of atopic diseases appear impressive. In a little over 10 years, many susceptibility genes have been identified as robust candidates, and the list keeps growing longer. However, conflicting results and recognition of the complexity of genetics in diseases like asthma and allergy, raised as many questions as it answered.

There is hope that genome-wide association (GWA) studies, if adequately designed and powered to detect gene-environment interactions, may prove to be effective tools, taking us closer to a comprehensive set of allergy genes. In 2007 the first GWA study for an allergy trait (childhood asthma) was published [155]. GWA studies rely on dense sets of SNPs across the genome to survey the most common genetic variants for a role in disease or to identify the heritable quantitative traits that are risk factors for disease [156]. The availability of dense genotyping chips, containing sets of thousands of SNPs that provide good coverage of much of the human genome, means that for the first time GWA studies for thousands of cases and controls are technically and financially feasible. The main strength of GWA studies is expected to lie in their ability to discover truly novel disease candidate genes, especially those associated with moderate risks [92].

The GABRIEL population provides an optimal framework in which GWA studies can be conducted. Indeed, genetic analyses in this population are ongoing and the first results are eagerly anticipated. The genes discovered through genome-wide approaches in analyses stratified by exposure level will also indirectly help to narrow the search for biologically active substances interacting with the products of the identified genes and thus might be an important step forward on the way to successful prevention of atopic diseases.

IX.IV Immunological data: A challenge for bioinformatics

Most important experimental data has to provide the biological and mechanistic knowledge to understand which immunological pathways are involved in the associations observed in epidemiological studies. The *CD14/-C1721T* SNP (Chapter 5) similarly modifies the effect of early farm milk consumption on atopic diseases and gene expression of CD14, strongly suggesting that the health effect is mediated by differential transcriptional activity among the genotypes. However, functional studies in cell lines or animals have to prove this hypothesis.

Our data and literature from experimental data (reviewed in [69]) suggest that the innate immune system plays a crucial role how microbial or even other environmental compounds exert their effect on the development of atopic diseases. The emergence of high-throughput genomic technologies allow large-scale gene expression profiles to be measured in a time-efficient way and these methodological advances have spread the range of potential applications and allow gene expression analyses to be used in the context of epidemiological studies (we report technical pitfalls and potential workarounds in Chapter 6). However, the enormous amounts of data these techniques generate create challenges in bioinformatics, e.g., how to identify true positive results (keyword “multiple testing”) or how to model complex immunological pathways by statistical means. Sophisticated statistical methods are available nowadays but scarcely used in immunological research [157]. Structural equation models (SEM) can handle multiple relationships among study variables [158] and are therefore eligible to deal with the complexity of immunological processes and their relationship with exposure and health outcome [157]. Using this kind of approach to analyze complex data in the context of gene expression measurements in

epidemiological studies might offer the opportunity to get a deeper insight into the biological mechanisms of the observed associations.

IX.V Clinical applications: From population to patient

One aim of epidemiological research is generating hypothesis that in turn have to be further investigated in experimental studies. However, the final goal is to come up with new therapeutical or preventive strategies that finally help patients by ameliorating or even preventing the manifestation of their disease. In the context of atopic diseases, aiming for the latter appears realistic since potential environmental factors were found that might prevent allergic disorders. Vaccination-like therapeutic challenge of the innate immune system by pharmacologically targeting PRR is one possibility and experimental studies are ongoing, e.g., application of TLR2, TLR4, and TLR9 ligands reduced the degree of allergic asthma through reduction of inflammation, total serum IgE and number of T helper cells in the lung in mice [69, 159]. Particularly for asthma, the use of CpG (cytosine-guanine) oligodeoxynucleotides contained in bacterial DNA promises to be a potential agent for prevention and therapy [160]. Furthermore, promising clinical studies with probiotic microbes are ongoing. Probiotic microbes are living microorganisms culturing the gut and thereby providing a beneficial stimulus to the innate immune system. Prenatal administration of probiotic microbes to children had preventive effects on atopic diseases at least until the age of 4 years in a Finnish trial [161] and other studies reported similar results (systematically reviewed in [125]).

In the context of our results, so far unidentified compounds contained in farm milk evolve as potential preventive measures for atopic diseases. The results presented in this thesis might add to evidence of the farm milk's beneficial effects and motivate future investigations, so that clinical trials exposing children to fresh cow's milk or its beneficial ingredients one day might come true.

Chapter 8 References

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Chapter 9 Abbreviations

ALEX	ALlergy and EndotoXin
APC	Antigen-Presenting Cells
CSR	class-switch recombination
DC	Dendritic Cells
EPS	Extracellular PolySaccharide
GWA	Genome-Wide Association
IFN	InterFeroN
Ig	ImmunoGlobulin
IL	InterLeukin
LPS	LipoPolySaccharide
PAMP	Pattern-Associated Molecular Pattern
PARSIFAL	Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle
PRR	Pattern-Recognition Receptor
SEM	Structural Equation Models
SNP	Single Nucleotide Polymorphism
TCR	T-Cell receptor
TGF	Transforming Growth Factor
Th	T helper cell
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	T regulatory cell

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Chapter 11 Curriculum Vitae

Christian Bieli, born on August 13th 1976 in Switzerland. Unmarried, native language is German, foreign languages are English, French and Spanish.

Education and professional experience

1983 – 1988	Primary and secondary school in Laufen, Switzerland
1989 – 1996	Gymnasium Laufen
1996 – 1997	Gardening and horticulture, Dalla Vecchia, Bärschwil
1997 – 2003	Medical school at the university of Basel, Switzerland - Internships in Basel, Berlin, Chur, Laufen and Vienna - Laboratory training at the Friedrich-Miescher-Institut, Basel
During medical school	Bike courier, Veloexpress, Basel
2003	Final examination (Staatsexamen)
2004 – 2007	PhD Student at the Institute for Social- and Preventive Medicine, University of Basel

Research projects

PARSIFAL (Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle)
Activitiy: Epidemiologic Research/Biostatistics

EuroPREVALL (The Prevalence, Cost, and Basis of Food Allergy across Europe)
Activitiy: Project management in collaboration with the

Department of Allergology, University Hospital, Zürich

FORALLVENT (Forum for Allergy Prevention)
Activitiy: Public/media relations and organisation of a International FORALLVENT symposium (April 2007)

2004 BOMS (basics of medical statistics), ISPM Basel

2004 Logistic regression in epidemiology, University of Lausanne (2 ECTS points)

2004 Introduction to STATA, University of Bern (1.5 ECTS points)

2004 Methods in epidemiology, University of Zürich (2.5 ECTS points)

2004 Environmental epidemiology, University of Basel (2 ECTS points)

2004 Genetic epidemiological approaches to pulmonary diseases, EAACI course, Paris (2 ECTS points)

2005 Applied regression modeling, University of Bern (2 ECTS points)

2005 – 2007 Advanced training (Weiterbildungs-Lehrgang) in applied statistics, Seminar for Statistics, ETH Zürich (30 ECTS points)

Clinical education

2005 Intern (3 months, 100%), emergency department, children's hospital, Zürich

2006 Intern (12 months, 10%), policlinic, allergology, children's hospital, Zürich

Since July 2007 Intern, continuing education FMH Pädiatrie, children's hospital, Zürich

Teaching activities

2004 – 2007	Several talks at colloquias ans research seminars at the ISPM, Basel
2005 – 2007	Tutorial „Humanwissenschaften“ for 1st year medical students, University of Basel
2006 – 2007	Tutorial “Biomedizinische Statistik” for 2nd year medical students, University of Basel
2006 – 2007	Statistical supervision of medical thesis of Lisbeth Stahlberger, University of Zürich
2007	Seminar „Scientifc Skills – Public Health“ for 2nd year medical students, University of Basel

List of publications

- Ege MJ*, Bieli C*, Frei R, van Strien RT, Riedler J, Ublagger E, Schram-Bijkerk D, Brunekreef B, van Hage M, Scheynius A, Pershagen G, Benz MR, Lauener R, von Mutius E, Braun-Fahrlander C, and The Parsifal Study Team.
- Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol 2006;117(4):817-23. *both authors contributed equally
- Bieli C*, Eder W*, Frei R, Braun-Fahrländer C, Klimecki W, Waser M, Riedler J, von Mutius E, Pershagen G, Doekes G, Lauener R, Martinez FD, and The Parsifal Study team. A polymorphism in CD14 modifies the effect of farm milk consumption on allergic diseases and CD14 gene expression. J Allergy Clin Immunol. 2007 Dec;120(6):1308-15. *both authors contributed equally
- Waser M, Michels KB, Bieli C, Flöistrup H, Pershagen G, von Mutius E, Ege M, Riedler J, Schram-Bijkerk D, Brunekreef B, van Hage M, Lauener R, Braun-Fahrländer C, and the Parsifal Study team.

- Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clin Exp Allergy. 2007 May;37(5):661-70.
- Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, Weiss G, Nyberg F, van Hage M, Pershagen G, Brunekreef B, Riedler J, Lauener R, Braun-Fahrlander C, von Mutius E, and The Parsifal Study Team.
- Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol. 2007 May;119(5):1140-7.
- Gene expression measurements in the context of epidemiological studies. Bieli C*, Frei R*, Schickinger V, Steinle J, Bommer C, Loeliger S, Braun-Fahrländer C, von Mutius E, Pershagen G, Lauener R, the PARSIFAL Study team. Allergy, 2008. 63(12): p. 1633-6. *both authors contributed equally
- Early Mobilization after Flexor Tendon Repair in Children. Moehrlen U, Mazzone L, Bieli C, Weber DM. Eur J Pediatr Surg. 2009 Feb 11. [Epub ahead of print]

Congress contributions

2005 Exposure to increasing numbers of different animal species is positively associated with gene expression of toll-like receptors.
Bieli Ch, Frei R, Lauener R, Sennhauser FH, Eder W, von Mutius E, Pershagen G, and Braun-Fahrländer C.
Talk, EAACI meeting (European Academy of Allergology and Clinical Immunology), Davos.

2005 Exposure to increasing numbers of different animal species is positively associated with gene expression of toll-like receptors.
Bieli Ch, Frei R, Lauener R, Sennhauser FH, Eder W, von Mutius E, Pershagen G, and Braun-Fahrländer C.
Poster presentation, ATS (American Thoracic Society) Congress, San Diego

2005 Exposure to increasing numbers of different animal species is positively associated with gene expression of toll-like receptors.

Bieli Ch, Frei R, Lauener R, Sennhauser FH, Eder W, von Mutius E, Pershagen G, and Braun-Fahrländer C.
Poster presentation, WAC (World Allergy Congress), Munich.

2006 Interaction between a polymorphism in CD14 and farm milk consumption modifies the frequency of asthma and allergies in children and alters CD14 gene expression.
Bieli Ch, Eder W, Lauener R, Frei R, Von Mutius E, Pershagen G, Riedler J, Waser M, Klimecki W, Martinez FM, and Braun-Fahrländer C.
Talk, EAACI congress (European Academy of Allergology and Clinical Immunology), Vienna.

2006 Interaction between a polymorphism in CD14 and farm milk consumption modifies the frequency of asthma and allergies in children and alters CD14 gene expression.
Bieli Ch, Eder W, Lauener R, Frei R, Von Mutius E, Pershagen G, Riedler J, Waser M, Klimecki W, Martinez FM, and Braun-Fahrländer C.
Talk, International Conference on Environmental Epidemiology & Exposure, Paris.

2007 Expression of genes related to Th1 and Th2 cell differentiation in farmers and non-farmers children.
Frei R, Bieli C, Pershagen G, Braun-Fahrlander C, and Lauener R.
Poster presentation, ATS (American Thoracic Society) Congress, San Francisco.

Awards and Distinctions

May 2009 This PhD-thesis was accorded the grade "summa cum laude".

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