# Exposure to indoor microbial agents, allergens and pets, and their relation to asthma and allergy prevalence in farmers' children and their peers from rural areas

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# Content

# Summary


## Background

In ‘westernised’ countries, the prevalence of childhood asthma and allergy have risen throughout the last three 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 more 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. Recent allergy research has focused on the interaction between the innate and adaptive immunity: innate immunity receptors of pathogens seem to modulate the activation of adaptive immunity mechanisms.

Three independent studies showed reduced prevalence of asthma and allergy among farmers’ children compared to their peers from the same rural areas from Switzerland, Austria, and Germany. A series of epidemiological studies in Europe, Canada, and Australia consistently confirmed and extended these findings. These results have been seen as an extension of the ‘hygiene hypothesis’, since a farm environment provides an enormous habitat for micro-organisms. A potential candidate that may explain these differences in the prevalence of childhood asthma and allergy is environmental exposure to endotoxin, a component of the outer membrane of gram-negative bacteria.

## Aim

To assess the exposure to indoor microbial agents, allergens and pets in farmers’ children and their peers of non-farming families, and to estimate whether these exposures are associated with the prevalence of childhood asthma and allergies.

## Methods

The ALEX (Allergy and Endotoxin) study was a cross-sectional survey in rural areas of Germany, Austria, and Switzerland. 2618 parents of 6-13 year-old children completed a standardised questionnaire including questions about asthma and allergy from the ISAAC study

and questions about the child's activities on farms, and characteristics of the home environment. Blood samples were obtained from selected children and tested for atopic sensitisation, specially for specific IgE and IgG4 antibodies to grass pollen and cat allergen. Endotoxin and cat allergen levels were measured in indoor dust samples and in settled dust from stables. Complete data were available for 812 children. In a subgroup of 553 children mattress dust muramic acid levels, another marker for exposure to bacteria, were determined.

### Results

Higher levels of indoor endotoxin exposure were associated with reduced allergen sensitisation, decreased prevalence of hay fever, atopic asthma and wheeze in a dose-dependent manner. The associations were equally strong among the sub sample children from non-farming families, indicating that even lower levels of endotoxin may favourably influence the risk of atopic diseases.

Endotoxin levels in stables were not correlated with the amount of endotoxin measured indoors, but a dose-dependent association between the child's activity on the farm and indoor home endotoxin levels was observed, both in farmers' and in non-farmers' children. Pet keeping, full time farming (compared to part time farming), and younger age of the children contributed additionally to increased indoor endotoxin levels. Endotoxin levels in stables increased with the number of cattle (but only up to the highest quartile), with hay feeding (compared to feeding of mainly silage), and additionally with provision of accommodation of horses, pigs, sheep or goats in the cattle stable. All these predictors might be surrogate measures for traditional dairy farming in hilly German speaking areas.

Children's mattress' muramic acid levels were significantly higher in farmers' children than in non-farmers' children. Mattress muramic acid and endotoxin levels were partially correlated, indicating that both substances are markers for the exposure to micro-organisms. Independent of being a farmers' child, mattress dust from homes heated with wood or coal and less frequently cleaned mattresses showed increasing muramic acid levels.

Independent of the endotoxin exposure, increasing muramic acid levels in mattress dust was associated with a lower frequency of current wheeze, but not with atopic sensitisation or hay fever. The protective effect on wheeze and diagnosed asthma was more pronounced in non-sensitised children. The different effect spectrum for muramic acid and endotoxin exposure suggest that different micro-organisms might contribute to the lower prevalence of asthma and allergy among farmers' children, compared to non-farmers' children.

Current contact to dogs was inversely associated with diagnosed hay fever, asthma, and specific sensitisation to grass pollen and to cat allergen, but not with increased IgG4 levels. Early and current exposure to cats – but not to dogs – was associated with lower frequency of wheeze and grass pollen sensitisation. None of these inverse associations were greatly affected by additionally taking into account the indoor endotoxin or cat allergen levels, but additionally adjustment for early or current exposure to farm animals attenuated the protective effects. Although pet exposure was frequent in this rural population, the protective effects of pet keeping observed in other peer-reviewed studies may be masked by frequent contact to farming environments.

### Conclusions and outlook

Endotoxin and muramic acid may be surrogate markers of a much broader spectrum of microbial compounds. Thus, further studies have not only to confirm the lower risk of children with contact to livestock or with higher exposure to micro-organisms early in life, but also to find the relevant mixture of protective components in the farm dust. In addition, as ingestion is another plausible route of exposure to micro-organisms, epidemiology may focus on differences in nutrition and their association with childhood asthma and allergy. The PARSIFAL (Prevention of allergy – Risk factors for sensitisation in children related to farming and anthroposophic lifestyle) study offers the opportunity to do so, as this study include children's populations growing up with different lifestyles. Experimental studies may focus on the relevant exposure route (inhalation, ingestion) of different patterns of micro-organisms. The genetic dimension has to be kept in mind in the discussion of environmental exposure to micro-organisms by identifying particularly sensitive groups through genetic investigations, as the available data in the ALEX study was limited.

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. Thus, the protective effect of a microbial environment on the development of asthma and atopy should be balanced against the benefits of established hygiene standards.

# 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. Die neuere Allergieforschung konzentriert sich auf die Interaktion zwischen dem angeborenen und dem erworbenen Immunsystem: Rezeptoren für Keime des angeborenen Immunsystems scheinen die Aktivität bestimmter Mechanismen des erworbenen Immunsystems zu beeinflussen.

Drei unabhängige Studien aus der Schweiz, Österreich, und Deutschland zeigten für Bauernkinder eine reduzierte Asthma- und Allergieprävalenz gegenüber Nichtbauernkindern aus den selben ländlichen Gegenden. Epidemiologische Studien aus Europa, Kanada und Australien bestätigten diese Befunde. Diese Resultate werden als eine Erweiterung der Hygienehypothese angesehen, weil das bäuerliche Umfeld als ein riesiges Biotop für Mikroorganismen betrachtet werden kann. Ein möglicher Ansatz für die Erklärung dieser Prävalenzunterschiede ist die Umweltexposition gegenüber Endotoxin, einer Komponente der äusseren Membran von Gram-negativen Bakterien.

## Ziel

Die Expositionsabschätzung von Bauernkindern und Nichtbauernkindern gegenüber häuslichen Keimen, Allergenen und Haustieren, und die Berechnung der Zusammenhänge dieser Expositionen mit den Asthma- und Allergieprävalenzen.

## Methoden

Die Querschnittsstudie ALEX (Allergy and Endotoxin) wurde in ländlichen Gegenden von Deutschland, Österreich und der Schweiz durchgeführt. 2618 Eltern von 6- bis 13-

jährigen Kindern füllten einen standardisierten Fragebogen zu Asthma und Allergien, zu Fragen nach der kindlichen Aktivität auf Bauernhöfen und zu häuslichen Merkmalen aus. Bei ausgewählten Kindern wurden Blutproben genommen und auf allergische Sensibilisierung, spezifische IgE- und IgG4-Antikörper gegenüber Graspollen und Katzenallergen getestet. Im häuslichen Staub und im Stallstaub wurden die Konzentrationen von Endotoxin und Katzenallergen gemessen. Von 812 Kindern war ein kompletter Datensatz verfügbar. In einer Untergruppe von 553 Kindern wurde die Konzentration von Muraminsäure im Matratzenstaub – ein weiterer Indikator für die Exposition gegenüber Bakterien – bestimmt.

**Resultate**

Die im häuslichen Umfeld gemessene Endotoxinbelastung war dosisabhängig mit einer reduzierten Prävalenz von allergischer Sensibilisierung, Heuschnupfen, allergischem Asthma und dessen Symptomen assoziiert. Die statistischen Zusammenhänge waren für Die Subgruppe der Nichtbauernkinder gleich stark. Das weist darauf hin, dass bereits relativ geringe Endotoxinbelastungen das Risiko von allergischen Erkrankungen vorteilhaft beeinflussen können.

Die Endotoxinbelastung im Stall war nicht mit der häuslichen Endotoxinbelastung korreliert, aber sowohl bei Bauern- als auch bei Nichtbauernkindern wurde eine dosisabhängige Beziehung zwischen der kindlichen Aktivität auf dem Bauernhof und der häuslichen Endotoxinbelastung festgestellt. Haustierhaltung, Vollzeitbauernbetriebe (verglichen mit Teilzeitbauernbetrieben) und jüngere Kinder trugen zusätzlich zu einer höheren häuslichen Endotoxinbelastung bei. Die Endotoxinbelastung in Ställen war erhöht mit der Anzahl Rindvieh (nur bis zum obersten Quartil), der Tierfütterung mit Heu im Vergleich zu hauptsächlich Silage-Fütterung, und wenn zusätzlich Pferde, Schweine, Schafe oder Ziegen im Kuhstall untergebracht waren. Alle diese Faktoren könnten Indikatoren für eine traditionelle Landwirtschaft in hügeligen deutschsprachigen Gegenden aller drei Länder sein.

Die Muraminsäurebelastung der Matratze war bei Bauernkindern signifikant höher als bei Nichtbauernkindern. Die Muraminsäurebelastung und die Endotoxinbelastung in der Matratze waren teilweise korreliert. Das deutet darauf hin, dass beide Substanzen Indikatoren für die Exposition gegenüber Mikroorganismen sind. Unabhängig vom Bauern / Nichtbauern-Unterschied waren Häuser, die mit Holz oder Kohle geheizt wurden, und weniger häufiges Reinigen der Matratzen Faktoren, die eine höhere Muraminsäurebelastung zeigten.

Die erhöhte Muraminsäurebelastung der Matratze war – unabhängig von der Endotoxinbelastung – assoziiert mit einer geringeren Häufigkeit von Asthmasymptomen, nicht aber von

allergischer Sensibilisierung oder Heuschnupfen. Der protektive Effekt in Bezug auf die Asthmasymptome war bei nicht sensibilisierten Kindern deutlicher ausgeprägt. Die Unterschiede in den Effekten von Muraminsäure und Endotoxin deuten darauf hin, dass verschiedene Mikroorganismen zu der tieferen Asthma- und Allergieprävalenz bei Bauernkindern im Vergleich zu Nichtbauernkinder beitragen.

Der gegenwärtige Kontakt zu Hunden war invers assoziiert mit Heuschnupfen, Asthma und einer spezifischen Sensibilisierung gegenüber Graspollen und Katzenallergenen, nicht aber mit einem erhöhten IgG4-Niveau. Der frühkindliche und der momentane Kontakt ausschliesslich zu Katzen war mit einer geringeren Häufigkeit von Asthmasymptomen und der Sensibilisierung auf Graspollen verbunden. Diese Beziehungen wurden durch zusätzliche Berücksichtigung der häuslichen Endotoxin- oder Katzenallergenbelastung nicht beeinflusst. Zusätzliches Adjustieren für frühkindliche oder momentane Exposition gegenüber Nutztieren schwächte diese Schutzeffekte aber ab. Obwohl diese ländliche ALEX-Population häufig Kontakt zu Haustieren pflegte, wurde der in anderen Studien beobachtete protektive Effekt vermutlich durch den ebenfalls häufigen Kontakt zu einem bäuerlichen Umfeld überdeckt.

### Schlussfolgerungen und Ausblick

Endotoxin und Muraminsäure sind Indikatoren für ein vermutlich viel grösseres Spektrum von Mikroorganismen. Deshalb werden zukünftige Studien nicht lediglich das tiefere Risiko von frühkindlichem Kontakt zu Nutztieren oder höherer Exposition gegenüber Mikroorganismen bestätigen müssen, sondern auch Mischung der relevanten protektiven Komponenten im Bauernhofstaub finden. Weil die Nahrungsaufnahme ebenfalls ein plausibler Expositionsweg von Mikroorganismen darstellt, könnte sich die epidemiologische Forschung auch auf Unterschiede in der Ernährung und dessen Einfluss auf das kindliche Asthma und Allergien fokussieren. Eine Möglichkeit bietet die PARSIFAL Studie, weil sie Kinder einbezieht, die in verschiedenen Lebensstilen aufwachsen. Experimentelle Studien könnten sich auf den relevanten Expositionsweg (Inhalation, Ingestion) von verschiedenen Mikroorganismen konzentrieren.

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. Deshalb sollte die Kenntnis des protektiven Effekts einer mikrobiellen Umwelt für die Entwicklung von Asthma und Allergien gegen den Nutzen der etablierten Hygienestandards abgewogen werden.

# General introduction and background

## Epidemiology of childhood asthma and allergy

In 'westernised' countries, the prevalence of childhood asthma and allergic diseases have risen throughout the last three decades (1, 2). In these countries, asthma is the most common chronic disease in childhood and accounts for substantial morbidity and health-care costs. Several recent studies suggested that the increase in childhood asthma and allergy may have stabilised however, at least in some societies (3-5). Epidemiological studies showed striking regional differences in the prevalence of atopic diseases worldwide (6). The incidence and prevalence of allergic diseases varies with age; whereas the peak incidence of childhood wheeze and atopic dermatitis is seen in the first years of life (7, 8), the manifestation of hay fever reaches its peak around school age and adolescence (9). It is well documented that a family history of atopic diseases is a strong risk factor for the development of asthma, hay fever, and atopic dermatitis (10-12).

Even though much recent research has focused on the determinants of childhood asthma and allergy, and many explanations have been proposed, a consensus with regard to most important factors has not been reached. Because the increase in asthma and allergic disease is too short for a shift in the human genome, changes in lifestyle and environmental factors are considered a more plausible explanation. In peer-reviewed publications, two major hypotheses can be identified which relate environmental exposures to the prevalence of allergic diseases in children. First, the increase of childhood asthma and allergy prevalence might be due to an increase in exposure to air pollutants, environmental tobacco smoke, or indoor allergens. Second, successive cohorts have become more inclined to develop allergies because of the lack of a reduction in exposure to protective factors.

### Increase in environmental risk factors for asthma and atopy

Contradictory data exist, however a clear causal association between the environmental exposure to air pollutants like particulate matter, $NO_2$, $SO_2$, or ozone in Europe and the development of childhood asthma and allergy has not been found (13, 14). 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 (15-17).

Among other factors, a westernised lifestyle has lead to increased indoor allergen exposure because of better house insulation and reduced indoor air ventilation, more carpeting on floors, and common pet ownership. So far, the increase in the prevalence childhood asthma and allergy could not consistently be explained by increased indoor allergen exposure (16, 18, 19) or pet ownership (20). Also, several peer-reviewed studies have demonstrated a lower prevalence of childhood allergy prevalence children exposed to pets early in life (21-24).

### The 'hygiene hypothesis'

In 1989, Strachan et al. reported a lower risk of hay fever and atopic sensitisation in children with more siblings (25). 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 he 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 (26). Support for the hygiene hypothesis was also provided by Ball et al. in a longitudinal study where growing up with older siblings and also early attendance of a day care centre was protective against the development of asthma later in childhood (27).

### Markers of poor hygiene

Reduced prevalence of allergy and asthma has also been associated with the presence of gastrointestinal pathogens. Italian military cadets exposed to orofaecal pathogens, which may be regarded as a marker of poor hygiene, showed a reduced risk of atopy (28). In a large survey in the US, serological antibody evidence of previous hepatitis A, *Toxoplasma gondii* and herpes simplex virus 1 infections (all orofaecal pathogens) were associated with reduced prevalence of asthma, hay fever, and allergen sensitisation in adults (29). A Danish study (30) reported that seropositivity to markers of poor hygiene were associated with a lower prevalence of atopy in adults.

The idea of a protective effect from early childhood infections against allergic diseases is supported by recent findings in immunology.

## Recent findings in immunology mechanisms of allergy

The human immune system is a complex defence mechanism that protects the body by destroying invading micro-organisms. Immune responses depend on the interaction between two major components: one innate and not antigen specific, the other adaptive and antigen specific. Among others, innate immunity uses a family of recognition receptors (known as Toll-like receptors) to act as an interface with both the external and the internal milieu. Innate immune cells such as antigen presenting dendritic cells sense the signals provided by environmental pathogens, which alert the system to the presence of potentially dangerous infectious agents. In a recent editorial Vercelli suggested (31) that through the release of inflammatory cytokines dendritic cells then communicate with naive $CD4^+$ T cells, instructing the cells to move along differentiation programmes dictated by the nature of the microbial threat either to T helper type 1 or 2 phenotype or to T regulatory cells (32).

The functions of Treg, Th1 and Th2 cells and their interactions with effector T and B cells in adaptive immunity is of great scientific interest. A Th2 inflammatory cytokine profile (IL-4, IL-5, IL-9, IL-13) is found in IgE mediated asthmatic children, whereas a Th1 cytokine profile (IL-12, IFN-γ) seems to inhibit the IgE synthesis (33). Treg cells mainly seem to control the balance of Th1 and Th2 cells, if their cytokine profile (IL-10, TGF-β) suppresses Th1 and Th2 functions (34, 35). TLRs not only detect virulent pathogens, but also non viable parts of microbial compounds. They control the activation of adaptive immune responses by antigen presenting cells. This mechanism might be responsible for the development of asthma and allergy (36). The recent models of the hygiene hypothesis go beyond the Th1-Th2 paradigm, and focus on the interaction between the environment, innate immunity and Treg cells (37).

## Environmental exposure to micro-organisms

Interest has also focused on the role of *Mycobaterium tuberculosis*, which is known to suppress the development of characteristic Th2 immune responses for atopic disorders. However, the association between tuberculin responses and asthma, allergic diseases and atopic sensitisation are still controversial (38, 39). Ecological analyses for example have consistently found an inverse association between the prevalence of tuberculosis in a given country and the prevalence of atopic disorders (40, 41). However, no consistent effects have been seen with rubella and measles infection (42-45).

### Early observations in farmers' children

It has been documented since the 19th century, that persons who work with hay rarely suffer from hay fever (46). Dr. med. M. Gassner, a Swiss paediatrician from a rural area was one of the first people to systematically collect serological data from 15 year old school children in his village since 1983 (47). Dr. Gassner was closely involved in epidemiological date collection for the large Swiss SCARPOL study, and discussed his observations that allergic diseases was rarely observed in farmers' children in his village with the study group. In the SCARPOL population, of 1620 6-15 year-old school children in Switzerland, the odds ratio of having seasonal symptoms of hay fever and of developing atopic sensitisation were significantly lower in children who were raised on a farm as compared to non-farmers' children from the same rural areas (48). 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 and extended in a series of epidemiological studies from Europe, Canada, and Australia. In a survey of Bavarian children entering school at the age 5-7 years, contact with stable animals was inversely related to the prevalence of diagnosed hay fever, asthma and wheeze among farmers' children (49), furthermore a dose-response relation could be observed. An Austrian survey of 8-10 year old children living in a rural area showed that even children from non-farming families who had regular contact to farm animals, suffered less often from allergic diseases and asthma (50). However, in all three surveys the development of atopic eczema was not related to the farming activities of the parents. Ernst et al. (51) from Canada and Kilpeläinen et al. (52) from Finland reported similar findings from rural secondary school students. Another study from Australia supported these results by showing the same protective effect in a region with livestock farming, but not in a crop farming region (53).

### Micro-organisms on a farming environment

These observations in farm populations were seen as an extension of the hygiene hypothesis, since a farm environment can be seen as a enormous habitat of micro-organisms. Viable or non viable micro-organisms or parts of them are ubiquitous in outdoor and indoor

environments. A potential candidate, among other factors, to explain differences in the prevalence of childhood asthma and allergy is exposure to bacterial products such as lipopolysaccharides (LPS). Endotoxin consists of a family of LPS, forming an intrinsic part of the outer membrane of gram-negative bacteria. LPS and other bacterial wall components engage with antigen-presenting cells eliciting interleucine-1, IL-6, IL-8, TNF-α, and strong IL-12 responses. IL-12, in turn, is regarded as an obligatory signal for the maturation of naive T cells into Th1 cells and thus favours Th1 type responses (54). LPS is known to occur in high levels in organic dust from animal stables (55). Thus, occupational exposure to high levels of endotoxin have been implicated as a cause of obstructive lung diseases in farm workers as well as neutrophilic airway inflammation (56).

## Farming environments as a scientific model: the ALEX study

A 'human model' of an environment rich in opportunities for exposure against microorganisms is the farming environment. Based on previous findings, in 1999, research teams from Germany, Austria, and Switzerland designed a cross-sectional study to assess the role of environmental and lifestyle factors that affect the prevalence of allergy among 6-13 year old farmers' children and their peers from the same rural area. In a pre-study, the methodology and practicability of field and laboratory work was developed to measure endotoxin from house dust. Even in this small study of 84 children, indoor endotoxin levels were found to be higher in the homes of farmers' children and children with regular contact to livestock as compared with the homes of non-farm children without animal contact (57).

A first analysis of ALEX questionnaire data showed that the risk of ever having asthma, current wheeze, and atopic sensitisation was significantly reduced if the child had first been exposed to animal stables during the first year of life, compared to the first exposure to stables during school age or with no exposure (58). In addition, the consumption of farm milk in the first year of life was independently associated with a risk reduction for asthma and atopic sensitisation. These results suggest that the time window for exposure to farming environmental factors seems important for a development of protection against allergies. Since the whole immune system is maturing during the first years of life, very early factors in life might play a key role in its development.

In this thesis, further analyses of the ALEX data, including the measurements of microbial agents and allergens in the child's living environment are investigated.

## Objectives and goal of the thesis

The overall goal of the thesis was to assess the exposure to indoor microbial agents, allergens and pets in farmers' children and their peers in non-farming families, and to assess whether these exposures are associated to the prevalence of childhood asthma and allergies.

In particular, the following research questions were addressed:

1. *Is there an association between environmental exposure to indoor endotoxin and the prevalence of childhood asthma or allergic diseases, independent of the observed effect of early farming exposure?*

To answer this question, associations were estimated between children's current exposure to indoor endotoxin levels (from the child's mattress and from the living room floor) and to farming exposure during the first year of life, and diagnosed asthma, wheeze, hay fever, and atopic sensitisation. The results are presented in chapter II.

As it is important to understand the factors that determine indoor endotoxin exposure, home and lifestyle characteristics, and the stable endotoxin levels were been related to endotoxin levels indoors. The following research questions were given in chapter III.

2. *Which home and lifestyle characteristics of farming and non-farming families are associated with indoor endotoxin levels?*
3. *Are indoor endotoxin levels associated with endotoxin levels found in settled dust in stables?*
4. *Which farm and stable characteristics determine endotoxin levels in stables?*

Endotoxin can be considered to be a marker for the occurrence of gram-negative bacteria in the environment. However, the relevant 'protective' exposure might include a much broader spectrum of micro-organisms. Peptidoglycan is a major component of the cell wall of all species of the domain bacteria. The amount of peptidoglycan found in environmental samples reflects the presence of environmental – gram-negative and gram-positive – bacteria. In the ALEX study muramic acid, a specific part of peptidoglycan, was measured. There also is

some evidence that peptidoglycan influences the immune system by activating innate immunity via TLR-2, which induces a functionally different cellular response compared to the endotoxin receptor TLR-4 (59). Chapter IV addresses the question whether other exposure measures of the microbial environment, specifically muramic acid, is associated with the prevalence of childhood asthma and allergy, independent of the endotoxin exposure. Specifically, the following questions were addressed:

5. *Are farmers' children exposed to higher muramic acid levels than their rural peers from non-farming families?*
6. *Which home and lifestyle characteristics determine the indoor muramic acid levels?*
7. *Is there an association between indoor muramic acid levels and childhood prevalence of asthma or allergy, independent of the indoor endotoxin exposure?*

The role of pet (allergen) exposure in the development of asthma and allergy is controversial. An increasing number of peer-reviewed studies support the notion of a protective 'pet effect' (22, 24) whereas others have found an increased risk of atopic sensitisation with pet exposure (20). It has been suggested that the protective effect of pet keeping on asthma and allergy might be explained by the higher endotoxin levels found in homes where cats and dogs are kept (60, 61). In chapter V, the relation between contact with pets and asthma and allergy in rural children has been considered. Specifically, the following questions were addressed:

8. *Is there a relation between exposure to pets or to their allergens and childhood asthma, hay fever or atopic sensitisation in the ALEX population?*
9. *Can the 'pet effect' be explained by current endotoxin or pet allergen exposures, or does the early or current farming exposure influence these associations?*

## Chapter II: Environmental exposure to endotoxin and its relation to asthma in school-age children

This article has been published: Braun-Fahrlander C, Riedler J, Herz U, Eder W, **Waser M**, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E, for the Allergy and Endotoxin Study Team. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002; 347: 869-77.

## Chapter III: Determinants of endotoxin levels in living environments of farmers' children and their peers from rural areas

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## Chapter IV: Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health

This article has been published: van Strien R, Engel R, Holst O, Bufe A, Eder W, **Waser M**, Braun-Fahrlander C, Riedler J, Nowak D, von Mutius E, and the ALEX Study Team. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. J Allergy Clin Immunol 2004; 113: 860-7.

## Chapter V: Exposure to pets, and the association with hay fever, asthma, and atopic sensitisation in rural children

This article has been published: **Waser M**, von Mutius E, Riedler J, Nowak D, Maisch S, Carr D, Eder W, Schierl R, Schreuer M, Braun-Fahrlander C, and the ALEX Study Team. Exposure to pets, and the association with hay fever, asthma, and atopic sensitisation in rural children. Allergy, 2005; 60(2): 177-84.

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# ENVIRONMENTAL EXPOSURE TO ENDOTOXIN AND ITS RELATION TO ASTHMA IN SCHOOL-AGE CHILDREN

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## ABSTRACT

***Background*** In early life, the innate immune system can recognize both viable and nonviable parts of microorganisms. Immune activation may direct the immune response, thus conferring tolerance to allergens such as animal dander or tree and grass pollen.

***Methods*** Parents of children who were 6 to 13 years of age and were living in rural areas of Germany, Austria, or Switzerland where there were both farming and nonfarming households completed a standardized questionnaire on asthma and hay fever. Blood samples were obtained from the children and tested for atopic sensitization; peripheral-blood leukocytes were also harvested from the samples for testing. The levels of endotoxin in the bedding used by these children were examined in relation to clinical findings and to the cytokine-production profiles of peripheral-blood leukocytes that had been stimulated with lipopolysaccharide and staphylococcal enterotoxin B. Complete data were available for 812 children.

***Results*** Endotoxin levels in samples of dust from the child's mattress were inversely related to the occurrence of hay fever, atopic asthma, and atopic sensitization. Nonatopic wheeze was not significantly associated with the endotoxin level. Cytokine production by leukocytes (production of tumor necrosis factor $\alpha$, interferon-$\gamma$, interleukin-10, and interleukin-12) was inversely related to the endotoxin level in the bedding, indicating a marked down-regulation of immune responses in exposed children.

***Conclusions*** A subject's environmental exposure to endotoxin may have a crucial role in the development of tolerance to ubiquitous allergens found in natural environments. (N Engl J Med 2002;347:869-77.)



ASTHMA is the most common chronic disease in childhood and accounts for substantial morbidity and health care costs. Although various environmental factors have been thought to play key parts in the development of asthma and allergies,[1-3] the causes of these diseases remain unclear.

One intriguing hypothesis is that changes in the type and degree of stimulation from the microbial environment associated with improvements in public health and hygiene may increase the predisposition to chronic allergic conditions during childhood.[4] Exposure to microbes can occur in the absence of infection. For example, viable and nonviable parts of microorganisms are found in varying concentrations in many indoor and outdoor environments. These microbial substances are recognized by the innate immune system in the absence of overt infection, and they induce a potent inflammatory response.[5] Therefore, environmental exposure to microbial products may have a crucial role during the maturation of a child's immune response, causing the development of tolerance to other components of his or her natural environment, such as pollen and animal dander.

We investigated the relation between exposure to microbial products and the occurrence of childhood asthma and allergies in an environment rich in op-

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portunities for such exposure — that is, a rural environment where some families engage in farming. We measured endotoxin — a cell-wall component of gram-negative bacteria — in samples of dust from the mattresses of children and then related the levels of endotoxin to the prevalence of asthma and allergies and to serum levels of specific IgE. We also assessed the cytokine-production profile of peripheral-blood leukocytes after activation of the innate immune system by stimulation with lipopolysaccharide and staphylococcal enterotoxin B.

## METHODS

### Study Population

This cross-sectional survey was conducted in rural areas of Austria, Germany, and Switzerland, as previously described.[6] Participating parents (2618 of 3504 potential participants [74.7 percent]) were asked to consent to the measurement of specific IgE in their children's serum, the assessment of the cytokine-production profile of the children's peripheral-blood leukocytes after stimulation with lipopolysaccharide and staphylococcal enterotoxin B, and the collection of dust samples from the children's bedding. The final analysis was restricted to 812 children with complete data and similar ethnic origin (categorized as German, Austrian, or Swiss nationality), in order to avoid potential confounding by ethnic background.[7]

Approval to conduct the survey was obtained from the three local ethics committees for human studies and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children.

### Dust Sampling

We collected dust by vacuuming each mattress for two minutes per square meter of surface area. The material obtained was divided in two for measurement of endotoxin and allergen content. Dust was collected on special filters provided by the Allergologisk Laboratorium Kopenhagen.[8] All field workers were centrally trained and certified to ensure similarity of sampling.

### Measurements of Endotoxin Levels

One dust sample was stored at room temperature and shipped within one week after collection to the central laboratory (in Munich, Germany). Endotoxin content was measured by a kinetic limulus assay, as described by Hollander et al.[9] Endotoxin results were expressed as endotoxin units per milligram of dust and as endotoxin units per square meter of surface area of the sampled mattress. All endotoxin levels were within the limits of detection of the assay.

### Measurements of Allergen Levels in Dust Samples

The second dust sample was frozen at −20°C for at least two days and then shipped to one central laboratory (University Children's Hospital Charité, Berlin, Germany) and stored at 4°C until it was analyzed for *Dermatophagoides pteronyssinus* (Der p1), *D. farinae* (Der f1), and *Felis domesticus* (Fel d1), as previously described.[3] The lower limit of detection was 10 ng per gram of dust for Der p1 and Der f1 and 16 ng per gram of dust for Fel d1; results are expressed in nanograms of major allergen per gram of mattress dust. For allergen levels below the limit of detection (9.7 percent for Der p1, 5.5 percent for Der f1, and 0.2 percent for Fel d1), the mean value between zero and the limit of detection was used.

### Questionnaire and Interview

The prevalence of diseases and symptoms and potential explanatory and confounding factors were assessed by a questionnaire given to the parents that included the questions of the International Study of Asthma and Allergies in Childhood,[10] as described previously.[6] Farmers' children were defined as children whose parents answered "yes" to the question "Does your child live on a farm?" In an interview with the parents as part of the home visit, we obtained details of the timing of the child's exposure to stables and to farm milk. Exposure to farming during the first year of life was defined as exposure to stables during the first year of life, consumption of milk directly from the farm during the first year of life, or both.

### Testing for Specific IgE in Serum

The level of specific IgE against airborne allergens in all serum samples was measured by fluorescence enzyme immunoassay in a central laboratory (University Children's Hospital Charité, Berlin). Atopy was defined by at least one positive test for specific IgE indicating a titer of at least 3.5 kU per liter for one or more of the six airborne allergens (house dust mites, storage mites, grass pollen, birch pollen, cat dander, and cow epithelium).

### Assessment of Cytokine Production by Peripheral-Blood Leukocytes

Venous blood was drawn at school from all 812 children. Heparinized blood was diluted in a ratio of 1:8 in RPMI culture medium supplemented with 10 percent heat-inactivated fetal-calf serum to a final volume of 1 ml. Cells were stimulated either with 10 $\mu$g of lipopolysaccharide per milliliter for 24 hours or with staphylococcal enterotoxin B for 72 hours at 37°C, in an environment of 5 percent carbon dioxide in humidified air. Cell-free supernatants were stored at −80°C and shipped to the central laboratory for measurement of interferon-$\gamma$ (limit of detection, 16 pg per milliliter), tumor necrosis factor $\alpha$ (limit of detection, 16 pg per milliliter), interleukin-10 (limit of detection, 8 pg per milliliter), and interleukin-12 (limit of detection, 8 pg per milliliter) by commercially available enzyme-linked immunosorbent assays (OptEIA, Pharmingen). Each sample was tested in duplicate by the serial dilution of a standard supplied by the company with a known cytokine level. Differential blood counts were also performed, and cytokine production was expressed in picograms per 1 million peripheral-blood leukocytes. To ensure consistent performance in sampling and culture procedures, laboratory personnel in the study centers participated in a one-week training and certification program.

### Statistical Analysis

Endotoxin levels were $\log_{10}$-transformed. Multivariate logistic-regression analyses, in which the endotoxin level was treated as a continuous variable, were performed with SAS software,[11] with adjustment for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of older siblings (the basic model). In addition, potential confounding by farming status, exposure to farming during the first year of life, exposure to cats or dogs during the first year of life, and allergen levels (log-transformed values for Der f1, Der p1, and Fel d1) was evaluated. We included an interaction term to assess whether the effect of endotoxin on asthma and wheeze in children with atopic sensitization (a specific IgE level of at least 0.35 kU per liter) would be different from the effect in children without atopic sensitization.

To evaluate potential threshold values or other nonlinearity in the relation between exposure and response, S-Plus software was used to perform local nonparametric smoothing.[12] The logit of the rates of symptoms was expressed as a continuous function of endotoxin level, obtained by local nonparametric smoothing with control for the covariates mentioned above. The smoothing parameter for each model was determined on the basis of Akaike's information criterion.[12] In the same way, the association between endotoxin levels and cytokine response was assessed. Cytokine levels were log-transformed, and the association of these levels with the level of endo-

toxin exposure was expressed as the ratio of the covariate-adjusted geometric mean cytokine level in children in the highest quartile of endotoxin exposure to the mean level in children in the lowest quartile. The regression analyses were repeated with a restricted sample of children from nonfarming households with adjustment for known allergy-avoidance measures (removal of pets or carpets because of allergies in the family), exposure to cats or dogs during the first year of life, and exposure to farming during the first year of life.

## RESULTS

Complete data were available for 812 children, 319 from farming families and 493 from nonfarming families. The mean (±SD) age was 9.5±1.2 years. The adjusted odds ratios for asthma and hay-fever symptoms in relation to the farming status did not differ significantly between the group with complete data and the group with only the self-administered questionnaire (0.59 vs. 0.48 for asthma and 0.44 vs. 0.32 for hay-fever symptoms).[6] The relations between farming status and environmental-exposure variables and health outcomes are shown in Table 1.

The results of multivariate logistic-regression analyses estimating the effect of the mattress endotoxin level and the endotoxin load on the rates of symptoms and disease, with adjustment for known covariates, are shown in Table 2. The data are presented as adjusted odds ratios for symptoms or disease with an increase from the lowest quartile to the highest quartile of endotoxin exposure. Current endotoxin exposure showed a strong inverse association with hay fever, hay-fever symptoms, and atopic sensitization. Smoothed plots of the prevalence of hay fever, hay-fever symptoms, and atopic sensitization in relation to the level of endotoxin exposure, with control for covariates, showed a largely monotonic decrease in prevalence with an increasing endotoxin load (Fig. 1). Similar results were obtained in analyses in which the endotoxin level was used as the exposure variable (data not shown).

An inverse relation was also found between the level of endotoxin exposure and the capacity of peripheral-blood leukocytes to produce inflammatory and regulatory cytokines after stimulation with lipopolysaccharide (Fig. 2). The associations between endotoxin exposure (in endotoxin units per square meter) and the production of tumor necrosis factor $\alpha$, interferon-$\gamma$, interleukin-10, and interleukin-12, expressed

**TABLE 1.** ENVIRONMENTAL EXPOSURE AND PREVALENCE OF HEALTH OUTCOMES, ACCORDING TO FARMING STATUS.*

| VARIABLE | CHILDREN FROM FARMING HOUSEHOLDS (N=319) | CHILDREN FROM NONFARMING HOUSEHOLDS (N=493) | P VALUE |
|---|---|---|---|
| | geometric mean exposure (5th–95th percentile) | | |
| Environmental exposure | | | |
| Endotoxin level (units/mg of dust) | 37.8 (14.4–88.9) | 22.8 (8.2–62.9) | <0.001 |
| Endotoxin load (units/m² of mattress surface area) | 29,897 (5452–157,208) | 14,456 (2915–75,730) | <0.001 |
| Der f1 (ng/g of dust) | 528.7 (5–51,990) | 610.3 (5–54,160) | 0.54 |
| Der p1 (ng/g of dust) | 7,092.4 (133–104,110) | 1,417.1 (5–104,060) | <0.001 |
| Fel d1 (ng/g of dust) | 5,405.6 (356–144,600) | 5,744.1 (204–434,460) | 0.69 |
| | no. (% [95% CI]) | | |
| Health outcomes | | | |
| Hay fever | 13 (4.1 [1.9–6.2]) | 52 (10.5 [7.8–13.5]) | <0.001 |
| Sneezing and itchy eyes during previous yr | 19 (6.0 [3.3–8.7]) | 62 (12.6 [9.7–16.0]) | 0.002 |
| Atopic sensitization | 55 (17.2 [13.1–21.4]) | 116 (23.5 [19.8–27.3]) | 0.03 |
| Atopic asthma | 10 (3.1 [1.2–5.0]) | 29 (5.9 [3.8–8.0]) | 0.07 |
| Nonatopic asthma | 5 (1.6 [0.2–2.9]) | 13 (2.6 [1.2–5.0]) | 0.31 |
| Atopic wheeze | 15 (4.7 [2.4–7.0]) | 29 (5.9 [3.8–8.0]) | 0.47 |
| Nonatopic wheeze | 5 (1.6 [0.2–2.9]) | 30 (6.1 [4.0–8.2]) | 0.002 |

*Children were considered to have hay fever if their parents reported a physician's diagnosis of hay fever; to have had sneezing and itchy eyes (symptoms of hay fever) during the previous year if their parents gave a positive response to a question about these symptoms; to have atopic sensitization if they had a specific IgE titer of at least 3.5 kU per liter; to have atopic asthma if their parents reported a physician's diagnosis of asthma or if they had recurrent asthmatic obstruction of the airway or spastic bronchitis and a specific IgE titer of at least 0.35 kU per liter; to have nonatopic asthma if their parents reported a physician's diagnosis of asthma or if they had recurrent asthmatic obstruction of the airway or spastic bronchitis and a specific IgE titer of less than 0.35 kU per liter; to have atopic wheeze if their parents reported that they had had wheezing or whistling in the chest during the previous 12 months and they had a specific IgE titer of at least 0.35 kU per liter; and to have nonatopic wheeze if their parents reported that they had had wheezing or whistling in the chest during the previous 12 months and they had a specific IgE titer of less than 0.35 kU per liter. CI denotes confidence interval, Der f1 *Dermatophagoides farinae*, Der p1 *D. pteronyssinus*, and Fel d1 *Felis domesticus*.

**TABLE 2.** ASSOCIATIONS BETWEEN CURRENT ENDOTOXIN EXPOSURE (LEVEL AND LOAD) AND ASTHMA, WHEEZE, HAY FEVER, AND ATOPIC SENSITIZATION IN THE TOTAL SAMPLE AND IN THE SUBGROUP OF CHILDREN FROM NONFARMING HOUSEHOLDS.

| HEALTH OUTCOME | TOTAL SAMPLE (N=812) | | CHILDREN FROM NONFARMING HOUSEHOLDS (N=493) | |
|---|---|---|---|---|
| | ENDOTOXIN LEVEL | ENDOTOXIN LOAD | ENDOTOXIN LEVEL | ENDOTOXIN LOAD |
| | adjusted odds ratio (95% CI)* | | | |
| Hay fever | 0.58 (0.39–0.85)† | 0.53 (0.35–0.81)† | 0.79 (0.52–1.19) | 0.56 (0.33–0.95)† |
| Sneezing and itchy eyes during previous yr | 0.61 (0.43–0.86)† | 0.50 (0.34–0.72)† | 0.70 (0.47–1.05) | 0.46 (0.28–0.76)† |
| Atopic sensitization‡ | 0.78 (0.60–1.01) | 0.76 (0.58–0.98)† | 0.80 (0.59–1.08) | 0.73 (0.51–1.04) |
| Atopic asthma | 0.73 (0.44–1.19) | 0.48 (0.28–0.81)† | 0.68 (0.39–1.19) | 0.52 (0.25–1.07) |
| Nonatopic asthma | 1.25 (0.62–2.51) | 1.13 (0.57–2.26) | 1.29 (0.62–2.68) | 1.00 (0.46–2.21) |
| Atopic wheeze | 0.89 (0.57–1.39) | 0.62 (0.39–0.99)† | 0.79 (0.46–1.33) | 0.64 (0.33–1.25) |
| Nonatopic wheeze | 0.97 (0.58–1.61) | 1.14 (0.68–1.90) | 1.36 (0.86–2.14) | 1.82 (1.04–3.18)† |

*Odds ratios are for the occurrence of the given symptom or disease with an increase in the endotoxin measure from the lowest quartile to the highest quartile; analyses were adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, and number of older siblings. The analysis of the subgroup of children from nonfarming households was also adjusted for allergen-avoidance measures, exposure to pets during the first year of life, exposure to stables during the first year of life, and consumption of milk directly from a farm during the first year of life.

†$P \leq 0.05$ for the comparison between children in the lowest quartile of endotoxin exposure and children in the highest quartile.

‡Atopic sensitization was defined by a specific IgE titer of at least 3.5 kU per liter.

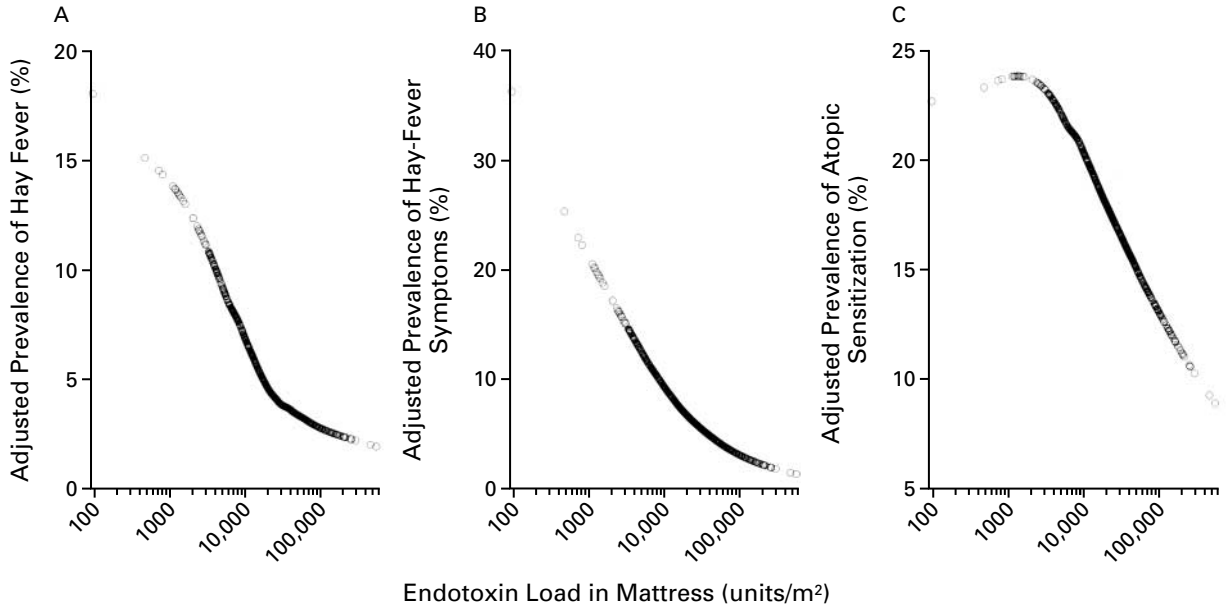


**Figure 1.** Smoothed Plots of the Prevalence of Hay Fever (Panel A), Hay-Fever Symptoms (Panel B), and Atopic Sensitization (Panel C) in Relation to the Log-Transformed Endotoxin-Load Values.

The analyses controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings. For each outcome, there was a monotonic decrease with increasing endotoxin load. A smoothing span of 0.9 was used for all three graphs.

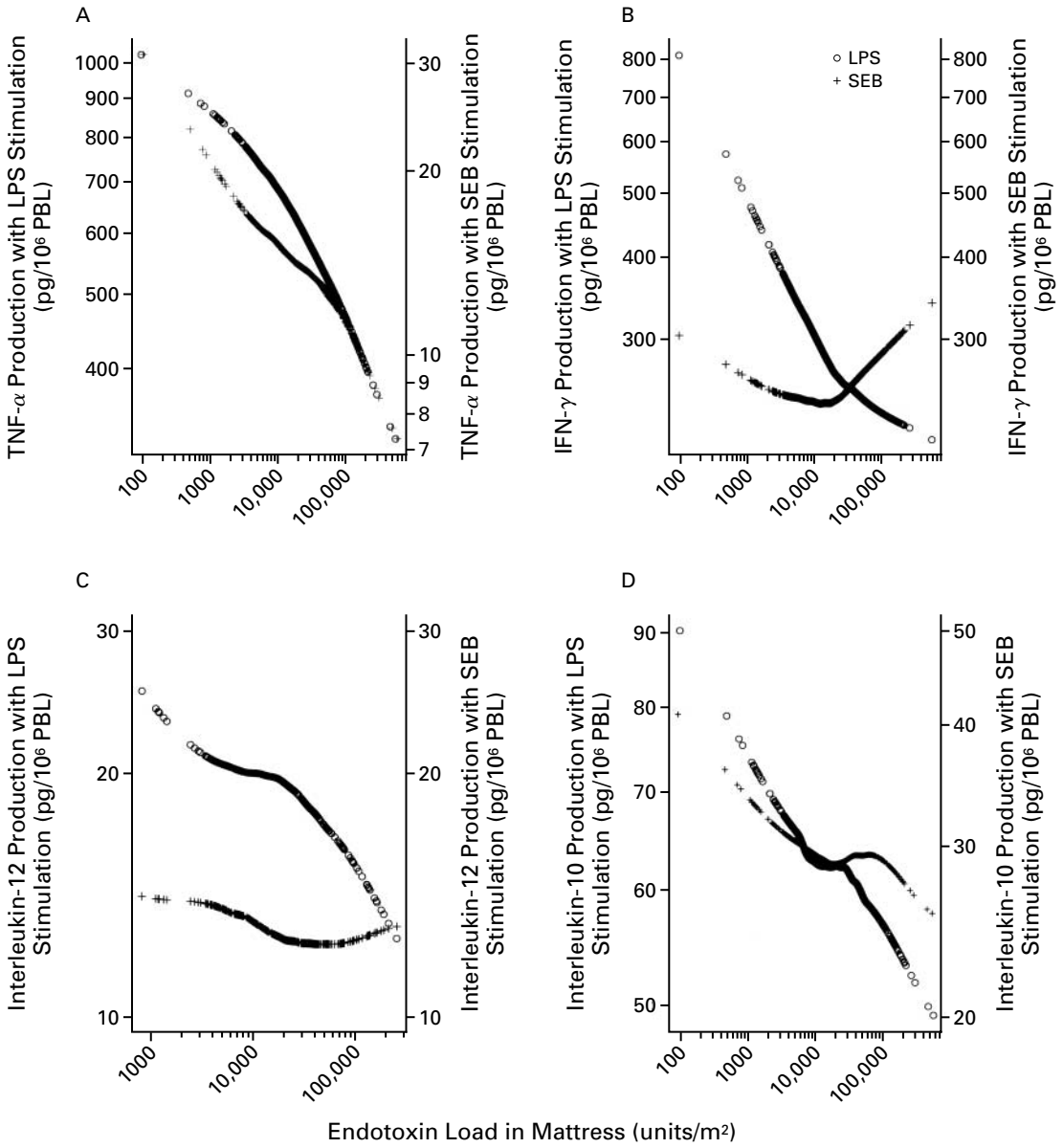


**Figure 2.** Smoothed Plots of the Log-Transformed Capacity of Peripheral-Blood Leukocytes (PBL) to Produce Tumor Necrosis Factor $\alpha$ (TNF-$\alpha$) (Panel A), Interferon-$\gamma$ (IFN-$\gamma$) (Panel B), Interleukin-12 (Panel C), and Interleukin-10 (Panel D) after Stimulation with Lipopolysaccharide (LPS) or Staphylococcal Enterotoxin B (SEB) in Relation to the Log-Transformed Endotoxin-Load Values.

Analyses were controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings; the analysis shows an inverse relation between the level of endotoxin exposure and cytokine response, except in the case of the production of IFN-$\gamma$ after SEB stimulation. A smoothing span of 0.9 was used for all four graphs.

as ratios of the mean level of cytokine production for children in the highest quartile of endotoxin exposure to the mean level for children in the lowest quartile, were 0.81 (95 percent confidence interval, 0.74 to 0.89), 0.80 (95 percent confidence interval, 0.70 to 0.92), 0.93 (95 percent confidence interval, 0.81 to 1.07), and 0.87 (95 percent confidence interval, 0.77 to 0.98), respectively. The corresponding results after stimulation with staphylococcal enterotoxin B were 0.83 (95 percent confidence interval, 0.74 to 0.93), 1.05 (95 percent confidence interval, 0.95 to 1.17), 0.97 (95 percent confidence interval, 0.84 to 1.11), and 0.96 (95 percent confidence interval, 0.86 to 1.06), respectively.

The association between endotoxin exposure and wheeze during the past year showed a different exposure–response pattern. There was a strong negative association for atopic wheeze and asthma, whereas for nonatopic wheeze and asthma, there was a nonsignificant trend toward increasing prevalence with increases in the current level of endotoxin exposure (Table 2 and Fig. 3). However, the term for the interaction between the level of endotoxin exposure and atopic status did not reach statistical significance. Exposure to farming in the first year of life showed a strong inverse association with all health outcomes, including nonatopic wheeze and asthma, independently of the current level of endotoxin exposure (Table 3). Additional adjustment for other potential confounders, including the levels of allergens (Der f1, Der p1, and Fel d1) in mattress dust, farming status, exposure to pets during the first year of life, and exposure to farming during the first year of life, did not change the results. To evaluate whether the results might be generalized to a nonfarming population and to adjust for potential uncontrolled confounding associated with a farming lifestyle, we restricted the sample to children from nonfarming households and also adjusted for exposure to stables and consumption of milk directly from the farm during the first year of life. Again, strong negative associations — albeit not all statistically significant (probably because of the sample size) — between the level of endotoxin exposure and atopic outcomes were observed, whereas positive associations were found for nonatopic wheeze (Table 2).

## DISCUSSION

These findings suggest that environmental exposure to microbial products, as measured by the endotoxin levels in mattress dust, is associated with a significant decrease in the risk of hay fever, atopic sensitization, atopic asthma, and atopic wheeze in child-

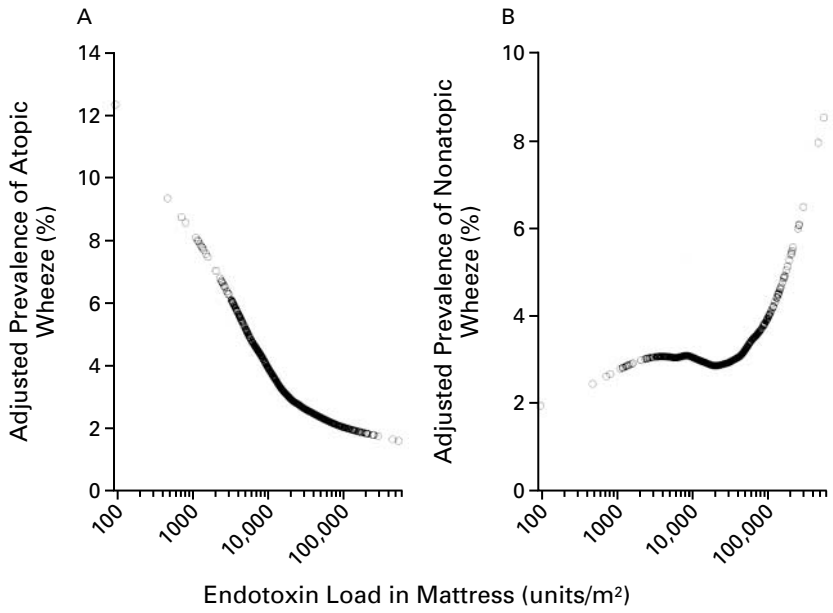


**Figure 3.** Smoothed Plots of the Prevalence of Atopic Wheeze (Panel A) and Nonatopic Wheeze (Panel B) in Relation to the Log-Transformed Endotoxin-Load Values.

The analyses were controlled for age, sex, study area, family history of asthma and hay fever, educational level of the parents, and number of siblings. There was a negative association for atopic wheeze, whereas for nonatopic wheeze, there was a nonsignificant positive trend with increasing levels of current endotoxin exposure. For Panel A, a smoothing span of 0.9 was used; for Panel B, a span of 0.5 was used.

**TABLE 3.** ASSOCIATION OF THE PREVALENCE OF SYMPTOMS AND DISEASE WITH THE CURRENT ENDOTOXIN LOAD AND EXPOSURE TO FARMING DURING THE FIRST YEAR OF LIFE.*

| HEALTH OUTCOME | EXPOSURE TO FARMING DURING THE FIRST YEAR† | CURRENT ENDOTOXIN EXPOSURE‡ |
|---|---|---|
| | odds ratio (95% CI) | |
| Hay fever | 0.26 (0.13–0.52)§ | 0.61 (0.40–0.95)§ |
| Sneezing and itchy eyes after 1 yr of age | 0.55 (0.31–0.97)§ | 0.53 (0.36–0.77)§ |
| Atopic sensitization¶ | 0.45 (0.30–0.68)§ | 0.83 (0.63–1.09) |
| Atopic asthma | 0.42 (0.18–0.96)§ | 0.52 (0.30–0.90)§ |
| Nonatopic asthma | 0.48 (0.16–1.41) | 1.22 (0.60–2.46) |
| Atopic wheeze | 0.59 (0.28–1.23) | 0.66 (0.41–1.07) |
| Nonatopic wheeze | 0.43 (0.19–0.97)§ | 1.23 (0.73–2.06) |

*Odds ratios are for the occurrence of the given symptom or disease with an increase in exposure to farming or the endotoxin load from the lowest quartile to the highest quartile. Analyses were adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, and number of older siblings.

†Odds ratios were also adjusted for current endotoxin exposure.

‡Odds ratios were also adjusted for exposure to farming during the first year of life.

§$P \leq 0.05$ for the comparison between children in the lowest quartile of the exposure variable and those in the highest quartile.

¶Atopic sensitization was defined by a specific IgE titer of at least 3.5 kU per liter.

hood. This protective effect was observed in children from farming and nonfarming households, indicating that even the lower levels of exposure that occur in nonfarming environments may favorably influence the risk of atopic diseases in childhood.

The mechanisms by which endotoxin exposure may protect against the development of atopic immune responses and diseases are not fully understood. Our findings suggest that by the time a child reaches school age, high levels of environmental exposure to endotoxin have resulted in a marked suppression of the capacity for cytokine production in response to activation of the innate immune system. Whereas lipopolysaccharide stimulation triggers an innate immune response by activating mainly antigen-presenting cells, staphylococcal enterotoxin B also activates T cells, resulting in a somewhat different pattern of cytokine production. Reduced responsiveness to stimulation with lipopolysaccharide after previous stimulation with lipopolysaccharide is a phenomenon referred to in the literature as lipopolysaccharide tolerance.[13,14] Our results suggest that such a down-regulation occurs in vivo as a consequence of long-term exposure to environmental endotoxin. Whether this down-regulation is merely a biologic marker of the exposure or is causally related to the decreased rate of atopy cannot be determined on the basis of our data; it is an area in which further exploration is needed. It has been suggested that the innate immune response has an instructive role in adaptive immunity.[15] Differential expression of lipopolysaccharide receptors in children from farming and nonfarming households has recently been reported,[16] suggesting that the innate immune system responds to the high microbial burden of the farming environment.

Although only current endotoxin exposure was measured, the levels are likely to reflect long-term exposure. Therefore, long-term, high-level environmental exposure may favor a state of tolerance,[14] which may prevent the development of allergic immune responses. We demonstrated that exposure during the first year of life to stables and other aspects of farm life that are likely to reflect exposure to microbial products has a strong protective effect against the occurrence of asthma and atopy at school age. However, independent of and in addition to this effect, endotoxin exposure at school age was associated with a markedly decreased risk of atopic outcomes. This protective effect was also seen in children with no exposure to farming whose mattress endotoxin levels were similar to levels found in urban homes in the Netherlands[17] and urban areas in the United States,[18,19] suggesting that exposure to ubiquitous microbial products strongly affects the development of atopy and childhood asthma. The increase in the frequency of asthma in inner-city areas of the United States, by contrast, may be related to other types of environmental exposure.

The protective effect of endotoxin exposure at school age was observed only for atopic wheeze and asthma, not for nonatopic wheeze. Childhood asthma is a complex syndrome with multiple illnesses involving wheezing that develop during the infant, toddler, school-age, and adolescent years, as has been shown in several long-term, prospective surveys in which children were followed from birth to adolescence and adulthood.[20-22] Although, in many cases, asthma is associated with atopic sensitization to a variety of allergens, illnesses involving wheezing also occur in the absence of increased IgE responses. Variations in genetic background, environmental factors, and the interplay among them are likely to account for the varying clinical presentations of wheeze. In studies of human exposure[23] and in studies of animals,[24] endotoxin has been shown to induce airway hyperresponsiveness in healthy, nonatopic subjects but to decrease airway responsiveness in sensitized animals, supporting the notion that the effect is modified by atopy, as we observed. In our study, exposure to farming in the first year of life had a protective effect against nonatopic wheeze, whereas exposure to endotoxin at school age was related to an increased risk. Therefore, not only an exposed subject's atopic status but also the timing of the exposure determines its beneficial or detrimental effects.

Endotoxin was measured in mattress dust, since children come into close contact with the microbial environment of their beds while sleeping and since the reproducibility of repeated endotoxin measurements is greater for dust from the bed than for dust from the floor.[25] Endotoxin measurements in dust from the bed have been reported to show little variation over time, with nonsignificant differences over a six-month period.[19] Environmental endotoxin levels are therefore likely to reflect longer-term exposure to microbial compounds. However, the cross-sectional design of our study limited our ability to determine precisely the duration of exposure represented by current endotoxin measurements, and prospective studies are clearly needed. We did not assess other bacterial components, such as nonmethylated cytidine phosphate guanosine dinucleotides specific for prokaryotic DNA (CpG motifs) or cell-wall components from atypical mycobacteria or gram-positive bacteria such as lipoteichoic acid, which are known to affect immune responses in ways similar to that of endotoxin.[26,27] The observed protective effect associated with endotoxin levels in mattress dust is therefore likely to reflect the effect of exposure to a much broader spectrum of microbial compounds than gram-negative bacteria alone.

The results of our study indicate that environmental exposure to microbial products as assessed by the measurement of endotoxin levels in mattress dust is associated with the development of tolerance toward ubiquitous allergens found in natural environments. Mechanisms relating to the recognition of these microbial compounds by the innate immune system and the regulation of the resulting inflammatory responses through adaptive immunity are likely to be of key importance for the development of atopic illnesses such as hay fever and childhood asthma and wheeze. These insights may foster the generation of novel strategies aimed at the prevention of these diseases.

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# Determinants of endotoxin levels in living environments of farmers' children and their peers from rural areas

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## Summary

*Background* Lower frequencies of asthma and hayfever have been observed in children with contact to livestock. At school age, the amount of endotoxin measured in the dust of children's mattresses is inversely related to the occurrence of atopic asthma, hayfever and atopic sensitization both in children from farming and non-farming households.

*Objective* The aim of the present study was to investigate which home and lifestyle characteristics of farm and non-farm families contribute to endotoxin levels measured in different indoor home environments.

*Methods* In the framework of the Allergy and Endotoxin (ALEX) Study, endotoxin was measured in dust samples from the living room floor and the child's mattress of 319 farmers' families and 493 non-farming families, and in settled dust from stables. Endotoxin content of all dust samples was determined by a kinetic Limulus assay (*Limulus-Amebocyte*-Lysate test). Information about the child's activities on farms, home characteristics and cleaning behaviours was obtained from parental questionnaires.

*Results* Endotoxin levels in stables did not predict the amount of endotoxin measured in floors or mattresses. However, a dose-dependent association between the child's activity on the farm and indoor home endotoxin levels was observed, both in farm and non-farm children. In non-farm children pet keeping and the frequency of floor cleaning were additionally associated with endotoxin levels, whereas in farm children parental farm activities, study area, time since last cleaning, the mattress type as well as younger age of the children contributed to increased microbial exposure.

*Conclusion* These results demonstrate that regular contact to farm animals increases indoor home endotoxin concentrations, both in farm and non-farm children, and might thus explain the protective effect of contact to livestock on atopic outcomes. To assess children's individual exposure to a microbial environment, measures of mattress dust exposure are needed as stable endotoxin concentrations were not associated with indoor home levels.

**Keywords** allergy, children, exposure assessment, farm animals and pets, farming, house and stable dust, indoor endotoxin, living room floor, mattress

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## Introduction

Lower frequencies of asthma, hayfever and atopic sensitization have been observed in children growing up on farms in Europe, Canada and Australia [1–7]. In comparison to children from non-farming families, farmers' children are exposed to higher concentrations of endotoxin both in stables and in dust from kitchen floors and mattresses [8]. We have recently shown that the timing of exposure to a farming environment is crucial for its protective effect on the development of asthma and allergy [9]. In addition, we could demonstrate that at school age the amount of endotoxin measured in the dust of children's mattresses is inversely related to the occurrence of atopic asthma, hayfever and atopic sensitization [10] both in children from farming and non-farming households. Thus, in addition to farm abode, other factors must be important in determining the levels of endotoxin in rural farm and non-farm households. However, little is known about these factors. Endotoxin is a stable glycolipid component of the outer membrane of Gram-negative bacteria that can be collected and assayed in settled dust from mattresses or floors [11–13]. From occupational studies, it is known that high levels of endotoxin can be measured in swine confinement and poultry houses [14–16], but it has not yet been investigated whether endotoxin levels

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measured in stables determine the indoor home environment of farmers' children. In urban environments, elevated levels of endotoxin have been reported from homes where cats and dogs were kept [17–20]. In US metropolitan homes, regular use of central air conditioning decreased the levels of indoor endotoxin concentrations, but other home characteristics for living conditions such as home dampness, number of inhabitants, cleaning frequency or presence of smokers did not influence endotoxin levels in house dust [18].

The aim of the present study was to investigate which home and lifestyle characteristics of farm and non-farm families might determine the endotoxin levels measured in the dust of the children's mattresses and in the living room floor dust, and to determine whether the same factors were relevant for both groups. For the farmers' subgroup, we evaluated whether the endotoxin levels measured in stables were directly associated with the respective levels in the dust of children's mattresses or the living room. In addition, we investigated which farm and stable characteristics determine the endotoxin levels in the stable. The analyses are based on the questionnaire data and endotoxin measurements of 812 farm and non-farm families which are part of the collaborative cross-sectional study *Al*lergy and *E*ndoto*x*in (ALEX) [9, 10].

## Materials and methods

### *Study population*

The ALEX Study was carried out in rural areas of Austria, Germany and Switzerland as previously described [9]. With respect to population density and farming characteristics, these regions were shown to be comparable concerning usually small, traditional farms, mainly run by members of the family and only occasionally by farmworkers. Ninety-eight percent of the farms have farm animals. The predominant farming type in these areas is traditional dairy farming with a mean number of 35 cows and calves per farm, and only small numbers of pigs, chicken, horses, sheep and goats.

Participating parents (2618 of 3504 potential participants; 74.7%) were asked to consent to blood sampling in their children and to collection of dust samples in different home environments. For these additional investigations, 1406 (53.7%) families accepted. Of these, all the children from farming families, all non-farming children with regular contact to a farming environment through neighbours and a random sample of children not being raised on a farm and not exposed to a farm environment were invited to participate ($n = 901$). The final analysis was restricted to 812 children with complete data and to children with German (471 of 511; 92.2%), Austrian (221 of 253; 87.4%) and Swiss nationality (120 of 137; 87.6%), to avoid potential confounding by ethnicity. They consisted of 319 farmers' children and 493 children from non-farming families. No relevant participation bias was found [9].

### *Questionnaire*

Demographic factors and home characteristics such as age, sex, number of siblings, house location, heating system, parents education, pets and indoor smoking, as well as information about the child's activities on farms (contact to stable animals, stay in stables and barns, helping with harvesting) and stable characteristics (type and amount of farm animals, feeding material, manure system) were assessed by parental questionnaire. 'Farmers' children' were defined as children whose parents answered 'yes' to the question 'Does your child live on a farm?'. For farm children, the time they assisted their parents with stable work was assessed. For non-farm children, a 'stable activity score' was defined combining regular contact to stable animals (No = 0/Yes = 1) and the frequency of staying in the stable (never = 0/rarely or several times per week = 1/at least daily = 2). The score ranges from 0 (no contact to stable animals and never in a stable) to 3. In addition, information on the date of dust sampling, the age and type (foam rubber, inner spring, or latex) of the mattress, the number of mattress covers, time since last cleaning the mattress, the type of floor, days since last cleaning the living room floor, and the presence of animals in stables during dust sampling were recorded in a sampling protocol during the home visit for dust collection.

### *Dust collection*

The dust collection was performed according to a standardized protocol. Seven fieldworkers sampled in three rural areas: three in Germany, two in Austria and two in Switzerland. Dust samples were taken using new vacuum cleaners of 1200 W (Miele Bodenstaubsauger S624, Miele AG Spreitenbach, Switzerland), calibrated with a flow of 200 L/min with a clean filter, operating power of about 800 W. ALK filters (ALK Allergologisk Laboratorium, Copenhagen, Denmark) were used for dust collection according to the International Study of Asthma and Allergic Disease in Childhood (ISAAC) phase II manual (ISAAC, 1998). To ensure comparability of the method of dust collection at the different study sites, all fieldworkers were centrally trained. Each fieldworker visited approximately an equal number of farmers' and non-farm families, and all samples were collected between November 1999 and February 2000.

The child's mattress was chosen by asking the parents 'Which mattress did your child sleep on most in the last four weeks?'. After removing all sheets apart from mite protecting covers or plastic sheets, the mattress was vacuumed for 2 min/$m^2$. In the living room, an area of $2m^2$ carpeted floor was chosen if there was any kind of carpet in this room. Otherwise, the fieldworker chose an area of $4\,m^2$ of smooth floor (without displacing the furniture). The living room floor was vacuumed for 4 min. The pre-weighed ALK-filter holders ($\pm$ 0.01 g) were re-weighed after dust collection with only particles bigger than 2 mm being removed by tweezers. For the stable dust sample, the stable was chosen, in which the child spent most time. Settled dust from dry surfaces in stables at a height between 0.5 and 1.5 m above ground level was collected with a spatula. Fine dust was separated from hay and straw with a household sieve.

All samples were stored at room temperature and transferred within 1 week after collection to the laboratory of the Institute for Occupational and Environmental Medicine, University of Munich, for endotoxin analyses.

### Measurements of endotoxin levels

Endotoxin content of all dust samples was determined by a kinetic Limulus assay (kinetic-QCL, BioWhittaker Inc., Walkersville, MD, USA). For extraction, 100 mg from each sample (for stable dusts two replicates) was extracted by rapid shaking with 7-mL endotoxin-free water for 1.5 h. Thereafter, the suspension was diluted 1 : 100 for samples from mattresses and floors, and 1 : 1000 for samples from stables. Directly after dilution, an aliquot of 100 μL was added to a microtitre plate (96 well, Falcon) and assayed with *Limulus-Amebocyte*-Lysate (LAL). To get information about possible enhancement or inhibition reactions of the LAL assay, replicate of each sample was spiked with an endotoxin standard. A standard calibration curve (0.05–0.5–5–50 EU/mL), a laboratory blank and an internal laboratory standard were performed on each plate. As recommended by the manufacturer, optical density at 405 nm was measured by an automatic reader (PowerWave$^{TM}$, MWG Biotech Inc., Mendelhall Oaks Parkway, NC, USA). If spike recovery was below 45%, the suspension was diluted higher and the analysis was repeated. The intra-assay variability (EU/mg dust) was less than 10%, whereas the inter-assay variability was lower than 20%. Resulting endotoxin levels were expressed as EU/mg dust (endotoxin concentration) and relative to the size of the sampling area and weight of the sampled dust, as EU/m$^2$ (endotoxin load). In case of dusts from stables, the mean of the two replicates was calculated. All measured endotoxin levels were above the limit of detection.

Repeated dust sampling from the same locations at 31 stables after at least 3 days yielded a Pearson correlation coefficient of 0.73, $P<0.001$ for log endotoxin concentrations. The corresponding mean coefficient of variation was 15.2%. To assess the variation in indoor home endotoxin concentrations over time, repeated dust sampling was obtained 2–4 months after initial sampling from 12 living room floors and seven mattresses. The Pearson correlation coefficients for log endotoxin concentrations in the living room floor dust were 0.66, *P*: 0.018, and 0.78, *P*: 0.040 for the mattress dust. The corresponding coefficients of variation were 20.1% and 12.9%, respectively.

### Statistical analyses

Endotoxin levels were best described by a lognormal distribution and therefore log transformed. Geometric means, geometric standard deviation, as well as *t*-tests on log scale data were calculated to describe differences in the endotoxin concentration (EU/mg dust), of mattress and living room floor dust of farm and non-farm families. For the subgroup of farm children, Pearson correlation coefficients were calculated to evaluate whether the log endotoxin concentrations measured in settled dust of stables were correlated with the respective levels in the living room floor and mattress dust. Likewise, for farm and non-farm families the correlation between log endotoxin levels in mattress dust and living room floor dust was determined.

Univariate associations between stable dust endotoxin concentrations ln(EU/mg) and farm or stable characteristics such as the number and type of farm animals kept in the respective stable, were calculated. All variables with a univariate association ($P\leqslant0.25$) were subsequently tested in multivariate regression models.

Univariate associations between endotoxin concentrations ln(EU/mg) in living room and mattress dust samples and potential predictive factors of indoor endotoxin levels were calculated separately for the subgroups of farmers' and non-farm children. We included factors related to the farm environment, and other factors that have been shown to influence the indoor endotoxin levels, such as pets [17–19, 21], house location, heating system, smoking [18] and the frequency of vacuum cleaning [21]. Available information on mattress types and floor covering [21] were also tested. To control for potential confounding all multivariate models were additionally adjusted for sex, age, amount of sampled dust, sampling area, fieldworker and the date of dust sampling. The association of a given predictor variable with endotoxin was expressed as the ratio of covariate-adjusted geometric means relating to a unit increase of the predictor variable (means ratio). All analyses were carried out using Intercooled Stata 8.0 (Stata Corporation, College Station, TX, USA).

## Results

The study population consisted of 319 farmers' children with a mean age of 9.42 years (SD 1.63), and 493 children from non-farming families, mean age 9.49 (SD 1.60). The characteristics of the study population and the geometric mean endotoxin concentrations in the measured compartments for farmers' and non-farm children are given in Table 1. No statistically significant correlation existed between stable endotoxin concentrations and the respective living room floor or mattress dust concentrations (Fig. 1a). However, there was a clear positive correlation between the endotoxin levels of living room floor dust and the respective mattress dust levels in both subgroups of children, and for both the endotoxin concentration (Fig. 1b) and the endotoxin load (Fig. 1c).

Crude and adjusted means ratios of the variables associated with endotoxin levels in settled stable dust are presented in Table 2. Full- and part-time farmers did not differ with respect to endotoxin levels in the stables, but increasing number of cattle kept in the stable increased the endotoxin level. However, there was no clear dose–response relationship. Stable animals had mostly been inside the stable for at least 1 month before dust sampling (93.1%). Sixty-five (23.3%) of the farms kept horses in addition to cattle. Keeping horses was independently associated with increased endotoxin levels. Feeding the cattle with hay was associated with increased endotoxin levels, compared to feeding with silage and hay which is a less traditional form of feeding. Using both solid and liquid muck in combination increased stable endotoxin levels. Stables in the Austrian and Swiss study areas yielded endotoxin levels higher than those in Germany. Other variables such as the number of people running the farm, the presence of additional pigs, goats or sheep in the stable did not independently increase the stable endotoxin levels. The adjusted model explained 21.0% of the variation in settled stable dust endotoxin concentration.

**Table 1.** Study population characteristics, stratified by farmers' children and non-farm children

| | | Farmers' children $N = 319$, *n* (%) | Non-farm children $N = 493$, *n* (%) | *P*-value* |
|---|---|---|---|---|
| Regular contact to stable animals | Any | 299 (93.7) | 188 (38.1) | < 0.001 |
| Regular stay in stable | Up to several times weekly | 153 (48.0) | 306 (62.1) | < 0.001 |
| | At least daily | 153 (48.0) | 30 (6.1) | |
| Regular stay in barn | Any | 301 (94.4) | 292 (59.2) | < 0.001 |
| Helping with harvesting | Any | 300 (94.3) | 199 (40.4) | < 0.001 |
| Pets regularly indoors | Only cats | 77 (24.1) | 153 (31.0) | 0.082 |
| | Cats and dogs | 36 (11.3) | 44 (8.9) | |
| Pets regularly on child's bed | Only cats | 52 (16.3) | 127 (25.8) | 0.003 |
| | Cats and dogs | 4 (1.3) | 11 (2.2) | |
| Sex | Girls | 147 (46.1) | 247 (50.1) | 0.263 |
| Number of older siblings, Ref: 0 | 1–2 | 160 (50.1) | 262 (53.1) | < 0.001 |
| | >2 | 56 (17.6) | 18 (3.7) | |
| Fathers education, Ref: None, Low | Medium | 132 (41.4) | 196 (39.8) | < 0.001 |
| | High | 40 (12.5) | 114 (23.1) | |
| Indoor smoking | Any | 46 (14.4) | 116 (23.5) | 0.001 |
| House location, Ref: Inside village | Outside village | 188 (58.8) | 45 (9.1) | < 0.001 |
| Heating system, Ref: Oil, gas or electricity | Wood, coal | 196 (61.4) | 82 (16.6) | < 0.001 |
| Age of the mattress (mean year ± SD) | | 8.7 ± 8.0 | 7.1 ± 6.8 | 0.002 |
| Type of mattress, Ref: Foam rubber | Inner spring | 159 (49.8) | 277 (56.2) | 0.071 |
| | Latex | 40 (12.5) | 77 (15.6) | |
| Type of floor, Ref: Smooth floor | Carpets | 277 (86.8) | 451 (91.5) | 0.034 |
| Days since last cleaning the living room floor (mean ± SD) | | 4.0 ± 3.3 | 4.2 ± 3.0 | 0.553 |
| Stable dust endotoxin concentration† | | 258 (1.86) | | |
| Living room floor dust endotoxin concentration† | | 81.8 (1.81) | 44.9 (2.00) | <0.001 |
| Mattress dust endotoxin concentration† | | 37.8 (1.77) | 22.8 (1.90) | <0.001 |

Reference if no other statement: Never.
**P*-value for farmers' children vs. non-farm children. †Geometric mean (EU/mg dust) (geometric standard deviation).

Table 3 shows the crude and adjusted means ratios of the variables associated with endotoxin levels in living room floor dust, both for farming and non-farming households. In farming households, the amount of farming activities of the parents (full-time vs. part-time) and the frequency of the child's assistance in the stable were positively associated with endotoxin concentrations, whereas the stable endotoxin concentration *per se* was not related to living room endotoxin levels. For non-farm children, contact to the farm environment was expressed as a stable activity score. A higher stable activity score was positively associated with endotoxin concentrations. Adjustment for all other variables somewhat reduced these effects.

Cats indoors, especially in combination with dogs, increased the endotoxin levels in floor dust in both farming and non-farming households. Father's education was inversely associated with endotoxin concentrations in farming households only. Austrian farm households showed the highest house dust endotoxin levels. Finally, although indoor smoking has been reported to contain biologically active endotoxin [22], we did not find an independent effect on endotoxin levels in the living room floor dust, nor could we detect an association with any of the other variables additionally included in the multivariate regression models such as the number of people running the farm, number of older siblings, the heating system, or the type of floor (smooth or carpeted). The adjusted models explained 34.0% and 20.1% of the variation in living room dust endotoxin concentration in farming and non-farming households, respectively.

Table 4 presents the crude and adjusted means ratios of the variables associated with endotoxin levels in mattress dust, stratified by farm and non-farm children. In non-farm children, the stable activity score increased mattress endotoxin concentrations in a dose-dependent way. Other farming activities such as helping with harvesting, or staying in barns were highly correlated with stable activities, and did not have an independent effect on mattress dust endotoxin levels. Allowing pets to stay on the child's bed also increased the measured endotoxin levels significantly.

In farm children, two or less people running the farm (mostly the parents only) was associated with increased endotoxin levels in the child's mattress, as was the amount of the child's own activity in the stable when assisting the parents. Again, no association was observed with stable endotoxin levels. Few farm children allowed their pets to stay on the bed. Cats and dogs on the bed increased endotoxin levels although not statistically significantly. Mattress endotoxin levels decreased with increasing age of the farm children, and were non-significant in non-farm children. Living on a farm in Austria or Switzerland was associated with increased endotoxin levels compared to the German study area. Inner spring mattresses in farming households were associated with higher endotoxin levels. Time since last cleaning only affected endotoxin levels in mattresses of farm children. The other potential predictors of mattress endotoxin concentration included in the regression models did not show independent additional effects, neither in the farm, nor in the non-farm subgroup. The adjusted models explained 31.7%

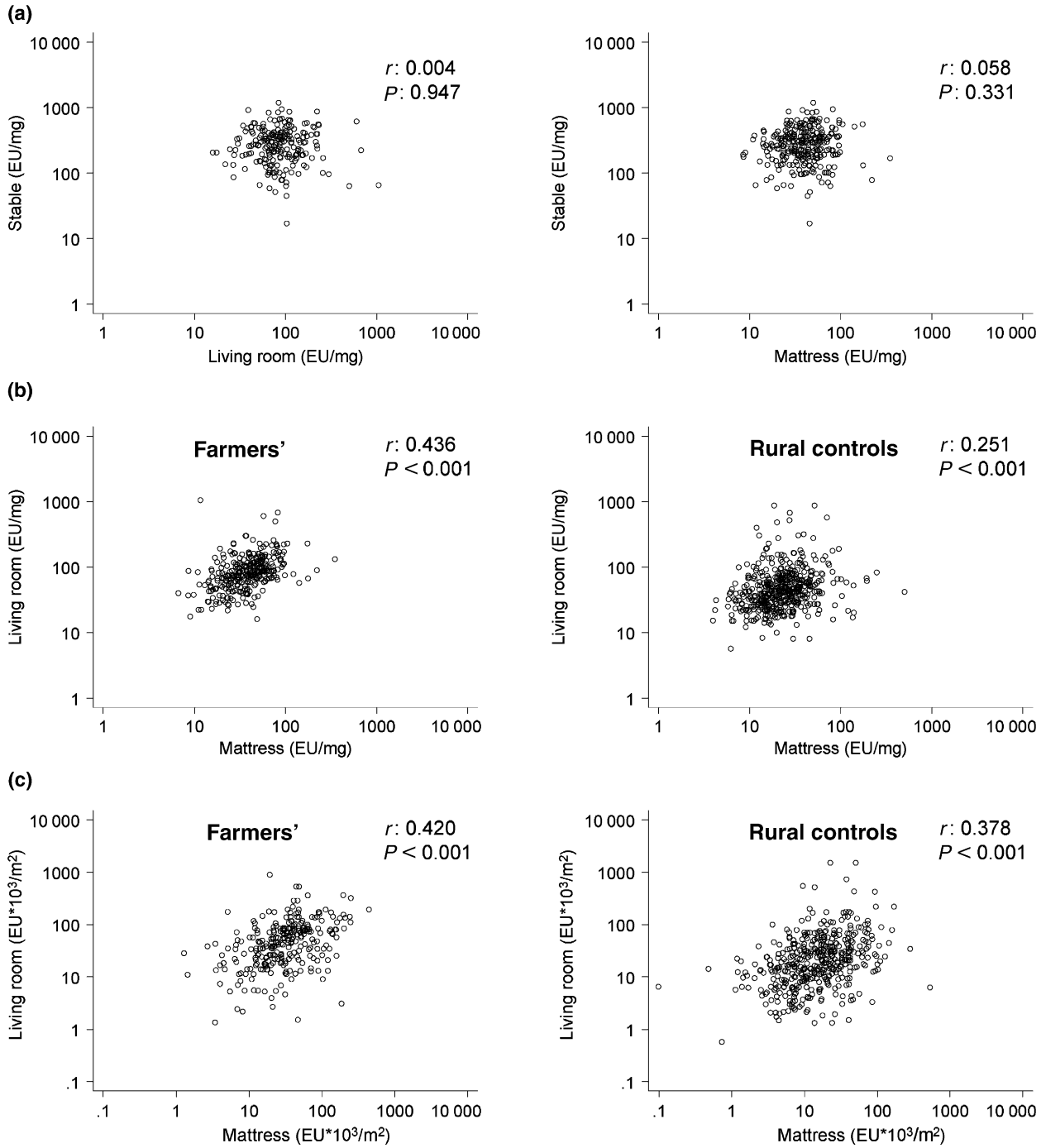


**Fig. 1.** Correlations of endotoxin levels in different home environments. (a) Stable vs. living room floor and mattress (endotoxin concentration). (b, c) Living room floor vs. mattress stratified by farmers and rural controls ((b) endotoxin concentration, (c) endotoxin load).

and 29.6% of the mattress dust endotoxin variation for farm and non-farm children, respectively.

## Discussion

The present analyses show that endotoxin concentrations measured in stables are not associated with the amount of endotoxin measured in dust samples from floors or from mattresses of farm children. However, a dose-dependent association between the child's activity on the farm and indoor home endotoxin levels was observed. This dose-dependent association was not limited to farmers' children but also seen in non-farm children with contact to the farming environment. Thus, to assess children's individual exposure to their microbial environment, measurements of mattress dust exposure is needed, as information about the number of stable animals or stable endotoxin levels does not predict

**Table 2.** Crude and adjusted† means ratios‡ (MRs) and 95% confidence intervals (CIs) of endotoxin concentrations in *settled stable dust*

| Predictor variables | | *n* | Crude MR (CI) | Adjusted* MR (CI) |
|---|---|---|---|---|
| Farming as parental occupation | Part-time | 105 | 1 | 1 |
| | Full-time | 180 | 1.07 (0.92–1.24) | 0.96 (0.79–1.17) |
| Keeping cattle | None | 30 | 1 | 1 |
| | 1–25 | 95 | 1.29 (0.91–1.83) | 1.51 (0.95–2.40) |
| | 26–50 | 87 | 1.51 (1.06–2.15) | 1.72 (1.04–2.84)* |
| | >50 | 73 | 1.15 (0.80–1.64) | 1.63 (0.96–2.77) |
| Keeping horses | No | 214 | 1 | 1 |
| | Yes | 65 | 1.31 (1.10–1.55) | 1.44 (1.20–1.72)** |
| Feeding material | Silage and hay | 218 | 1 | 1 |
| | Only hay | 66 | 1.27 (1.07–1.50) | 1.26 (1.03–1.53)* |
| Mucking system | Only solid muck | 122 | 1 | 1 |
| | Only liquid muck | 46 | 0.87 (0.70–1.07) | 1.09 (0.82–1.44) |
| | Both solid and liquid | 109 | 1.11 (0.95–1.31) | 1.23 (1.01–1.51)* |
| Study area | Germany | 128 | 1 | 1 |
| | Austria | 98 | 1.31 (1.12–1.54) | 1.45 (1.16–1.82)** |
| | Switzerland | 59 | 1.43 (1.18–1.72) | 1.56 (1.20-2.04)** |

**P*-value <0.05, ***P*-value <0.01 (*P*-values are only given for the adjusted model). †All MRs were adjusted for part-time/full-time farming, number of people running the farm, stable animals: number of cattle, presence of horses, sheep, goats, pigs, animals in stable during dust sampling, feeding material, mucking system, study area, date of dust sampling. ‡Back-transformed regression coefficient: $e^{\beta}$.

**Table 3.** Crude and adjusted means ratios (MRs) and 95% confidence intervals (CIs) of endotoxin levels in *living room floor dust*

| | | Farming households | | | Non-farming households | | |
|---|---|---|---|---|---|---|---|
| Predictor variables | | *n* | Crude MR (CI) | Adjusted† MR (CI) | *n* | Crude MR (CI) | Adjusted‡ MR (CI) |
| Farming as parental occupation | Part-time | 132 | 1 | 1 | | | |
| | Full-time | 186 | 1.41 (1.24–1.61) | 1.29 (1.08–1.53)** | | | |
| Stable endotoxin level (EU/mg) | Per unit change | 285 | 1.00 (0.90–1.12) | 0.95 (0.84–1.07) | | | |
| Current assistance in stable | Never | 46 | 1 | | | | |
| | At least once per week | 199 | 1.30 (1.08–1.56) | 1.15 (0.92–1.43) | | | |
| | At least daily | 68 | 1.50 (1.21–1.86) | 1.44 (1.11–1.87)** | | | |
| Stable activity score | 0 | | | | 137 | 1 | 1 |
| | 1 | | | | 163 | 1.20 (1.02–1.40) | 1.15 (0.97–1.37) |
| | 2 | | | | 162 | 1.31 (1.12–1.53) | 1.24 (1.04–1.48)* |
| | 3 | | | | 22 | 1.49 (1.09–2.03) | 1.28 (0.91–1.80) |
| Pets regularly indoors | No indoor pets | 205 | 1 | 1 | 293 | 1 | 1 |
| | Only cats | 77 | 1.23 (1.06–1.44) | 1.17 (0.98–1.39) | 151 | 1.39 (1.21–1.58) | 1.35 (1.16–1.58)** |
| | Cats and dogs | 36 | 1.33 (1.08–1.64) | 1.46 (1.08–1.97)* | 44 | 1.63 (1.32–2.02) | 1.40 (1.09–1.80)** |
| Father's education | None or low | 139 | 1 | 1 | 169 | 1 | 1 |
| | Medium | 132 | 0.97 (0.84–1.12) | 0.81 (0.66–1.00)* | 192 | 0.87 (0.75–1.00) | 0.89 (0.75–1.06) |
| | High | 39 | 0.86 (0.69–1.06) | 0.64 (0.48–0.86)** | 114 | 0.95 (0.80–1.12) | 0.90 (0.75–1.08) |
| Study area | Germany | 155 | 1 | 1 | 314 | 1 | 1 |
| | Austria | 104 | 1.13 (0.98–1.31) | 1.77 (1.17–2.68)** | 113 | 0.93 (0.81–1.08) | 0.91 (0.65–1.26) |
| | Switzerland | 59 | 1.45 (1.22–1.73) | 1.25 (0.73–2.15) | 61 | 0.73 (0.60–0.88) | 0.81 (0.60–1.10) |
| Last floor cleaning | <4 days | 182 | 1 | | 254 | 1 | 1 |
| | 4–5 days | 73 | 0.90 (0.76–1.06) | 0.87 (0.73–1.04) | 137 | 1.08 (0.94–1.25) | 1.20 (1.02–1.40)* |
| | >5 days | 58 | 0.97 (0.81–1.15) | 1.10 (0.87–1.38) | 87 | 1.21 (1.02–1.44) | 1.28 (1.06–1.54)** |

**P*-value < 0.05, ***P*-value < 0.01 (*P*-values are only given for the adjusted models). †MRs were adjusted for all the variables in the table, and additionally for number of people running the farm, age, sex, older siblings, house location, heating system, fieldworker, date of dust sampling, indoor smoking, amount of sampled dust, type of floor. ‡MRs were adjusted for all the variables in the table, and additionally for age, sex, older siblings, house location, heating system, fieldworker, date of dust sampling, indoor smoking, amount of sampled dust, type of floor.

indoor home exposure. Parental farming activities, assessed as full- or part-time farming, independently contributed to mattress endotoxin levels of farm children. Other farming activities, such as helping with harvesting or staying in barns, were highly correlated with stable activities and had no independent effect on house dust endotoxin levels. Since endotoxin (lipopolysaccharides) was shown to be very sticky to different fatty acid binding proteins [23], it is likely that children carry dust loaded with bacteria and endotoxin on clothes and shoes from the stables into the indoor environment, similar to the role of clothing as a means of distributing cat allergen into cat-free environments [24]. In addition, it is conceivable that the amount of endotoxin in the mattress also depends on the child's personal cleanliness. In farm children,

**Table 4.** Crude and adjusted† means ratios (MRs) and 95% confidence intervals (CIs) of endotoxin levels in *mattress dust*

| | | Farmers' children | | | Non-farm children | | |
|---|---|---|---|---|---|---|---|
| Predictor variables | | *n* | Crude MR (CI) | Adjusted MR (CI) | *n* | Crude MR (CI) | Adjusted MR (CI) |
| Farming as parental occupation | Part-time | 133 | 1 | 1 | | | |
| | Full-time | 185 | 1.21 (1.06–1.37) | 1.08 (0.90–1.29) | | | |
| People running the farm | >2 | 145 | 1 | 1 | | | |
| | 1 or 2 | 155 | 1.15 (1.01–1.30) | 1.18 (1.00–1.40)* | | | |
| Stable endotoxin level (EU/mg) | Per unit change | 284 | 1.05 (0.95–1.17) | 0.96 (0.84–1.09) | | | |
| Current assistance in stable | Never | 46 | 1 | 1 | | | |
| | At least once per week | 198 | 1.16 (0.96–1.39) | 1.11 (0.87–1.41) | | | |
| | At least daily | 69 | 1.38 (1.12–1.71) | 1.33 (1.00–1.76)* | | | |
| Stable activity score | 0 | | | | 140 | 1 | 1 |
| | 1 | | | | 164 | 1.11 (0.96–1.28) | 1.08 (0.91–1.27) |
| | 2 | | | | 162 | 1.19 (1.03–1.37) | 1.23 (1.05–1.44)** |
| | 3 | | | | 22 | 1.38 (1.03–1.83) | 1.35 (1.00–1.83)* |
| Pets regularly on child's bed | No pets on childs bed | 261 | 1 | 1 | 354 | 1 | 1 |
| | Only cats | 52 | 1.15 (0.97–1.37) | 1.07 (0.87–1.32) | 127 | 1.14 (1.00–1.30) | 1.20 (1.04–1.38)* |
| | Cats and dogs | 4 | 1.60 (0.91–2.81) | 1.81 (0.94–3.50) | 11 | 1.49 (1.01–2.18) | 1.68 (1.14–2.46)** |
| Child's age (years) | ⩽8 | 103 | 1 | 1 | 158 | 1 | 1 |
| | 9 | 64 | 0.89 (0.74–1.06) | 0.85 (0.68–1.05) | 93 | 1.03 (0.87–1.21) | 1.02 (0.86–1.23) |
| | 10–11 | 116 | 0.82 (0.71–0.95) | 0.74 (0.61–0.89)** | 187 | 0.89 (0.78–1.02) | 0.91 (0.79–1.06) |
| | >11 | 35 | 0.67 (0.54–0.83) | 0.65 (0.51–0.84)** | 51 | 0.83 (0.68–1.02) | 0.82 (0.66–1.03) |
| Study area | Germany | 154 | 1 | 1 | 316 | 1 | 1 |
| | Austria | 105 | 1.24 (1.08–1.42) | 1.31 (1.00–1.70)* | 115 | 1.03 (0.90–1.18) | 1.22 (0.96–1.57) |
| | Switzerland | 59 | 1.54 (1.30–1.82) | 1.42 (1.04–1.94)* | 61 | 0.98 (0.82–1.17) | 1.08 (0.80–1.45) |
| Type of mattress | Foam rubber | 98 | 1 | 1 | 121 | 1 | 1 |
| | Inner spring | 158 | 0.97 (0.84–1.12) | 1.22 (1.01–1.48)* | 277 | 1.01 (0.88–1.16) | 1.05 (0.89–1.24) |
| | Latex | 40 | 0.91 (0.74–1.13) | 1.11 (0.82–1.50) | 76 | 0.98 (0.82–1.17) | 1.09 (0.87–1.36) |
| Last cleaning the mattress | ⩽20 weeks | 173 | 1 | 1 | 273 | 1 | 1 |
| | >20 weeks | 132 | 0.98 (0.86–1.11) | 1.19 (1.01–1.40)* | 216 | 0.99 (0.88–1.11) | 1.00 (0.88–1.13) |

*$P$-value $< 0.05$, **$P$-value $< 0.01$ ($P$-values are only given for the adjusted models). †MRs were adjusted for all the variables in the table, and additionally for parents education, sex, older siblings, heating system, fieldworker, date of dust sampling, indoor smoking, amount of sampled dust, age of mattress, additional covers.

continuously living in an environment rich in microbial products, endotoxin concentrations in mattress dust decreased with increasing age of the children indicating that cleanliness or other personal habits may change with age and, therefore, differentially influence mattress endotoxin levels. Alternatively, it may indicate that older farm children spend less time on the farm due to school activities.

It might be argued that the lack of an association between stable endotoxin concentrations and indoor home endotoxin levels was due to a poor assessment of stable endotoxin levels. However, in this study, endotoxin concentrations of dust samples taken in a subset of stables on two separate occasions were highly correlated and thus seem to be reliable. In addition, a series of farm characteristics predicted stable endotoxin levels as expected. But it has to be kept in mind that the ALEX Study focuses on children's exposure to endotoxin and, therefore, only a limited number of variables that might be associated with stable endotoxin levels were available for analysis. The endotoxin concentrations increased with the number of cattle, but only up to the highest quartile. This might be due to the fact that in these colline to subalpine areas, farms with larger numbers of cattle usually own modern stables with better ventilation systems that are known to reduce airborne endotoxin concentrations in swine stables [25]. Similarly, the positive association between hay feeding and endotoxin levels compared to feeding of silage and hay might be interpreted as indicating more traditional farming. Likewise, running the farm by two or less people compared to more than two people, which was associated with higher endotoxin levels, may be a surrogate measure of a traditional, less advanced family business. Horse keeping was also associated with increased endotoxin levels. Farms that kept horses did not differ from non-horse keeping farms with respect to study area, part- or full-time farming activity, or number of cattle kept, but they were more likely to accommodate additional farm animals such as pigs, sheep or goats. Hence, either horse keeping truly increases endotoxin levels or it might be a surrogate for a range of other animals contributing to endotoxin levels in stables. Other farm characteristics such as the distance of the stable from the farm house or whether the farm house and the stable were built under the same roof were not available. None of the farms in our study was a pure pig or poultry farm business, thus, endotoxin was essentially measured in cattle stables. This might also explain why the variability of the endotoxin measurements was rather limited.

Stable endotoxin levels and indoor home endotoxin concentrations were significantly higher in Austrian and Swiss farm homes compared to German ones. The reasons for these differences remain unclear, with current European studies

comparing air contaminants in occupational farm environments, focused on greenhouse workers and on pig and poultry farms [26]. However, Swiss and Austrian farms might have been smaller and more traditional, as the number of cattle in German stables were higher than in Austria and Switzerland (mean of 43 (95% CI: 38–48), 27 (22–31), 29 (25–32), respectively.

As previously reported in the literature we found that pet keeping indoor increased the indoor endotoxin levels in floor dust [17, 18, 20]. The association with pets and rodents in the home with higher house dust endotoxin levels suggest that animal species whose gastrointestinal tracts are typically colonized with Gram-negative bacteria contribute substantially to indoor endotoxin levels. Pets staying on the child's bed contributed even more to mattress endotoxin levels. Although farmers' families were less likely to have pets on a bed, the association between endotoxin levels and pets on beds was seen in farm and non-farm families. While the number of dogs on beds was small, it still had a strong impact on mattress endotoxin concentration indicating that dogs specifically increase endotoxin concentration. Several factors such as time since last mattress cleaning, mattress age and type, and the number of mattress covers, were not associated with endotoxin concentration in non-farm children, but farm children who slept on old inner spring mattresses and whose mattresses had not been cleaned for several months were exposed to slightly higher endotoxin levels.

The role of carpeting and cleanliness as determinants of indoor endotoxin is controversial. In a small study of US homes, Gereda et al. found no association between cleanliness and carpeting and house dust endotoxin concentrations [18]. Park et al., however, reported mattress type, type of rug on floor and operating humidifiers to be significant determinants of airborne endotoxin concentrations in homes in the Boston area [19]. Although we found a series of significant determinants of indoor endotoxin levels in these rural populations, only about one-third of the variability of indoor endotoxin levels could be explained by the multivariate models suggesting that many other, yet unknown sources, contribute to house dust endotoxin levels.

In conclusion, the present analyses demonstrate that regular contact to livestock, which has previously been shown to be inversely related to the occurrence of allergic diseases [3, 4] is indeed associated with increased mattress endotoxin concentrations, both in farm and non-farm children. In non-farm children, pet keeping and floor cleaning additionally increased endotoxin levels, whereas in farm children, parental farm activities, the study area, younger age and mattress cleaning, also contributed to increased microbial exposure. However, it must be kept in mind that other bacterial components such as CpG-motifs, or cell-wall components from atypical mycobacteria or Gram-positive bacteria which are known to affect immune responses in ways similar to that of endotoxin were not measured. Endotoxin may thus be a surrogate marker of a much broader spectrum of microbial compounds than Gram-negative bacteria alone. By adjusting for the total amount of dust in our analyses, we aimed to taking into account additional microbial exposure, and were thus determining specific predictors of endotoxin exposure. It therefore seems premature to translate current knowledge on the 'protective' effect of microbial exposure on allergic outcomes into immediate preventive action or therapy.

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# Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health

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**Background: Endotoxin exposure has been shown to be associated with a decreased prevalence of atopic sensitization and symptoms. Yet endotoxin represents only a part of the indoor microbial exposure. Muramic acid, a constituent of peptidoglycan, is present in gram-negative and gram-positive bacteria in the environment and may therefore serve as an additional marker of microbial exposure.**
**Objective: To study the factors determining the level of indoor exposure to muramic acid/peptidoglycan, as well as its potential association with respiratory health.**
**Methods: In 553 farm and nonfarm school children from Austria, Switzerland, and Germany, mattress dust muramic acid concentrations were determined, and health was assessed by using IgE measurements and questionnaire information.**
**Results: The muramic acid concentration was found to be significantly higher in dust from farm children's mattresses than in dust from nonfarm children's mattresses (157 vs 131 ng/mg). Children with higher mattress dust muramic acid concentrations had a significantly lower prevalence of wheezing (odds ratio of highest vs lowest tertile of muramic acid concentration, 0.3; 95% CI, 0.1-0.9), regardless of farming status and endotoxin exposure. The association for asthma was similar, and no association was found with atopic sensitization.**
**Conclusion: Next to endotoxin, muramic acid provides us with an independent marker of microbial exposure. Unlike endotoxin, muramic acid was inversely associated with wheezing rather than with atopic sensitization. (J Allergy Clin Immunol 2004;113:860-7.)**

***Key words:*** *Farming, allergy, asthma, peptidoglycan, muramic acid, microbial exposure, hygiene hypothesis*

Ever since the hygiene hypothesis proposed a mechanism explaining the increased development of allergies and hay fever in children,[1] efforts have been made to find agents responsible for the proposed mechanism. Household size,[1,2] early life day care,[3] farming,[4,5] and keeping pets in the first year of life[6,7] were found to be related to a decreased prevalence of sensitization and possibly asthma. Increased exposure to infections early in life[8,9] or an increased microbial burden early in life[10] were put forward as other possible causes. One of the substances currently under investigation is endotoxin. Several studies linked increased endotoxin exposure to decreased sensitization rates[11,12] and possibly lower prevalences of hay fever, asthma, and wheezing.[13] Determinants of endotoxin exposure have also been studied. The presence of pets,[14,15] an increasing number of household occupants, older vacuum cleaners, a high relative humidity, and the absence of floor insulation were associated with increased endotoxin concentrations in settled household dust.[15]

Endotoxin, however, represents only a part of the total microbial burden, because it occurs only in gram-negative bacteria. Peptidoglycan, in turn, is a major component of the cell wall of all species of the domain Bacteria, but not in species of the domain Archaea. Gram-positive bacteria have a thicker cell wall compared with gram-negative bacteria. The amount of peptidoglycan found in environmental samples reflects the presence of the greater part of environmental bacteria more precisely. However, it may be influenced more strongly by the presence of gram-positive bacteria than by the presence of gram-negative bacteria.

A major component of bacterial peptidoglycan is *N*-acetyl-muramic acid (2-acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose), which is not found elsewhere in nature.[16] In this article, we refer to this substance as *muramic acid*. Different research groups developed methods to determine the amount of muramic acid in

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*Abbreviations used*
ALEX: Allergies and Endotoxin
GLC: Gas-liquid chromatography
TLR: Toll-like receptor

environmental samples.[17-19] Several occupational environments, like swine confinement buildings,[17] a dairy,[18] 2 schools,[20] and several other places with exposure to organic dust,[19] have been studied. Few studies are available on determinants of muramic acid in house dust,[21-23] and no data are available on health effects of muramic acid/peptidoglycan. The determination of muramic acid may be more representative than endotoxin in estimating the total microbial burden; there is also some evidence that peptidoglycan influences the innate immune system. Endotoxin has been shown to act as a ligand for the CD14/Toll-like receptor (TLR)−4 pathway,[24] which may result in modulation of the T helper cell response. In contrast, peptidoglycan was reported to activate innate immunity via TLR-2, which induces a functionally different cellular response compared with TLR-4.[24] Therefore, muramic acid/peptidoglycan is not only a specific marker for microbial burden, but also a biologically active substance, influencing cellular immune response in a different way compared with endotoxin.

For 812 previously described[25] school children from farms and their peers living in the same villages but not on a farm, dust samples were taken.[13] We analyzed the muramic acid content of a subset of the mattress dust samples and studied the association between muramic acid/peptidoglycan exposure and sensitization, respiratory symptoms, and the diagnoses of asthma and hay fever.

## METHODS

### Subjects

The Allergies and Endotoxin (ALEX) Study was performed in rural areas of Austria, Germany, and Switzerland, as previously described.[13,25] Approval to conduct the survey was obtained from the 3 local ethics committees for human studies and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children. Participation and consent are shown in Fig 1. Mattress dust samples from the homes of 812 children with German, Swiss, or Austrian nationality were analyzed for endotoxin. The study group was representative of the rural population in the participating countries.[25] The muramic acid content of the mattress dust was analyzed for 553 children for whom enough dust was still available after measurement of endotoxin and allergens. Dust samples were collected from November 1999 through February 2000.

### Questionnaire

Demographic factors, information about the parents, information about the children's health, and information about their activities on farms were assessed by parental questionnaire. The questionnaire contained questions on sneezing with itchy eyes and wheezing in the past 12 months and on physician-diagnosed hay fever and asthma according to the International Study of Asthma and Allergies in Childhood questionnaire. The children's activities on farms were categorized into rarely (not at all, or rarely) and frequently (now and then, or frequently) by questions on frequency of visits to the barn and the stable and providing help during hay harvesting. The questionnaire also contained questions about behavior during pregnancy and the first year of life of the child. Furthermore, questions were asked about the age and type of mattress (foam rubber, inner spring, or latex), the cleaning of the mattress, and the presence of pets.

### Dust collection

Dust collection was performed by 7 centrally trained field workers (3 in Germany, 2 in Austria, and 2 in Switzerland) following a standardized protocol. New vacuum cleaners (Miele S624; Miele, Gütersloh, Germany) were calibrated to have a flow of 200 L/min using a clean filter, which resulted in an operating power of approximately 800 W. ALK filters (Allergisk Laboratoreit Københavns, Hørsholm, Denmark) were used for dust collection, following the International Study of Asthma and Allergies in Childhood phase II protocol (http://isaac.auckland.ac.nz/). Dust samples were collected by vacuuming the mattress of the child for 2 min/$m^2$, after removal of all sheets apart from mite-impermeable mattress encasings and plastic sheets. Dust was collected on preweighed ALK filter holders. Particles larger than 2 mm (paper clips, stones, and so forth) were removed with tweezers after dust sampling, and subsequently, the filter holders were reweighed. Samples were stored at 20°C to 22°C until transport to a central location, where the samples were stored at 20°C to 22°C. The endotoxin analysis was described elsewhere.[13]

### Muramic acid analysis

Ten milligram dust was methanolyzed (85°C, 18 hours) in 3 mL 4-mol/L methanolic HCl in a glass tube. The tube was cooled at 20°C to 22°C and then transferred to a 10-mL vial in liquid nitrogen. Subsequently, the sample was dried in a vacuum centrifuge (speed vac UV S400; Life Sciences International, Frankfurt/Main, Germany). Two hundred microliters pyridine and 100 μL acetic anhydride were added, and after ultrasonication, the sample was incubated at 85°C for 30 minutes and dried in a vacuum centrifuge. The sample was suspended in 1 mL 50% ethyl acetate in hexane and allowed to precipitate. The supernatant was transferred to a small column (approximately 1-1.5 mL in a small Pasteur pipette) of silica gel 60 (230-400 mesh) in 50% ethyl acetate in hexane, which before this step had been washed twice with this solvent mixture. Then the column was washed 5 times with 50% ethyl acetate in hexane (1 mL each time). The sample was then extracted twice with 0.5 mL ethyl acetate. The combined supernatants of this step were transferred to the column of silica gel, which was finally eluted with 8 mL ethyl acetate. The eluted sample was dried in a stream of nitrogen, dissolved in chloroform, and analyzed by combined gas-liquid chromatography (GLC)/mass spectrometry (HP5890/MSD 5970; Hewlett-Packard, Böblingen, Germany). GLC separation was achieved on a column (12 m × 0.33 μm in diameter) of Ultra-1 (Hewlett-Packard), applying a temperature program of 120°C over a period of 3 minutes, then increasing by 5°C/min to 320°C, which was maintained for 10 minutes. For quantification of *N*-acetyl-muramic acid in dust samples, authentic *N*-acetyl-muramic acid (10 μg; Sigma, Deisenhofen, Germany) was methanolyzed (0.5 mL, 4-mol/L methanolic HCl), acetylated (50 μL pyridine and 25 μL acetic anhydride), and purified on a column of silica gel 60 as described for the dust samples above. The standard was dissolved in 100 μL chloroform, and for determination of a calibration curve, volumes equal to 5, 10, 25, 50, and 100 ng were analyzed by GLC/mass spectrometry and quantified by determination of the specific ion at *m/z* 187. Areas increased linearly with the amounts injected; thus, the calibration was verified and could subsequently be used for muramic acid analyses. The assay coefficient of variation was 10.6%, which was determined by analyzing 3 samples 6 times on different days. The detection limit was 50 ng/mg dust, and all samples with

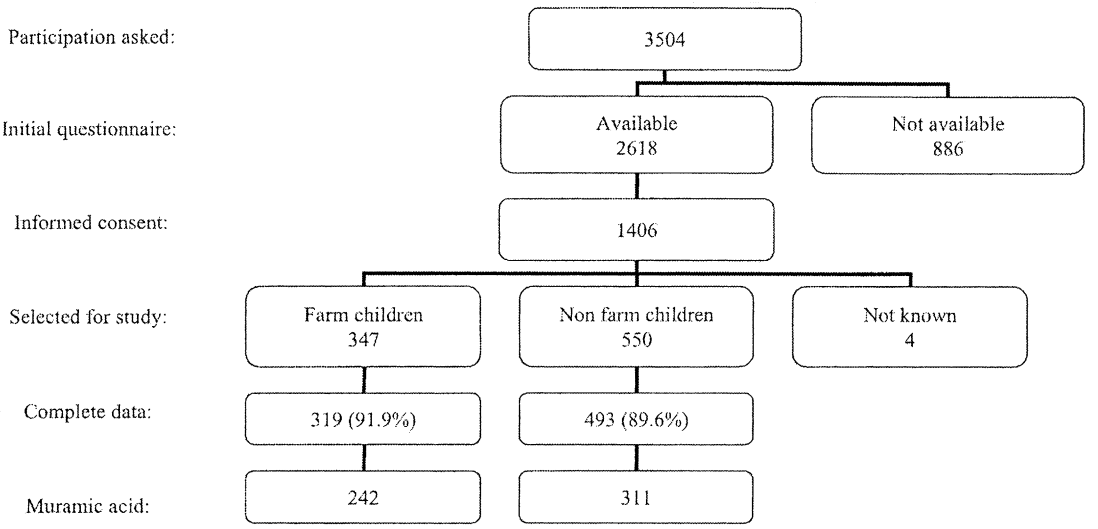


**FIG 1.** Participation and consent in the ALEX Study.

nondetectable muramic acid concentrations (n = 25) were assigned a value of 25 ng/mg. Mattress dust muramic acid load (μg/m$^2$) was calculated by multiplying with the total amount of dust collected and dividing by the surface area of the mattress.

## Sensitization

Serum was collected from the children and tested for specific IgE to a mixture of aeroallergens containing grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, and *Cladosporium herbarum* (sx1 Immunocap, Pharmacia, Uppsala, Sweden). Results were obtained in kilounits per liter. Cut-off values of 0.35 kU/L (comparing RAST class 1 or higher with RAST class 0) and 3.5 kU/L (comparing RAST class 3 or higher with RAST classes 0, 1, and 2) were both used to study sensitization, and the cut-off value of 0.35 kU/L was used to stratify the children by sensitization.

## Statistical analyses

The muramic acid and endotoxin concentrations as well as the muramic acid, endotoxin, and dust loads were log-normally distributed, and therefore ln-transformed. Pearson correlation coefficients were calculated. Differences for subject or home characteristics between farming and nonfarming children were tested with $\chi^2$ tests. Adjusted geometric means and 95% CIs of the muramic acid concentration for different subject or home characteristics were calculated as least-square means by using linear regression analysis in SAS version 8.2 (Cary, NC). The Tukey-Kramer method was used to adjust for multiple comparisons. Locally weighted regression smoothers were used to study the unadjusted association of respiratory symptoms and diagnoses with increasing muramic acid exposure. Adjusted odds ratios were calculated by using multiple logistic regression analysis. Odds ratios are shown for an increase in muramic acid concentration or load equal to the interquartile range of the distribution (108 ng/mg and 145 μg/m$^2$, respectively), and in the case of nonlinearity, for the 2 highest tertiles of the muramic acid concentration (98.5-167.5 ng/mg and >167.5 ng/mg) versus the lowest (<98.5 ng/mg). Significance was assumed at $P < .05$.

# RESULTS

Only mattress dust samples for which a sufficient amount of dust was still available (553 of 812) were analyzed for muramic acid. The analyzed samples contained initially on average about twice as much dust compared with the samples that were not analyzed (respective geometric means, 0.86 g/m$^2$ vs 0.39 g/m$^2$; *t* test: $P < .05$). On the other hand, the endotoxin concentration in analyzed samples was not significantly different from the concentration in samples that were not analyzed. Farm children were represented more often in the group for whom the dust samples were analyzed than in the group for whom the dust samples were not analyzed (44% vs 30%; $\chi^2$ test, $P < .05$), whereas the prevalence of sensitization, respiratory symptoms, or diagnoses of asthma or hay fever was not significantly different for children with analyzed versus nonanalyzed samples.

The geometric mean muramic acid concentration in dust from mattresses of rural children (median age, 9 years; range, 6-14 years) was 128 ng/mg (95% CI, 121-135; range, 25-2011). Prevalences of different symptoms and diagnoses are described elsewhere for the whole group (n = 812) and were not significantly different for the subgroup described here. There was some correlation between the muramic acid and endotoxin concentration of the dust ($r = 0.44$; Fig 2, *A*), whereas the correlation between the muramic acid and the endotoxin loads ($r = 0.74$; Fig 2, *B*) was high. The latter is most probably caused by the strong influence of the amount of dust collected from the mattress. The muramic acid concentration of the dust was not correlated with the amount of dust ($r = 0.14$; Fig 2, *C*), and the muramic acid concentration and the muramic acid load were correlated highly ($r = 0.77$).

In Table I, adjusted geometric means and 95% CIs of the mattress dust muramic acid concentration are shown for separate categories of each potential predictor of the muramic acid concentration. For 439 children, complete data were available. Furthermore, Table I shows the distribution of each potential predictor for farm children and nonfarm children. Apart from the variables listed in

Table I, the analysis was adjusted for field worker (and thereby also for country, because field workers operated only in 1 country), month of sampling, endotoxin concentration, and dust load. The results show that independently from all other variables, farm children had a higher mattress dust muramic acid concentration compared with nonfarm children. Furthermore, the mattress dust muramic acid concentration was increased in homes heated with wood or coal (compared with gas, oil, or electric heating) and in dust from mattresses that were cleaned less often. When taking multiple comparisons into account, results did not change. When looking at farm children only, children who often helped with the work in the stables had increased mattress dust muramic acid concentrations (data not shown). Samples taken in Switzerland, and samples taken in January and February compared with November and December, had higher muramic acid concentrations (data not shown).

Table II shows odds ratios (adjusted for potential confounding, including mattress dust endotoxin concentration or mattress endotoxin load) for sensitization, wheezing, sneezing with itchy eyes, hay fever, and asthma. In bivariate analyses, an increasing muramic acid concentration was associated with a decreasing sensitization rate, but this association disappeared after adjusting for the endotoxin concentration. No relation was found between muramic acid load or concentration and sneezing with itchy eyes, hay fever, and asthma. For wheezing in the past 12 months, however, a decreased risk was seen with increasing mattress dust muramic acid concentration (ng/mg), and a similar trend was seen with increasing mattress muramic acid load ($\mu g/m^2$). All odds ratios in Table II were calculated for an increase of the mattress dust muramic acid concentration, or the mattress muramic acid load of a magnitude equal to the interquartile range of the distribution. Locally weighted regression smoothers were used to study the assumption of a linear risk increase, whereas subjects were stratified according to sensitization status (data not shown). Among nonsensitized children, the inverse association appeared to be roughly linear, but among sensitized children, the association followed an inverse J-shaped trend. Therefore, the association between tertiles of the mattress dust muramic acid concentration and the prevalence of wheezing or an asthma diagnosis, stratified by sensitization, was investigated.

Fig 3 shows the prevalence (in blue) of wheezing and asthma for the 3 tertiles of the mattress dust muramic acid concentration. The corresponding adjusted odds ratios (and 95% CIs) are shown in black, comparing the 2 highest tertiles with the lowest, while adjusting for the mattress dust endotoxin concentration, the child living in a farming family, and other potential confounders. A decreasing risk of wheezing was seen for an increasing mattress dust muramic acid concentration. This trend was mostly seen for wheezing and asthma among nonsensitized children. Among sensitized children, the highest tertile seemed to follow the same trend, whereas children in the middle tertile tended to have a higher risk of wheezing and asthma compared with children in the lowest tertile. This association was also seen in the subgroup of nonfarming children (data not shown).

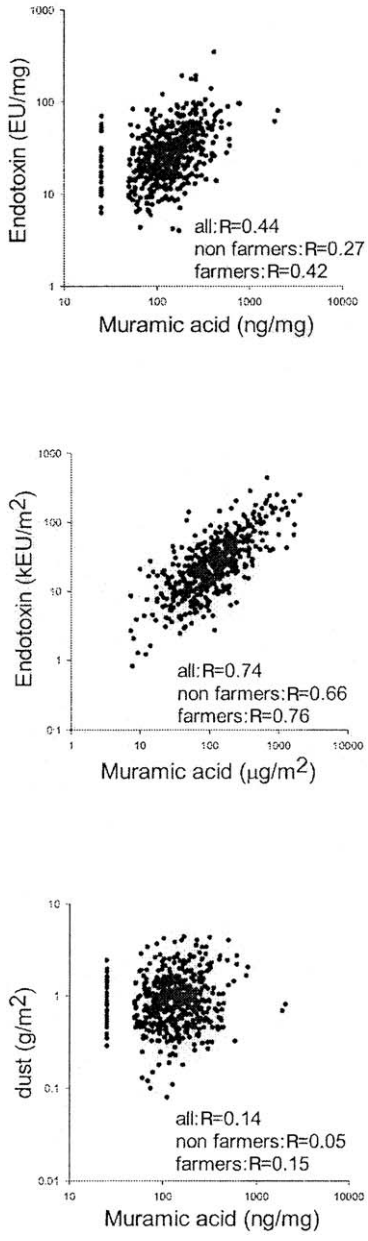


**FIG 2.** Correlation among muramic acid, dust, and endotoxin in mattress dust.

## DISCUSSION

Our study shows that muramic acid, in addition to endotoxin, can be considered as a marker for microbial exposure. Independent of the endotoxin concentration, increasing mattress dust muramic acid concentrations

**TABLE I.** Characteristics and percentage of children in each category; the geometric mean muramic acid concentration (and 95% CI) is shown for each category, n = 439.

| | Category | Nonfarming (n = 260) % | Farming (n = 179) % | $\chi^{2}$* | Mass spectrometry muramic acid (all children) (ng/mg)§ |
|---|---|---|---|---|---|
| Farming family | No | 100.0 | 0.0 | — | 131 (113-152) |
| | Yes | 0.0 | 100.0 | | 157 (135-182)† |
| Frequency of child's barn visits (present) | Rarely | 83.9 | 29.1 | ‡ | 146 (127-169) |
| | Frequently | 16.2 | 71.0 | | 141 (120-165) |
| Child helps with hay harvesting (present) | Rarely | 83.1 | 20.1 | ‡ | 140 (122-161) |
| | Frequently | 16.9 | 80.0 | | 147 (126-171) |
| Frequency of child's stable visits (present) | Rarely | 72.7 | 16.8 | ‡ | 137 (117-160) |
| | Frequently | 27.3 | 83.2 | | 151 (131-173) |
| Raw milk during pregnancy | No | 72.3 | 34.6 | ‡ | 147 (129-168) |
| | Yes | 27.7 | 65.4 | | 140 (121-161) |
| Heating | Gas, oil or electric | 85.8 | 38.0 | ‡ | 133 (116-152) |
| | Wood or coal | 14.2 | 62.0 | | 155 (134-179)† |
| Age of mattress | ≤6 y (median) | 53.1 | 41.3 | † | 140 (123-160) |
| | >6 y | 46.9 | 58.7 | | 147 (128-169) |
| Mattress material | Foam rubber | 21.2 | 33.0 | † | 155 (134-178) |
| | Inner spring | 66.2 | 57.0 | | 139 (122-158) |
| | Latex | 12.7 | 10.1 | | 138 (113-168) |
| Extra mattress cover | No | 49.6 | 45.3 | | 146 (128-168) |
| | Yes | 50.4 | 54.8 | | 141 (123-161) |
| Weeks ago mattress last cleaned | ≤20 (median) | 50.0 | 54.2 | | 135 (119-154) |
| | >20 | 50.0 | 45.8 | | 152 (133-174)† |
| Indoor pets | None | 57.7 | 71.0 | † | 132 (119-146) |
| | Cat only | 31.9 | 19.6 | | 145 (127-165) |
| | Dog only | 3.5 | 3.9 | | 160 (119-215) |
| | Cat and dog | 6.9 | 5.6 | | 138 (111-172) |

*$\chi^{2}$ Test for difference between farming and nonfarming (†$P < .05$; ‡$P < .01$).
§Adjusted for fieldworker, month of sampling, endotoxin concentration, dust load, and all other variables in the table (†$P < .05$).

**TABLE II.** Adjusted* odds ratios for symptoms with increasing† muramic acid concentration and muramic acid load.

| | Muramic acid concentration (ng/mg) | Muramic acid load (μg/m$^2$) |
|---|---|---|
| Sensitization (SX1 screening) | | |
| >0.35 kU/L | 0.90 (0.68-1.18) | 0.92 (0.63-1.35) |
| >3.50 kU/L | 0.94 (0.69-1.30) | 1.01 (0.64-1.60) |
| Sneezing and itchy eyes (past 12 mo) | 0.80 (0.52-1.24) | 0.73 (0.38-1.40) |
| Hay fever | 0.87 (0.54-1.39) | 0.74 (0.37-1.50) |
| Wheezing (past 12 mo) | 0.62 (0.41-0.94)§ | 0.55 (0.29-1.04)‡ |
| Asthma | 1.17 (0.69-2.00) | 1.08 (0.51-2.26) |

*Adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, number of older siblings, and endotoxin concentration or load.
†Odds ratios were calculated for an increase of the exposure by a magnitude of the interquartile range (108 ng/mg and 145 μg/m$^2$).
‡$P < .10$.
§$P < .05$.

were associated with a lower frequency of wheezing and possibly asthma among rural school children.

The analysis of muramic acid in mattress dust samples of the original ALEX Study subjects[13] could be performed in only 553 of 812 subjects because no dust was available for the remaining 259 subjects. This raises the question of whether selection bias occurred. The following arguments sustain the fact that the occurrence of selection bias was unlikely. First, the endotoxin concentration in analyzed samples was similar compared with the endotoxin concentration in samples that were not analyzed for muramic acid. Second, the low correlation between the amount of dust and the muramic acid concentration ($r = 0.14$) indicated that this

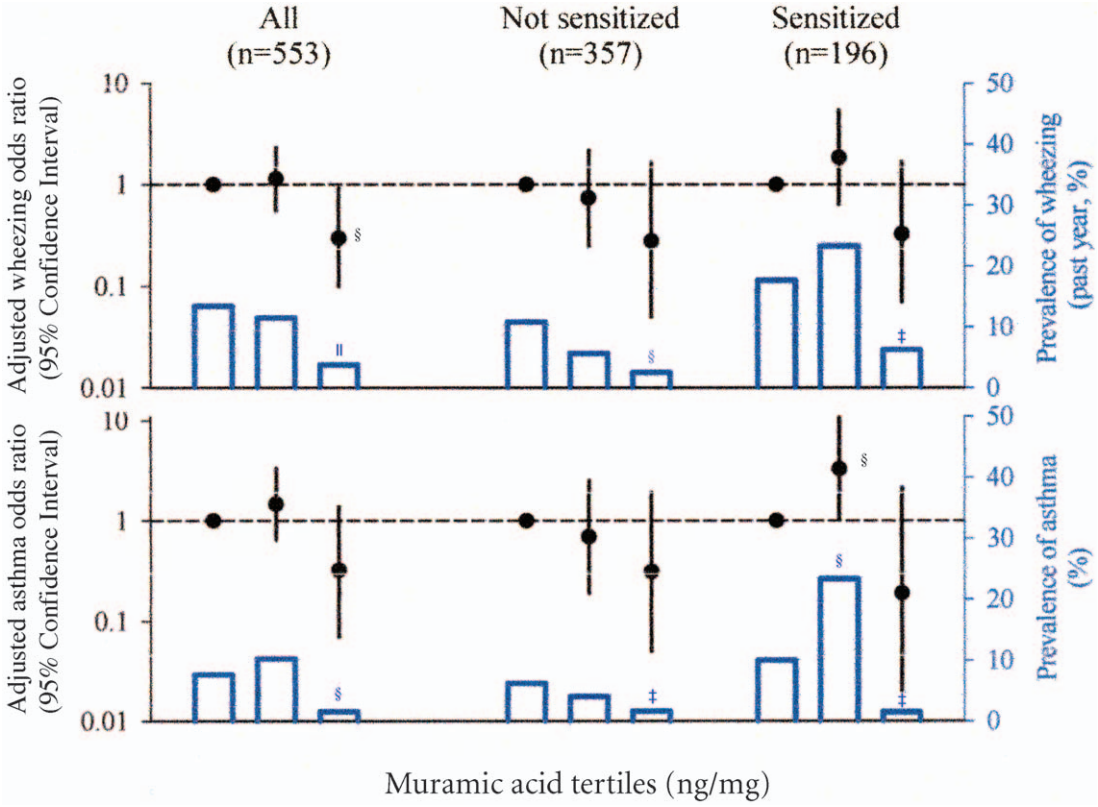


**FIG 3.** Prevalence (in *blue*) and adjusted* odds ratios (in *black*) for wheezing (past 12 months) and asthma diagnosis with increasing† muramic acid concentration (ng/mg; ‡$P < .10$; §$P < .05$; ‖$P < .01$). *Adjusted for age, sex, study area, family history of asthma or hay fever, educational level of the parents, number of older siblings, living on a farm, and mattress dust endotoxin concentration. †Odds ratios compare children in the second (98.5-167.5 ng/mg) and third tertile (>167.5 ng/mg) with children in the first tertile (<98.5 ng/mg) of the mattress dust muramic acid concentration.

forced selection of subjects with relatively high dust exposure was most likely not biased. Third, the prevalence of sensitization, respiratory symptoms, and diagnoses of asthma and hay fever was also not significantly different when comparing the two groups, indicating that bias because of this selection was not to be expected. Therefore, and keeping earlier considerations[25] in mind, we concluded that the observations made here were applicable to the rural population in the countries in which the study took place, and may not be extrapolated to urban populations. Muramic acid concentrations found in our study (geometric mean, 128 ng/mg) were somewhat higher when compared with previously reported concentrations of 23.1 ng/mg and 50.5 ng/mg dust from 2 beds,[22] and were much higher than concentrations found by Liu et al[20] in floor dust from schools. These differences are probably in part attributable to methodological differences in sampling or analysis and to differences between school floors and mattresses. In addition, some studies reported airborne muramic acid concentrations in several different occupational environments[17,19]; however, the outcomes are difficult to compare with our results.

There was some correlation between endotoxin and muramic acid ($r = 0.44$), indicating that both substances are markers for microbial exposure, but because the correlation coefficient was not so high, indicating a partial correlation, they seem to occur largely independent of each other. Endotoxin is only present in gram-negative bacteria, whereas muramic acid is a constituent of peptidoglycan, which forms the rigid backbone of the bacterial cell wall of gram-negative and gram-positive bacteria. The peptidoglycan of the gram-positive cell wall is much thicker than that of the gram-negative cell wall, and this may result in concentration differences between the 2 dust contaminants. The high correlation between the muramic acid load and endotoxin load of the mattress could be a cause of concern, and therefore, the muramic acid concentration was used instead. To avoid reporting spurious associations between health outcomes and muramic acid concentrations, all analyses were adjusted for the endotoxin concentration.

Several factors that may influence microbial exposure were studied for their relation to the muramic acid concentration. Farm children had higher muramic acid exposure than non-farm children, as was shown for endotoxin.[26] This finding is probably explained by higher

exposure to microbial contaminants in general, because most of the farms in the study area were traditional dairy farms, where the children are in close contact with the farm animals. Independent of being a farm child or not, mattress dust from homes heated with wood or coal had increased muramic acid concentrations. Using wood or coal for heating may be indicative of a more traditional way of life, possibly associated with increased microbial exposure. Using wood or coal for heating may also spread airborne dust through the house more easily. Furthermore, dust from less frequently cleaned mattresses also contained more muramic acid, pointing to the fact that muramic acid exposure from the mattress may also be influenced by cleaning.

Some studies have shown that peptidoglycan, of which muramic acid is a major component, activates the innate immune response via TLR-2 and stimulates the production of TNF-α, IL-1, and IL-6 by human monocytes, although the dose necessary for induction was very high compared with lipopolysaccharide.[24,27,28] Dust containing muramic acid was furthermore shown to stimulate the production of IL-6 and IL-8 in lung epithelial cells, although the effect could not be separated from the effects of other constituents of the dust.[22]

Because of limited numbers of samples and subjects, no previous epidemiological studies have investigated the potential health effects of muramic acid exposure. In this study, the prevalence of wheezing in the past year decreased with increasing mattress dust muramic acid concentrations. The effect persisted after adjusting for potential confounders, after adjusting for the endotoxin concentration or stratifying by the median of the endotoxin concentration, and also after adjusting for being part of a farming family. Moreover, exclusion of families who reported taking measures to reduce allergen exposure (n = 105) did not considerably change the results (data not shown). Sensitivity analyses separate for farm and nonfarm children showed that the inverse association between muramic acid exposure and wheezing was apparent for both groups of children (data not shown), suggesting that the effect was not limited to a highly exposed group (farm children) but was also seen among rural children not living on a farm.

Muramic acid exposure was most strongly associated with the prevalence of wheeze in the past 12 months. In Germany, Austria, and Switzerland, atopic wheezing children are more likely to be labeled as asthmatic patients than nonatopic wheezing children. We therefore stratified the analyses for wheeze and asthma into children with atopic and nonatopic phenotypes as measured by sensitization. It is intriguing that microbial exposure as assessed by muramic acid concentrations of mattress dust was inversely related to wheeze and asthma, whereas no association with atopy was seen. This observation contrasts with the assumption of 1 interpretation of the hygiene hypothesis, proposing that the potential beneficial effect of infectious or "unhygienic" exposure is mediated via $T_H1$-like immune responses, resulting in reduced atopic responses. Yet asthma is only in part attributable to atopy,[29] and the regulation of immune responses other than those eventually leading to the production of IgE antibodies is likely to be involved. Peptidoglycan has been shown to modulate the innate immune response on a pathway other than endotoxin, acting as a ligand for TLR-2 functioning in innate immunity.[24] TLR-2 genes were also more strongly expressed in farm children compared with nonfarm children.[30] It is thus interesting to speculate that exposure to endotoxin and muramic acid/peptidoglycan modulates different pathways of the innate immune system and thus different phenotypes of asthma, although the molecular processes behind this observation are as yet unknown.

The results reported herein therefore suggest that ubiquitous environmental exposure to bacteria, in particular gram-positive bacteria, is inversely associated with the prevalence of asthmatic symptoms, supporting the hygiene hypothesis. Thereby, the inception of asthma may relate to the lack of activation of innate immune responses as triggered by certain microbial products.

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## Original article

# Exposure to pets, and the association with hay fever, asthma, and atopic sensitization in rural children

**Background:** An increasing number of studies report pet exposure to be associated with lower risk of asthma and allergies. This 'protective pet effect' has been suggested to result from a modified T-helper (Th)2-cell response, or because of increased microbial load in homes where pets are kept. We examined the associations between pet contact and the occurrence of asthma and allergies in children of the rural Allergy and Endotoxin (ALEX) population, taking farm animal contact, endotoxin and cat allergen levels in mattress dust into account.

**Methods:** Information about contact with pets and farm animals, asthma and allergy were collected for 812 children by a standardized parents' questionnaire and an interview. Mattress dust endotoxin and cat allergen levels as well as specific IgE and IgG4 antibodies to Fel d1 were determined.

**Results:** Current contact with dogs was inversely associated with diagnosed hay fever (OR 0.26, 95% CI 0.11–0.57), diagnosed asthma (OR 0.29, 95% CI 0.12–0.71), sensitization to cat allergen (OR 0.48, 95% CI 0.23–0.99) and to grass pollen (OR 0.55, 95% CI 0.33–0.94), but not with increased IgG4 levels. Early and current contact with cats were associated with reduced risk of wheezing (OR 0.48, 95% CI 0.23–1.00, and OR 0.49, 95% CI 0.26–0.92, respectively) and grass pollen sensitization. Adjustment for farm animal contact but not for endotoxin and cat allergen exposure attenuated these associations and the effect of pet was stronger among farmers' children.

**Conclusion:** Although pet exposure was very frequent in this rural population, the inverse relation between current dog contact, asthma and allergy was mostly explained by simultaneously occurring exposure to stable animals or was restricted to farm children. In addition, a subtle form of pet avoidance may contribute to the protective effect of pet.

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Key words: allergen; allergy; asthma; children; endotoxin; farming; hygiene; pet.

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Regular contact with stable animals has been shown to confer protection against the development of asthma and allergy in children (1–3). The amount of family farming activities, and the degree of the child's presence in stables and contact with farm animals have been shown to predict endotoxin levels in the mattresses of these children (4). Several studies suggest, that high endotoxin levels in mattress or floor dust of children are associated with a reduced risk for atopic diseases (5–8).

Yet, children in rural environments are not only exposed to farm animals but have frequent contact with pets as well. The role of pet exposure in the development of asthma and allergy is still controversial. An increasing number of studies, including a series of cohort studies support the notion of a protective 'pet effect' (9–13). Others, however, found an increased risk of sensitization associated with pet exposure (14–16) or no association between cat allergen exposure early in life and the occurrence of asthma (17). It has been suggested that the 'protective effect' of pet keeping on asthma and allergy might be the result of a modified T-helper (Th)-2 cell response as exposure to cat allergen has been shown to produce IgG and IgG4 antibody response without sensitization or risk of asthma (18). Others have speculated that the inverse relation between pet exposure and the development of atopic sensitization, hay fever and asthma might be explained by the higher endotoxin levels found in homes where cats and dogs are kept (19). This view has been challenged by a recent longitudinal study from the US (20), reporting exposure to high levels of cat allergen and having a dog in the home to be associated with decreased risks for wheezing, independent of the effect of endotoxin in house dust.

The Allergy and Endotoxin (ALEX) study (5, 21) including school-aged children from rural areas of Germany, Switzerland and Austria provides a good

opportunity to evaluate these hypotheses as we not only measured endotoxin and the major allergen of cat dander, Fel d1, in the mattresses of the children but ascertained the details of timing, frequency, and intensity of children's exposure to pet as well as farm animals in a interview with the child's mother. In addition to specific IgE measurements in the serum samples of the children, IgG4 measurements were performed as well.

The aim of the present analysis was to evaluate the role of contact with cats or to dogs in this rural, partially farming population on the occurrence of asthma and allergy and to examine whether contact with farm animals, increased levels of cat allergen or endotoxin in house dust might explain the pet effect.

## Materials and methods

### Study population

The cross-sectional survey ALEX (Allergy and Endotoxin) was performed in rural areas of Austria, Germany and Switzerland as previously described (5, 21). In brief, 2618 of 3504 (74.7%) participating families consented to the measurement of specific IgE and IgG4 in their children's serum, and to the collection of dust samples from the respective child's mattress. Of these 1406 (53.7%), all children from farming families, all children from nonfarming families who reported regular contact with a farm environment, and a random sample of children from nonfarming families who were not exposed to a farm environment were invited to participate ($n = 901$). The final analysis was restricted to 812 children (319 farmers' children and 493 children from nonfarming families) with complete data and similar ethnic origin – as German, Austrian and Swiss nationality. The study population was representative of the rural population in the participating areas (21).

Approval to conduct the study was obtained from the three local ethics committees for human studies and from the principals of the schools attended by the children. Written informed consent was obtained from the parents of all children.

### Parents' questionnaire and interview

Demographic factors, potential explanatory and confounding factors, and the prevalence of diseases and symptoms were assessed by a questionnaire given to the parents that included the questions of the International Study of Asthma and Allergies in Childhood (ISAAC) (22), as described previously (21). In an interview with the parents as part of the home visit, we obtained details of the timing of the child's exposure to pets and to farming activities. Exposure to pets was defined as contact with cats only, to dogs only and to dogs and cats. As few children were reported to have contact with dogs only, a variable combining any contact with dogs was used in the regression analyses. Contact with pets during the past 12 months (current) was distinguished from pet contact during the first year of life. Pet avoidance because of asthma or someone having an allergy in the family was assessed by asking 'Did you ever implement any action to reduce allergen levels in your house? If yes, did you remove any pets?' Exposure to farming during the first year of life was defined as exposure to stables during the first year of life, consumption of milk directly from the farm during the first year of life, or both. The child's current exposure to farm animals was expressed as a 'stable activity score', combining regular contact with stable animals (No/Yes) and the frequency of staying in a stable (never/rarely or several times per week/at least daily). The score ranged from 0 (no contact with stable animals and never in a stable) to 3 (regular contact with stable animals and daily stable visit).

### Dust collection

We collected with ALK filters (ALK, Copenhagen, Denmark) by vacuuming every mattress for 2 min/$m^2$ of surface area after removal of all sheets apart from mite impermeable mattress encasings and plastic sheets following the ISAAC phase II protocol (http://isaac.auckland.ac.nz). The material obtained was divided into two parts for the measurement of endotoxin and allergen content. All seven field workers (three in Germany, two in Austria, and two in Switzerland) were trained to ensure uniformity in sampling, using a standardized protocol.

### Endotoxin and allergen analysis

Of the dust samples, one was stored at room temperature and transported in endotoxin-free vials within 1 week after collection to the central laboratory of the Institute for Occupational and Environmental Medicine of the University of Munich, Germany. Endotoxin content was measured by a kinetic limulus assay, and the details are given in Ref. (4). All endotoxin levels were within the limits of detection of the assay. The second dust sample was frozen at −20°C for at least 2 days and then transported to the Allergy Laboratory of the Department of Paediatric Pneumology and Immunology University Children's Hospital, Charite, Berlin, Germany (S. Lau), and stored at 4°C until it was analysed for *Felis domesticus* Fel d1 as previously described (14). The lower limit of detection was 16 ng Fel d1/g of dust. For allergen levels below the detection limit (0.2% of all measures), half the detection limit was considered.

### Testing for specific IgE and IgG4 in serum

The level of specific IgE against airborne allergens in all serum samples was measured by fluorescence enzyme immunoassay (Pharmacia CAP System; Pharmacia Diagnostics AB, Uppsala, Sweden) in a central laboratory (Allergy Laboratory of the Department of Paediatric Pneumology and Immunology University Children's Hospital, Charite, Berlin, Germany). The serum concentration of specific IgE raised against a panel of aeroallergens (mixed-grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander and *Cladosporium herbarum*) was measured in all samples by fluorescence enzyme immunoassay (SX1, CAP; Pharmacia). In children with a positive SX1 result, responses to specific allergens (grass pollen, birch pollen, *D. pteronyssinus*, and cat dander) were measured. We defined atopic sensitization as at least one positive specific IgE test result of 0.35 kU/l or greater for the eight aeroallergens but additionally considered a cut-off level of 3.5 kU/l [corresponding to radioallergosorbent test (RAST) class 3 or higher] in the analyses.

IgG4 antibodies specific to cat dander were measured using fluorescent-enzyme immunoassay (FEIA) (CAP; Pharmacia) and diluting samples 1 : 10 in diluents provided with the kit. All measurements were conducted in a central laboratory (Respiratory Center, Tucson, AZ, USA). Allergen-specific IgG4 were expressed as microgram per litre. The limit of sensitivity of the assay was 15 μg/l, and undetectable samples (1.4% of all measures) were assigned a value of 10 μg/l. The mean IgG4 antibodies to Fel d1 was

121 kU/l (SD 170). IgG4 antibody levels were dichotomized into high and low levels using the median of 86.3 kU/l as cut-off.

### Statistics

Chi-square statistics, geometric means, 95% confidence intervals, and *t*-test on log-scale data were calculated to describe differences in exposure characteristics of children exposed or not exposed to pets. Logistic regression models were performed to study the association between pet exposure, and asthma, hay fever and atopic sensitization. In our basic model we adjusted for sex, age, study area, family history of asthma or hay fever, parents' educational levels, number of older siblings, and pet avoidance because of asthma or allergies in the family. In addition, sensitivity analyses of the association between current pet exposure and allergy outcomes were performed by sequentially including pet exposure in the first year of life, endotoxin levels in mattress dust (log-transformed, $EU/m^2$), cat allergen levels in mattress dust (log-transformed, μg Fel d1/g), farming exposure in the first year of life, and the child's current stable activity. To evaluate a potential interaction between pet exposure and the child's farming status, we ran the basic model for farmers' children and nonfarmers' children separately. We also included an interaction term for pet exposure and farming status in the logistic regression analyses and calculated likelihood ratio tests (LRT) for interaction. Besides including a variable for avoidance of pets because of asthma or allergies in the family into the basic model, we also restricted the analyses to families who did not report pet avoidance to evaluate the effect of residual confounding. As families with a history of asthma or hay fever might be less prone to keep pets, the analyses were repeated restricting the sample to children without a family history of asthma or hay fever. Data analysis was performed with STATA 8 (Stata corporation, College Station, TX, USA). A two-sided alpha level of 5% was considered significant.

Table 1. Exposure characteristics of the study population

| | Farmers' children [$n = 319$; $n$ (%)] | Nonfarmers' children [$n = 493$; $n$ (%)] | $P$-value* |
|---|---|---|---|
| Contact with pets in the first year of life | | | |
| No | 109 (34.2) | 330 (67.1) | <0.001 |
| Cats only | 92 (28.8) | 97 (19.7) | |
| Dogs only | 26 (8.2) | 25 (5.1) | |
| Dogs and cats | 92 (28.8) | 40 (8.1) | |
| Current contact with pets | | | |
| No | 30 (9.4) | 179 (36.5) | <0.001 |
| Cats only | 129 (40.6) | 166 (33.8) | |
| Dogs only | 12 (3.8) | 36 (7.3) | |
| Dogs and cats | 147 (46.2) | 110 (22.4) | |
| Pet avoidance due to asthma or allergies in the family | 2 (0.6) | 12 (2.4) | 0.034 |
| Exposure to farming in the first year of life† | 274 (85.9) | 203 (41.2) | <0.001 |
| Current exposure to farm animals‡ | | | |
| 0 | 4 (1.3) | 141 (28.8) | <0.001 |
| 1 | 22 (6.9) | 164 (33.5) | |
| 2 | 138 (43.5) | 162 (33.1) | |
| 3 | 153 (48.3) | 22 (4.5) | |
| Current mattress endotoxin levels (EU/mg of dust) [geometric mean (95% CI)] | 37.8 (35.5–40.2) | 22.8 (21.5–24.1) | <0.001 |
| Current mattress cat allergen levels (μg Fel d1/g of dust) [geometric mean (95% CI)] | 5.41 (4.40–6.63) | 5.80 (4.61–7.30) | 0.673 |

The values are $n$ (%).

* $P$-value for farmers' children *vs* nonfarmers' children.

† First stay in stable and farm milk consumption in the first year of life.

‡ Stable activity score: regular contact with farm animals (no, yes) and frequency of staying in stable (never, at least weekly, at least daily).

## Results

The study population consisted of 319 farmers' children with a mean age of 9.42 years (SD 1.63), and 493 children from nonfarming families, living in the same rural areas with a mean age of 9.49 years (SD 1.60). Fifty-one per cent of all children were boys. Farmers' families and nonfarming families of the respective study area differed in many socio-demographic and lifestyle aspects as previously described (1, 4, 23). Table 1 presents the exposure characteristics of the study population, stratified by the child's farming status. Farmer's children were significantly more likely to have contact with pets currently and during the first year of life and to be involved in farming activities. However, because of our selection procedure, contact with the farm environment was also quite common among nonfarmers' children. Only 1.7% (14/812) of the families reported avoidance of pets because of someone having allergy in the family. Although higher levels of endotoxin were found in mattress dust of farm children, no significant differences were observed with respect to Fel d1 levels.

Figure 1A,B displays the levels of Fel d1 and endotoxin in mattress dust of school-aged children exposed to pets in the first year of life and currently. Contact with cats in the first year of life and currently specifically increased Fel d1 levels, whereas endotoxin levels were associated with pet keeping, in general.

The results of the basic multivariate regression model examining the association between pet exposure and allergy endpoints are given in Table 2. Exposure to cats only during the first year of life tended to be associated with a reduced risk for wheezing ($P = 0.050$), sensitization to cat allergen (RAST class ≥ 1) ($P = 0.061$), and grass pollen sensitization (RAST class ≥ 3) ($P = 0.021$), but not with overall sensitization (SX1). There was also a negative association with diagnosed asthma in sensitized children, however, because of the reduced numbers, the confidence intervals were wide. Exposure to dogs in the first year of life was not associated with any of the clinical outcomes, but was inversely associated with sensitization to grass pollen (RAST class ≥ 3) ($P = 0.039$) and non-significantly with cat dander. Current contact with dogs, however, was inversely associated with most of the clinical outcomes as well as with sensitization to cat allergen (RAST class ≥ 1) ($P = 0.047$) and grass pollen (RAST class ≥ 3) ($P = 0.029$). Current exposure to cats

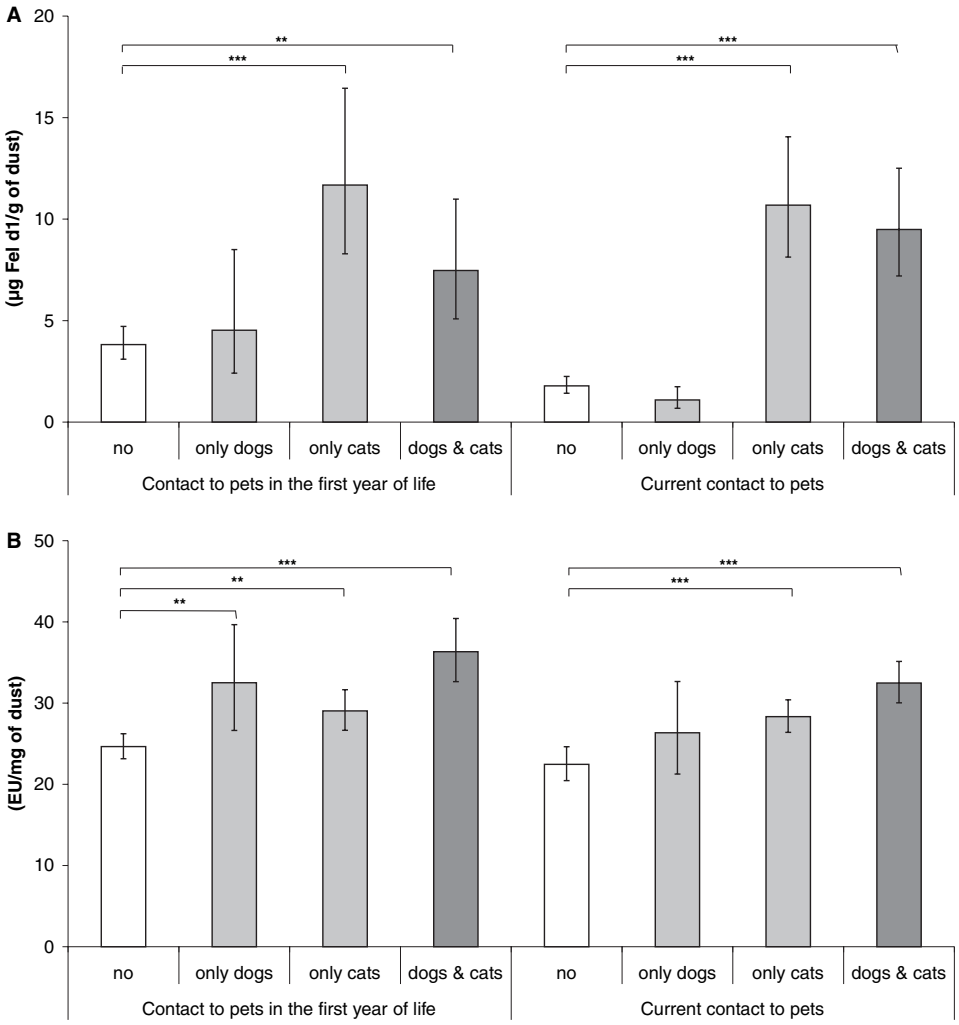


*Figure 1.* Mattress cat allergen (µg Fel d1/g) and endotoxin (EU/mg) levels (geometric means and 95% CI) of children with contact with pets.

only showed no association with hay fever and diagnosed asthma. Yet, an inverse association was observed for diagnosed asthma in sensitized children ($P = 0.035$), for wheezing ($P = 0.025$) and sensitization to grass pollen (RAST class ≥ 3) ($P = 0.027$). IgG4 against cat allergen was slightly but nonsignificantly increased in children with exclusive early ($P = 0.261$) and current ($P = 0.131$) contact with cats. When the analyses were repeated excluding all children from families who reported pet avoidance, or restricted to children without a family history of atopic diseases, the results remained essentially the same (data not shown).

To evaluate the effect of a series of variables that might explain or confound the association between current exposure to dogs and allergy outcomes we added these variables sequentially to the basic model as shown in Table 3. Adjustment for pet exposure in the first year of life strengthened the inverse association with hay fever and diagnosed asthma but attenuated the association with sensitization to grass pollen. Inclusion of current endotoxin and cat allergen exposure did not greatly attenuate the association between current contact with dogs and allergic symptoms. However, adjustment of farming exposure in the first year of life, and particularly current exposure to stable and farm animals reduced the effect of dog exposure for most outcomes, although the inverse association between dog exposure and diagnosed hay fever and diagnosed asthma remained statistically significant. The analyses were then repeated for the subsamples of children from farming and nonfarming families. Among farmers' children current exposure to dogs was associated with a reduced risk for all investigated health endpoints, although due to the small sample size the confidence intervals were very wide. Among

Table 2. Associations between pet exposure and asthma and allergy

| | Raw prevalence [*n* (%)] | Exposure to pets in the first year of life* [adj. OR (95% CI)§] | | Current exposure to pets† [adj. OR (95% CI)§] | |
|---|---|---|---|---|---|
| | | Cats only | Dogs‡ | Cats only | Dogs‡ |
| Diagnosed hay fever | 65 (8.0) | 0.69 (0.34–1.41) | 0.64 (0.30–1.36) | 0.84 (0.45–1.57) | 0.26 (0.11–0.57) |
| Current hay fever symptoms | 81 (10.1) | 0.97 (0.51–1.85) | 0.94 (0.50–1.79) | 0.86 (0.47–1.56) | 0.39 (0.20–0.78) |
| Diagnosed asthma | 57 (7.0) | 0.85 (0.40–1.81) | 0.90 (0.39–2.05) | 0.85 (0.43–1.66) | 0.29 (0.12–0.71) |
| Asthma in sensitized | 39 (4.9) | 0.58 (0.21–1.59) | 0.64 (0.21–1.90) | 0.39 (0.16–0.93) | 0.22 (0.08–0.63) |
| Current wheeze | 79 (9.9) | 0.48 (0.23–1.00) | 0.76 (0.39–1.46) | 0.49 (0.26–0.92) | 0.57 (0.31–1.04) |
| Wheezing in sensitized | 44 (5.8) | 0.60 (0.23–1.55) | 0.99 (0.43–2.30) | 0.45 (0.19–1.04) | 0.56 (0.25–1.27) |
| Atopic sensitization SX1 | | | | | |
| RAST class ≥ 1 | 289 (35.6) | 0.94 (0.63–1.39) | 0.77 (0.51–1.16) | 1.27 (0.84–1.91) | 0.91 (0.60–1.38) |
| RAST class ≥ 3 | 171 (21.1) | 0.81 (0.50–1.30) | 0.72 (0.44–1.19) | 0.94 (0.59–1.51) | 0.78 (0.48–1.26) |
| IgE against grass allergen | | | | | |
| RAST class ≥ 1 | 208 (25.7) | 0.87 (0.56–1.34) | 0.75 (0.48–1.17) | 1.01 (0.65–1.56) | 0.75 (0.48–1.18) |
| RAST class ≥ 3 | 119 (14.7) | 0.50 (0.28–0.90) | 0.55 (0.31–0.97) | 0.55 (0.32–0.94) | 0.55 (0.33–0.94) |
| IgE against cat allergen | | | | | |
| RAST class ≥ 1 | 69 (8.5) | 0.47 (0.21–1.04) | 0.59 (0.27–1.29) | 0.76 (0.39–1.47) | 0.48 (0.23–0.99) |
| RAST class ≥ 3 | 20 (2.5) | 0.42 (0.10–1.73) | 0.39 (0.08–1.92) | 0.51 (0.16–1.71) | 0.34 (0.09–1.35) |
| IgG4 levels against cat allergen above Median | 398 (49.8) | 1.23 (0.86–1.76) | 1.07 (0.73–1.55) | 1.34 (0.92–1.95) | 1.17 (0.80–1.70) |

* Reference category: no contact with cats or to dogs in the first year of life.

† Reference category: no current contact with cats or to dogs.

‡ Combined category of dogs and cats + dogs only.

§ Adjusted for sex, age, study area, family history of asthma or hay fever, parent's education level, number of older siblings, and pet avoidance due to asthma or allergies in the family.

nonfarmers' children, current exposure to dogs remained inversely related to hay fever and hay fever symptoms and sensitization to grass pollen, whereas associations with wheeze, asthma and sensitization to cat dander were much weaker. The interaction term between dog exposure and farming status was of borderline significance for diagnosed asthma (LRT; $P = 0.058$) and for wheezing (LRT; $P = 0.159$) but nonsignificant for sensitization to cat dander (LRT; $P = 0.415$).

## Discussion

The present analyses examined the role of pet keeping in a rural, partially farming population on the occurrence of childhood asthma and allergy. In contrast to several other recent publications (9, 11, 12, 24–26) no clear associations between pet exposure in the first year of life and asthma or allergy endpoints were observed. Early exposure to a farming environment which has previously been shown to confer protection from the development of asthma and allergy (21) remained independently associated with a reduced risk of hay fever, asthma and atopic sensitization. Current contact with dogs was associated with a reduced risk of hay fever, asthma, wheezing, and sensitization to cat allergen and grass pollen. The inverse association between exclusive exposure to cats was limited to wheezing, atopic asthma and grass pollen sensitization. The stronger protective effect associated with dog contact may simply reflect exposure to more than one pet as in most cases exposure to dogs coincided with contact with cats as well. In a recent prospective cohort study (11) a reduced risk of allergic sensitization was only observed in children exposed to two or more dogs or cats in the first year of life. We cannot determine whether this interpretation also holds true for the present study because the number of pets a child was exposed to has not been recorded.

Several studies have shown that keeping a dog is associated with higher indoor endotoxin levels (4, 19) and it has been postulated that this higher microbial exposure might explain the 'protective' effect of dog on asthma and allergy. However, when current indoor endotoxin levels were added to the regression models as a potential explanatory variable, the inverse association between dog exposure, asthma and hay fever was not greatly affected. Similarly, a recent longitudinal study from the US found exposure to dog to be associated with a reduced risk of wheezing in young children independent of house dust endotoxin levels (20). These findings support the notion, that the effects of pet exposure and endotoxin on allergic outcomes work through different albeit unknown immunological mechanisms. It has been postulated that the observed inverse association between exposure to cat allergen and asthma may result from a modified Th2 immune response, inducing IgG4 antibodies which are not associated with an increased risk of asthma (18). Although, in the present study, current and early contact with cats was associated with a decreased risk of wheezing and atopic asthma, no significant increase in IgG4 levels

Table 3. Sensitivity analysis of the association between current contact with dogs* *vs* asthma and allergy outcomes

| | Diagnosed hay fever | Current hay fever symptoms | Diagnosed asthma | Current wheeze | SX1, RAST ≥ 3 | IgE grass, RAST ≥ 3 | IgE cat, RAST ≥ 1 |
|---|---|---|---|---|---|---|---|
| Basic models† (BM) | 0.26 (0.11–0.57) | 0.39 (0.20–0.78) | 0.29 (0.12–0.71) | 0.57 (0.31–1.04) | 0.78 (0.48–1.26) | 0.55 (0.33–0.94) | 0.48 (0.23–0.99) |
| BM + pet exposure in the first year of life | 0.23 (0.09–0.58) | 0.31 (0.14–0.67) | 0.17 (0.06–0.51) | 0.59 (0.29–1.18) | 0.85 (0.50–1.44) | 0.67 (0.37–1.20) | 0.50 (0.22–1.15) |
| BM + current mattress endotoxin level | 0.24 (0.10–0.61) | 0.42 (0.20–0.90) | 0.31 (0.12–0.83) | 0.54 (0.27–1.07) | 0.80 (0.48–1.33) | 0.59 (0.34–1.05) | 0.59 (0.28–1.27) |
| BM + current mattress cat allergen level | 0.26 (0.11–0.63) | 0.34 (0.16–0.74) | 0.26 (0.10–0.67) | 0.48 (0.25–0.94) | 0.78 (0.46–1.34) | 0.64 (0.35–1.16) | 0.51 (0.23–1.14) |
| BM + farming exposure in the first year of life‡ | 0.31 (0.14–0.71) | 0.45 (0.23–0.90) | 0.35 (0.14–0.85) | 0.64 (0.34–1.19) | 0.92 (0.56–1.50) | 0.71 (0.41–1.24) | 0.52 (0.25–1.10) |
| BM + current stable activity§ | 0.42 (0.18–0.97) | 0.57 (0.28–1.18) | 0.29 (0.12–0.74) | 0.70 (0.36–1.36) | 0.95 (0.56–1.60) | 0.79 (0.45–1.41) | 0.66 (0.31–1.43) |
| BM for farmers' children ($n = 319$) | 0.28 (0.03–2.83) | 0.48 (0.08–2.81) | 0.03 (0.00–0.47) | 0.19 (0.04–0.86) | 0.64 (0.22–1.89) | 0.54 (0.14–2.01) | 0.22 (0.04–1.33) |
| BM for nonfarmers' children ($n = 493$) | 0.34 (0.14–0.82) | 0.46 (0.21–1.02) | 0.51 (0.19–1.35) | 0.93 (0.46–1.87) | 0.86 (0.48–1.54) | 0.64 (0.34–1.20) | 0.69 (0.30–1.57) |

The values are adj. OR (95% CI), adjusted for sex, age, study area, family history of asthma or hay fever, parent's education level, number of older siblings, pet avoidance because of asthma or allergies in the family, and current contact with cats only.

* Reference category: no current contact with cats or with dogs.

† Basic models from Table 2 additionally adjusted for potential confounding factors.

‡ First stay in stable and farm milk consumption in the first year of life.

§ Regular contact with farm animals (no, yes) and frequency of staying in stable (never, at least weekly, at least daily).

against cat dander was observed. Thus, the proposed mechanism behind the 'protective' effect of cat on asthma does not seem to be of importance in this study population. The hypothesis of a modified Th2 immune response has also been challenged by a recent Swedish study indicating that although all children had an immune response to cat, the presence of IgG4 antibodies was not associated with less allergy (27).

Deliberate avoidance of pets might be an other explanation for the protective 'pet effect' observed in many studies. In the present study, only a small proportion of families (1.7%) reported that pets had been given away because of presence of allergic disease in one of the family members. Adjustment for pet avoidance did not influence our results. Yet, as many families with a history of allergic diseases are counselled to avoid keeping pets, we cannot discount an unmeasured bias in the association between pet exposure and allergic diseases. A Swedish study reported that one-fourth of the Swedish population reported avoidance behaviour towards pets and concluded that this might explain the protective effect of exposure to pets during childhood on asthma and allergies (28). When we restricted our sample to children without a family history of asthma and allergy to address the issue of primary pet avoidance we still found an inverse relation between contact with dogs and allergy outcomes making primary pet avoidance an unlikely explanation for the observed pet effect in our population. Nevertheless, the discrepancy between the effects on asthma and allergy observed in the present study for current and early pet exposure might indicate parental avoiding behaviour which was not measured in the questionnaire. To address this question we compared the 30 children (8%) who had contact with pets in the first year of life but no current pet contact with children who had early and current contact with pets and found the latter to be significantly less likely to suffer from asthma and to be sensitized to grass pollen and cat dander. Thus, the association between current pet contact, asthma and allergic sensitization might at least partially be explained by a sort of pet avoidance of which parents are not aware.

Most but not all of the inverse association between current dog exposure and allergic sensitization, wheezing and hay fever symptoms was attenuated and no longer statistically significant when current contact with stable animals was introduced into the regression models. Only the association with diagnosed hay fever and asthma remained statistically significant. Conversely, current exposure to endotoxin in mattress dust and early farm exposure remained independently associated with a reduced risk of asthma and allergy as previously reported (5, 21).

In addition, a stronger association between current dog contact and asthma or sensitization to cat dander was observed in farmers' children. In nonfarmers' children only the inverse relation between contact with dogs and hay fever remained statistically significant. We can only

speculate about the reason for such differential effects as the mechanism behind a protective effect of pet exposure is still unknown. Pet contact of farm children might be more intense as many more cats and dogs are usually found on a farm compared with a nonfarming household. It is also conceivable that compared with pets from nonfarming environments, pets living on farms carry a broad spectrum of microbes leading to a different and more intense stimulation of a farm child's immune system.

Based on the present analyses we conclude that although pet exposure was very frequent in this rural population, the inverse relation between current dog contact, asthma and allergy was mostly explained by simultaneously occurring exposure to stable animals or restricted to farm children. In addition, a subtle form of pet avoidance may contribute to the protective pet effect. However, the cross-sectional design of our study limits our ability to draw firm conclusions. Prospective studies are needed to definitely clarify the role of pet exposure in a farming environment.

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# General discussion and outlook

In the following, the research questions put forward in chapter I are answered in form of short summaries of the main findings. Specific findings of all papers included in this thesis have been discussed in detail in the respective chapters.

## Summary of the main findings

1. *Is there an association between indoor endotoxin exposure and the prevalence of childhood asthma or allergic diseases, independent of the observed effect of early farming exposure?*

Higher levels of indoor endotoxin exposure were associated with reduced allergen sensitisation, decreased prevalence of hay fever, atopic asthma, and atopic wheeze in a dose-dependent manner. This effect was observed in addition to, and independent of, the effect of first-year-in-life exposure to farm characteristics, assessed as contact to farm animals and farm milk consumption. These associations were equally strong among children from farming families and among children from non-farming families, indicating that even lower levels of endotoxin, as encountered in non-farming environments, may favourably influence the risk of atopic diseases in childhood. TNF-α, IL-12, and IL-10 production by leukocytes was inversely associated with indoor endotoxin levels, indicating a down-regulation of immune responses in exposed children.

2. *Which home and lifestyle characteristics of farm and non-farm families determine the indoor endotoxin levels?*

3. *Are indoor endotoxin levels associated to endotoxin levels found in settled dust in stables?*

Endotoxin levels in stables were not correlated with the amount of endotoxin measured indoors, but a dose-dependent association between the child's activity on the farm and indoor home endotoxin levels was observed, both in farmers' and in non-farmers' children. In non-farmers' children, pet keeping and lower frequency of floor cleaning contributed additionally to increased indoor endotoxin levels. In farmers' children, parental farm activities (full time more than part time farming), the study area (Austria and Switzerland more than Germany), and younger age of the children were independently associated with higher indoor endotoxin

levels. Thus, assessment of children's individual exposure to their microbial environment requires measurements of indoor dust exposure since information about the number of stable animals or stable endotoxin levels is insufficient to predict indoor exposure to endotoxin.

4. *Which farm and stable characteristics determine endotoxin levels in stables?*

Endotoxin levels in stables increased with the number of cattle, but only up to the highest quartile. This might be due to the fact that farms with larger numbers of cattle usually have modern stables with ventilation systems better able to reduce airborne endotoxin levels. Similarly, the positive association between hay feeding and endotoxin levels compared to feeding of mainly silage might be interpreted as indicating more traditional farming. Horse keeping was also associated with increased endotoxin levels. Farmers who kept horses were more likely to accommodate additional farm animals such as pigs, sheep or goats. This predictor might also be a surrogate measure for traditional dairy farming in German speaking partially mountainous areas.

5. *Are farmers' children exposed to higher muramic acid levels than their rural peers from non-farm families?*

6. *Which home and lifestyle characteristics determine the indoor muramic acid levels?*

Children's mattress' muramic acid level were found to be significantly higher in farmers' children than in non-farmers' children. Muramic acid level analogous to endotoxin level might be an indicator for exposure to microbial agents. There was a partial correlation between endotoxin and muramic acid levels, indicating that both substances are markers for the exposure to micro-organisms. Independent of being a farmers' child, mattress dust from homes heated with wood or coal showed increased muramic acid levels, and the levels in dust from less frequently cleaned mattresses were also higher.

7. *Is there an association between indoor muramic acid levels and the childhood prevalence of asthma or allergy, independent of the indoor endotoxin exposure?*

Independent of the endotoxin exposure, increasing muramic acid levels in mattress dust was associated with a lower frequency of current wheeze but not with atopic sensitisation or hay fever. After stratifying the group of wheezing children into groups of children with and without atopic sensitisation, the protective effect of muramic acid exposure on wheeze and diagnosed asthma was more pronounced in non-sensitised children.

8. *Is there a relation between exposure to pets or to their allergens and childhood asthma, hay fever or atopic sensitisation in the ALEX population?*

9. *Can the 'pet effect' be explained by current endotoxin or pet allergen exposures, or does the early or current farming exposure influence these associations?*

No clear pattern of associations between pet exposure in the first year of life and childhood asthma or allergy were observed. Current contact to dogs was inversely associated with diagnosed hay fever, asthma, and specific sensitisation to grass pollen and to cat allergen. Early and current exposure to cats – but not to dogs – was associated with lower frequency of wheeze and grass pollen sensitisation. The stronger protective effect associated with dog contact may reflect exposure to more than one pet as in most cases exposure to dogs coincided with contact to cats. None of these inverse associations were greatly affected by additionally taking into account the endotoxin levels or cat allergen exposure. However, if the logistic models were additionally adjusted for current contact to stable animals or for farming exposure in the first year of life, only inverse associations with diagnosed hay fever and asthma remained statistically significant. In addition, a stronger association between current dog contact and asthma was observed in farmers' children.

In conclusion, it has to be mentioned that although pet exposure was frequent in this rural population, the inverse relation between current pet contact, asthma and allergy was mostly explained by simultaneously occurring exposure to stable animals or was restricted to farmers' children.

## General aspects of the results from the ALEX study

This discussion includes more general aspects of the ALEX study results, and addresses major limitations of the study. Finally, possible scientific questions arising from the ALEX study are outlined.

### Exposure to indoor microbial agents

Farming environments are characterised by containing a large variety of micro-organisms, as well as a diversity of moulds and fungi. The ALEX study is, so far, the only epidemiological study in farming environments that has included objective markers of exposure to micro-organisms, namely endotoxin and muramic acid. We found endotoxin in abundance not only in stables but also in indoor environments of farm houses ((57) and chapter III, Table 1). Results from the ALEX study suggest that exposure to indoor endotoxin decreases the risk of hay fever, atopic sensitisation, and atopic asthma in childhood, thus particularly affecting the atopic phenotypes (chapter II, Table 2). In contrast, levels of muramic acid were not related to hay fever and atopic sensitisation but were inversely related to non-atopic wheeze and asthma in the ALEX population (chapter IV, Figure 3). This different effect spectrum suggest that different micro-organisms might contribute to the 'farm effect'.

It has to kept in mind that other bacterial components, that are known to affect immune responses – non-methylated cytodine phosphate guanosine dinucleotide motifs (CpG DNA), heat shock protein (HSP), cell-wall components from atypical mycobacteria, lipoteichoic acid, or cell-wall components of fungi, such as β-1,3-glucan or extracellular polysaccharides (EPS) (62-65) –, were not measured in the ALEX study. Concentrates of some of these components were correlated with endotoxin in house dust. Endotoxin and muramic acid may thus be surrogate markers of a much broader spectrum of microbial compounds.

An other limitation of the chosen exposure assessment was that it was based on measurements made at only one point in time of the child's life. Even though these point measures were validated with some repeated dust sampling from the same locations (chapter III, measurements of endotoxin levels), little information about seasonal variation, dust sampling variation, or other influences on the exposure levels is known. To confirm the inverse association of exposure to endotoxin or other markers of the microbial burden to respective health outcomes, longitudinal studies with repeated measurements during the child's life are necessary. Such a study design would offer the possibility of a more detailed exposure assessment.

### Effects of micro-organisms were not restricted to farmers' children

The protective effects of dose-dependent exposure to endotoxin and to muramic acid were also observed among children from non-farming families, indicating that a protective effect against the development of childhood asthma and atopic diseases may be attained at the relatively low levels of exposure observed in non-farming environments. This contention is supported by other peer-reviewed studies, that show that indoor exposures to endotoxin are inversely related to atopic sensitisation in urban environments also (66, 67).

One point of discussion has been that the observed 'farm effect' could be a consequence of a selection process in the farming population. However, in the ALEX study, adjustment for giving up farming during the last or last but one generation due to allergy in the family did not attenuate the 'farm effect'. This variable might not reflect the whole dimension of the question, as the protective factors 'contact to stable animals, consumption of farm milk' are highly correlated with being a farmers' child. The observed dose-dependent protective effect of exposure to micro-organisms as a proxy for the contact to a farming environment among non-farmers' children is a additional valid argument against a genetic selection in farmers' children.

### Timing of exposure

Exposure to stables and consumption of farm milk during the first year of life conferred an additional protective effect over and above that of the current endotoxin exposure (chapter II, Table 3). Thus, exposure to bacteria and to other typical farming-associated characteristics early in life, during the development of the immune system, seems to be important in providing protection against the development of allergic disease. One important limitation of the ALEX study is its cross-sectional design. It was impossible to detect causal relationships between certain environmental exposures and the various phenotypes of childhood asthma and allergy. We assessed the exposure to endotoxin at the same time as the prevalence of health outcomes, but retrospective information was gathered with regard to exposure to stables and farm milk consumption.

The standards of epidemiologic evidence offered 1965 by Hill (68), such as the strength of the association, consistency of the findings, specificity of the effects, temporality, a dose-response relationship, plausibility, coherence of the interpretation, experimental evidence, and analogy are very difficult to achieve in any scientific field, and they include many exceptions and reservations (69). The only obligatory condition is temporality, which refers to the neces-

sity that a cause precedes the effect in time. In the ALEX study attempts were made to consider the timing of exposure, but retrospective assessment of the relevant factors are vulnerable to recall bias. To address effects of timing against microbial exposure early in life, it is necessary to establish studies in birth cohorts and follow individuals from before the development of the immune system to the periods of development of allergic diseases.

### Route of exposure

In the ALEX study endotoxin was measured in two indoor home environments; dust from children's mattresses and the dust from the living room floor as a proxy for the exposure to micro-organisms. In chapter II and IV we focused on the measures in mattress dust, since children come into close contact with the microbial environment of their beds while sleeping. But with this exposure assessment we are not able to make a conclusion in respect of the route of exposure. We cannot disentangle whether the internal exposure depends on the exposure to inhaled parts of micro-organisms, or whether the child took the relevant load through licking contaminated fingers or toys. Further, we showed an independent protective effect of farm milk consumption in the first year of life on atopic sensitisation in the ALEX study. As farming families usually drink their self-produced milk often unpasteurised, the relevant route of exposure might also be ingestion of non-infectious parts of micro-organisms through home-made foods.

However, the correlations of mattress endotoxin levels with the *in vivo* measured leukocytes activity (chapter II, Figure 2) give a hint that the internal exposure to endotoxin might be related to measured mattress dust endotoxin levels. However, the relevant route of exposure to micro-organisms that protects children from developing asthma or allergies remains unclear and needs to be investigated with further studies.

### Exposure to pets

The ALEX analyses of the child's early or current exposure to pets showed that additionally adjusting for farming characteristics attenuated the observed negative associations on hay fever, specific sensitisation to grass and cat allergens, and diagnosed asthma (chapter V, Table 3). However, the current endotoxin exposure did not influence these effects. This result suggest, that the protective effects of pet keeping observed in other peer-reviewed studies may be masked by frequent contact to farming environments in this rural population.

### The innate immunity

In a subset of the ALEX study population it was shown that blood cells of farmers' children express higher amounts of CD14 and TLR-2 compared with non-farmers' children (70). An increased expression of CD14 as well as TLR-2 was also observed *in vitro* in human leukocytes after treatment with LPS (71), which suggested that the differences found *in vivo* between farmers' children and non-farmers' children mirror different degrees of exposure to such microbial components in the environment.

It would be interesting to investigate whether markers of a different spectrum of the exposure against micro-organisms, in particular muramic acid or others, might influence different gene expressions of innate immunity receptors, or whether endotoxin and muramic acid may activate different cells of the innate signalling way. Some components of the signalling pathways downstream of TLRs are of recent scientific interest: IRAK-4 and IRAK-M have been found to regulate TLR dependent functions (72, 73). IRAK-4 was shown *in vivo* to enhanced susceptibility to pyogenic bacterial infections (74). SOCS-1, a molecule known to act as a negative regulator of cytokine receptor signalling, also negatively regulates LPS responses (75).

### Gene-environment interaction

The genetic dimension has to be kept in mind in the discussion of environmental exposure against microbial agents, because asthma and allergy have a strong genetic background. Susceptibility to various microbial exposures might depend on the make-up of different elements of the innate immune response. Polymorphisms in genes encoding these receptors might therefore modulate the protective effects observed in farming populations and thereby modify the exposure effects.

In an other subset of the ALEX population it has been found that a polymorphism in the gene encoding TLR-2 significantly interacts with a farming environment (76). Only farmers' children with a T allele for TLR-2-16934 were susceptible to the protective factors on the farm, whereas children homozygotic for the A allele had prevalence of asthma and atopy comparable with those of non-farmers' children. Further, among non-farmers' children, no effect of TLR-2 polymorphisms was seen. These results strongly point to a gene-environment interaction and suggest that genetic variation in the TLR-2 interacting with an environment rich in microbial products is a major determinant of the susceptibility to asthma and allergies

in farmers' children. Gene-environment interactions should be addressed in further studies, as the available data in the ALEX study was limited.

### Generalisation of the ALEX data

In a comment on the publication in chapter II, two researchers from the US misunderstood the extension of the hygiene hypothesis with epidemiologic studies involving farmers' children (77) as a trying to explain the global increase in the prevalence of asthma. Epidemiological studies with partially farming families do not primarily intend to explain this global increase but offer a unique opportunity to investigate the role of microbial compounds in the development of asthma and allergies. The rural regions involved in the ALEX study are quite similar with respect to farming; mainly livestock farming in a hilly region restrict the cultivation of a relatively small area, and due to climatic conditions, farm animals are mainly kept in stables during wintertime.

It is known from studies in New Zealand (78) and southern Europe (79), that the 'farm effect' is not confirmed in temperate climate zones. In these regions animals, on large farm holdings, stay outdoors throughout the year. Children's exposure to microbial products from farm animals might therefore be very different on such a farm as compared to a central European farm. Indeed, the New Zealand study reported that indoor home endotoxin levels of farming families were lower than those of non-farming families. Even in central Europe the 'farm effect' has to be confirmed in other studies.

## What scientific questions arise from the ALEX study?

Finally, some ideas of further scientific actions in the field of childhood asthma and allergy research and the use of farming environments as a 'human model' will be discussed. For future preventive action or therapy it will be crucial to understand the types of micro-organisms that might be protective against atopy, how diverse the microbial stimulation of the immune system has to be, and which route of exposure might be most relevant.

### Studies who may confirm the 'farm effect'

The protective effect of exposure to microbial agents on asthma and allergy from the ALEX study has to be confirmed in other rural areas in Europe and in longitudinal studies. The cross-sectional study PARSIFAL (Prevention of allergy – Risk factors for sensitisation in children related to farming and anthroposophic lifestyle) involved other rural areas in Switzerland, Austria and Germany, and additional to the ALEX study, rural areas from the Nether-

lands and from Sweden. The prospective PASTURE (Protection against Allergy: Study in Rural environments) study has already initiated as a birth cohort study to confirm the important role of timing of exposure to microbial agents proposed in the ALEX study. Infants from farming families and from non-farming families from Germany, Austria, Switzerland, France and Finland will be followed from birth until two years of age. In this study again other European areas except the German speaking areas are involved.

### Finding the relevant mixture of protective dust

As described above, endotoxin and muramic acid might be surrogate markers of a much broader spectrum of microbial compounds. One can expect an enormous biotope of different micro-organisms and fungi especially in animal farms. Because it is still unclear which microbial agents are responsible for the lower prevalence of asthma and allergy in farmers' children, and it seems unlikely to be a single agent, bacterium or mould, one possibility is to collect stable dust and treat it with different methods to preserve different active components of micro-organisms like (lipo)polysaccharides or oligosaccharides, (lipo)-teichoic acids, or (parts of) peptides. These fractions with different spectra of active compounds could be tested *in vitro* with human cell cultures or *in vivo* with established allergy mouse models. The different potential of each fraction to generate dendritic cells from monocytes, the activation of dendritic cells or Treg cells could be studied. Inhibition of allergic sensitisation or bronchial hyperresponsivness could be tested through mice studies. The most potent fractions may then be analysed in detail to find a relevant mixture of allergy preventing micro-organisms.

### Inhaled exposure of stable dust

Another possibility would be to study the relevant exposure route to different patterns of micro-organisms more precisely. The inhaled absorption of micro-organisms in farmers' children is assumed to be high in animal stables, and it depends on the type of stable (amount and type of animals, ventilation system) and the activity in the stable. In a pilot project within PASTURE airborne dust samples from different animal stables will be collected before, during and several time after foddering. It is known that the air contamination in stables increases substantially during agricultural operations (80), and different micro-organisms sediment with different rates. In an epidemiological study these exposure measurements could be related to the parental reports of the timing and duration of their child's visit to the stables.

### Other lifestyle patterns

As lifestyle factors related to farming strongly correlate, it is difficult to find subgroups with a different pattern of lifestyle factors with a potential to prevent from allergic diseases. Farmers' children are not only exposed to higher levels of micro-organisms, they also drink more unpasteurised milk and other home-made foods than non-farmers' children. Alm et al. showed 1999 (43) in a cross-sectional study, that children of families with an anthroposophic background had a lower prevalence of atopy compared with their peers from the same area. Members from the anthroposophic community cultivates a different lifestyle compared to other local families, and thus this group may be an other interesting scientific model for epidemiology. Potential explanatory factors related to the observed inverse association of atopy in anthroposophic children were less use of antibiotics early in life, less children with immunisation against measles, more children who had had measles, and more children who had consumed fermented vegetables, containing live lactobacilli.

While also nutritional behaviour changes have been identified in westernised lifestyles – the consumption of fresh food decreased, adipose consumption increased, and breastfeeding becoming more common (81, 82) –, the role of nutrition in the development of asthma and allergy is not yet clarified. Although dietary habits were distinctly different between farming and non-farming families in a Finnish study (83), they did not explain the 'farm effect'. Research is needed on the intriguing phenomenon that the consumption of yoghurt, unpasteurised milk or fresh vegetables might confer protection from atopy. It is important to know which components of these products are responsible for the observed protective effect. In a farming or in an anthroposophic community discrete, these questions may be difficult to answer, because of high colinearity with other lifestyle characteristics. That results in a small exposure range in the study group. The PARSIFAL study currently focuses not only on farming environments, but also children growing up in an anthroposophic environment. The role of diet in the development of childhood asthma and allergy will be a substantial component of this cross-sectional study.

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 pathogenic micro-organisms continue to be a serious public health problem, and the protective effect of a microbial environment on the development of asthma and atopy should be balanced against the benefits of established hygiene standards.

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# Curriculum vitae

Marco Waser, born on 27 June 1967 in Switzerland. Married, two children, native language is German, foreign languages are French, English, and Italian.

## Education and professional experience

1974-1983 Primary and secondary school in Zug, Switzerland

1983-1987 Apprenticeship as a precision mechanics draughtsman, at the 'Gewerblich industrielle Berufsschule Zug' (GIBZ) in Switzerland

1987-1991 Member of an engineering team for robotics and product development. Main task: Project management and design of a self-controlled machine to assembly Swiss Army knifes, for the Landis & Gyr AG in Zug

1988-1990 Part-time teaching course at the 'Schweizerisches Institut für Berufspädagogik' in Brugg, Switzerland. At the same part-time school teacher of descriptive geometry, physics, and engineering with computer-aided design, at the GIBZ

1989-1992 Matura Typus E (economics), at the 'Kantonale Maturitätsschule für Erwachsene' (KME) in Zürich, Switzerland

1992-1998 Studies of Environmental Sciences at the Swiss Federal Institute of Technology Zurich (ETHZ). Main focus on Environmental Hygiene and Anthroposphere:

- 1996 Development and evaluation of an instrument with which credit accounting clerks can assess potential ecological risks of firms, at the Credit Suisse Group (CSG)
- 1997 Case study 'Environmental hygiene assessment of a restaurant and a disco' (Umwelthygienische Beurteilung eines Restaurants und einer Disco in Zürich), at the ETHZ Institute for Hygiene and Applied Physiology (IHA) (Supervision: PD Dr. sc. nat. Ch. Monn)
- 1998 Case study 'Environmental management and ecological product design in practice', at the ETHZ-Center Enterprise Sciences (BWI) (Supervision: PD R. Züst, PhD)
- 1998 Diploma thesis 'Impact of plastics additives on indoor air quality' (Die Beeinflussung der Luftqualität von Innenräumen durch Kunststoff-Additive), at IHA-

ETHZ and the University of Düsseldorf, Germany (Supervision: Prof. Th. Koller)

1996-2000 High-school teaching training for biology and environmental science, at the ETHZ 'Institut für Verhaltenswissenschaften' (Head: Prof. K. Frey)

1998-2004 Research assistant at the Institute of Social and Preventive Medicine, University of Basel (ISPMBS), Switzerland (Head: Prof. U. Ackermann-Liebrich):

1998 Project assistance of the $PM_{10}$ and $PM_{2.5}$ exposure assessment project in different SAPALDIA and SCARPOL study areas

1999 Fieldwork, data base management, statistical analysis, publications, and presentations of the tri-national study 'Allergy and Endotoxin' ALEX

2000 Project management, questionnaire design, fieldwork, data base management, and statistical analysis of the Swiss part of the international study 'Prevention of allergy – Risk factors for sensitisation in children related to farming and anthroposophic lifestyle' PARSIFAL

2002 Methodical study to measure endotoxin on $PM_{2.5}$ outdoor filters in selected SAPALDIA and SCARPOL study areas

Educational activities comprising: supervisor of a MSc student 'Erfassung der Endotoxinbelastung in Staubproben des häuslichen Umfeldes von Bauern- und Nichtbauernkindern'; Tutor of several case studies for 2$^{nd}$ year medical students; internal colloquia and statistical work shops

2000-2004 Doctoral student at the ISPMBS (Supervision: Prof. Ch. Braun-Fahrländer):

2001 New England Epidemiology summer school at the Epidemiology Research Institute (ERI) in Boston, MA, USA (Lecturers: P. Cole, A. Ahlborn, H. Austin, K. Rothman, D. Harrington, H. Chekoway)

2001 Short course in epidemiology 'Biometry for epidemiologists' at the Swiss Tropical Institute (STI); Self-study in a statistical package (STATA)

2002 Master of public health (MPH) postgraduate course in 'Environmental Epidemiology' in Basel, Switzerland (Lecturers: U. Ackermann-Liebrich, Ch. Braun-Fahrländer, D. Dockery, N. Künzli, J. Schwartz)

2002 Workshop in cooperation, leadership and team work management at the university of Basel (Lecturer: M. Graf)

2003 Short course 'Basic and Clinical Allergy', at the National Heart & Lung Institute, Imperial College, London, UK (Organiser: A.B. Kay)

2003 MPH postgraduate course in 'Longitudinal Studies' in Berne, Switzerland (Lecturers: J. Sterne, M. Egger, B. Ledergerber)

Various colloquia at the ISPM and 'Reading Epidemiological Papers' (Lecturers: N. Künzli, PhD, Prof. U. Ackermann-Liebrich)

## List of publications


- Frei M, Waser M, Züst R. Schnittstelle zwischen Umweltmanagement und Produktentwicklung. Resultate einer Umfrage unter ökologisch führenden Unternehmen der Schweizer Maschinen-, Elektro- und Metallindustrie. uwf Unternehmenspraxis 1998; 6: 58-61.
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### Congress contributions

- Waser M, Riedler J, Nowak D, von Mutius E, Braun-Fahrlander C. Bäuerliches Leben schützt Kinder vor allergischer Sensibilisierung. Abstract and speech at the Swiss Society for Public Health (SGPG) Junitagung 2000.
- Waser M, Braun-Fahrlander C, Schierl R, von Mutius E, Maisch S, Carr D, Riedler J, Eder W, Schreuer M, Nowak D. Environmental determinant of endotoxin concentrations in mattresses of farmers' children and rural controls. Abstract and speech at the ISEE 2001.
- Waser M, von Mutius E, Carr D, Nowak D, Schierl R, Eder W, Riedler J, Braun-Fahrlander C. Current exposure to dogs is inversely associated with hay fever independent of endotoxin exposure and being a farm child. Abstract and speech at the SGPG and the ERS 2003.

### Reports

- Waser M, Hunziker J, Rust A, Walter J. Umweltbildung in einem Kinderlager. ETH Zürich 1996.
- Scholz R, Bösch S, Carlocci L, Oswald J, Waser M. Fallstudie 1998: Nachhaltige Regionalentwicklung. Chancen der Region Klettgau. UNS-ETH Zürich 1998.
- Monn C, Junker M, Riediker M, Waser M. Bericht Messpraktikum Umwelthygiene: Umwelthygienische Beurteilung eines Restaurants und einer Disco in Zürich. IHA-ETH Zürich 1997.
- Waser M. Die Beeinflussung der Luftqualität von Innenräumen durch Kunststoff-Additive. IHA-ETH Zürich, Institut für Hygiene und Arbeitsphysiologie 1998.

- Röösli M, Waser M, Oglesby L, Neu U, Künzli N, Braun-Fahrlander C. Schlussbericht 'PM2.5-Projekt'. Konzentrationen verschiedener Schwebestaubfraktionen an den SCAR-POL- und SAPALDIA Standorten. Auftrag des Bundesamtes für Umwelt, Wald und Landschaft BUWAL 2002; FE/BUWAL/810.98.50.

### Awards

Award for the best scientific speech: 'Current exposure to dogs is inversely associated with hay fever independent of endotoxin exposure and being a farm child', at the 'Workshop Public Health Switzerland' (Werkschau Public Health Schweiz), from the SGPG, 2003.

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