Respiratory effects of endotoxin exposure: individual susceptibility and gene-environment interactions

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Blootstelling aan endotoxine en respiratoire gezondheidseffecten:
individuele gevoeligheid en gen-omgeving interacties

(met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

Endotoxin: an occupational health hazard

Already in 1555, Olaus Magnus wrote that grain dust inhalation was a health hazard for threshers,[1, 2] and in 1700, Bernardino Ramazzini reported in the first systematic treatise on occupational diseases that workers developed acute airway symptoms when exposed to grain, hemp or flax dust. In workers exposed to textile dust, he observed that "…a foul and poisonous dust flies out of these materials, enters the mouth, then the throat and lungs, makes the workmen cough incessantly, and by degrees brings on asthmatic troubles…".[3] Since then, many authors reported respiratory and flu-like illnesses related to occupational organic dust exposures, especially in the textile industry.[4] In 1942, it was proposed for the first time that inhaled endotoxins, cell wall components of Gram-negative bacteria commonly occurring on plants and plant products from which the dust originated, may have a role in the pathogenesis of these diseases.[5, 6] This hypothesis was corroborated by human exposure experiments. Inhalation of cotton dust or purified endotoxin resulted in fever, acute respiratory symptoms, and a reduction of the forced expiratory volume in one second (FEV_1).[6-8] Rylander et al.[9] reported a significant correlation between endotoxin levels in cotton dust and acute pulmonary and systemic responses among cotton workers in an experimental setting. A dose-response relationship between endotoxin exposure and an acute decrease in FEV_1 was also found in naïve, healthy volunteers.[10] In contrast, there was no association between the dust concentration and the change in FEV_1.[10]

Gram-negative bacteria are ubiquitous in the environment and, as a result, a large variability in airborne endotoxin exposure levels has been measured in diverse occupational environments.[11] Epidemiological studies in occupational populations have shown exposure-response relationships between endotoxin exposure and a lower FEV_1, across-shift lung function decline, respiratory symptoms, and airway hyperresponsiveness.[12-23] A longitudinal study in pig farmers has demonstrated a long-term effect of exposure by showing that daily work at high endotoxin levels was associated with an accelerated lung function decline[24] and similar observations have been reported in workers exposed to cotton and grain dust.[25, 26] These findings indicate that workers chronically exposed to high levels of endotoxin may be at risk to develop chronic obstructive pulmonary disease (COPD). Increased endotoxin levels have also been associated with asthma and asthma severity in domestic settings,[27-29] although airborne levels of house dust and endotoxin levels per mg house dust are relatively low compared with occupational environments.[11, 30, 31]

Endotoxin-related airway obstruction and respiratory symptoms most probably result from acute and chronic airway inflammation. Endotoxin is a lipopolysaccharide (LPS),

and its lipid part (lipid A) is a well-described, potent pro-inflammatory agent. Systemic and airway inflammatory responses to inhaled endotoxin are characterized by increased levels of neutrophils and proinflammatory cytokines such as IL8 and TNFα as shown in blood, nasal lavage fluid, induced sputum and bronchoalveolar lavage fluid.[32-35]

The proinflammatory properties of endotoxin, its ubiquitous presence in dust, established dose-response relationships in experimental and occupational settings, and experiments with endotoxin-sensitive and -resistant mice all argue for a major causal role of endotoxin in organic dust induced airway disease.[36, 37] However, organic dust contains other non-allergenic microbial agents such as fungal β(1→3)-glucans, which may contribute to the observed health effects as well.[38]

Endotoxin: protection against allergic sensitization

The global increases in asthma and allergy prevalence in recent decades have caused a shift of attention from risk factors for asthma to the possible role of microbial exposure as a protective factor.[39] Two decades ago, Strachan[40] was the first to suggest a causal relationship between improved standards of hygiene and the concurrent increase in the prevalence of hay fever over the last century. Since then, numerous studies have investigated the "hygiene hypothesis", according to which bacterial and viral infections, and exposure to proinflammatory, noninfectious microbial compounds such as endotoxin may protect from the development of allergic disease by modifying the immune responses to allergens.[41, 42] The initial interpretation was that growing up in a more hygienic environment with less microbial exposure would allow continuation of the predominance of the "atopic" T-helper 2 (Th_2) phenotype of fetal and perinatal immune responses, whereas microbial pressure would drive the responses of the immune system to environmental allergens into a Th_1 direction with ensuing suppression of atopic immune responses.[41] More recently, an alternative interpretation has been offered which involves a reduction in activity of T regulatory cells resulting in a reduced immune suppression and subsequently an up-regulation of both Th_1 and Th_2 immunity.[43]

In addition to studies on specific agents with potential protective effects, research on the increase of asthma has focused on subpopulations with low atopy and asthma rates compared with general populations.[39] In particular, children of farming parents appeared to have a lower risk of atopic disease than their peers living in the same rural community.[44] Remarkably, in 1873, Blackley[45] already observed in his influential treatise on hay fever that "…It would seem that hay-fever has, of late years, been

considerably on the increase..." and "...One very curious circumstance in connection with hay-fever is that the persons who are most subjected to the action of pollen belong to a class which furnishes the fewest cases of the disorder, namely, the farming class...". The reduced prevalence of atopic disease observed in children and adults who lived on a farm during childhood has, in line with the hygiene hypothesis, been attributed to high environmental exposures to microbial components among farm children, for instance through contact with livestock.[44, 46-50] Indeed, several studies have shown inverse associations between domestic endotoxin exposure and atopy and atopic asthma in children from farming families, but also in children living in urban areas.[51-54] An increasing number of studies has shown that childhood farm exposures continue to protect against atopic sensitization into adulthood. It has been argued that especially microbial exposure in early childhood contributes to the reduction of allergic sensitization,[49] and that this protective effect continues in early adulthood. However, recent studies suggest that exposures during adulthood still protect against atopy and (atopic) asthma (see Table 1 for a literature overview). Interestingly, two of these studies found in populations of adult farmers a lower prevalence of atopy and atopic asthma at higher endotoxin exposure levels, but this was paralleled by an increased risk of non-atopic asthma, respiratory symptoms, and non-specific airway hyper-responsiveness.[60, 62] These studies clearly illustrated the dual nature of endotoxin, which seems to serve as both "friend and foe".[67] However, the observed effects of current endotoxin exposure might have been partly due to confounding by childhood exposure, which was not taken into account. Other studies that investigated both farm exposures during childhood and adulthood used "contact with livestock" or simply "being a farmer" as a proxy for current microbial exposures.

Individual susceptibility to endotoxin

Evidently, not all endotoxin-exposed workers develop a symptomatic respiratory response, even at very high exposure levels. Genetic and non-genetic susceptibility factors may interact with organic dust exposure, resulting in inter-individual heterogeneity in type and magnitude of responses within groups of individuals with apparently identical exposures.[68] Castellan et al.[10] screened naïve, healthy volunteers with a normal baseline FEV_1 for inclusion in cotton dust inhalation experiments. After precautionary exclusion of those with a decrease in FEV_1 >30% on their first dust exposure, the authors found that endotoxin exposure elicited a measurable response in approximately one third of the remaining subjects, whereas others appeared unresponsive.[10]

Table 1 Studies showing a significant inverse association between farm life or endotoxin exposure and atopy or allergic disease in adults

Population	Phenotype	Protective factor	Reference
		During childhood	
6,251 adults from five European countries	Atopy, sensitization to pollen and cat	Farm childhood	Leynaert et al. 2001[48]
805 French adults	Atopy, total IgE, asthma	Rural childhood	Kauffmann et al. 2002[55]
296 Finnish students	Asthma, sensitization to cat	Farm childhood	Kilpelainen et al. 2002[56]
1,309,652 Swedish conscripts	Asthma, allergic rhinitis, eczema	Farm childhood	Braback et al. 2004[57]
137 university employees, Illinois	Sensitization to occupational (rodent) allergens	Rural childhood	Dorevitch et al. 2007[58]
		During adulthood	
350 German urban adults	Atopy	House dust endotoxin	Gehring et al. 2004[59]
1 614 Norwegian farmers	Atopic asthma	Occupational exposure to endotoxin and/or fungal spores ≥2 types of livestock	Eduard et al. 2004[60]
2,106 Norwegian farmers and 727 non-farmers	Atopic and non-atopic asthma	Being a farmer	Eduard et al. 2004[61]
194 Dutch pig farmers	Atopy	Occupational endotoxin exposure	Portengen et al. 2005[62]
578 Canadian farmers and 1,503 non-farmers	Atopy	Being a farmer	Chen et al. 2007[63]
		Throughout life	
988 Danish farming students and 399 conscripts	Atopy, total and specific IgE, BHR	Farm childhood and being a farming student	Portengen et al. 2002[50]
231 Finnish women who were living on a farm and 202 non-farm women	Sensitization to pollen and pets	Farm childhood and current contact with livestock	Koskela et al. 2005[64]
2,678 German rural adults	Atopy in combination with rhinitis symptoms	Contact with livestock during childhood and adulthood	Radon et al. 2006[65]
4,288 farmers and 1,328 non-farmers from New-Zealand	Asthma, wheeze, shortness of breath, asthma medication	Farm childhood and being a farmer Years of farm exposure	Douwes et al. 2007[66]

In another experimental human provocation study by Kline et al.,[69] pronounced and reproducible differences in the FEV_1 response were found in 72 non-atopic healthy volunteers challenged with increasing doses of LPS. In the same study, *ex vivo* LPS-stimulated peripheral blood monocytes from hyporesponsive subjects released less IL6 and IL8 than LPS-stimulated cells of sensitive subjects, thus pointing to a possible underlying mechanism of the inter-individual differences in responsiveness.[69]

Some studies have suggested an increased susceptibility for the physiologic or inflammatory response to endotoxin in atopic and/or asthmatic subjects,[70, 71] but others found no marked differences in responsiveness between normal and atopic or asthmatic subjects.[72, 73] In a Dutch population-based study, only atopic subjects had an increased risk of asthma if occupationally exposed to organic dust,[74] but there is no compelling evidence from studies with measured exposure data that atopy modifies the effect of occupational endotoxin exposure.

It is well established that the ability to respond to endotoxin can be influenced by genetic factors. During the last decade, rapid advances in the field of genetics have led to the identification of several candidate genes that may be associated with endotoxin responsiveness. Since 1965, genetic LPS-hyporesponsiveness has been described in the C3H/HeJ mouse strain,[75] and in 1998, positional cloning revealed that hyporesponsive mice have a point mutation that modifies a conserved residue in the intracellular domain of Toll-like receptor 4 (TLR4).[76] This study identified TLR4 as an essential membrane-bound receptor for LPS on inflammatory cells.[76] Subsequently, two co-segregating missense mutations in the extracellular domain of TLR4 were the first genetic polymorphisms that have been associated with endotoxin responsiveness in humans.[77] Another receptor protein, CD14, binds LPS and transfers it to TLR4. A functional[78] single nucleotide polymorphism (SNP) in the *CD14* promoter, *CD14/-260* C to T, has been shown to interact with house dust endotoxin exposure in the inverse association with allergic sensitization and asthma. Inverse associations between endotoxin and allergic sensitization,[79, 80] total serum IgE,[81] and asthma[82] have been shown to be strongest in *CD14/-260* CC homozygotes. Although this appears to be a replication of a gene-environment interaction, it should be interpreted with caution, given the different phenotypes, age and ethnicity of study subjects, and diverse exposure definitions across these studies. *CD14* SNPs were also related to lung function and wheeze in a small study among 97 agricultural workers, suggesting that variation in *CD14* could be involved in responsiveness to occupational endotoxin exposure.[83] Another candidate gene for organic dust susceptibility is *TLR2*, which encodes a pattern recognition receptor involved in the recognition of multiple products of Gram-positive organisms,

mycobacteria, and yeast. The *TLR2/-16934* SNP was associated with asthma and allergy among children of farmers, but not among non-farm children, thus suggesting another gene-environment interaction.[84] Increased sensitivity to agricultural exposures has also been shown in young farmers with rare S and Z α1-antitrypsin alleles.[85] In Chinese cotton workers, SNPs in genes encoding tumor necrosis factor (*TNF* and *LTA*),[86] and microsomal epoxide hydrolase *(mEH)*[87] were associated with endotoxin-associated longitudinal lung function decline.

Aims and outline of this thesis

The main aim of this thesis is to investigate associations between endotoxin exposure and the occurrence of respiratory and allergic effects in adults. Cytokine responsiveness in an *ex vivo* whole blood assay and genetic variation in innate immunity genes were evaluated as susceptibility factors in associations between exposure and respiratory and allergic outcomes.

Chapter 2 describes a questionnaire survey among conventional and organic farmers in The Netherlands. In this study, associations between current and childhood farm exposures and respiratory health effects are investigated.

Chapter 3 to 6 present results of a study investigating exposure-response relationships between endotoxin exposure and respiratory and allergic outcomes in Dutch farmers and agricultural industry workers.

Chapter 3 describes the assessment of endotoxin exposure and associations between exposure and self-reported respiratory and allergic symptoms.

In **Chapter 4**, relationships between endotoxin exposure and atopic sensitization and bronchial hyper-responsiveness to methacholine are investigated, and the role of atopy in endotoxin-related respiratory effects is evaluated.

Chapter 5 presents associations between endotoxin exposure and exhaled nitric oxide, a marker of airway inflammation.

Chapter 6 investigates the hypothesis that individual differences in respiratory responses to endotoxin exposure can partly be explained by *ex vivo* cytokine production in response to LPS in a whole blood assay. Associations between cytokine release and respiratory symptoms and FEV_1 are studied, and the cytokine response is investigated as an effect modifier of associations between endotoxin exposure and respiratory outcomes.

Chapter 7 and 8 describe two studies on genetic factors and gene-environment interactions in asthma and allergy.

Chapter 7 describes a nested case-control study in young Danish farmers investigating associations between polymorphisms in *CD14* and *TLR* genes and atopy and new onset asthma.

Chapter 8 investigates whether polymorphisms in *CD14* and *TLR* genes are associated with asthma, and whether these polymorphisms modify associations between living in the country and asthma in adult subjects from the French Epidemiological study on the Genetics and Environment of Asthma.

In **Chapter 9**, the General Discussion, the influence of susceptibility factors in associations between occupational exposure to endotoxin and respiratory health effects is evaluated in more detail.

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Chapter 2

Hay fever and asthma symptoms in conventional and organic farmers in The Netherlands

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Abstract

Background: Farming has been associated with respiratory symptoms and with protection against atopy. To date, effects of organic farming on respiratory health have not been studied.

Aims: To (1) compare hay fever and asthma-like symptoms in organic and conventional farmers and (2) assess associations between current and childhood farm exposures and respiratory health effects by conducting a survey.

Methods: Questionnaire data from 1205 conventional and 593 organic farmers were evaluated. Associations between health effects and farm exposures were assessed by logistic regression analyses.

Results: Organic farmers reported less wheezing with shortness of breath and slightly more hay fever than conventional farmers. However, organic farming was not an independent determinant of hay fever when adjusted for farming practices and potential confounders. Livestock farmers who grew up on a farm had a threefold lower prevalence of hay fever than crop farmers without a farm childhood (odds ratio (OR) 0.3, 95% confidence interval (CI) 0.1 to 0.5). Both crop farmers who grew up on a farm and livestock farmers who did not grow up on a farm had a reduced prevalence, although less pronounced and not statistically significant. Use of disinfectants containing quaternary ammonium compounds was positively related to hay fever (OR 2.1, 95% CI 1 to 4.4). No effects of farming practices were found for asthma.

Conclusions: Our study adds to the evidence that a farm childhood in combination with current livestock farming protects against allergic disorders. This effect was found for both organic and conventional farmers.

Introduction

Farmers have been shown to have an increased risk of respiratory diseases, including chronic obstructive pulmonary disease, accelerated lung function decline, and organic dust toxic syndrome.[1-5] Conversely, living on a farm during childhood has been associated with a reduced risk for atopic sensitisation, allergic asthma, and hay fever as shown in children, adolescents and young adults.[6-13] Recent studies on adult farmers have shown that protection against atopy and atopic asthma may continue into adulthood.[14-16] Exposure to livestock and microbial agents, in particular bacterial endotoxin, has been suggested to have a critical role. Current exposure to endotoxins may also protect highly exposed adult pig farmers against atopic sensitisation.[17] In the same study, however, endotoxin exposure was also positively associated with respiratory symptoms, bronchial hyperresponsiveness, and a lower lung function.

To date, effects of organic farming on respiratory health have not been studied. In The Netherlands, organic farming has grown considerably in the past few years,[18] and a similar development has occurred in other Western countries. The principal features that distinguish organic farming from conventional farming are complete rejection of the use of chemical pesticides, artificial fertilizers, and genetically modified organisms.[19] Organic livestock housing criteria must meet strict criteria, and livestock farmers are not allowed to carry out practices such as tail docking, cutting of teeth, and dehorning unless it is necessary for safety or welfare reasons. Furthermore, certain disinfectants with established respiratory and immunological effects such as chloramine-T, and quaternary ammonium compounds (QACs) are prohibited.[19] Consequently, organic and conventional farmers are likely to have different exposure patterns for both chemical and biological compounds. In addition, organic farmers may also differ from conventional farmers in socioeconomic status and lifestyle factors such as education, farm childhood, and diet. Some organic farmers may have also adopted an anthroposophic lifestyle, which has been associated with lower occurrence of childhood atopy.[13, 20]

In this study, we conducted a questionnaire survey to compare the prevalence of respiratory symptoms among adult organic farmers and conventional farmers. Symptoms in farmers were compared with a general non-farming population. Furthermore, we investigated to what extent farming exposures during childhood combined with current farming practices affect asthma-like symptoms and hay fever prevalence in organic and conventional farmers.

Methods

Study Population

Questionnaires for both principal farm operators and also their spouses when working on the farm were sent to 1013 organic and 1846 conventional farms in March 2001. Organic farms were selected from the records of the inspection body for organic production in The Netherlands (Skal, Zwolle, The Netherlands). All companies certified by Skal were selected, excluding those owned by a non-profit organization, those in transition from conventional to organic production, and those processing only agricultural products. Conventional farms were selected using a commercial database (Prosu, Dronten, The Netherlands). Questionnaires were sent to all mid-sized pig farms (100-200 sows or 400-600 finishing pigs; n = 617), dairy farms (around 50 cows; n = 647) and crop farms (between 22 and 45 hectares; n = 582) from four central and eastern provinces.

A major outbreak of foot and mouth disease occurred a few weeks after the questionnaire had been mailed,[21] which affected our response rates. Between April and June 2001 we attempted to remind all farmers by telephone. Those who declined to complete the questionnaire were requested to answer a limited number of questions on respiratory symptoms and allergy to test for non-response bias. However, telephone reminders were not effective as many farmers were distressed about the foot and mouth epidemic and unwilling to cooperate. Therefore, telephone reminders were limited to all organic farmers and approximately half of the conventional farmers (mainly crop farmers). Of the 2859 farm owners initially approached, 96 replied that they had left farming and were excluded from the study. A total of 1923 questionnaires were returned, 1054 by principal farmers (response rate 38.1%) and 869 by spouses who were working on the farm. Hereafter, all responders are called farmers. Response rates for organic and non-organic farms were comparable (37.9% and 38.3%, respectively). Responders were excluded if they were aged >70 years (n = 12), or if their production was only partly organic, or in transition from conventional to organic production (n = 113). Altogether, data from 1798 farmers (975 men and 823 women) were used for statistical analysis.

Of these, 1557 were enrolled before the telephone reminder, 241 responded after the reminder. Another 105 farmers answered a few questions on symptoms by telephone. Information on lack of response was obtained from 168 farms, either by telephone or because the questionnaire was returned with a reason for lack of response. The main reasons for not participating included a lack of time or lack of interest. The foot and mouth epidemic was another commonly mentioned reason for not participating.

Items regarding general respiratory symptoms were compared with data from the Dutch part of the European Community Respiratory Health Survey (ECRHS).[22, 23] In The Netherlands, subjects aged 20-70 years were investigated (those aged 20–44 years were included in the ECRHS). Twenty-nine agricultural workers and three subjects aged >70 years were excluded from this general population sample, and data of the remaining 2679 subjects were used.

Questionnaire

The questionnaire consisted of questions on respiratory symptoms, family history of asthma or allergy, personal characteristics, occupational characteristics, and smoking habits. The questionnaire for the principal farmer also included questions about farm characteristics. Questions on respiratory symptoms were adopted from the Dutch version of the ECRHS questionnaire.[22, 24] According to the ECRHS definition, asthma was defined as a positive response to any of the following questions: "Have you had an attack of asthma in the last 12 months?", "Have you been woken by an attack of shortness of breath at any time in the last 12 months?", and "Are you currently taking any medicine for asthma?.[24] A positive response to the question " Have you ever had any allergies?" was followed by a list of possible allergens (house dust, food, animals, pollen, or others) and symptoms (sneezing or runny nose, dyspnoea, itchy skin, or itchy or watery eyes). Hay fever was defined as self-reported pollen allergy accompanied by itchy or watery eyes or sneezing.

Statistical Analysis

Data were analysed using SAS statistical software V.8.2. The crude prevalence of respiratory symptoms was compared between early and late responders (those who returned a questionnaire after the telephone reminder or answered a limited number of questions by telephone). The late response group included more organic farmers and more crop farmers, therefore when comparing early and late responses, weighted prevalences were also calculated to correct for differences between the initial sample and late-response group - that is, the prevalence of symptoms among organic/conventional farmers or crop/livestock farmers in the late-response group was given the weight of their proportion in the early-response group. The prevalence of respiratory symptoms was compared between farmers and a general non-farming Dutch population sample by logistic regression analysis, adjusting for age, sex, and smoking habits. In further analyses, we studied farming characteristics in association with asthma and hay fever in univariate logistic regression models for organic farmers and conventional farmers separately, and for all farmers together. All variables that were associated with the outcome variables in the univariate models (p<0.2) along

with age, sex, and smoking habits were included in the multiple regression models. As 97% of principal farmers were men, we only adjusted for sex and not for principal farmer/spouse status. We also repeated the analyses by using logistic regression with generalized estimating equations (GEE) to adjust for possible correlation in each household (farm was included as cluster). Estimates and standard errors were not different from those obtained by the initial models. Variables not originally selected from the univariate models were added one at a time into the model to assess significance in the multiple regression models. Goodness-of-fit of the multiple regression models was assessed by the Hosmer-Lemeshow test.

Results

Table 1 summarises the demographic and farming characteristics of 1205 conventional and 593 organic farmers. Organic farmers were slightly younger, had been working as farmers for a shorter period of time, had a higher level of education, and had lived on a farm during childhood less often than conventional farmers ($p<0.05$; t-test, χ^2 test). Hay fever in parents or siblings was reported more commonly by organic farmers ($p<0.05$; χ^2 test), whereas the prevalence of asthma in parents or siblings was equal among both groups. In addition, differences existed between both farming populations with respect to farm type (livestock and crop types), and disinfectant use. Female farmers less often grew up on a farm than male farmers (59% vs. 91%), and 65% of women worked part-time on the farm when compared with 21% of men.

Early or late response was not associated with the prevalence of self-reported respiratory symptoms or allergy, both before and after adjustment for the proportion of organic farmers or crop farmers in the late response group (Table 2; $p>0.1$, χ^2 test).

Mean age was comparable in farmers and the general population (45.1 vs. 45.4 years), but among the general population were more current smokers (36.7% vs 17.9%) and women (49.7% vs. 45.8%). Farmers reported significantly less often that they had woken up due to cough or shortness of breath, wheezing, wheezing with shortness of breath, wheezing without a cold and asthma (adjusted odds ratios between 0.2 and 0.6; Table 3). Adjustment for age, smoking habits, and sex did not change the results. Almost all asthma symptoms were less prevalent in organic farmers than in conventional farmers. This difference was significant for waking up due to shortness of breath (OR 0.4, 95% CI 0.2-0.9) and wheezing with shortness of breath (OR 0.7, 95% CI 0.4-1.0).

Table 1 Demographic and farming characteristics of conventional farmers and organic farmers.

	Conventional farmers (n = 1205)	Organic farmers (n = 593)
Male, n (%)	658 (54.6)	317 (53.5)
Mean (SD) age, years	45.5 (9.7)	44.2 (8.4)
Mean (SD) years of farm work	21.8 (10.8)	16.6 (9.9)
Education, n (%)		
Low or medium	965 (80.8)	325 (55.5)
High (at least higher secondary education)	229 (19.2)	261 (44.5)
Smoking habits, n (%)		
Current	228 (19.1)	91 (15.4)
Ever	380 (31.8)	210 (35.6)
Asthma in parents or siblings, n (%)	193 (16.1)	96 (16.3)
Hay fever in parents or siblings, n (%)	204 (17.1)	130 (22.0)
Childhood farming environment, n (%)		
No	202 (16.8)	226 (38.1)
Crops only	234 (19.4)	80 (13.5)
Livestock	769 (63.8)	287 (48.5)
Current farm type, n (%)		
Crops only	428 (35.5)	201 (33.9)
Livestock and crops	137 (11.4)	121 (20.4)
Livestock only	640 (53.1)	271 (45.7)
Farm childhood, adulthood livestock farming, n (%)		
No, No	86 (7.1)	98 (16.5)
No, Yes	116 (9.6)	128 (21.6)
Yes, No	342 (28.4)	103 (17.4)
Yes, Yes	661 (54.9)	264 (44.6)
Livestock type, n (%)		
Dairy	558 (46.3)	254 (42.8)
Pigs	372 (30.9)	43 (7.3)
Sheep	79 (6.6)	90 (15.2)
Poultry	33 (2.7)	47 (7.9)
Beef or veal	33 (2.7)	43 (7.3)
Goats	8 (0.7)	39 (6.6)
Crop type, n (%)		
Arable farming	548 (45.5)	167 (28.2)
Horticulture	120 (10.0)	256 (43.2)
Disinfectant use, n (%)		
Quaternary ammonium compounds (QACs)	183 (15.2)	34 (5.7)
Other disinfectants	541 (44.9)	197 (33.2)

Table 2 Prevalence of respiratory symptoms in 1557 farmers who responded early, and in 346 farmers who returned a questionnaire after the telephone reminder or answered a few questions by telephone (late-response group).

	Early response	Late response	Late response [¶]	Late response [§]
Daily cough up phlegm	8.6	9.0	9.1	9.4
Woken due to shortness of breath	2.1	3.2	3.6	2.4
Wheezing	11.5	11.1	11.8	10.0
Asthma attack last year	1.8	2.1	2.4	1.6
Asthma medication use	3.2	4.1	4.1	3.5
Asthma (ECRHS)	4.6	5.9	6.2	4.4
Any allergy	24.0	25.8	25.4	25.1
Hay fever	7.6	7.9	7.8	7.5

ECRHS, European Community Respiratory Health Survey.
$p > 0.1$ for all symptoms; χ^2 test.
Values are expressed in percentage.
[¶] Weighted for proportion of organic farmers in early-response group.
[§] Weighted for proportion of livestock farmers in early-response group.

Hay fever was reported more commonly among organic farmers than among conventional farmers (9.3% vs. 6.9%), although this difference was only of borderline statistical significance (univariate analysis, Table 4). Current livestock farming and childhood farming environment (both crop and livestock) were inversely associated with hay fever, whereas hay fever heredity (hay fever in parents or siblings) strongly increased the risk. Asthma heredity and QAC or other disinfectant use were associated with an increased risk of asthma. Stratified analysis by conventional and organic farming showed only minor differences between organic and conventional farmers with regard to risk factors both for hay fever and asthma (Table 4). The prevalence of hay fever was significantly lower in conventional pig farmers (OR 0.6, 95% CI 0.3-1.0), whereas this was not the case for the small group of organic pig farmers. Other specific types of livestock farming were not associated with hay fever or asthma in univariate analyses (data not shown).

The association between organic farming and hay fever decreased after adjustment for confounders in a multiple logistic regression model (Table 5). The same model confirmed that currently keeping livestock and growing up on a crop farm or livestock farm were associated with a two times lower prevalence of hay fever. Use of QACs was associated with a two times higher prevalence of hay fever. Farming characteristics were not significantly associated with asthma in the multiple regression model. Potential confounders such as level of education and working part-time, and other farming characteristics such as pig farming were also considered in the multiple regression models, but none of these were related to hay fever or asthma and they were therefore not included in the final model.

Table 3 Prevalence (%) and adjusted OR of respiratory symptoms between farmers (and farmer subpopulations) and a general non-farming Dutch population sample of the European Community Respiratory Health Survey.

	General population (n = 2679)	All farmers (n = 1798)		Conventional farmers (n = 1205)		Organic farmers (n = 593)	
	%	%	OR (95% CI)	%	OR (95% CI)	%	OR (95% CI)
Cough symptoms							
Daily cough	15.8	12.7	0.9 (0.8-1.1)	13.1	0.9 (0.8-1.2)	11.7	0.9 (0.7-1.2)
Daily cough up phlegm	9.9	8.6	0.9 (0.7-1.1)	8.5	0.9 (0.7-1.2)	8.7	0.9 (0.7-1.3)
Woken due to cough	32.0	17.1	**0.5 (0.4-0.6)**	17.7	**0.5 (0.4-0.6)**	16.1	**0.5 (0.4-0.6)**
Shortness of breath, wheezing							
Woken due to SOB	6.3	2.4	**0.4 (0.3-0.5)**	2.9	**0.4 (0.3-0.7)**	1.4	**0.2 (0.1-0.4)** [*]
Wheezing	23.7	11.7	**0.5 (0.4-0.6)**	12.3	**0.5 (0.4-0.6)**	10.6	**0.5 (0.4-0.6)**
Wheezing with SOB	15.7	7.7	**0.5 (0.4-0.6)**	8.6	**0.5 (0.4-0.7)**	5.8	**0.4 (0.3-0.6)** [*]
Wheezing without a cold	13.0	5.5	**0.4 (0.4-0.6)**	5.5	**0.4 (0.3-0.6)**	5.3	**0.4 (0.3-0.7)**
Asthma							
Doctor diagnosed asthma	4.7	5.5	1.0 (0.8-1.4)	6.0	1.1 (0.8-1.6)	4.4	0.9 (0.5-1.3)
Asthma attack last year	1.6	1.9	1.2 (0.7-1.9)	2.1	1.3 (0.8-2.2)	1.5	1.0 (0.5-2.2)
Asthma medication use	2.5	3.3	1.2 (0.8-1.7)	3.8	1.4 (0.9-2.0)	2.4	0.8 (0.5-1.6)
Asthma (ECRHS)	7.6	4.7	**0.6 (0.4-0.8)**	5.2	**0.6 (0.5-0.9)**	3.9	**0.5 (0.3-0.8)**

ECRHS, European Community Respiratory Health Survey; SOB, shortness of breath.
Data are presented as OR for farmers versus general population with 95% CI, adjusted for age, smoking habits and sex.
[*] Prevalence of symptoms differs significantly between conventional and organic farmers (p<0.05).

Table 4 Univariate models for hay fever and asthma (ECRHS definition) in conventional farmers (n = 1205) and organic farmers (n = 593) (stratified analysis), and in all farmers (n = 1798).

	Hay fever, crude OR (95% CI)			Asthma, crude OR (95% CI)		
	All farmers	Conventional farmers	Organic farmers	All farmers	Conventional farmers	Organic farmers
Organic farming						
No	1	-	-	1	-	-
Yes	1.4 (1.0-2.0)	-	-	0.7 (0.5-1.2)	-	-
Current farm type						
Crops only	1	1	1	1	1	1
Livestock	0.7 (0.5-1.0)	0.7 (0.5-1.2)	0.7 (0.4-1.2)	1.5 (0.9-2.4)	1.5 (0.8-2.6)	1.5 (0.6-3.8)
Childhood farming environment						
No	1	1	1	1	1	1
Crops	**0.4 (0.3-0.7)**	**0.5 (0.2-0.9)**	0.3 (0.1-1.0)	0.5 (0.2-1.1)	0.6 (0.2-1.6)	0.2 (0.0-1.8)
Livestock	**0.4 (0.3-0.6)**	**0.3 (0.2-0.6)**	**0.5 (0.3-0.9)**	0.9 (0.6-1.5)	1.0 (0.5-2.0)	0.6 (0.3-1.5)
Disinfectant use						
None	1	1	1	1	1	1
QACs	1.0 (0.6-1.8)	1.2 (0.6-2.2)	0.7 (0.2-2.9)	**1.9 (1.0-3.6)**	2.1 (1.0-4.4)	NE
Other disinfectants	0.9 (0.6-1.3)	0.8 (0.5-1.2)	1.3 (0.8-2.4)	**1.6 (1.0-2.6)**	1.5 (0.8-2.7)	1.7 (0.7-4.0)
Asthma in parents or siblings						
No	1	1	1	1	1	1
Yes	**1.9 (1.3-2.9)**	1.6 (0.9-2.7)	**2.6 (1.4-4.8)**	**3.8 (2.4-6.0)**	**4.5 (2.7-7.6)**	2.4 (0.9-5.9)
Hay fever in parents or siblings						
No	1	1	1	1	1	1
Yes	**4.7 (3.3-6.8)**	**5.4 (3.4-8.5)**	**3.7 (2.1-6.6)**	**2.1 (1.3-3.3)**	**1.8 (1.0-3.3)**	**2.9 (1.2-6.7)**

NE, not estimable.

Table 5 Multiple logistic regression analysis of hay fever and asthma (ECRHS definition) in farmers (n = 1798).

	Hay fever adjusted OR (95% CI)	Asthma adjusted OR (95% CI)
Organic farming		
No	1	1
Yes	1.2 (0.8-1.7)	0.7 (0.4-1.2)
Current farm type		
Crops only	1	1
Livestock	**0.5 (0.3-0.9)**	1.0 (0.5-2.2)
Childhood farming environment		
No	1	1
Crops only	**0.5 (0.3-0.9)**	0.5 (0.2-1.2)
Livestock	**0.4 (0.3-0.7)**	0.6 (0.4-1.2)
Disinfectant use		
None	1	1
QACs	**2.1 (1.0-4.4)**	1.7 (0.7-3.9)
Other disinfectants	1.5 (0.9-2.8)	1.4 (0.7-2.9)
Hay fever in parents or siblings		
No	1	
Yes	**4.4 (3.0-6.3)**	-
Asthma in parents or siblings		
No		1
Yes	-	**3.6 (2.3-5.7)**

Odds Ratios are adjusted for age, smoking habits, sex, and all other variables in the model.
- Variable not included in the model.

Hay fever and asthma were approximately four times more prevalent in farmers with a family history of hay fever or asthma. Female farmers had somewhat less hay fever and asthma than male farmers, but differences were not significant (OR 0.9, 95% CI 0.6-1.3 and OR 0.7, 95% CI 0.4-1.2 respectively). Removing family history variables, age, smoking habits, or sex from the model did not influence the results. The Hosmer-Lemeshow test showed adequate fit for both models (p>0.2).

Asthma-like symptoms that differed significantly between organic and conventional farmers (waking up due to shortness of breath and wheezing with shortness of breath) were studied in the same multiple regression models. Only wheezing with shortness of breath was significantly less prevalent in organic farmers after adjustment for potential confounders (OR 0.6, 95% CI 0.4-0.9). Asthma heredity was a strong determinant for these and other asthma-like symptoms such as wheezing and doctor diagnosed asthma (OR between 3.1 and 4.2). No other clear determinants for asthma-like symptoms were identified.

Finally we investigated whether current farming practices and farm childhood were independently associated with respiratory or allergic outcomes. Figure 1 presents a regression model that includes a variable combining farm childhood (yes or no) and

adulthood livestock farming (yes or no), along with all other variables that were included in the earlier model. Hay fever was significantly less prevalent in livestock farmers who grew up on a farm, compared with crop farmers who did not grow up on a farm (OR 0.3, 95% CI 0.1-0.5). The effect of farm childhood in combination with current livestock farming is stronger than addition of both separate effects, i.e. significant interaction (p<0.03) between farm childhood and current livestock farming. Living on a farm during childhood without adulthood livestock farming, and vice versa, was also associated with a lower prevalence of hay fever, but this was less pronounced and not statistically significant. No significant differences were found for asthma.
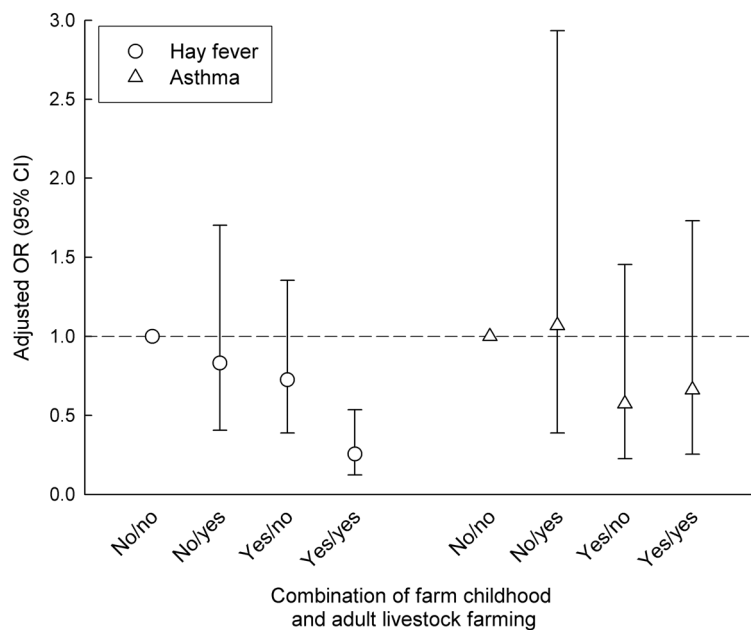
Figure 1 Odds Ratios (OR; 95% CI) for hay fever and asthma (ECRHS definition) in farmers (n = 1798) by farm childhood and adult livestock farming. ORs are adjusted by logistic regression for age, smoking habits, sex, organic farming, disinfectant use, and hay fever or asthma in family members.

Discussion

Organic and conventional farmers reported significantly less asthma-like symptoms in comparison to a general Dutch population. The adjusted prevalence of wheezing with shortness of breath was lower in organic farmers than in conventional farmers, suggesting a lower risk of asthma-like symptoms in organic farmers. In conventional

and organic farmers with both a farm childhood and current livestock exposure a strongly decreased prevalence of hay fever was seen, compared with crop farmers who did not grow up on a farm. The prevalence of hay fever seemed slightly higher in organic farmers than in conventional farmers. However, this difference disappeared after adjustment for other variables such as having lived on a farm during childhood, which was less often the case in organic farmers. Effects of farm childhood and farming characteristics on asthma and asthma-like symptoms were small and mostly non-significant.

A limitation of our study was the low response rate of <40%. An important reason for this low response was the foot and mouth disease epidemic that occurred shortly after the questionnaires had been mailed. The serious nature of the epidemic and the psychological impact this had on many of the study participants prevented us from conducting a more rigorous follow-up that would involve multiple reminders and telephone interviews. The non-response owing to this epidemic is unlikely to have significantly biased our results as it is not expected to be associated with asthma and/or hay fever symptoms in the participants. To test this, we compared early responders (n=1557) with late responders and non-responders who completed a few questions on asthma symptoms by telephone (n=346). These results showed no significant differences in the prevalence of symptoms (Table 2), suggesting that self-selection and subsequent bias was indeed minimal. However, we cannot exclude the possibility that some bias has occurred due to lack of response. Nonetheless, this is unlikely to explain the protective effects observed in farmers compared with the general population as it implies that the high lack of response in the farming population would have led to an overestimation of the prevalence of symptoms in that population and consequently to an underestimation of the protective effect. In our study, non-response bias would thus have resulted in a bias towards the null for the comparison between the farming and the general population, and the reported protective effects are therefore a conservative estimate. For bias to explain the differences in the prevalence of symptoms in the group of farmers, lack of response should have resulted in an overestimation of symptoms in crop farmers without a farm childhood (or an underestimation of symptoms in livestock farmers and farmers who grew up on a farm) which is unlikely. Similarly, it is unlikely that non-response resulted in an overestimation of the prevalence of hay fever in those farmers who reported using disinfectants, particularly because, as mentioned above, there were no indications that symptom reports were different between early responders, and late responders and non-responders.

Another limitation was that only self-administered questionnaire data on health outcomes and farming exposures were used and no objective data on dust,

endotoxin, muramic acid, fungal glucans, ammonia, etc, were collected. On the other hand, self-reported hay fever symptoms have been shown to be strongly associated with more objective skin prick tests or specific radio-allergosorbent tests in Dutch populations,[23, 25] and questions on asthma and other respiratory symptoms were derived from the validated and widely used ECRHS Questionnaire. It could be argued that it may not be appropriate to use the ECRHS population as it was studied nine years before the farming population. However, a more recent general population study on chronic diseases was undertaken in The Netherlands between 1993 and 1997, and only relatively small differences regarding the prevalence of asthma or chronic obstructive pulmonary disease symptoms were found between both population studies.[26] Nevertheless, we cannot exclude the possibility that changes in respiratoy symptom prevalence occurred in the Dutch population between 1997 and 2001, leading to under- or overestimation of the difference between farmers and the general population. The ECRHS Questionnaire was not specifically validated for farmers, but the same questionnaire has been used in many other occupational groups including both white and blue collar workers. Moreover, it has been suggested that farmers might underreport symptoms.[16] However, the evidence for this is very weak. Also, a recent study in farmers' and control children in Europe concluded that the reliability of questionnaire responses on asthma and wheeze was comparable between farmers' and control children.[27]

Use of disinfectants, particularly QACs, was an independent risk factor for hay fever which is in accordance with an earlier study in Dutch pig farmers that showed a high prevalence of IgE sensitisation to common allergens in farmers using QACs.[28] Atopic pig farmers who used QACs or were exposed to high levels of endotoxin had a higher risk of asthma-like symptoms,[28] moreover the use of QACs and other disinfectants was associated with mild bronchial hyperresponsiveness.[29] In our study, asthma was elevated in farmers using QACs and other disinfectants; however, it did not appear a significant risk factor for asthma in the multiple regression analysis. As organic farmers are not allowed to use disinfectants such as QACs and chloramine-T they may be protected against disinfectant-induced atopy or asthma. However, longitudinal studies are necessary to study such effects in more detail.

Interestingly, the reduced risk for hay fever was similar for farmers who were raised on a livestock farm or on a crop farm. Previous studies in farm children focused mainly on livestock farms, as increased bacterial endotoxin levels in house dust were found in households where children had regular contact with farm animals.[6, 30, 31] Children from non-farming families (with or without contact to livestock) served as reference groups; information on exposure for crop farmers and their children is therefore scarce. A comprehensive study among Dutch farmers and other agricultural

workers has shown that endotoxin exposure levels >200 EU/m^3 are often present during crop production,[32] which might suggest that farmers who were raised on a crop farm benefited from microbial exposures during childhood in a similar way as those raised on a livestock farm. Nonetheless, we cannot rule out the possibility that farmers who were raised on a crop farm also had regular contact to livestock as a child. Recall bias regarding the type of farm during childhood could also have affected the association, although it is unlikely that the observed protection is entirely attributable to bias.

Our findings on hay fever confirm previous results of a Finnish study showing a similarly decreased OR for pollen sensitisation in women who lived on a farm during childhood.[14] A study among adult Norwegian farmers showed a reduced prevalence of atopic asthma in farmers with two or more types of livestock, whereas non-atopic asthma was increased in pig farmers and in farmers with two or more types of livestock.[15] In the same study, exposure to endotoxin and fungal spores during farm work was inversely associated with atopic asthma and positively with non-atopic asthma. In our study, we observed a negative association between hay fever, an atopic condition, and livestock farming, confirming these results to a certain extent, but as serum IgE or skin prick test data were not available, we could not differentiate between atopic and non-atopic asthma. In the present study, only 21 of 85 asthmatic farmers reported hay fever which suggests that non-allergic asthma may be quite common in our farming population. However, as numbers are too small, we did not calculate ORs for asthma after stratifying for reported hay fever. In the Norwegian study, 80% of the farmers with asthma had a non-atopic phenotype, which the authors attributed to high levels of microbial exposures such as endotoxin that can cause non-allergic, non-eosinophilic inflammatory responses leading to reversible airways obstruction.[33, 34] These findings are supported by a recent study on Swedish farmers that found an increased risk for adult onset asthma despite a low prevalence during childhood.[35]

Farmers with respiratory health problems may seek a job outside agriculture leading to a "healthy farmer effect" which could (at least partly) explain the differences between farmers and the general population in our and other similar studies.[36, 37] Similarly, young adults with asthma or allergy may not take over their parents' farm, and adults without a farm childhood may only start farming if they have no health problems.[38, 39] To what extent such selection processes play a part in this study is difficult to establish, but a healthy farmer effect and selection into the population over multiple generations cannot be excluded. Nonetheless, differential avoidance of livestock farming because of hay fever does not appear to be likely, i.e. in our study 23 principal farmers reported that they had changed production of their farm because

of respiratory health reasons, and only three of them (two livestock farmers and one crop farmer) had hay fever. Farmers who grew up on a farm less often had a family history of hay fever (16.7% versus 25.1%), which suggests that selection processes over time might have occurred. However, farm childhood was a protective factor for hay fever independently of family history. Thus, our data seem to support the growing evidence that a farm childhood continues to protect against atopic disease in adult farmers.

In conclusion, a lower prevalence of asthma-like symptoms was found in organic farmers than in conventional farmers. Living on a farm during childhood combined with current livestock farming is associated with a lower prevalence of hay fever in both conventional and organic farmers.

Acknowledgments

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Chapter 3

Exposure-response analysis of allergy and respiratory symptoms in endotoxin-exposed adults

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Abstract

The objective of the present study was to investigate exposure-response relationships between current endotoxin exposure and allergic and respiratory symptoms in adults, taking into account farming exposures during childhood.

A cross-sectional study was conducted among 877 Dutch farmers and agricultural industry workers in 2006. Based on 249 full-shift personal airborne endotoxin samples, a job-exposure matrix was constructed to assign endotoxin exposure levels to all participants. Associations between endotoxin exposure and questionnaire data on symptoms were studied by multiple logistic regression.

Adjusted odds ratios (OR) for an interquartile range increase of endotoxin level were elevated for respiratory symptoms such as wheezing (OR 1.41, 95% confidence interval (1.16-1.72)), wheezing with shortness of breath (1.50 (1.18-1.90)) and daily cough (1.29 (1.03-1.62)). In contrast, endotoxin was strongly associated with a decreased prevalence of hay fever (0.62 (0.49-0.78)). Workers who had grown up on a farm had a lower prevalence of hay fever, but no evidence was found of effect modification by farm childhood.

In conclusion, occupational endotoxin exposure in adulthood is associated with an increased risk of asthma-like symptoms, but a reduced prevalence of hay fever.

Introduction

Numerous studies have shown a reduced prevalence of atopic disease in children and adults who lived on a farm during childhood, and this observation has been attributed to high microbial exposures in farm children.[1-8] Indeed, several studies have reported protective effects of increased domestic endotoxin exposure on the development of atopy and (atopic) asthma in children not only from farming, but also from urban environments.[9-11] Although it has been argued that especially microbial exposure in early childhood contributes to the reduction of allergic sensitisation,[1, 5] there are studies suggesting that exposure during adulthood continues to protect against atopy. Gehring et al.[12] found an association between house-dust endotoxin levels and a decreased prevalence of allergic sensitization in adults. In addition, two studies in farmers found inverse relationships between airborne endotoxin exposure and atopic asthma[13] and atopy.[14] A protective effect of exposure throughout life has also been suggested by studies that used current contact with livestock as a representative measure for high microbial exposure in adulthood, and found the lowest prevalence of atopy or hay fever in subjects with both a farm childhood and current contact with farm animals.[5, 7, 8]

Paradoxically, although microbial agents may protect against atopic disease, house-dust endotoxin has also been associated with asthma, asthma severity, and non-atopic wheeze.[10, 15-17] Moreover, a large number of epidemiological studies in occupational environments have consistently shown exposure-response relationships between exposure to endotoxin and other microbial agents such as fungal spores, and non-atopic asthma, airway obstruction, accelerated lung function decline, bronchial hyperresponsiveness, and organic dust toxic syndrome.[13, 14, 18-20] These two aspects of microbial exposure were well illustrated in studies among farmers, demonstrating an inverse association between airborne endotoxin exposure and atopy or atopic asthma, but also an increased risk of airway responsiveness, a lower lung function, and non-atopic asthma.[13, 14] However, these effects of current endotoxin exposure might be partly due to childhood exposure, which has not been taken into account.

Therefore, the objective of the present cross-sectional study was to investigate the role of airborne endotoxin exposure in adulthood and farming exposures during childhood on the occurrence of allergic and respiratory symptoms in adults exposed to a wide range of work-related endotoxin levels.

Methods

Study population

The study population consisted of workers involved in processing and trade of agricultural products who were recruited in collaboration with their Product Boards, and farmers who were recruited through the records of the inspection body for organic production in the Netherlands (Skal, Zwolle, The Netherlands). The study was carried out between February and December 2006.

Questionnaires were sent to all employees of 23 participating companies, a representative sample of companies in four agricultural sectors (onion trade, flower bulb trade, animal feed industry and vegetable seed industry) around 2 weeks before the companies were visited for exposure measurements. In total, 376 completed questionnaires were received, a response rate of 90%. Farmers were recruited by sending questionnaires to 748 farm owners, and everyone aged >18 yrs and working on the farm was invited to complete a questionnaire. Up to two postal reminders were sent to nonresponders. Of the 748 farm owners approached initially, 59 had left farming and were excluded from the study. In total, 525 completed questionnaires were received from 418 farms, thus the farm owners' response rate was 61%. The majority of farm owners (78%) had shifted from conventional to organic farming, on average 7.1 years ago, whereas 22% had never practiced conventional farming.

Overall, 23 subjects aged < 18 or > 65 yrs and one subject with missing data on farm childhood were excluded from the study. Altogether, data from 877 questionnaires were used for statistical analysis.

Of the 525 farmers who completed the questionnaire, 340 (65%) indicated that they would not object to being contacted for exposure measurements. A sample of 122 farmers was approached by telephone on the basis of distance from the present authors' laboratory Utrecht, The Netherlands), and 82 farmers were able to participate in personal exposure measurements on one of the proposed dates. During visits to the agricultural companies, 116 workers across all different worksites were asked to participate in exposure measurements; all invited workers agreed to participate. Single measurements were obtained from 65 workers, and from 51 workers two samples were obtained on two consecutive days, resulting in a total of 249 full-shift inhalable dust and endotoxin measurements. Farms and agricultural companies were visited on random days and, therefore, measurements reflected average exposures rather than exposures related to specific tasks.

Questionnaire

Questions about respiratory symptoms were adopted from the Dutch version of the European Community Respiratory Health Survey (ECRHS) questionnaire.[21, 22] Current wheezing was defined as wheezing at any time in the last 12 months. Doctor-diagnosed asthma was defined as ever having had asthma that was confirmed by a doctor, and was stratified by time of onset (before or after 18 yrs of age). Questions about personal characteristics, allergy, smoking habits, farm childhood and farm characteristics were obtained from an earlier questionnaire survey among farmers.[7] A farm childhood was defined as a positive answer to the question "Did you grow up on a farm?". A positive response to the question "Have you ever had any allergies?" was followed by a list of possible allergens (house dust, food, animals, pollen) and symptoms (sneezing or runny nose, dyspnea, itchy skin, or itchy or watery eyes). Hay fever was defined as self-reported pollen allergy experienced as itchy or watery eyes or sneezing.[7] Job title, workplace, and tasks of employees of agricultural companies were assessed by questions developed in collaboration with the respective Product Boards.

Endotoxin exposure assessment

Full-shift inhalable dust samples were collected using Gilian GilAir portable pumps (Gilian, West Caldwell, NJ, USA) at a flow rate of 3.5 l/min using Gesamt Staub Probenehmer (GSP) sampling heads and 37-mm glass-fibre filters (Whatman GF/A, Maidstone, UK). Filters were extracted in pyrogen-free water with 0.05% Tween 20.[23] Supernatants were analysed by the quantitative kinetic chromogenic Limulus amebocyte lysate assay (lysate lot no. EL004V, standard lot no. 4L3560; Cambrex, Verviers, Belgium).[23] Endotoxin levels were above the limit of detection in all samples. Results were expressed as endotoxin units (EU)/m^3 (18 EU = 1 ng).

Data analysis

Data analysis was performed on natural log-transformed endotoxin concentrations. Mixed effects models that assume correlation between repeated exposure measurements in the same worker were applied for each sector separately, including worker as random effect and job title as fixed effect, to calculate geometric mean (GM) exposure levels for different job titles.[24] The resulting job-exposure matrix was combined with the job title of all participating subjects to assign endotoxin exposure. Exposure measurements were available for each of the 30 combinations of sector or farm type and job title. Modeled endotoxin exposure showed good correlation with measured exposure (r=0.76; p<0.001) in the subjects with measurement data available. The shape of the relationship between modelled endotoxin exposure and

health outcomes was studied by means of generalised additive modelling (smoothing) as described before.[14] If the smoothed relationship was not significantly different from a linear fit, multiple linear logistic regression analyses were applied to calculate odds ratios (ORs) for an interquartile increase in endotoxin exposure. ORs were adjusted for age, sex, smoking habits, and farm childhood. To investigate effect modification by farm childhood or self-reported allergy, interactions between farm childhood and endotoxin exposure, and allergy and endotoxin exposure were explored and tested.

Results

Table 1 shows characteristics of the study population, stratified by farm childhood. Workers who had grown up on a farm were more often working in primary agricultural production, male and older, and were less often current smokers than workers without a farm childhood ($p < 0.05$; Chi-square test, t-test). The average endotoxin exposure levels were, however, practically identical (GM 265 EU/m^3), and a wide range of exposure levels was found in both subgroups.

Table 1 Characteristics of 877 agricultural workers, stratified by farm childhood.

	Farm childhood	
	No	Yes
Total subjects n	366	511
Male, n (%)	269 (73.5)	455 (89.0)
Age (yrs), mean (SD)[#]	40.5 (10.5)	46.0 (9.6)
Smoking habits, n (%)		
Never	138 (37.7)	246 (48.1)
Former	117 (32.0)	177 (34.7)
Current	111 (30.3)	88 (17.3)
Agricultural sector, n (%)		
Flower bulb trade	72 (19.7)	54 (10.6)
Animal feed industry	82 (22.4)	32 (6.3)
Onion trade (modernised)	38 (10.4)	5 (1.0)
Onion trade (traditional)	39 (10.7)	15 (2.9)
Vegetable seed industry	19 (5.2)	17 (3.3)
Crop farming	50 (13.7)	87 (17.0)
Dairy farming	62 (16.9)	252 (49.3)
Pig farming	4 (1.1)	49 (9.6)
Modelled endotoxin exposure (EU/m^3), GM (GSD)[#]	265.0 (5.4)	264.7 (3.7)

[#] SD, standard deviation; EU, endotoxin unit; GM, geometric mean; GSD, geometric standard deviation.

Table 2 Measured endotoxin exposure levels in agricultural workers, by job title.

Sector and job title	N1[#]	N2[#]	Endotoxin (EU/m^3) GM (GSD)[¶]
Flower bulb trade			
Office worker	40	3	15 (1.1)
Lifttruck operator	10	2	86 (1.3)
Technician	4	2	93 (1.5)
Packer	8	9	170 (3.4)
Crop grower	9	1	180 (-)
Operator	55	14	690 (2.9)
Animal feed industry			
Office worker	9	6	35 (2.1)
Lab worker	3	4	59 (2.1)
Controlroom worker	31	19	250 (4.1)
Truckdriver	6	10	250 (8.9)
Operator	44	27	320 (6.8)
Technician	10	7	400 (2.5)
Crane driver	4	5	950 (7.5)
Cleaner	2	3	1000 (4.4)
Unloader	5	5	9500 (2.8)
Onion trade (modernised)			
Office worker	8	3	380 (3.7)
Packer	5	1	630 (-)
Lifttruck operator	13	2	1100 (2.4)
Operator	19	6	1300 (3.3)
Onion trade (traditional)			
Technician	3	2	800 (3.5)
Lifttruck operator	13	6	4100 (3.7)
Packer	4	4	5300 (1.4)
Operator	32	15	10000 (1.6)
Vegetable seed industry			
Lab worker	12	2	22 (1.3)
Packer	2	2	25 (2.1)
Crop grower	5	2	36 (1.7)
Operator	17	5	280 (4.8)
Farming			
Crop farmer	137	30	63 (2.2)
Livestock farmer, mainly dairy	314	46	220 (4.6)
Pig farmer	53	6	3400 (6.9)

[#] N1, number of participants in questionnaire study; N2, number of personal endotoxin measurements;
[¶] EU, endotoxin unit; GM, geometric mean; GSD, geometric standard deviation.
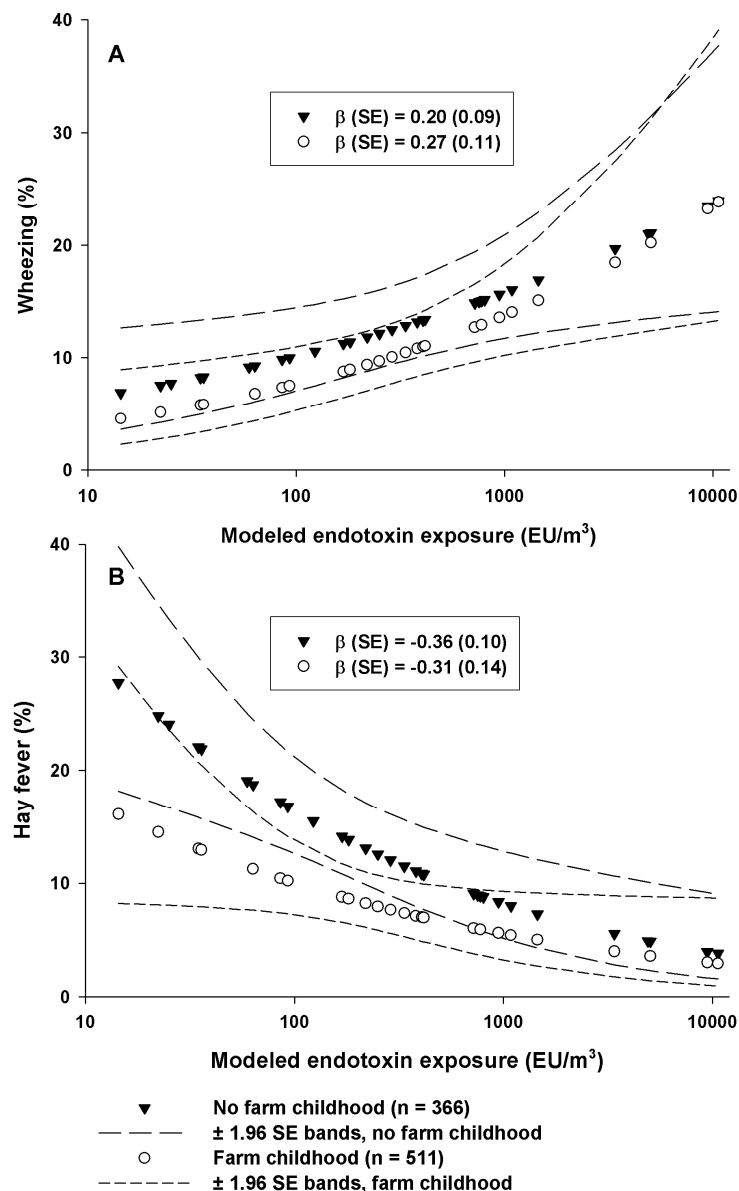
Figure 1 Logistic regression plots with pointwise ± 1.96 SE bands representing associations between modeled current endotoxin exposure and wheezing in the last 12 months (A), and between endotoxin exposure and hay fever (B), in 877 adult agricultural workers, stratified by farm childhood. Each symbol represents a group of workers with the same estimated exposure level. EU: endotoxin unit.

The exposure data underlying the job-exposure matrix used to assign endotoxin exposure levels to all subjects is presented in Table 2. Exposure levels were comparable with those in recent studies in agricultural industries.[25, 26] Highest endotoxin levels were found among onion workers and animal feed unloaders (GM

~10,000 EU/m^3). Levels among crop farmers (GM 63 EU/m^3) were lower than levels in dairy farmers or pig farmers (GM 220 EU/m^3 and 3,400 EU/m^3 respectively).

Hay fever prevalence declined with increasing endotoxin exposure, but a positive relationship between endotoxin and wheezing was found (Figure 1). The slope of exposure-response relationships was similar in workers who had grown up on a farm, and those without a farm childhood, although the prevalence of hay fever was lower in the former group (Figure 1). Comparison of smoothed and parametric models showed that associations could be described satisfactorily with parametric logistic regression models, and ORs were, therefore, calculated for an interquartile increase in endotoxin exposure with multiple logistic regression models. Increased endotoxin exposure was significantly associated with cough symptoms, shortness of breath and wheezing, but inversely related to allergy and hay fever (Table 3). The prevalence of doctor diagnosed asthma was low (both childhood and adult onset asthma) and was not related to endotoxin level. Associations between endotoxin exposure and health outcomes were not modified by farm childhood, i.e. no significant interaction was observed for any of the symptoms (p>0.1). Subjects with a farm childhood reported significantly less often that they had woken up due to cough than workers without a farm childhood (OR 0.64, 95% confidence interval (CI) 0.42-0.98). A farm childhood was also associated with less reported hay fever, although this difference was not statistically significant (0.65 (0.41-1.03)). Female workers reported more cough (1.91 (1.06-3.46)) and waking up due to cough (2.46 (1.54-3.91)) compared with male workers. Adjustment for sex, age, smoking habits and farm childhood, however, did not meaningfully change ORs for the relationships between current endotoxin exposure and health outcomes.

To disentangle associations between allergy, respiratory symptoms, and endotoxin, the present authors investigated whether self-reported allergy modified associations between endotoxin exposure and respiratory symptoms, such as wheezing. As expected, the prevalence of all respiratory symptoms was higher in subjects who reported allergy (Table 4). However, ORs for the relationship between endotoxin exposure and respiratory symptoms were essentially equal in both strata, and there was no significant interaction (p>0.2). Self-reported allergy was strongly associated with respiratory symptoms (p-values <0.001-0.09), and controlling for confounding by allergy resulted in higher ORs for respiratory symptoms (model 2, Table 4). Thus, adverse effects of endotoxin might be somewhat underestimated due to confounding by atopy.

In a subgroup of 429 workers, self-reported allergy was validated against specific serum IgE levels, which were measured by means of enzyme immunoassays, and atopy was defined as serum IgE to one or more of the common allergens grass

pollen, house-dust mite, cat, and dog.[27] Strong associations were observed between allergy and atopy (OR 8.5, 95% CI (5.0-14.5)), and between hay fever and IgE to grass pollen (18.2 (9.1-36.6)).

In addition, the present authors investigated whether the associations between endotoxin and health effects differed between the various agricultural sectors included in the study. Endotoxin was dichotomised using the median exposure level for each sector as a cut-off. Highly consistent results were found across the different sectors, although statistical significance was only reached within the relatively large group of farmers (Table 5). Endotoxin exposure above the median level led in all groups to an approximately two-fold higher risk of wheezing, whereas the risk of hay fever was two to five times lower. Only in the small group of vegetable seed workers was no association between endotoxin exposure and hay fever observed. Although similar ORs were found, the prevalence of symptoms was not equal in groups with different exposure levels, which explains the monotonous dose-response curves presented in Figure 1.

Table 3 Prevalence of respiratory and allergic symptoms and association with modelled endotoxin exposure in 877 adult agricultural workers, stratified by farm childhood.

	All (n = 877)		No farm childhood (n = 366)		Farm childhood (n = 511)	
	%	OR (95% CI)	%	OR (95% CI)	%	OR (95% CI)
Cough symptoms						
Daily cough	8.1	1.29 (1.03-1.62)	10.2	1.10 (0.82-1.48)	6.7	1.58 (1.11-2.25)
Daily cough up phlegm	7.3	1.60 (1.27-2.03)	8.0	1.40 (1.01-1.93)	6.9	1.84 (1.31-2.58)
Woken due to cough	14.1	1.36 (1.13-1.64)	19.3	1.18 (0.93-1.50)	10.4	1.67 (1.25-2.25)
Shortness of breath, wheezing						
Woken due to SOB	3.3	1.47 (1.05-2.07)	4.4	1.51 (0.99-2.32)	2.5	1.41 (0.82-2.43)
Wheezing	11.4	1.41 (1.16-1.72)	12.8	1.33 (1.02-1.73)	10.4	1.48 (1.10-1.99)
Wheezing with SOB	7.4	1.50 (1.18-1.90)	8.5	1.42 (1.04-1.95)	6.7	1.55 (1.09-2.21)
Wheezing without a cold	6.6	1.60 (1.25-2.05)	7.9	1.70 (1.23-2.36)	5.7	1.44 (0.98-2.12)
Asthma						
Doctor diagnosed asthma, age <18	4.1	0.79 (0.58-1.10)	5.8	0.84 (0.58-1.24)	2.9	0.62 (0.33-1.19)
Doctor diagnosed asthma, age ≥18	0.9	0.99 (0.48-2.03)	1.1	0.95 (0.39-2.31)	0.8	0.90 (0.28-2.82)
Asthma attack last year	0.9	0.81 (0.41-1.63)	1.1	0.87 (0.36-2.14)	0.8	0.79 (0.26-2.44)
Current asthma medication use	2.3	0.71 (0.45-1.14)	3.0	0.71 (0.41-1.24)	1.8	0.58 (0.25-1.37)
Allergy						
Any self-reported allergy	24.9	0.82 (0.70-0.96)	26.2	0.75 (0.60-0.93)	23.9	0.89 (0.70-1.12)
Hay fever	10.5	0.62 (0.49-0.78)	13.7	0.59 (0.44-0.80)	8.2	0.64 (0.43-0.95)
House dust allergy	10.4	0.86 (0.69-1.07)	12.6	0.76 (0.57-1.00)	8.8	1.03 (0.74-1.45)

Data are presented as the odds ratio (OR) for an interquartile range ($\exp^{1.45}$ =factor 4.3) increase in modeled endotoxin exposure with 95% confidence interval (95% CI). OR are adjusted for age, smoking habits, sex, and (in unstratified analysis) farm childhood.

Table 4 Prevalence of respiratory symptoms and association with modelled endotoxin exposure in 877 adult agricultural workers, stratified by self-reported allergy.

	All (n = 877)			No allergy (n = 659)		Allergy (n = 218)	
	%	OR (95% CI) Model 1[¶]	OR (95% CI) Model 2[¶]	%	OR (95% CI) Model 1[¶]	%	OR (95% CI) Model 1[¶]
Cough symptoms							
Daily cough	8.1	1.29 (1.03-1.62)	1.32 (1.05-1.66)	7.3	1.39 (1.06-1.82)	10.6	1.14 (0.70-1.84)
Daily cough up phlegm	7.3	1.60 (1.27-2.03)	1.64 (1.29-2.08)	6.7	1.64 (1.23-2.18)	9.2	1.58 (1.02-2.43)
Woken due to cough	14.1	1.36 (1.13-1.64)	1.42 (1.17-1.71)	12.4	1.38 (1.10-1.72)	19.4	1.48 (1.02-2.13)
Shortness of breath, wheezing							
Woken due to SOB	3.3	1.47 (1.05-2.07)	1.56 (1.10-2.20)	2.4	1.38 (0.88-2.17)	6.0	1.76 (1.04-2.98)
Wheezing	11.4	1.41 (1.16-1.72)	1.55 (1.26-1.90)	7.6	1.51 (1.15-2.00)	22.9	1.66 (1.20-2.31)
Wheezing with SOB	7.4	1.50 (1.18-1.90)	1.65 (1.29-2.11)	4.4	1.88 (1.33-2.67)	16.6	1.46 (1.03-2.07)
Wheezing without a cold	6.6	1.60 (1.25-2.05)	1.72 (1.34-2.21)	4.6	1.80 (1.28-2.54)	12.8	1.70 (1.16-2.49)
Asthma							
Doctor diagnosed asthma, age <18	4.1	0.79 (0.58-1.10)	0.87 (0.62-1.21)	2.0	0.94 (0.57-1.53)	10.7	0.77 (0.49-1.22)
Doctor diagnosed asthma, age ≥18	0.9	0.99 (0.48-2.03)	1.14 (0.55-2.40)	0.3	1.04 (0.27-3.97)	2.8	0.99 (0.44-2.25)
Asthma attack last year	0.9	0.81 (0.41-1.63)	0.89 (0.43-1.82)	0.3	1.05 (0.30-3.73)	2.8	0.83 (0.35-1.96)
Current asthma medication use	2.3	0.71 (0.45-1.14)	0.82 (0.51-1.32)	0.8	0.90 (0.41-1.97)	6.9	0.65 (0.35-1.19)

Data are presented as the odds ratio (OR) for an interquartile range ($exp^{1.45}$=factor 4.3) increase in modeled endotoxin exposure with 95% confidence interval (95% CI).
[¶] Model 1: OR are adjusted for age, smoking habits, sex, and farm childhood.
[¶] Model 2: OR are adjusted for age, smoking habits, sex, farm childhood, and self-reported allergy.

Table 5 Prevalence of wheezing and hay fever and association with high endotoxin exposure within five agricultural sectors.

	Subjects n	Group median endotoxin exposure (EU/m^3)[#]	Wheezing		Hay fever	
			%	OR (95% CI)[¶]	%	OR (95% CI)[¶]
Flower bulb workers	126	180	7.1	2.13 (0.48-9.38)	8.7	0.38 (0.09-1.59)
Animal feed workers	114	320	15.8	1.78 (0.56-5.67)	9.7	0.52 (0.14-1.95)
Onion workers	97	4100	16.5	1.95 (0.62-6.15)	6.2	0.18 (0.02-1.62)
Vegetable seed workers	36	280	11.1	2.67 (0.14-50.3)	30.6	1.05 (0.12-9.18)
Farmers	504	220	10.5	2.41 (1.08-5.39)	10.5	0.52 (0.28-0.95)

EU, endotoxin unit.

¶ Data are presented as the odds ratio (OR) for high (above group median) versus low (below group median) endotoxin exposure with 95% confidence interval (95% CI). OR are adjusted for age, smoking habits, sex, and farm childhood.

Discussion

The present study found that current occupational exposure to endotoxin was associated with a reduced prevalence of hay fever in adults, regardless of childhood exposure. However, in the same population a positive dose-dependent association between endotoxin exposure and adverse respiratory effects such as wheezing, shortness of breath and cough was found. To the present authors' knowledge, this is the first study showing an inverse association between measured airborne endotoxin exposure and allergy in adults, while taking farming exposures during childhood into account.

The pro-inflammatory properties of endotoxin can explain the increased risk of airway symptoms such as wheezing and cough. On the other hand, endotoxin is thought to reduce the risk of allergic sensitisation by inducing a shift from allergic T-helper cell (Th) type 2 responses to Th1-dominated responses, through stimulation of the innate immune system and regulatory T-cells.[28] The results of the present study suggest that inhaled endotoxin may exert such immune-modulating effects beyond childhood, and similar conclusions were previously derived from a longitudinal study showing that young adult dairy farmers may lose allergic sensitisation over time, especially to grass pollen.[29]

Instead of actually measured endotoxin levels, many studies have used contact with farm animals as a representative measure for increased endotoxin exposure.[1, 5-8] Although, in the present study, animal farmers were indeed exposed to higher airborne endotoxin levels than crop farmers, the highest levels of endotoxin exposure were found among workers who handled plant products such as grains for animal feed and onions in an industrial setting. In previous studies it has been shown that plant products can be heavily contaminated with Gram-negative bacteria and their endotoxins, leading to high exposures among workers processing plant materials like cotton, grain, and seeds.[25, 26, 30] The present study showed that the source of endotoxin did not influence risk estimates, whereas health effects depended strongly on measured exposure levels. This clearly demonstrates the importance of quantified endotoxin exposure estimates over a crude classification, and potential misclassification, of environments according to the presence of farm animals.

For some of the job titles only a limited number of measurements was available, and it could be argued that this would result in exposure misclassification. However, analyses excluding groups that were measured less than five times (14 job titles, 119 subjects), resulted in very similar ORs (hay fever: OR 0.53, 95% CI (0.38-0.74); wheeze: 1.35 (1.08-1.69)). In addition, on theoretical grounds, this type of grouping

strategy is not very sensitive to the effects of exposure misclassification, and measures of association usually show very little bias.[31]

The agricultural sectors that were included in the present study were selected based on the expected heterogeneity of endotoxin exposure levels.[26] Since a monotonous dose-response relationship was found for all environments, there is no reason to believe that the associations found in the present study can not be extrapolated to other endotoxin-exposed workers. In previous studies, exposures to agricultural chemicals such as pesticides and disinfectants have also been reported to contribute to wheeze, atopic sensitization, and hay fever.[7, 32, 33] Confounding by chemical exposures in this study is unlikely, as organic farmers are not allowed to use chemical pesticides or toxic disinfectants, such as chloramine-T and quaternary ammonium compounds. An earlier survey showed only small differences in respiratory health status between conventional and organic farmers, especially after adjusting for potential confounders, including disinfectant use and farm childhood.[7] Thus, the current authors assume that the relationships between endotoxin exposure and health outcomes are representative for conventional farmers as well.

Endotoxin was measured as a marker for microbial exposure. However, most of the workers in the present study were probably exposed to mixed bioaerosols, which may have contained other microbial agents including β-1,3-glucan and fungal spores, as well as gases such as ammonia. In farmers, it has been shown that not only endotoxin, but also fungal spores and ammonia are positively associated with non-atopic asthma, but inversely with atopic asthma;[13] the individual agents were, however, significantly correlated, which prevented the authors from drawing a definite conclusion regarding the causative agent.[13] In the present study, using inhalable dust as an explanatory variable yielded similar findings to those obtained with endotoxin (data not shown), which is not surprising given the high correlation between both agents (r=0.83, p<0.001). Thus it cannot be excluded that the observed relationships were at least partially due to associated exposures to other agents. However, given its well-known potency as inducer of inflammatory reactions and activator of immunoregulatory pathways, a major role for endotoxin itself seems highly likely.

Consistent with earlier reports, workers with a farm childhood had a lower prevalence of hay fever, whereas the protective and adverse effects of current exposure to airborne endotoxin were demonstrated in all subjects and no evidence was found of effect modification by farm childhood. By selecting a study population of not only crop and livestock farmers, but also workers involved in processing of plant products, a wide range of endotoxin exposure levels was obtained, with relatively balanced numbers of subjects with and without a farm childhood. This enabled investigation of

effect modification by farm childhood, which would have been more difficult to achieve in a study consisting only of farmers.

Although the response was lower in farmers (61%) than in other agricultural workers (90%), results were similar in the different environments (Table 5). It is unlikely that nonresponse bias explains the results, because in that case nonresponse should have had opposite effects in low-exposed farmers (overestimating hay fever and underestimating asthma-like symptoms) and high-exposed farmers (underestimating hay fever and overestimating asthma-like symptoms). Agricultural processing workers were allowed to complete the questionnaire during the working day, which resulted in a high response rate. This health survey was not initiated because of health concerns raised by employees or participating companies; therefore, it does not seem likely that the high response is associated with overestimation of symptoms.

In cross-sectional studies among occupational populations, it is difficult to eliminate healthy worker selection effects. Avoidance of exposure to endotoxin by subjects with any allergic history would have led to an overestimation of the protective effect of endotoxin on hay fever. However, if strong endotoxin-dependent selection effects had taken place, one would expect associations between endotoxin exposure and asthma-like symptoms to be attenuated in the same population, which did not appear to be the case. Moreover, in the subgroup of 429 subjects for whom IgE serology was available, a significant inverse association was observed between endotoxin exposure and allergic sensitisation in subjects who did not report any allergic symptoms; thus, self-selection is unlikely to explain the present results. Nevertheless, a longitudinal study would be necessary to exclude selection effects completely.

In the present study, self-reported data on symptoms was relied upon. Questions on respiratory symptoms such as current wheezing, were derived from the validated ECRHS Questionnaire, and self-reported wheeze and shortness of breath have been shown to be independent predictors for pulmonary obstruction in dust exposed workers in The Netherlands.[34] In the present study and in other Dutch populations it has been demonstrated that self-reported allergy and hay fever were strongly associated with atopy, as assessed by skin-prick tests or specific IgE against common allergens.[35, 36]

In conclusion, the results of the present study demonstrated an inverse exposure-response relationship for hay fever in agricultural workers currently exposed to airborne endotoxin, regardless of childhood farming exposures. At the same time, endotoxin exposure was associated with an increased prevalence of asthma-like symptoms. Although endotoxin might protect workers against allergies, the present

study emphasises the need to lower endotoxin exposure levels in the agricultural environment to protect workers against chronic respiratory health effects.

Acknowledgments

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Chapter 4

Occupational endotoxin exposure reduces the risk of atopy in adults without a farm childhood

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Abstract

Background: Farm exposures in both childhood and adult life are protective against atopy, allergic rhinitis, and atopic asthma, but in adults also paralleled by an increased prevalence of non-atopic asthma.

Objective: To investigate associations between occupational endotoxin exposure and atopy and bronchial hyperresponsiveness to methacholine (BHR) in agricultural workers, and to study the role of atopy in endotoxin-related respiratory effects.

Methods: Data were available for 427 farmers and agricultural industry workers, for whom airborne endotoxin exposure levels were determined with a model based on 249 personal exposure measurements. Atopy was assessed as specific serum IgE to common inhalant allergens, and respiratory symptoms and personal characteristics by standardized questionnaires. BHR was determined in 113 subjects.

Results: Endotoxin exposure was inversely associated with atopy and IgE to grass pollen ($P<0.001$), but only in workers who had not grown up on a farm. Endotoxin exposure was positively associated with wheeze and BHR ($P<0.05$), irrespective of growing up on a farm. The proportions of wheeze and BHR that were attributable to atopy were only 14.7% and 26.4% respectively.

Conclusion: Occupational endotoxin exposure is inversely related to the risk of atopic sensitization in adults who had not grown up on a farm. It is unlikely that this is a result of healthy worker selection, as significant associations between endotoxin and atopy were found regardless of reported allergic symptoms. At the same time, high endotoxin exposure is a risk factor for wheeze and BHR, which were characterized by a predominantly non-atopic phenotype.

Introduction

There is increasing evidence that farm exposures throughout life are protective against atopy, allergic rhinitis, and atopic asthma in adults.[1] Several studies have observed a strongly decreased prevalence of allergic sensitization,[2-4] hay fever,[5] and asthma[6] among adults with both childhood and current farm exposures. Farmers and children growing up on farms are exposed to high levels of microbial agents, such as bacterial endotoxin and fungal $\beta(1\rightarrow3)$-glucans.[7, 8] It has been hypothesized that exposure to such agents may induce a shift from atopic Th-2 responses to Th-1 responses through stimulation of the innate immune system and regulatory T cells.[9] Protective effects of house dust endotoxin on the development of atopy and asthma have been shown in children,[10-13] and more recently, studies among adults have shown similar inverse relationships between endotoxin exposure and atopic asthma,[7] allergic sensitization,[14, 15] and hay fever.[16]

Since endotoxin is also a potent proinflammatory agent, the downside of increased exposure is a well described elevated risk of non-allergic respiratory health effects, as shown in occupational and domestic environments.[17] A few studies have clearly illustrated the dual nature of endotoxin by showing protective effects of exposure on atopic disease, but an increased risk of non-atopic asthma, non-atopic wheeze, and non-specific airway hyperresponsiveness.[7, 11, 14, 16]

We recently investigated the relationship between occupational endotoxin exposure and respiratory symptoms in a large adult population of farmers and workers in the agricultural industry, and found a significantly increasing prevalence of wheeze at higher exposure, but an inverse association between endotoxin exposure and the risk of self-reported hay fever.[16] The latter finding is compatible with a presumed down-regulatory effect of endotoxin exposure on atopic immune responses, since hay fever symptoms are usually strongly associated with demonstrable atopic sensitization to grass, tree, or weed pollen.[18] Since wheeze is generally also associated with atopy and bronchial hyperresponsiveness,[19, 20] the higher prevalence at high endotoxin exposure may seem paradoxical. Dose-response relationships between endotoxin and wheeze were found regardless of self-reported allergy, which was used as a surrogate for atopy[16] and this suggested that much of the wheeze reported in this population may not be due to atopic airway inflammation.

The aim of the present study was to further elucidate this apparent paradox by using objectively measured health data, namely serum IgE to common allergens and non-specific bronchial hyperresponsiveness. Specific objectives were to investigate associations between occupational endotoxin exposure and atopy and airway hyperresponsiveness in agricultural workers, and to study the role of atopy in

association with endotoxin-related respiratory effects. We also took into account whether growing up on a farm influenced associations between endotoxin exposure and allergic and respiratory health effects later in life.

Methods

Study population

In total, 434 subjects participated. The study population was derived from a questionnaire survey in Dutch farmers and workers from agricultural processing industries, which has been described earlier.[16] A total of 342 agricultural industry workers (91% of 376 eligible) consented to blood sampling. A total of 340 farmers (65% of 525 eligible) indicated that they would not object being contacted for a medical examination. For logistical reasons, however, only approximately 100 farmers could be included. A random sample of 122 farmers was approached by telephone, and 92 farmers participated. Venous blood samples for IgE analysis were taken during a visit to the worksite as part of the medical examination. Data from 427 of 434 participating subjects were used (338 industry workers, 89 farmers), as we excluded four subjects aged <18 or >65 yrs, and three subjects with incomplete data. Farmers who participated in the medical examination did not differ significantly from farmers in the original sample with respect to endotoxin exposure, potential confounders and wheeze, but hay fever was more prevalent in the 89 farmers who participated (17% vs 9%; *P*=0.02, χ^2-test). Selection bias was a potential concern and, therefore, sensitivity analyses were performed to see whether results changed after excluding farmers. The study protocol was approved by the institutional ethics committee and all participants gave written informed consent.

Endotoxin exposure

Endotoxin exposure assessment and modeling have been reported in detail earlier.[16] Personal airborne endotoxin exposure (249 full-shift measurements) was measured in a sample of participants. Endotoxin exposure was modeled on the basis of work site and job title to assign exposure levels to each participant. Endotoxin exposure levels were expressed as endotoxin units (EU) per m^3. Individual cumulative occupational exposure levels were calculated by multiplying exposure level and years of work and were expressed as EU/m^3 x year.

IgE serology

Specific IgE to common allergens house dust mite, grass pollen (mix of timothy and perennial ryegrass), cat and dog, and total serum IgE were determined by enzyme immunoassays as described previously.[14, 21] Total IgE levels were dichotomized by using 100 IU/ml as cut-off level. Atopy was defined as the presence of specific serum IgE antibodies to one or more of the common allergens.

Bronchial hyperresponsiveness

Bronchial hyperresponsiveness (BHR) to methacholine was assessed in subjects from 7 randomly selected companies that were visited on a second occasion. In total, 113 of 119 subjects who were present again on the second occasion consented to BHR (response 95%). Due to logistical constraints, BHR was not assessed in farmers. Forced expiratory lung function and BHR were assessed according to European Respiratory Society guidelines.[22, 23] Methacholine was inhaled from 0.019 mg up to a cumulative dose of 2.5 mg as described before.[24] The test was stopped after the maximum cumulative dose was reached, or when FEV_1 fell by 20% or more. BHR was defined as a fall in FEV_1 of at least 20% at a methacholine dose of 2.5 mg or less.

Data analysis

Associations between natural log-transformed endotoxin levels and dichotomous health outcomes were assessed by multiple linear logistic regression analysis. Odds ratios for an interquartile increase in endotoxin exposure were calculated and were adjusted for age, sex, smoking habits, and farm childhood, unless stated otherwise. Interactions between farm childhood and endotoxin exposure were explored and tested. Exposure-response relationships between endotoxin exposure and health outcomes were further studied by assessing the shape of the exposure-response relationship by means of generalized additive modeling (smoothing) as described before.[14]

Results

Characteristics of the study population are presented in Table 1. Subjects who had grown up on a farm were more often male, somewhat older, and less often current smokers than workers without a farm childhood. Geometric mean (GM) endotoxin levels were almost identical in both groups (~319 EU/m^3), but cumulative occupational endotoxin levels were significantly higher in workers with a farm

childhood. Specific serum IgE to one or more common allergens was found in 81 subjects (19%). Agricultural workers with a farm childhood were less often atopic, and had less often specific IgE to grass pollen than workers without a farm childhood (Table 1).

Table 1 Characteristics of 427 agricultural workers, by farm childhood.

	No farm childhood (n=234)	Farm childhood (n=193)
Male, n (%)	193 (82.5)	178 (92.2)[*]
Age (years), mean (SD)	40.0 (10.7)	44.0 (9.7)[*]
Smoking habits, n (%)		
Never	83 (35.5)	90 (46.6)
Former	69 (29.5)	65 (33.7)
Current	82 (35.0)	38 (19.7)[*]
Endotoxin exposure (EU/m^3), GM (GSD)	319 (6.5)	318 (4.5)
Cumulative endotoxin exposure (EU/m^3 x y), GM (GSD)	2407 (8.0)	4355 (6.5)[*]
Atopy, n (%)	53 (22.7)	28 (14.5)[*]
Grass pollen IgE, n (%)	34 (14.5)	15 (7.8)[*]
House dust mite IgE, n (%)	31 (13.3)	15 (7.8)[#]
Cat IgE, n (%)	6 (2.6)	1 (0.5)[#]
Dog IgE, n (%)	1 (0.4)	2 (1.0)
Total IgE (≥100 IU/ml), n(%)	58 (24.8)	46 (23.8)
Total IgE (IU/ml), GM (GSD)	26.9 (7.8)	24.2 (7.3)
Hay fever	30 (12.8)	21 (10.9)
Wheezing	32 (13.7)	23 (11.9)
BHR [§]	17 (24.3)	13 (30.2)

* p<0.05, # p<0.10 (χ^2 test or t-test); SD, standard deviation; EU, endotoxin unit; GM, geometric mean; GSD, geometric standard deviation; [§] BHR was measured in a subgroup of 113 workers (70 without and 43 with farm childhood).

The prevalence of both reported wheeze and hay fever, and their associations with endotoxin exposure levels, were very similar to what we found in the larger questionnaire study[16] (Table 2). Endotoxin exposure was positively associated with wheeze (OR=1.30 per interquartile range of exposure, compared to 1.41 in the earlier study), whereas a negative association was observed for hay fever (OR=0.57 vs. 0.62 in the previous report). Moreover, there was no evidence of effect modification by farm childhood: point estimates for the ORs in the two strata were very similar. Endotoxin exposure was significantly associated with less atopy and less grass pollen sensitization, with ORs of 0.67 and 0.59, respectively (Table 2). However, further stratified analyses revealed that the inverse associations with atopy and IgE

to grass pollen were restricted to subjects without a farm childhood (*P*<0.001). In contrast, for workers who had grown up on a farm, no association between endotoxin and sensitization was found (ORs close to unity). Associations between occupational endotoxin exposure and atopy were significantly different for the groups with and without a farm childhood (*P*-value interaction=0.02).

Table 2 Associations of atopy, elevated total IgE, reported respiratory symptoms and bronchial hyperresponsiveness (BHR) with endotoxin exposure in agricultural workers, stratified by farm childhood.

	All (n = 427)	No farm childhood (n = 234)	Farm childhood (n = 193)
	Endotoxin exposure OR (95% CI)[#]	Endotoxin exposure OR (95% CI)[#]	Endotoxin exposure OR (95% CI)[#]
Atopy	0.67 (0.54-0.84)	0.56 (0.42-0.74)	1.01 (0.68-1.50)
Grass pollen IgE	0.59 (0.45-0.79)	0.52 (0.37-0.73)	0.89 (0.52-1.52)
House dust mite IgE	0.84 (0.65-1.09)	0.75 (0.56-1.02)	1.10 (0.66-1.83)
Total IgE (≥100 IU/ml)	0.87 (0.72-1.05)	0.79 (0.63-1.01)	1.05 (0.76-1.45)
Hay fever	0.57 (0.43-0.75)	0.56 (0.40-0.79)	0.57 (0.35-0.95)
Wheezing	1.30 (1.01-1.67)	1.26 (0.92-1.71)	1.39 (0.90-2.14)
BHR [§]	1.55 (1.03-2.35)	1.53 (0.84-2.79)	2.21 (1.04-4.69)

[#] Data are presented as the odds ratio (OR) for an interquartile range ($\exp^{1.45}$ = factor 4.3) increase in modeled endotoxin exposure levels (EU/m^3) with 95% confidence interval (95% CI). ORs are adjusted for age, smoking habits, sex, and (in unstratified analysis) farm childhood.
[§] Bronchial hyperresponsiveness (BHR) was measured in a subgroup of 113 workers (70 without and 43 with farm childhood). OR are adjusted for age, smoking habits, sex, farm childhood, and atopy.

In contrast to atopy, BHR showed a positive and significant association with endotoxin exposure (Table 2), and no evidence of effect modification by farm childhood was found. In BHR positive subjects, PD_{20} was lower in those exposed to high endotoxin levels (levels above GM), with a GM PD_{20} of 0.37 mg vs. 0.57 mg in workers exposed to lower endotoxin levels. Although this suggests a dose-response relationship, the difference was not significant (*P*=0.3). Similar and significant ORs were found for all health outcomes described in Table 2 when cumulative occupational endotoxin exposure was used instead of current endotoxin exposure levels (data not shown).

Sensitivity analyses were performed to see whether results changed after excluding farmers. Analysis restricted to agricultural industry workers showed very similar results for all health outcomes. For example, an OR of 0.57 (0.43-0.76) was found for the association between endotoxin and atopy in workers without a farm childhood

(n=220), and 1.11 (0.71-1.75) for workers with a farm childhood (n=118). Adjusting ORs for occupational group (farmer/non-farmer) did also not change results, thus, the impact of selection seemed minimal.

Hay fever was strongly associated with grass pollen sensitization (OR 18.1, 9.0-36.3). Despite the high OR, only a relatively small proportion of subjects reporting hay fever had a positive test for grass pollen IgE (53%) or were atopic (61%). These proportions were not different at high and low levels of endotoxin exposure. Wheezing and BHR were strongly related (OR 6.4, 2.1-19.8), and were both significantly associated with atopy, showing ORs of 2.2 (1.1-4.1) and 4.1 (1.6-10.5) respectively. Still, the majority of wheezers (69%) and subjects with a positive BHR test (57%) had a non-atopic phenotype. At higher levels of endotoxin exposure (levels above GM), even larger proportions of wheezers (77%) and BHR positive subjects (65%) were non-atopic. Associations between endotoxin exposure and wheeze and BHR were similar in non-atopic and atopic subjects, also when ORs were further stratified for farm childhood (Table 3). When hay fever was subdivided by atopic status and analyzed as atopic and non-atopic rhinitis, no association between endotoxin exposure and non-atopic rhinitis was found in workers without a farm childhood (OR 0.89), whereas an inverse association was found in workers with a farm childhood (OR 0.54). In atopic subjects, rhinitis was inversely associated with endotoxin irrespective of farm childhood (Table 3).

The shape of the exposure-response relationships was investigated by nonparametric models (smoothing), suggesting a non-linear relationship between endotoxin exposure and atopy in workers without a farm childhood (Figure 1). At very low endotoxin exposures (<35 EU/m^3, and <100 EU/m^3 x y), the highest prevalence of atopy and grass pollen sensitization was seen, after which a steep decline in the predicted prevalence was observed up to levels of around 1000 EU/m^3 and 10,000 EU/m^3 x y. At higher endotoxin exposure levels a constant and very low prevalence was seen (Figure 1A-D). Somewhat less pronounced exposure-response relationships were seen for house dust mite sensitization (Figure 1E-F). For workers with a farm childhood, the prevalence of sensitization was low over the whole range of current or cumulative occupational endotoxin exposure.

To examine the possibility that healthy worker selection bias was (partly) responsible for these observations, we investigated whether the inverse associations between endotoxin and atopy were also present in workers not reporting any allergy symptoms. As shown by Table 4, significant inverse associations between endotoxin exposure and atopy/grass pollen IgE were observed for both non-symptomatic and symptomatic subjects without a farm childhood.

Table 3 Association between endotoxin exposure and wheezing, rhinitis, and BHR, stratified by atopy and farm childhood.

		All (n=427)		No farm childhood (n = 234)		Farm childhood (n=193)	
		n/N (%)	Endotoxin exposure OR (95% CI)	n/N (%)	Endotoxin exposure OR (95% CI)	n/N (%)	Endotoxin exposure OR (95% CI)
Wheezing	No atopy	38/346 (11.0)	1.43 (1.04-1.97)	23/181 (12.7)	1.40 (0.95-2.06)	15/165 (9.1)	1.48 (0.85-2.57)
	Atopy	17/81 (21.0)	1.46 (0.87-2.45)	9/53 (17.0)	1.31 (0.71-2.41)	8/28 (28.6)	1.53 (0.55-4.25)
Rhinitis	No atopy	20/346 (5.8)	0.73 (0.48-1.12)	10/181 (5.5)	0.89 (0.51-1.55)	10/165 (6.1)	0.54 (0.27-1.10)
	Atopy	31/81 (38.3)	0.52 (0.31-0.87)	20/53 (37.7)	0.57 (0.33-1.00)	11/28 (39.3)	0.38 (0.13-1.12)
BHR	No atopy	17/87 (19.5)	1.94 (1.11-3.41)	8/52 (15.4)	1.46 (0.73-2.92)	9/35 (25.7)	3.05 (1.08-8.60)
	Atopy	13/26 (50.0)	2.12 (0.71-6.30)	9/18 (50.0)	1.70 (0.32-9.09)	4/8 (50.0)	2.15 (0.53-8.70)

n/N: Subjects in stratum reporting wheeze, hay fever, or having a positive test for BHR (n) / All subjects in stratum (N).
Data are presented as the odds ratio (OR) for an interquartile range ($\exp^{1.45}$ = factor 4.3) increase in modeled endotoxin exposure levels (EU/m^3) with 95% confidence interval (95% CI). ORs are adjusted for age, smoking habits, sex, and (in unstratified analysis) farm childhood.
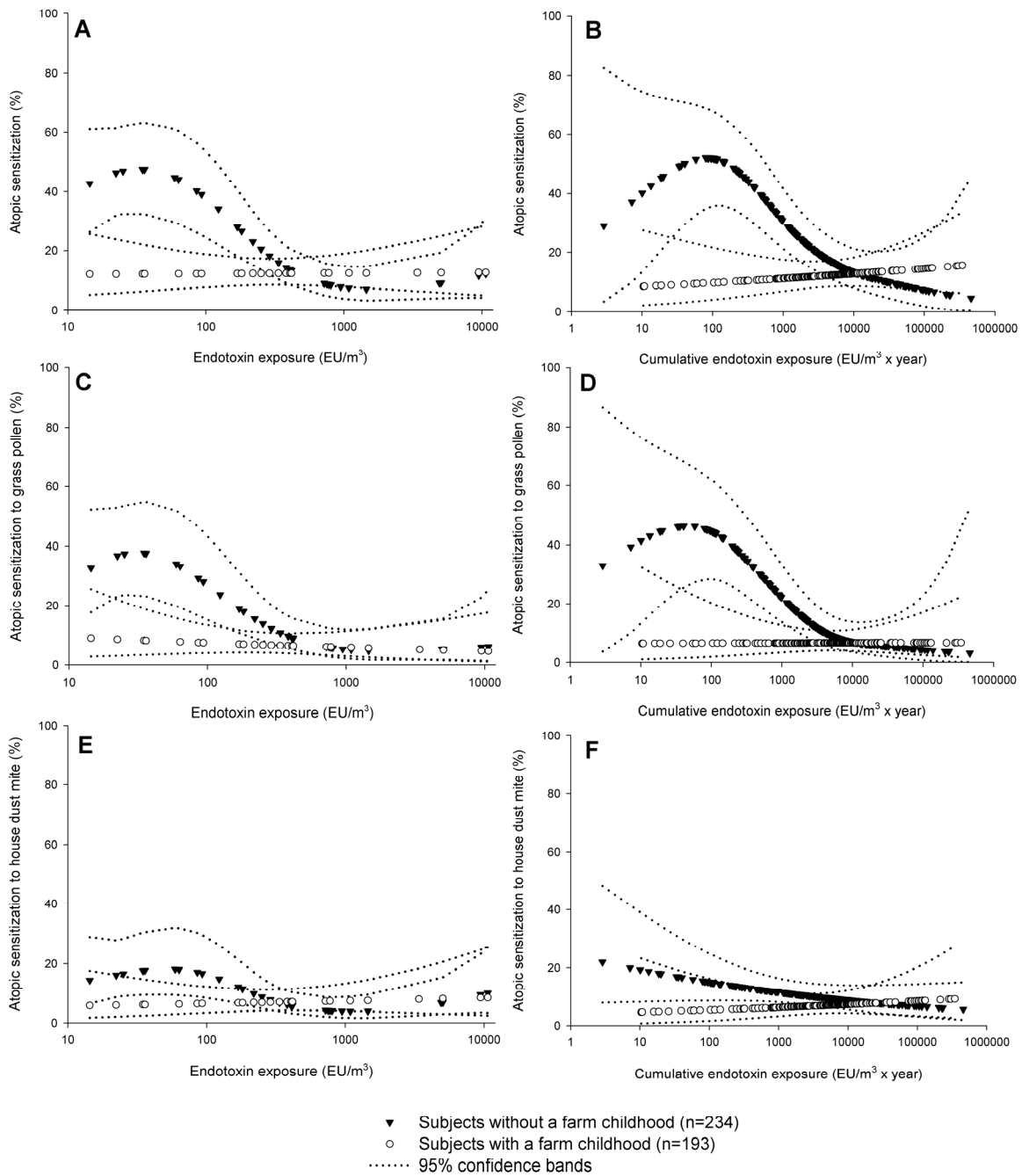
Figure 1 Smoothed plots with 95% confidence bands representing associations of endotoxin exposure (A,C,E) and cumulative endotoxin exposure (B,D,F) with atopic sensitization (A,B), atopic sensitization to grass pollen (C,D), and atopic sensitization to house dust mite (E,F) in 193 agricultural workers with a farm childhood (black triangles) and 234 workers without a farm childhood (white circles). Associations were adjusted for age, sex, and smoking.

Table 4 Association between endotoxin exposure and atopy or grass pollen sensitization, stratified by self-reported allergy and farm childhood.

	No farm childhood (n=234)		Farm childhood (n=193)	
	N	Endotoxin exposure OR (95% CI)[#]	N	Endotoxin exposure OR (95% CI)[#]
Non-symptomatic subjects [§]	178		147	
Atopy	22	0.61 (0.42-0.88)	10	1.77 (0.96-3.26)
Grass pollen IgE	10	0.50 (0.29-0.86)	5	1.00 (0.41-2.43)
Symptomatic subjects	56		46	
Atopy	31	0.45 (0.24-0.84)	18	0.82 (0.46-1.47)
Grass pollen IgE	24	0.58 (0.33-0.99)	10	1.00 (0.52-1.95)

[#] Data are presented as the odds ratio (OR) for an interquartile range ($exp^{1.45}$ = factor 4.3) increase in modeled endotoxin exposure (EU/m^3) with 95% confidence interval (95% CI). OR are adjusted for age and sex.
[§] Symptomatic subjects are those who gave a positive answer to the question "Have you ever had any allergies?".

Discussion

According to the hygiene hypothesis, bacterial and viral infections, and environmental exposures to microbial compounds may protect from the development of allergic disease by influencing immune responses. It has been argued that the immune system is most susceptible to the influence of microbial exposures during infancy or early childhood, and as a result it seems plausible that timing of exposure plays a critical role.[25] Several studies have shown that farm exposures during childhood may exert long-lasting protective effects on allergy or allergic asthma,[3, 26] and the lower prevalence of atopic disease that is often found among farmers is therefore likely to be partly a result of early microbial exposures.[2-6, 27] In accordance with the hypothesis that effects of early exposures can be long-lasting, we observed a significantly lower prevalence of atopy in agricultural workers with a farm childhood (Table 1, Figure 1). Nevertheless, others have argued that immune deviation may take place throughout life,[6] and exposure in adulthood to endotoxin and other microbial compounds has indeed been associated with a lower prevalence of allergy or allergic asthma.[7, 14-16] Our study substantiates the hypothesis that also exposures at adult age may be associated with a protective effect by showing strong inverse associations between occupational endotoxin exposure and atopy, taking into account whether subjects had grown up on a farm. In workers without a farm childhood, steep inverse dose-response relationships between endotoxin exposure and atopy were observed for exposure levels above 35 EU/m^3 or 100 EU/m^3 x y. The protective effect of endotoxin exposure was much stronger for grass pollen

sensitization than for house dust mite sensitization or total IgE, which is consistent with other studies in children and adults.[14, 15, 28, 29] It seems therefore that microbial factors may have a strong influence on grass pollen sensitization, whereas levels of allergen exposure or genetic factors may be more important for other allergies and total IgE levels. As we argued before,[16] we can not exclude that the observed relationships were at least partially due to other microbial agents than endotoxin. Given its well-known potency as an inducer of inflammatory reactions and activator of immunoregulatory pathways, a major role for endotoxin itself seems, however, highly likely.

The present study population was derived from a questionnaire survey in 901 subjects (877 were included in data analysis),[16] showing significant inverse associations between endotoxin exposure and hay fever in workers with and without a farm childhood. Results of the questionnaire survey were confirmed in the 427 subjects with IgE data available, also when farmers, where selection effects might have occurred, were excluded from analyses. Hence, the fact that occupational endotoxin exposure in workers with a farm childhood was associated with less hay fever, but not with less atopy or grass pollen IgE, did not seem to be caused by selection of the subpopulation. A more likely explanation might be the only partial concordance of self-reported allergy with positive IgE serology. The proportion of hay fever that was attributable to atopy was no higher than 52% (i.e. population attributable risk). Although this proportion appears to be very low, in a review of large population-based surveys it has been shown that the average percentage of self-reported seasonal rhinitis or allergen related rhinitis attributable to atopy was only 48%.[30] In workers with a farm childhood the proportion of self-reported hay fever attributable to atopy or grass pollen IgE was lower than among workers without a farm childhood (44% versus 57% for atopy, and 38% versus 53% for grass pollen IgE). It is unclear why workers with a farm childhood more often reported hay fever without having serologic evidence of grass pollen allergy than workers without a farm childhood. It has been suggested that the perception of asthma symptoms might differ between subjects with and without a farm childhood.[31] However, evidence is conflicting and differences in the perception of hay fever have not been studied.[31, 32] When we defined hay fever as a combination of reported symptoms and grass pollen IgE, we still observed a highly significant inverse association between endotoxin exposure and hay fever in workers without a farm childhood (OR 0.44, 95%CI 0.28-0.69). On the other hand, in workers who had grown up on a farm, only a weak inverse association was found (OR 0.81, 0.42-1.58), hence the earlier reported inverse association between endotoxin and hay fever in this subgroup was driven by an association between endotoxin exposure and rhinitis symptoms without

demonstrable IgE (OR 0.51, 0.28-0.96). The inverse association between endotoxin and non-atopic rhinitis in this subgroup may be partly explained by selection effects, although the strongly positive associations between endotoxin exposure and wheeze and BHR among the same workers seem a strong argument against a major influence of health-based selection.

Healthy worker effects are likely to be important in agriculture, either because less healthy subjects would prefer not to take over a farm or be employed in a dusty agricultural processing company, or because less healthy workers are more likely to leave high-exposure jobs.[33] Moreover, in western countries migration out of rural areas and agricultural occupations is going on for more than a century. However, one would not expect strong selection effects to have taken place among workers who never had any allergic complaints. Thus, the inverse exposure-response relationships between endotoxin exposure and atopy in the subgroup of non-symptomatic subjects without a farm childhood (Table 4) supports the hypothesis that microbial exposures in adulthood might be causally related to less atopic sensitization. However, more and especially longitudinal studies are needed before drawing conclusions about causality.

In contrast with the inverse association between endotoxin and atopy, we found that endotoxin exposure was a risk factor for wheeze and BHR. Since atopy was positively associated with these respiratory outcomes, the opposite effects of endotoxin exposure may seem paradoxical. However, the proportions of wheeze and BHR in our population that were attributable to atopy were only 14.7% and 26.4% respectively. These apparently low proportions are consistent with data presented by Pearce et al. who demonstrated that the proportion of asthma-related outcomes in population based studies that is attributable to atopy is on average lower than 40%.[34] Obviously, in adults exposed to high levels of proinflammatory agents such as endotoxin, one may expect that the attributable risk of atopy is even lower.

In occupational and domestic studies, endotoxin exposure has been shown to be a dose-dependent risk factor for wheeze and other asthma symptoms in adults.[35, 36] Conversely, in a study among 12-19 year old secondary school students in Quebec, a lower prevalence of wheeze and BHR was found in students who had grown up on a farm compared to those without regular farm exposures.[37] This effect was paralleled by a protective effect on allergic sensitization, and it was assumed that the asthma-related outcomes were also of an atopic phenotype. Our results suggest that prolonged exposure to high levels of endotoxin may override the advantage of a farm childhood on asthma-related effects, most likely through non-allergic mechanisms. Therefore, it is important that related - but not synonymous - phenotypes such as atopy, BHR, and wheeze are carefully distinguished and their mutual relations

disentangled to reveal underlying mechanisms and associations with microbial exposures.

In conclusion, a significant inverse dose-response relationship between occupational endotoxin exposure and atopy was observed in adults who had not grown up on a farm. Workers with a farm childhood had a lower prevalence of atopy than those without farm childhood, but no additional effect of current exposure was found in this subgroup. In both groups of workers, endotoxin exposure was a risk factor for wheeze and bronchial hyperresponsiveness, which were characterized by a predominantly non-atopic phenotype.

Acknowledgments

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Chapter 5

Exhaled nitric oxide in endotoxin-exposed adults: effect modification by smoking and atopy

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Abstract

Background: Occupational exposure to endotoxin is associated with non-allergic asthma and other airway inflammatory reactions. Little is known about the role of mucosal nitric oxide (NO) production in endotoxin-induced airway inflammation. The objective was to explore exposure-response relationships between occupational endotoxin exposure and fractional concentrations of exhaled NO (FE_{NO}), and study the role of FE_{NO} as an intermediate factor in the relationship between endotoxin exposure and asthma-like symptoms.

Methods: FE_{NO} was measured online in 425 farmers and agricultural industry workers. For each participant (cumulative) endotoxin level was modelled on the basis of 249 personal measurements and job history. Atopy was assessed as specific serum IgE to common inhalant allergens, and other health data and personal characteristics by standardized questionnaires.

Results: A significant positive exposure-response relationship was found between endotoxin and FE_{NO}, but only in non-atopic, non-smoking subjects (p=0.001). FE_{NO} was significantly associated with current wheeze and other asthma-like symptoms irrespective of atopy and current smoking. Associations between endotoxin exposure and symptoms changed slightly after adjusting for FE_{NO}.

Conclusions: A positive association was found between occupational endotoxin exposure and exhaled nitric oxide in non-smoking, non-atopic adults. Increased FE_{NO} was associated with asthma-like symptoms, but the role of FE_{NO} as an intermediate factor between endotoxin exposure and airway symptoms appears to be limited.

Introduction

Epidemiological studies in occupational environments have shown associations between endotoxin exposure and health effects such as respiratory symptoms, non-allergic asthma, accelerated lung function decline, and organic dust toxic syndrome (ODTS).[1-4] Inflammatory responses to inhaled endotoxin are characterized by increased levels of neutrophils and proinflammatory cytokines as shown in nasal lavage, induced sputum and bronchoalveolar lavage studies.[5-8] Animal models and in vitro cell studies have demonstrated that endotoxins can induce the expression of nitric oxide (NO) synthase which may result in increased levels of the fractional concentration of exhaled NO (FE_{NO}).[9, 10]

Endotoxin inhalation challenge in human experimental studies caused an acute and temporary increase in FE_{NO} in asthmatics, but not in healthy volunteers.[11, 12]

Exhaled NO is a noninvasive and reproducible indicator of airway inflammation, and chronically increased FE_{NO} has been associated with respiratory disease and atopy in both children and adults.[13-18] To date, only few studies have measured exhaled NO as a marker of airway inflammation in occupational populations. Some of these studies involved populations exposed to occupational sensitizers, and resulted in conflicting evidence regarding the usefulness of exhaled NO in relation to occupational asthma.[19, 20] A few studies have reported FE_{NO} measurements in subjects exposed to mixed bioaerosols and ammonia in pig confinement buildings. Von Essen et al.[21] observed a small elevation in mean exhaled NO in swine confinement workers compared with urban controls. Elevated FE_{NO} has also been demonstrated in previously unexposed, healthy volunteers, 5 hours after exposure in a pig confinement building.[22] However, no effect of acute bioaerosol exposure on FE_{NO} was found in three other studies among healthy volunteers or asthmatic subjects.[23-25]

The current authors recently reported significant associations between endotoxin exposure and respiratory symptoms such as wheeze, shortness of breath, and cough in a large adult population occupationally exposed to high levels of endotoxin.[1] In the present study, exposure-response relationships between endotoxin exposure and FE_{NO} as a marker of airway inflammation, and as a putative intermediate factor in the relationship between endotoxin and respiratory symptoms, were investigated in the same population.

Methods

Study population
The study population was recruited among farmers and workers from agricultural processing industries in 2006. A detailed description of the enrollment of the study population is given elsewhere.[1] In short, questionnaires including items on asthma and asthma-like symptoms of the European Community Respiratory Health Survey (ECRHS) questionnaire[26] were sent to farm owners and to employees of participating agricultural processing companies. The questionnaire was completed by 525 farmers, a farm owner response rate of 61%, and 376 agricultural industry workers, a response rate of 90%.

A total of 358 agricultural industry workers, 95% of questionnaire responders, agreed to participate in a medical examination. Of the 525 farmers who completed the questionnaire, 340 (65%) indicated that they would not object being contacted for a medical examination. For logistical reasons, a random sample of 122 farmers was approached by telephone, and 96 farmers were able to participate on one of the proposed dates. Data from 425 of 454 participating subjects were used, because we excluded subjects who did not consent to blood sampling (n=20), subjects who failed to perform an acceptable FE_{NO} measurement (n=2), subjects aged <18 or >65 yrs (n=4), and subjects who had incomplete questionnaire data (n=3). The study protocol was approved by the local medical ethics committee and all participants gave written informed consent.

Endotoxin exposure
Endotoxin exposure assessment and modelling has been reported in detail earlier.[1] Briefly, a job-exposure matrix was constructed using 249 personal airborne endotoxin exposure measurements. For each participant, individual (cumulative) endotoxin level was calculated by multiplying modelled exposure level - in endotoxin units (EU)/m^3 - and years of exposure. Subjects had worked on average for 15.4 years (SD 11.4 y) in agriculture.

FE_{NO} measurement
All measurements took place on-site at the beginning of the workday. Thus, FE_{NO} was measured as an indicator of chronic inflammation rather than as an effect of acute endotoxin exposure. Subjects performed a single online measurement for 10 s at an exhalation flow rate of 50 ml/s and a pressure of 10 cm H_2O using a portable NO sampling device (NIOX Mino, Aerocrine AB, Solna, Sweden) that was recently evaluated and found to be directly comparable with a conventional

chemiluminescence analyzer.[27] The same NIOX Mino device was used for all measurements.

Specific IgE

Specific immunoglobulin E (IgE) antibodies to the common allergens house dust mite, grass pollen (mix of timothy and perennial ryegrass), cat, and dog were measured by enzyme immunoassays as described previously.[28, 29] Atopy was defined as serum IgE to one or more common allergens.

Data analysis

Associations between modelled cumulative endotoxin exposure and FE_{NO}, and associations between respiratory symptoms and FE_{NO} were studied by univariate and multiple linear regression analyses, using natural log-transformed FE_{NO} and endotoxin levels. Geometric mean FE_{NO} ratios (GMR) and 95% confidence intervals (CI) were calculated by exponentiating regression coefficients and their CI. Associations between modelled cumulative endotoxin exposure and FE_{NO} are represented as GMR for subjects at the 75-percentile of endotoxin exposure versus subjects at the 25-percentile. Regression coefficients for ln-transformed endotoxin exposure were first multiplied by the interquartile range of ln-transformed endotoxin exposure (2.03, corresponding to a $exp^{2.03}$ = 7.6-fold increase in endotoxin exposure), and then exponentiated to obtain the GMR for an interquartile range increase in exposure. Comparison between parametric models and models including smoothed functions showed that parametric linear regression models adequately described associations between endotoxin exposure and FE_{NO}. Effect modification by current smoking and atopy was explored. Unless stated otherwise, results were adjusted for age, sex, and height.

Results

GM FE_{NO} levels (ppb), 25th and 75th percentiles, and crude GM FE_{NO} ratios for personal characteristics are shown in Table 1. Unadjusted FE_{NO} levels were slightly higher among workers exposed to endotoxin above the median level (17.6 vs. 15.9 ppb NO), but this difference was not statistically significant (p=0.12). Male sex, height, and atopy were significantly associated with increased FE_{NO} levels. Current smoking was associated with strongly reduced FE_{NO} levels (12.0 ppb NO in smokers versus 19.1 ppb NO in ex-smokers and never smokers, p<0.001).

Table 1 GM FE$_{NO}$ levels (ppb) and crude GMR (95% CI) in 425 agricultural workers.

Predictor	N	GM	25th - 75th Percentile	GMR (95% CI)
Cumulative endotoxin exposure				
< 4117 EU/m^3 x yr (median)	212	15.9	10 - 22	1
≥ 4117 EU/m^3 x yr	213	17.6	12 - 27	1.10 (0.97-1.25)
Sex				
Female	55	11.4	7 - 16	1
Male	370	17.7	12 - 26	1.56 (1.30-1.87)
Age				
< 42.3 yr (median)	212	16.2	11 - 23	1
≥ 42.3 yr	213	17.2	11 - 26	1.06 (0.94-1.20)
Smoking habits				
Non-smoker	306	19.1	12 - 28	1
Current smoker	119	12.0	8 - 19	0.63 (0.55-0.72)
Height				
< 1.79 m (median)	212	15.3	10 - 24	1
≥ 1.79 m	213	18.2	12 - 27	1.19 (1.05-1.35)
Atopy				
No	345	15.4	11 - 22	1
Yes	80	23.6	13 - 38	1.53 (1.31-1.79)

GMR: geometric mean ratio.

Table 2 Adjusted GMR for FE$_{NO}$ in 425 agricultural workers, stratified by current smoking.

Predictor	Non-smokers (n = 306) GMR (95% CI) [†]	Current smokers (n = 119) GMR (95% CI) [†]
Cumulative endotoxin exposure [‡]	1.08 (1.01-1.16)[*]	0.99 (0.88-1.11)
Male sex	1.21 (0.95-1.53)	1.26 (0.87-1.84)
Age (per 10 yr)	1.11 (1.03-1.19)[*]	0.99 (0.90-1.10)
Height (per 10 cm)	1.12 (1.01-1.24)[*]	1.11 (0.97-1.28)
Atopy	1.64 (1.39-1.93)[**]	1.08 (0.79-1.49)

[*] p <0.05; [**] p < 0.001.
[†] Geometric mean ratio (GMR, 95% CI) adjusted for all variables in the model.
[‡] Per interquartile range (exp$^{2.03}$ = factor 7.6) increase in endotoxin exposure (EU/m^3 x yr).

FE$_{NO}$ was associated with endotoxin exposure in non-smoking subjects (p=0.02; Table 2). Age, height, and atopy were also independently and positively associated with FE$_{NO}$ levels, but only in non-smoking workers. Further analyses were limited to non-smokers. A significant exposure-response relationship was found between endotoxin exposure and FE$_{NO}$ in non-atopic, but not in atopic subjects (Figure 1). The

unadjusted GMR for non-atopic, non-smoking subjects at the 75-percentile of endotoxin exposure versus subjects at the 25-percentile was 1.15 (95%CI 1.07-1.23; p=0.0003). Adjustment for sex, age, and height resulted in a slightly lower GMR (1.13, 95%CI 1.05-1.21; p=0.001). The influence of caffeine intake, use of inhaled or oral corticosteroids (reported by 5 subjects), and having had a cold or flu last week was also assessed in the analyses, but these variables did not change associations between endotoxin exposure and FE_{NO}. The accuracy of the regression models was further examined by removing the most influential data points (identified by Cook's distance), which slightly increased the adjusted GMR for the association between endotoxin exposure and FE_{NO} in non-smoking, non-atopic workers (1.15, 95%CI 1.08-1.22; p<0.0001).
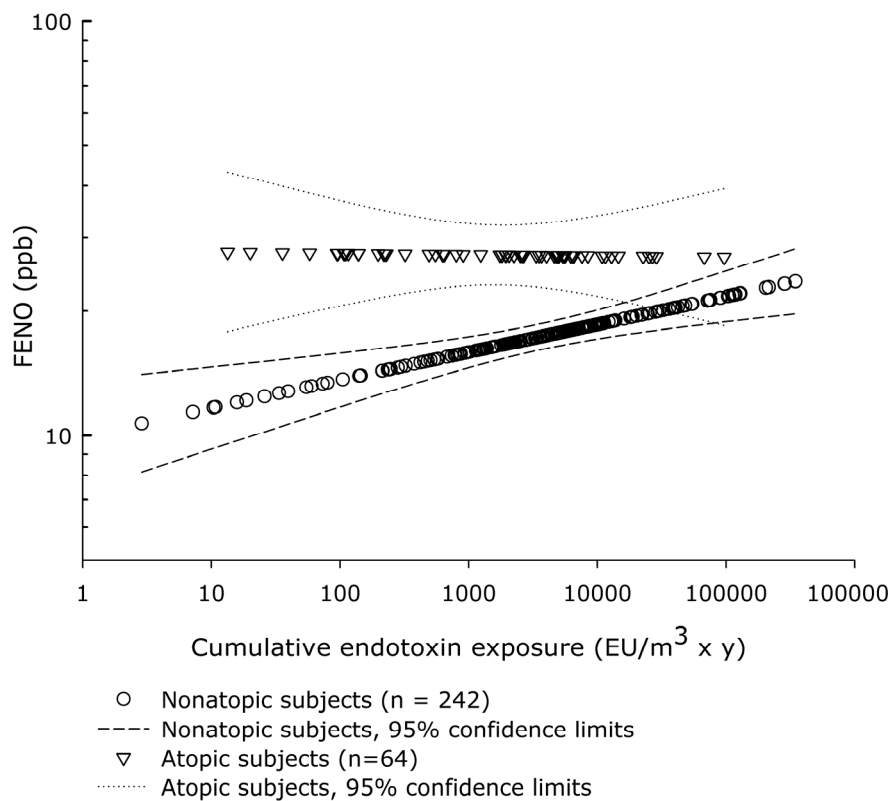
Figure 1 Linear regression plots with pointwise 95% CI representing associations between modeled cumulative endotoxin exposure and FE_{NO} in 242 non-smoking, non-atopic subjects (circles; β (SE) = 0.068 (0.019)) and 64 non-smoking, atopic subjects (triangles; β (SE) = -0.003 (0.04)).

In non-smoking, non-atopic workers, a statistically significant association was also found between present endotoxin exposure levels (EU/m^3) and FE_{NO} (GMR 1.09, 95% CI 1.03-1.17; p=0.006). In a regression model that contained both present endotoxin levels (EU/m^3) and years of exposure as two separate variables, the same

GMR was found for present exposure level (GMR 1.09, (1.02-1.16); p=0.007), and a positive but not significant association was found between years of exposure (GMR per 10 y: 1.05 (0.97-1.13); p=0.21) and FE_{NO}. The earlier presented model with cumulative endotoxin exposure (EU/m^3 x yr) was, however, the best-fitting model (log-likelihood: -200.5, versus -201.77 for the model with present exposure and -201.0 for the model with present exposure and years of exposure as two separate variables).

Current wheeze was significantly associated with FE_{NO}, irrespective of atopy and current smoking (Figure 2). Adjustment for age, sex, and height did not change the observed associations. Similar associations with FE_{NO} were found for wheezing without a cold, wheezing with shortness of breath, and shortness of breath during the night, but not for cough symptoms (data not shown).

In the subgroup of non-atopic non-smokers, we studied the role of FE_{NO} as an intermediate factor in the association between endotoxin exposure and symptoms. As shown in Table 3, associations between endotoxin exposure and symptoms changed only slightly after adjusting for FE_{NO}, suggesting that exhaled NO is not an essential intermediate factor for endotoxin-induced respiratory symptoms.
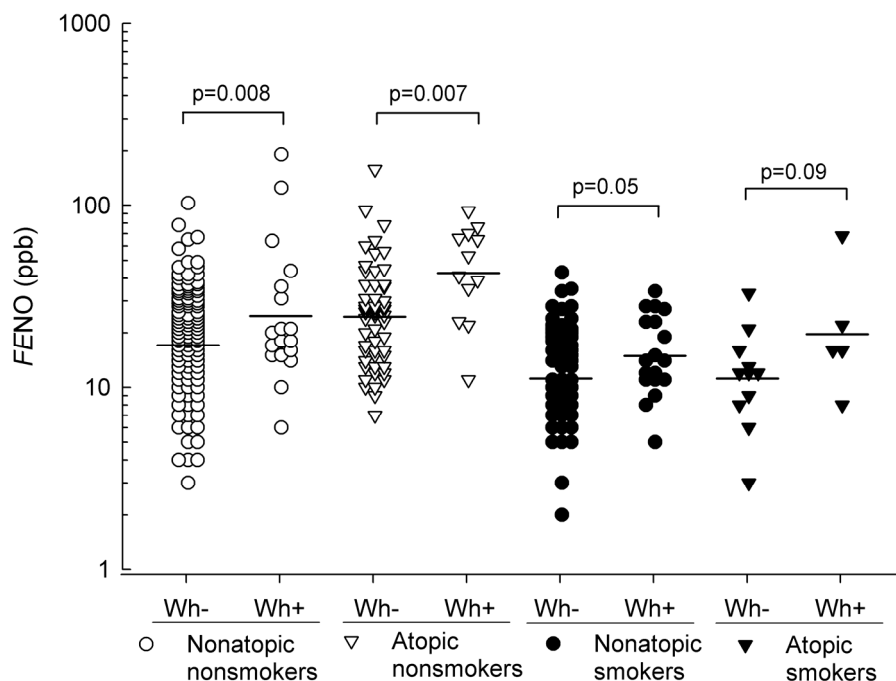
Figure 2 FE_{NO} values in non-atopic non-smokers (white circles), atopic non-smokers (white triangles), non-atopic smokers (black circles), and atopic smokers (black triangles), for non-wheezers and current wheezers (Wh- and Wh+). Horizontal lines represent geometric means. Geometric mean FE_{NO} ratios for subjects reporting wheeze versus non-wheezing subjects were 1.46, 1.73, 1.32, and 1.78 for non-atopic non-smokers, atopic non-smokers, non-atopic smokers, and atopic smokers, respectively.

Table 3 Associations between cumulative endotoxin exposure and symptoms in non-smoking, non-atopic agricultural workers (n=242), with and without adjustment for FE_{NO}.

	OR (95% CI), Model 1[‡]	OR (95% CI), Model 2[‡]
Wheezing	1.45 (0.85-2.48)	1.34 (0.77-2.34)
Wheezing with shortness of breath	1.60 (0.89-2.86)	1.50 (0.82-2.73)
Wheezing without a cold	2.39 (1.08-5.31)	2.27 (1.02-5.06)
Awakened due to shortness of breath	2.26 (0.82-6.27)	2.16 (0.79-5.94)

[‡] Odds ratio (OR) for an interquartile range ($exp^{2.03}$ = factor 7.6) increase in endotoxin exposure (EU/m^3 x yr).
Model 1, adjusted for sex and age.
Model 2, adjusted for exhaled NO, sex and age.

Discussion

In the present study, a significant exposure-response relationship was found between exposure to endotoxin and exhaled NO in non-smoking, non-atopic agricultural workers. Current wheeze and other respiratory symptoms were significantly associated with FE_{NO}, irrespective of atopy and current smoking. However, results suggest that exhaled NO has only a limited role in the pathway between endotoxin exposure and respiratory symptoms.

A few studies on organic and inorganic dust and chemical exposures have reported higher FE_{NO} levels among swine farmers, underground construction workers, aluminium potroom workers, and ozone exposed bleachery workers as compared with control groups.[21, 30-32] Differences in FE_{NO} levels between exposed and unexposed groups were usually relatively small (between 15% and 63% higher in exposed subjects), but may indicate subclinical inflammation due to pro-inflammatory exposures in the environment. However, within these populations no dose-response relationships with measured exposures were established. In the present study, measured endotoxin exposure data were available, and a high contrast in individual exposure levels was obtained by including workers from different agricultural processing industries.[1] Moreover, the large size of the study population allowed to control for potential confounders or effect modifying factors like smoking and atopy, and the results showed that effect modification by smoking and atopy indeed had a major impact on the dose-response relationships. Still, results showed that even in the subgroup with the strongest association, average exhaled NO levels increased only twofold over a range of five orders of magnitude of cumulative endotoxin exposure. This may explain why in many other studies with less pronounced

exposure gradients and/or no adequate adjustments an exposure-response relationship will easily be missed.

Exposure-response relationships between endotoxin and FE_{NO} were observed only when analyses were limited to non-smokers. It is well-known that smokers exhibit strongly reduced FE_{NO},[33] and as a result the usefulness of exhaled NO as a tool to assess airway inflammation among smokers appears to be questionable. Therefore, in the present study and in previous studies in occupational settings, smokers were excluded, or results were stratified by current smoking.[21, 22, 30-32, 34, 35] In addition to the inhibitory effect of current smoking on FE_{NO} values and on endotoxin-associated enhanced FE_{NO}, we also found that endotoxin exposure was only associated with increased FE_{NO} in non-smoking subjects without sensitization to common allergens. Avoidance of high endotoxin exposure by atopic workers with increased FE_{NO} could have obscured an underlying exposure-response relationship. However, wheezing was significantly associated with endotoxin exposure in atopic workers (OR 2.08, 95%CI 1.09-3.97), which argues against a strong effect of health-based selection. Instead, one could speculate that endotoxin exposure does not further increase the already elevated FE_{NO} values among chronically exposed atopic subjects.

An acute but transient increase of exhaled NO after a single endotoxin provocation has been observed in asthmatic volunteers, but not in healthy subjects.[11, 12] However, results of the present study in long-term exposed subjects and observations in naïve individuals after acute endotoxin exposure are difficult to compare. In the present study, the vast majority of subjects (95%) had been working in agriculture for more than one year, and exhaled NO was measured before the workday. The relationship between cumulative endotoxin exposure (EU/m^3 x yr) and FE_{NO} was stronger than the relationship between present endotoxin levels (EU/m^3) and FE_{NO}. Results of the present study thus suggest that elevated exhaled NO in endotoxin exposed workers may represent mild chronic inflammation, rather than an acute effect of current endotoxin exposure. However, elucidation of the underlying mechanisms of chronically increased exhaled NO levels would help to interpret the biological relevance of the present epidemiological observations.

In all subgroups, self-reported wheeze and other asthma-like symptoms were associated with increased FE_{NO} levels. In a cross-section of agricultural workers, one would expect that current wheeze (wheeze at any time during the last 12 months) represents relatively mild airways obstruction. It is likely that many subjects were not experiencing symptoms during the week before FE_{NO} measurements, which is supported by the fact that only 5 subjects reported the use of inhaled corticosteroids during the last week. Therefore, subjects reporting wheeze are likely to exhibit increased but relatively stable exhaled NO values as has been shown in mild asthma

patients, who showed no diurnal or day-to-day variation in FE_{NO}.[18] Whether FE_{NO} acts as an intermediate factor in the relationship between endotoxin and asthma-like symptoms was only investigated in non-atopic non-smokers, since the strong relationships between exhaled NO, smoking and atopy would complicate further interpretation of findings in smokers and atopic subjects. Associations between endotoxin exposure and symptoms decreased marginally after correction for FE_{NO}, suggesting that exhaled NO is not of major importance in the pathway between endotoxin exposure and associated respiratory symptoms.

Although we have shown that measuring FE_{NO} may be a useful tool in an epidemiological survey investigating health effects of endotoxin exposure, a question for future research in exposed workers would obviously be whether FE_{NO} can be used to identify subjects with a higher risk to develop more severe airway inflammation. This question is difficult to answer using cross-sectional data, and a longitudinal study would be needed to investigate the potential of FE_{NO} for predicting which workers would be at risk of occupational lung disease. Another potential of the use of exhaled NO in an occupational setting was demonstrated recently by Dressel et al.[20] who measured exhaled NO measurements before and after an educational intervention programme for farmers with occupational asthma, showing the efficacy of preventive measures.

In conclusion, the present study was the first to show a significant dose-response relationship between endotoxin exposure and exhaled NO. Increased FE_{NO} was associated with current wheeze and other asthma-like symptoms, but the role of FE_{NO} as an intermediate factor between endotoxin exposure and symptoms appears to be limited.

Acknowledgments

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Chapter 6

Susceptibility to endotoxin-related respiratory effects: the role of LPS-induced cytokine responses

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Abstract

Rationale: The presence of respiratory symptoms and FEV_1 reductions due to occupational endotoxin exposure may partly be attributed to cytokine responsiveness of inflammatory cells.

Objectives: To investigate associations between LPS-induced cytokine release in a whole blood assay and respiratory effects in endotoxin-exposed workers, and to study whether the cytokine response modifies associations between endotoxin exposure and respiratory outcomes.

Methods: We studied 412 farmers and agricultural industry workers. Endotoxin exposure levels were determined based on 249 personal exposure measurements. Lung function was measured, and subjects answered a questionnaire on respiratory symptoms. TNFα, IL1β, and IL10 release was measured in *ex vivo* LPS-stimulated whole blood. For each cytokine, the population was dichotomized into low and high responders according to median cytokine concentrations.

Results: High IL10 responders had a higher prevalence of airway symptoms than low IL10 responders (ORs between 2.03 and 5.10, $P<0.05$). The TNFα response was also positively, but not significantly, associated with symptom prevalence, whereas no relationship was found between the IL1β response and symptoms. For all three cytokines, subjects with responses above the median showed significant positive dose-response relationships between endotoxin exposure and asthma symptoms, and significant associations between endotoxin exposure and a lower FEV_1 ($P<0.05$). In contrast, exposure-response relationships were attenuated and statistically non-significant for low responders. The TNFα response was a significant effect modifier for the association between endotoxin exposure and wheeze (interaction $P<0.05$).

Conclusions: *Ex vivo* cytokine responses to LPS are associated with susceptibility to endotoxin exposure-related respiratory illness.

Introduction

Occupational exposure to airborne endotoxins, cell wall components of Gram-negative bacteria, is associated with respiratory health effects in a dose-dependent manner in farmers and agricultural workers. Epidemiological studies in occupationally exposed populations have shown exposure-response relationships between endotoxin exposure and acute lung function changes, accelerated chronic lung function decline, and respiratory symptoms.[1-8] Airway inflammatory responses to inhaled endotoxin are characterized by increased levels of infiltrating neutrophils and production of pro-inflammatory cytokines as shown by nasal lavage, induced sputum and bronchoalveolar lavage studies.[9, 10]

We recently reported significant exposure-response relationships between occupational endotoxin exposure and asthma-like symptoms in a large adult population of farmers and workers in the agricultural industry.[8] The majority of exposed subjects did, however, not report any respiratory symptoms, even at very high endotoxin exposure levels. Individual differences in sensitivity to inhaled endotoxin are likely to play a role and have already been described more than two decades ago.[11-13] Castellan et al.[11] found that short-term, experimental endotoxin inhalation by naïve subjects elicited a measurable, acute response in one third of eligible subjects, whereas others did not respond at all.

The precise biological mechanism which explains the observation that some individuals appear to be more sensitive to inhaled endotoxin than others is not known, but differences in cytokine production of inflammatory cells in response to LPS might play a role.[13] We therefore tested the hypothesis that heterogeneity in respiratory responses to occupational endotoxin exposure can partly be explained by cytokine responsiveness of LPS-stimulated inflammatory cells. We measured cytokine responses to purified endotoxin (lipopolysaccharide, LPS) in an *ex vivo* whole blood assay (WBA). Production of inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin 1β (IL1β) were measured as these cytokines are known to play a key role in endotoxin-induced inflammatory responses. In addition, the regulatory cytokine IL10 was measured. The specific objectives of the present study were to investigate associations between LPS-induced cytokine release and respiratory health outcomes in a population with medium and high exposure to airborne endotoxin, and more specifically, to study whether the thus defined cytokine responsiveness modifies associations between endotoxin exposure and respiratory health parameters.

Methods

Study population
In total, 434 subjects participated. The study population was sampled from a questionnaire survey in Dutch farmers and workers from agricultural processing industries, which has been described earlier.[8] A total of 342 agricultural industry workers (91% of 376 eligible) consented to blood sampling. A total of 340 farmers (65% of 525 eligible) indicated that they would not object being contacted for a medical examination. For logistical reasons, however, only approximately 100 farmers could be included. A random sample of 122 farmers was approached by telephone, and 92 (75%) participated. Venous blood samples were taken and lung function was assessed according to European Respiratory Society guidelines[14] during a visit to the worksite at the start of the workday. Specific serum IgE levels were measured by enzyme immunoassays, and atopy was defined as serum IgE to one or more of the common allergens: grass pollen, house-dust mite, cat and dog.[15, 16]

In total, data from 412 of 434 participating subjects were used (323 agricultural industry workers, 89 farmers), because WBA could not be performed with blood from 15 subjects, and we excluded four subjects aged <18 or >65 yrs and three subjects with incomplete data. Farmers who participated in the medical examination did not differ significantly from farmers in the original sample with respect to endotoxin exposure, personal characteristics and the prevalence of reported wheeze (P>0.05; t-test or χ^2). Nevertheless, selection bias in farmers was a potential concern and, therefore, sensitivity analyses were performed by repeating all statistical analyses in the subpopulation of agricultural industry workers only. The study protocol was approved by the institutional ethics committee and all participants gave written informed consent.

Endotoxin exposure
Personal airborne endotoxin exposure (249 full-shift measurements) was measured in a sample of participants and endotoxin exposure was modeled on the basis of work environment and job title to assign exposure levels to each participant in the whole population, as described in detail earlier.[8]

Whole blood assay
Heparinized blood samples were kept on ice until use in the WBA. Average time between blood collection and start of the cell culture was 4.1±1.4 h. Whole blood samples (80 µl aliquots) were transferred into sterile round bottom 96-well microtiter

plates (Greiner, Alphen a/d Rijn, The Netherlands) and diluted with an equal volume of RPMI 1640 supplemented with 100U penicillin/100µg streptomycin per ml (Gibco, Breda, The Netherlands). LPS (Fluka, Buchs, Switzerland) was added to the culture medium to a final concentration of 1 ng/ml. Cells were incubated during 18h at 37°C, 5% CO_2, and 96% relative humidity. After incubation, microtiter plates were centrifuged for 15 min at 100xg and supernatants were stored at -20°C. TNFα, IL1β, and IL10 were measured in supernatants using a Bio-plex assay as described before.[17] Before full-scale application of the WBA in the present study population, a pilot in 12 subjects was conducted to optimize the assay,[18] and on the basis of the pilot results it was decided not to measure IL6 and IL8, as intra-individual variation exceeded inter-individual variation for these cytokines.

LPS-induced cytokine production was calculated by subtracting cytokine concentrations in non-stimulated samples from corresponding LPS-stimulated samples. Smoothed plots showed a statistically significant inverse log-linear relationship between the level of cytokine production and the time between blood collection and start of the cell culture ($P<0.01$). TNFα, IL1β, and IL10 production decreased with 32%, 12%, and 8%, respectively, per hour that elapsed between venapuncture and start of the cell culture. Therefore, the ln-transformed cytokine concentration was standardized to t=0h by applying a linear correction factor.

Data analysis

For each cytokine, the population was dichotomized into low and high responders according to median cytokine concentrations. Associations between cytokine production and respiratory symptoms were assessed by multiple logistic regression analysis.

Odds ratios (ORs) for associations between endotoxin exposure and respiratory symptoms were estimated in separate strata of low and high cytokine responders. Associations between ln-transformed endotoxin exposure levels and respiratory symptoms were presented as ORs and 95%CI for an interquartile range (IQR) of endotoxin exposure as in our previous study.[8] The null hypothesis was tested that effects of cytokine response and endotoxin exposure on respiratory symptoms were independent by including an interaction term in the model. The shape of the exposure-response relationships was further analyzed by means of generalized additive modeling (smoothing) as described in the literature.[16] Associations between endotoxin exposure and FEV_1 in low and high cytokine responders were analyzed by multiple linear regression analysis in the 363 male subjects of the study population.

Results

Determinants of cytokine response

Figure 1 shows LPS-induced cytokine levels after adjustment for time until incubation. High variability in cytokine release between participants was observed (2-3 orders of magnitude), in particular for TNFα. Production of the three cytokines was significantly correlated, with pairwise correlations between ln-transformed cytokine levels of 0.57 for IL1β and IL10, 0.63 for IL1β and TNFα, and 0.61 for IL10 and TNFα (Pearson correlation coefficients, $P<0.001$). As the main objective of this study was to assess whether cytokine responses influence the relations between endotoxin exposure and respiratory health outcomes, subjects were dichotomized into low and high responders using the median cytokine concentrations for each cytokine separately as a cut-off level. In addition, sensitivity analyses were performed for other cut-points, for instance with the upper and lower tertiles, or with cytokine concentrations as continuous variables.
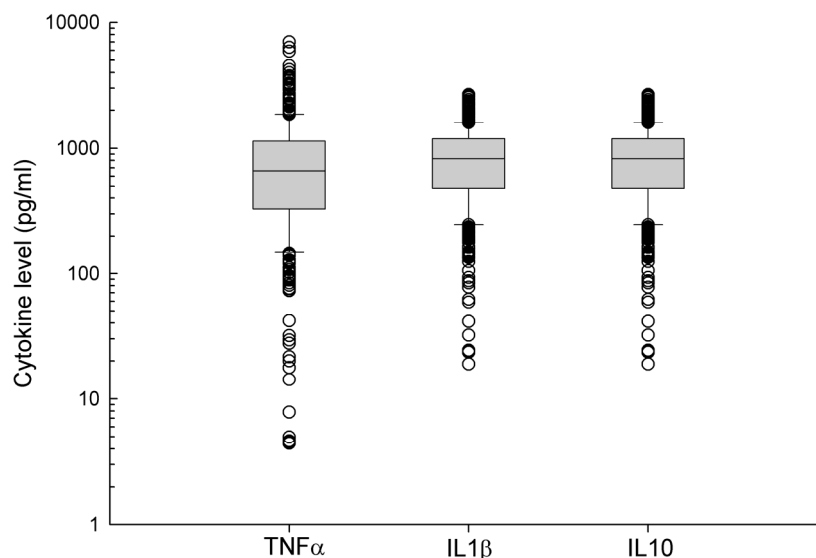
Figure 1 Box plots representing cytokine production in response to 1 ng/ml LPS in all study participants (n=412). Boxes delineate the 25th and 75th percentiles with a horizontal line at the median and whiskers depict the 10th and 90th percentiles.

Women (n=49) had a significantly lower LPS-induced cytokine production than men, in particular for IL1β (factor 0.78; P=.04) and TNFα (factor 0.56; P=.001). The sex difference in cytokine production was independent of other factors such as age or endotoxin exposure. Therefore, correction for time and dichotomization into low and high responders was performed separately for men and women. Categorization of

cytokine response was hardly influenced by the correction for time until incubation: categorizations on the basis of adjusted or unadjusted levels were ~90% concordant. Table 1 shows the distribution of potential determinants over high and low TNFα, IL1β, and IL10 responders. There was no association with smoking habits or atopy for any of the cytokines. High IL10 responders were on average 3.5 yr younger and had less often grown up on a farm than low IL10 responders. These relations could also be confirmed among non-farmers (*P*<0.05). Higher age and a farm childhood were also in a multiple logistic regression model independent determinants of a lower IL10 response with ORs of 0.97 (0.95-0.99) and 0.57 (0.38-0.85), respectively. Analyses with cytokine concentrations as continuous variables or upper and lower tertiles yielded very similar results.

Table 1 Determinants of cytokine response.

| | TNFα response | | IL1β response | | IL10 response | |
	Low	High	Low	High	Low	High
Age (yrs), mean (SD)	42.6 (10.2)	41.6 (10.6)	42.9 (9.7)	41.2 (11.0)	43.9 (9.5)	40.3 (10.9)[*]
Smoking habits,%						
Never	42.0	38.8	41.0	39.8	43.4	37.4
Former	29.3	33.5	29.8	33.0	28.3	34.5
Current	28.8	27.7	29.3	27.2	28.3	28.2
Farm childhood,%	47.8	44.2	47.8	44.2	54.2	37.9[*]
Atopy,%	20.0	18.5	21.5	17.0	16.1	22.3
Endotoxin exposure (EU/m^3), GM (GSD)	276 (4.9)	307 (5.5)	238 (5.1)	356 (5.2)[*]	247 (5.2)	343 (5.2)[*]

[*]*P*<0.05, t-test or χ^2-test.
EU: endotoxin unit; GM: geometric mean; GSD: geometric standard deviation.

A weak but statistically significant positive association between occupational endotoxin exposure and high IL1β and IL10 response was found. ORs for an interquartile range increase in endotoxin exposure were 1.25 (1.05-1.49) for IL1β and 1.20 (1.01-1.42) for IL10. When cytokine production was treated as a continuous variable, a significant but low correlation was also found between endotoxin exposure and IL1β (ln-transformed values, Pearson's r=0.11, *P*=0.02), but not between exposure and IL10 or TNFα (*P*>0.2). Although endotoxin exposure was associated with cytokine levels, the difference in average exposure levels between high and low responders was small and a strongly overlapping range of exposure levels was found in each subgroup.

Table 2 Prevalence of respiratory symptoms and association with cytokine responses above the median.

	TNFα response			IL1β response			IL10 response		
	Low	High		Low	High		Low	High	
	%	%	OR (95% CI)	%	%	OR (95% CI)	%	%	OR (95% CI)
Wheezing	10.7	15.1	1.45 (0.80-2.63)	13.2	12.6	0.91 (0.50-1.65)	8.8	17.0	**2.03 (1.08-3.83)**
Wheezing with SOB	7.3	9.7	1.32 (0.65-2.67)	9.3	7.8	0.77 (0.38-1.57)	4.9	12.1	**2.58 (1.18-5.66)**
Wheezing without a cold	4.9	8.8	1.86 (0.83-4.17)	6.3	7.3	1.13 (0.52-2.46)	2.4	11.2	**5.10 (1.85-14.1)**
Awakened due to SOB	2.4	4.4	1.77 (0.58-5.41)	3.4	3.4	0.95 (0.32-2.79)	1.5	5.3	3.51 (0.93-13.2)

SOB: shortness of breath. ORs are adjusted for sex, age, smoking habits, and farm childhood. Bold ORs represent significant associations between cytokine response and symptoms (*P*<0.05).

Associations between cytokine response and respiratory symptoms
High IL10 responders had wheeze symptoms more often than low IL10 responders (Table 2). TNFα response was also positively but non-significantly associated with symptom prevalence (ORs between 1.32 and 1.86), whereas no relationship between IL1β response and symptoms was found. Cough symptoms (daily cough, cough up phlegm, nocturnal cough) were not associated with cytokine production (data not shown). No evidence of confounding or effect modification by variables such as age, farm childhood, smoking or atopy was found, and exclusion of farmers or women did not influence results. As expected on the basis of the weak associations between endotoxin exposure and cytokine responses, endotoxin exposure was no confounding factor for the association between cytokine response and respiratory outcomes. Similar patterns were observed when cytokine production was treated as a continuous variable, or when the lowest and highest tertiles of cytokine production were compared.

Cytokine responses as effect modifiers
In the previously reported study we found a clear dose-response relationship between endotoxin exposure and the prevalence of respiratory symptoms,[8] and these results were confirmed in the present study: e.g. ORs for wheeze (per interquartile range increase in endotoxin exposure) were 1.41 (1.16-1.72) in the larger population and 1.39 (1.06-1.83) in the population in which the WBA was performed. In stratified analyses these dose-response relationships appeared to be largely restricted to the subjects with high cytokine responses (Table 3). Significant positive relationships were found between occupational endotoxin exposure and wheeze, wheeze with shortness of breath, and wheeze without a cold for high responders (ORs between 1.54 and 2.13), and no or much weaker and non-significant associations in low responders (ORs between 0.98 and 1.26). ORs for wheeze and wheeze with shortness of breath were significantly different between low and high TNFα responders (interaction $P<0.05$). Exposure-response relationships between endotoxin exposure and cough symptoms were not modified by cytokine response (data not shown).

The shape of the relationships between endotoxin and wheeze seemed log-linear for both high and low TNFα responders (Figure 2A). A non-linear relationship was suggested for IL1β and IL10 high responders, who showed a steep exposure-response relationship, but only at very high endotoxin exposure levels (>1000 EU/m^3), whereas curves for low responders flattened at high exposures (Figure 2B and 2C; spline $P<0.1$).

Table 3 Association between occupational endotoxin exposure and respiratory symptoms in 206 low cytokine responders and in 206 high cytokine responders.

| | TNFα response | | IL1β response | | IL10 response | |
| | Low | High | Low | High | Low | High |
	Endotoxin exposure OR (95% CI)	Endotoxin exposure OR (95% CI)	Endotoxin exposure OR (95% CI)	Endotoxin exposure OR (95% CI)	Endotoxin exposure OR (95% CI)	Endotoxin exposure OR (95% CI)
Wheezing	1.02 (0.66-1.56)	**1.82 (1.24-2.67)** **	1.14 (0.76-1.72)	**1.66 (1.13-2.45)**	1.12 (0.70-1.78)	**1.54 (1.08-2.20)**
Wheezing with SOB	1.02 (0.63-1.66)	**2.13 (1.33-3.41)** **	1.26 (0.79-2.00)	**1.74 (1.08-2.81)**	1.24 (0.68-2.28)	**1.56 (1.04-2.34)**
Wheezing without a cold	0.98 (0.54-1.77)	**1.92 (1.21-3.04)** *	1.20 (0.70-2.06)	**1.69 (1.05-2.72)**	1.05 (0.45-2.49)	**1.63 (1.07-2.47)**
Awakened due to SOB	1.79 (0.73-4.35)	1.86 (0.99-3.49)	**2.16 (1.01-4.61)**	1.55 (0.82-2.95)	0.88 (0.29-2.68)	**2.28 (1.22-4.28)** *

SOB: shortness of breath.
* P interaction <0.10; ** P interaction <0.05; Bold ORs represent significant associations between endotoxin exposure and symptoms (P<0.05).
Data are presented as the odds ratio (OR) for an interquartile range increase in modeled endotoxin exposure levels (EU/m^3) with 95% confidence interval (95% CI). ORs are adjusted for sex, age, smoking habits, and farm childhood.
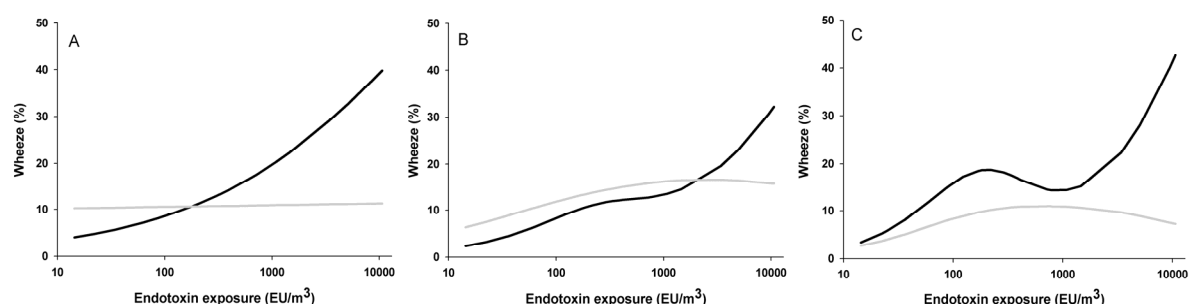
Figure 2 Smoothed plots representing dose-response relationships of endotoxin exposure and current wheeze in 206 high cytokine responders (—— black curve) and 206 low cytokine responders (—— grey curve) for TNFα (A), IL1β (B), and IL10 (C).

Results changed only marginally when female workers were excluded, or when farmers were excluded: in agricultural industry workers, ORs between 0.78 and 1.29 were found for wheeze-related symptoms in low cytokine responders, and between 1.55 and 2.05 for high responders.

FEV_1 was not significantly different in low and high responders (P>0.2, data not shown). In high TNFα, IL1β and IL10 responders, however, a significantly lower FEV_1 was found at increasing levels of endotoxin (80 to 92 ml lower for an IQR increase in endotoxin exposure, P<0.05), whereas in low responders a much smaller and non-significant difference in FEV_1 (18 to 30 ml lower) was found at the same increase in exposure (Table 4). Formal tests for interaction were, however, not significant for any of the cytokines.

Table 4 Association between occupational endotoxin exposure and FEV_1 (ml) in 363 male subjects according to cytokine response.

	Low responders		High responders	
	β (95%CI)	*P*	β (95%CI)	*P*
TNFα	-30 (-111 to 51)	0.47	-80 (-154 to -6)	0.03
IL1β	-18 (-97 to 62)	0.66	-80 (-157 to -3)	0.04
IL10	-28 (-103 to 47)	0.46	-92 (-170 to -14)	0.02

Data are presented as the reduction in FEV_1 at an interquartile range increase in modeled endotoxin exposure levels (EU/m^3). Regression coefficients (β) and 95%CI are adjusted for standing height, age, atopy, smoking habits and farm childhood.

Sensitivity analyses with combined cytokine response

As LPS-induced production of the three cytokines showed highly significant correlations, an alternative data analysis approach could be considered, in which the three cytokines are combined and treated as a single entity, e.g. by comparing subjects with a low or high response to LPS for all three cytokines (low: n=124; 30.1% and high: n=123; 29.9%), and ignoring subjects with discordant responses (n=165; 40.1%). Following this approach showed that a thus defined high cytokine production was positively associated with symptom prevalence, with ORs between 1.69 and 3.43 ($P<0.05$ for wheeze without a cold). Effect modification of the exposure-response relationships between endotoxin exposure and symptoms or FEV_1 was very similar to results shown in Table 3 and 4. High responders had ORs between 1.68 and 2.07 for respiratory symptoms ($P<0.05$ for wheeze, wheeze with SOB, wheeze without a cold) and a 172 ml lower FEV_1 ($P=0.002$) for an IQR increase in endotoxin exposure. In contrast, corresponding ORs for low responders were between 0.80 and 1.17 for symptoms ($P>0.05$) and their FEV_1 was on average only 27 ml lower when endotoxin exposure increased over an IQR. The interaction between the clustered cytokine response and endotoxin exposure was in formal tests significant for the association with wheezing (P-value interaction=0.03).

Discussion

In the present study we found that susceptibility to adverse respiratory effects induced by high occupational endotoxin exposure is determined by the inflammatory response to LPS, measured as *ex vivo* TNFα, IL1β, and IL10 responses in a WBA. Exposure-response relationships were relatively steep and statistically significant for high cytokine responders, whereas in low cytokine responders only weak, non-significant associations were found between occupational endotoxin exposure and FEV_1 or respiratory symptoms. We also found positive associations between cytokine responses and respiratory symptoms (especially for IL10, and to a lesser extent for TNFα), but the key message of the present study is the finding that cytokine responsiveness to LPS acts as an effect modifier of the association between occupational exposure and respiratory health effects.

On theoretical grounds, dichotomization of subjects into high and low responders according to median cytokine production would be in agreement with the finding that more than one third of individuals appeared responsive to acute, experimental endotoxin inhalation.[11] Sensitivity analyses showed that results were robust when tertiles were used or when cytokine production was treated as a continuous variable.

Heterogeneous results were observed for the three cytokines under study, in particular for associations between cytokine production and symptom prevalence. A possible biological meaning of this difference is, however, not directly available, and should be interpreted with caution. Alternatively, categorization based on all three cytokine responses together is expected to be less sensitive for measurement error of individual cytokines, and thus strengthened the finding that *ex vivo* LPS-induced cytokine response is associated with susceptibility to endotoxin-induced respiratory disorders. On the basis of our findings it can, however, not be concluded whether the three different cytokine responses should be interpreted as three separate biologically meaningful responses, or as a single correlated response to LPS.

It has been shown before that the time between blood collection and start of the incubation in the WBA is a critical variable for cytokine release.[19, 20] Although we made every effort to minimize this time period by optimizing fieldwork and laboratory procedures, we could not start the *in vitro* incubation of all blood samples within two hours after collection, and we therefore adjusted all measured cytokine levels for the time-until-incubation. However, the variation in time-until-incubation was small, and it was shown that correction had only a minor impact on the categorization of high and low responders, i.e. a concordance of ~90%.

We found lower TNFα and IL1β production from whole blood samples of women compared with men. A similar sex difference has been observed previously in a study among volunteers, and was attributed to the influence of sex hormones.[21] We therefore categorized men and women separately into high and low responders, and we also showed that results were very similar when we excluded women from analyses.

Prolonged exposure to high levels of endotoxin has been shown to lead to persistent airway symptoms and obstruction, probably as a result of chronic mucosal airway inflammation. Apparently, high occupational exposure may also have a systemic effect by enhancing the ability of blood monocytes to produce cytokines, as suggested by the weak but positive association between endotoxin exposure and IL1β and IL10 (Table 1). Also when cytokine responses were combined (all low vs. all high), we found a positive association between endotoxin exposure and the cytokine response (OR 1.31 (1.04-1.66)). In a previous study among grain workers, TNFα release from unstimulated and stimulated peripheral blood monocytes (3 ng/ml LPS) increased significantly across the workweek in exposed workers, and the increase in TNFα levels was associated with grain dust exposure levels.[22] In our study, blood was collected from Monday to Wednesday at the start of the workday, and there was no association or trend between day of the week and cytokine production (*P*>0.3). A lower response in the WBA has been described in mice who had developed

tolerance to LPS,[23] but in human studies including the present one, there is no evidence of development of tolerance at adult age to occupational endotoxin exposure, at least not at the level of cytokine responses.[22]

Childhood exposure to microbial agents in a farm environment has, on the other hand, been associated with lower LPS-induced IL10 responses by Braun-Fahrländer et al.[24] who found an inverse association between endotoxin load in children's mattress dust and LPS-induced IL10. Our finding that workers who had grown up on a farm had a lower IL10 response (Table 1) appears to be in accordance with that study. Whether our observation relates to a long-lasting down-regulation of IL10 responses as a result of childhood exposures to endotoxin and other farm-associated microbial agents, or to other factors correlated to a farm childhood, however, is not clear.

LPS-induced release of IL6 and IL8 by alveolar macrophages and peripheral blood monocytes correlates with airflow obstruction in response to experimental LPS inhalation.[13] This suggests that cytokine release from inflammatory cells is predictive of *in vivo* susceptibility. A WBA is a simple alternative to stimulating isolated monocytes or alveolar macrophages, especially in large-scale population studies with complicated logistics. The WBA reproducibly measures an individual's cytokine responsiveness, with considerably larger differences in cytokine release between individuals than within individuals when repeated during a period between one and six weeks.[19, 20, 25] It is as yet unknown whether individual responses are reproducible over a longer period of time. Moreover, longitudinal studies are needed to assess the predictive value of cytokine responsiveness.

In conclusion, we have shown that TNFα, IL1β, and IL10 production by *ex vivo* LPS-stimulated blood monocytes is associated with *in vivo* susceptibility to occupational endotoxin exposure-associated respiratory effects. Exposure-response relationships between endotoxin exposure and respiratory symptoms and FEV_1 were relatively steep and statistically significant for high cytokine responders, whereas weak, non-significant associations were found for low cytokine responders.

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Chapter 7

Atopy and new-onset asthma in young Danish farmers and *CD14*, *TLR2*, and *TLR4* genetic polymorphisms: a nested case-control study

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Abstract

Background: Evidence exists that exposure to high levels of microbial agents such as endotoxin in the farm environment decreases the risk of atopic sensitization. Genetic variation in innate immunity genes may modulate the response to microbial agents and thus influence susceptibility to asthma and atopy.

Objective: To study potential associations between single nucleotide polymorphisms (SNPs) in *CD14*, Toll-like receptor 2 (*TLR2*), and *TLR4* genes, and atopy and new-onset asthma in young farmers.

Methods: A nested case-control study was conducted within a cohort of 1901 young Danish farmers. We genotyped 100 new-onset asthma cases and 88 control subjects for three *CD14* SNPs, three *TLR2* SNPs, and two *TLR4* SNPs. Atopy at baseline (defined as a positive skin prick test (SPT) to one or more common inhalant allergens) was found in 17 asthma cases (17.0%) and in 17 controls (19.3%).

Results: The *CD14*/-260T allele was significantly associated with less atopy (Odds Ratio (OR) 0.39; 95% confidence interval (CI) 0.21-0.72, additive genetic model), whereas the *CD14*/-651T allele was positively associated with atopy (OR 2.53; 95%CI 1.33-4.80). Similar results were obtained by haplotype analysis. Stratified analysis by farm childhood showed stronger effects of both *CD14* SNPs on atopy among farmers who were born and raised on a farm, although no significant interaction was found. No associations between *CD14*, *TLR2*, or *TLR4* genotypes and new-onset asthma were found.

Conclusion: The *CD14*/-260 and *CD14*/–651 promoter polymorphisms are associated with atopy prevalence among young adults exposed to farm environments.

Introduction

Evidence exists that exposure to high levels of microbial agents in the farm environment decreases the risk of atopic sensitization in children and adults.[1-5] Although the precise immunological mechanism is yet to be elucidated, it has been hypothesized that increased microbial exposure induces a shift from atopic T-helper type 2 (Th2) responses to Th1-dominated responses through stimulation of the innate immune system. In addition, it has been suggested that regulatory T cells play a role in suppressing allergic and non-allergic immune responses. Toll-like receptors (TLRs) present on the cell surface of innate immune cells recognize microbial motifs called pathogen-associated molecular patterns (PAMPs). In response to interaction of lipopolysaccharide (LPS) as well as other PAMPs with TLRs, IL-12 and IFN-γ are produced which induce a Th1 response.[6, 7] The capacity of PAMPs to induce inflammation may also result in adverse respiratory health effects including non-atopic asthma, accelerated lung function decline, and organic dust toxic syndrome.[7-10]

Variation in innate immunity genes such as *TLR2*, *TLR4,* or *CD14* may modulate responsiveness to LPS and other PAMPs and thus play a role in the development of atopy and respiratory disease. TLR4 functions predominantly, if not exclusively, as a receptor for LPS whereas TLR2 is involved in the recognition of multiple products of Gram-positive organisms, mycobacteria, and yeast.[11] CD14 is the receptor that binds LPS and transfers it to TLR4, thus forming the CD14-TLR4 complex. Arbour et al.[12] demonstrated an association between the Asp299Gly polymorphism in *TLR4* and a blunted response to inhaled endotoxin in humans. However, subsequent studies on *TLR4* polymorphisms as a risk factor for atopy or asthma in adults or children have shown conflicting results.[13-17] Carriage of the wild-type *TLR2*/-16934T allele was found to be associated with less asthma and allergies among German and Austrian farmers' children, whereas such an effect was absent in non-farmers' children.[15] A single nucleotide polymorphism (SNP) in the promoter region of *CD14* (C -260T, also reported as C -159T) has been associated with increased levels of soluble CD14 (sCD14),[18, 19] and with lower levels of total serum IgE or atopic sensitization in farming and non-farming populations.[17, 18, 20, 21] Moreover, functional studies revealed increased transcriptional activity of the T allele.[22] On the other hand, other investigators did not find an association between *CD14*/-260 and atopy or allergic disease, or obtained opposite results.[19, 23-26] Farming is associated with high levels of exposure to a wide range of micro-organisms and PAMPs;[27] therefore we hypothesized that the susceptibility to asthma and atopy in farmers might be associated with polymorphisms in innate immunity genes. We carried out a nested

case-control study to explore associations between atopy and new-onset asthma, and *CD14*, *TLR2*, and *TLR4* genetic polymorphisms in young farmers from the Danish SUS cohort.[28]

Methods

Study population and design

In 1992-1994, a cohort of 1901 Danish farming school students (1691 men and 210 women, mean age 19.2 years, range 16-26 years, response rate 81%) was recruited into a longitudinal study to investigate the incidence of asthma in relation to farm exposures. Details of the baseline population have been reported previously.[4, 28, 29] Within this cohort, we conducted a nested case-control study. Each consecutive year, up to 1999, all participants received a questionnaire, and subjects with new onset of respiratory symptoms were contacted by phone for an interview. Those who fulfilled the questionnaire criteria for new-onset asthma were invited to a clinical evaluation together with a randomly selected asthma-free control subject.[30, 31] For each new case, one control subject was selected by incidence density sampling to obtain odds ratios (ORs) that are unbiased estimates of the relative risk. Among the young farmers, 106 cases of new asthma were identified, and 102 controls were included at the same time. DNA samples were available for 100 cases and 88 controls. The study was approved by the ethics committee and all participants gave written consent.

Health outcomes

The clinical evaluation included an interview that consisted of questions about onset of asthma symptoms, smoking habits, and occupational history. Asthma was diagnosed if the subjects answered yes to at least one of the group A questions and two of the group B questions in Table 1.[28] A skin prick test (SPT) was performed at baseline with a panel of eight common inhalant allergens; house dust mite, cat, dog, pollen from grass (mix of five species), birch, mugwort (*Artemisia*), and moulds (*Alternaria alternata* and *Cladosporium herbarum*) (Soluprick, ALK, Copenhagen, Denmark). The panel was extended with six 'farm-related' allergens from three different storage mites (*Acarus siro*, *Tyrophagus putrescentia*, and *Lepidoglyphus destructor*), cow, pig, and horse.[4, 28] As we wanted to compare our results with other studies, we focused mainly on the common allergens. Atopy was defined as a positive SPT to one or more of the eight common inhalant allergens (weal diameter of at least 3 mm).

Table 1 Questions on asthma.

Group A	Group B
Have you been told by a doctor that you have asthma?	Do you ever have chest tightness?
Do you have asthma?	Do you wake in the morning with chest tightness?
Have you ever had asthma?	Do you wake in the night with wheeze?
Do you ever wheeze?	Do you cough when you wake up in the morning?
	Do you wake in the morning with cough?
	Do you wheeze by exposure to cold air?
	Do you wheeze when you exercise?
	Do you wheeze by exposure to pollen?
	Do you wheeze by exposure to animals?
	Do you use asthma drugs?

Table 2 Identification numbers and primer sequences (SSP-PCR) for the identification of biallelic single nucleotide polymorphisms in the *CD14*, *TLR2*, and *TLR4* genes.

Polymorphism	dbSNP[†]	Specific primer	Consensus primer
CD14/-651 C/T	rs5744455	[‡]	
CD14/-260 C/T	rs2569190	5'CAGAATCCTTCCTGTTA CGG C/T	5'CTGAGGTTCGGAGAAGT TGC
CD14/+1342 C/A	rs2563298	[‡]	
TLR4 Asp299Gly A/G	rs4986790	[‡]	
TLR4 Thr399Ile C/T	rs4986791	5'TCTCAAAGTGATTTTGG GACAA C/T	5'GAGAGAGGTCCAGGAAG GTC
TLR2/-16934 A/T	rs4696480	5'ATTGAAGGGCTGCATC TGG A/T	5'GTGTGCCCCAAAGCTCA TG
TLR2 Pro631His C/A	rs5743704	5'CTGCTGGGAGCTTTCC TG G/T	5'AGCAAGCACTGGCCAAA GTCT
TLR2 Arg753Gln C/T	rs5743708	5'AGGTCTTGGTGTTCATT ATCTTC T 5'GGTCTTGGTGTTCATTA TCTTC C	5'ATGATGTGGGCCTGGCT C

[†] The SNP loci were identified using accession numbers according to the SNP database at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp.
[‡] SNPs determined using TaqMan® SNP genotyping assay.

Genotyping

We selected two SNPs in the 5' flanking region of the *CD14* gene (C-651T and C-260T), and a SNP in the 3' untranslated region of the *CD14* gene (C+1342A). *CD14* polymorphisms C–651T and C–260T are also reported as C–550T and C–159T in the literature. For the *TLR4* gene, we genotyped the Asp299Gly and Thr399Ile polymorphisms. In addition, three SNPs in the *TLR2* gene were selected: A-16934T,

Pro631His, and Arg753Gln. DNA was extracted from whole-blood samples using standard procedures. *CD14/–260*, *TLR4* Thr399Ile, and the three *TLR2* SNPs were determined using sequence-specific primer (SSP) and PCR.[32] Primers were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in Table 2. The other SNPs (*CD14/-651*, *CD14/+1342*, and *TLR4* Asp299Gly A/G) were determined using TaqMan® SNP Genotyping Assays using real-time PCR (7500 FAST; Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands).

Statistical analysis

Haplotypes were inferred using Phase, version 2.1 with a 90% probability threshold.[33, 34] Data were analysed using SAS statistical software V.8.2. Differences in genotype frequencies or descriptive characteristics between case and control groups were determined by X^2 test or by Fisher's exact test when the expected count for any cell was less than five. Allele frequencies were tested for Hardy-Weinberg equilibrium by X^2 test. Univariate and multiple logistic regression analysis were used to further study associations between health outcomes and genotypes. Genotypes were coded as dummy variables using wild-type homozygotes as a reference group. In addition, an additive genetic model was considered by categorizing genotypes into a 3-level variable for the number of minor alleles.[35] In the haplotype analysis, calculations were based on the number of copies of the haplotype (0,1,2), assuming an additive model.[36] The most frequent haplotype was used as the reference. When atopy was studied as an outcome variable, data were analysed as cross-sectional data. In order to do so ORs and 95% confidence intervals (CIs) were adjusted for the original case-control status to prevent confounding by the selection process. Potentially confounding factors such as being born and raised on a farm, and smoking habits were controlled for. A p-value <0.05 was considered statistically significant.

Results

Table 3 summarizes general characteristics and genotype frequencies of *CD14*, *TLR4*, and *TLR2* among new asthma cases and controls. No differences in genotype frequencies were found between asthma cases and control subjects for any of the *CD14*, *TLR4*, and *TLR2* SNPs. Atopy as well as a positive SPT to one or more farm-related allergens were found as frequently in new asthma cases as in control

subjects. In 18 individuals (11 cases and 7 controls) a positive SPT to one or more farm-related allergens did not coincide with common atopy. Stratified analysis for non-atopic subjects also did not show differences in genotype frequencies between asthma cases and controls. Allele frequencies for each SNP were in Hardy-Weinberg equilibrium (p>0.1). Pairwise linkage disequilibrium (LD, expressed as r^2) between the SNPs in the *CD14* gene was as follows: -651 and -260, 0.24; -260 and +1342, 0.34; -651 and +1342, 0.14. High LD (0.74) was found between the two studied *TLR4* SNPs. LD between the SNPs in the *TLR2* gene was: -16934 and Pro631His, 0.05; Pro631His and Arg753Gln, 0.00; -16934 and Arg753Gln, 0.03.

When atopy was considered as an outcome variable, the *CD14*/-260 T allele appeared significantly associated with less atopy, whereas the *CD14*/-651 T allele showed a significant positive association with atopy (Table 4). Potential confounders (being born and raised on a farm, smoking, and case-control status) had ORs close to 1 in multiple logistic regression models, and adjusted ORs showed comparable results (Table 4). ORs for both SNPs remained significant in a multiple regression model that included both *CD14*/-260 and *CD14*/-651 as independent variables (additive genetic model for the minor T alleles; OR 0.45; 95% CI (0.21-0.97), and OR 2.42; 95% CI (1.09-5.37), respectively). Similar patterns were observed when one or more positive SPT for farm-related allergens was used as an outcome variable, however those associations were not significant (additive genetic model for the minor T alleles; *CD14*/-260, OR 0.59; 95% CI (0.32-1.06), and *CD14*/-651, OR 1.91; 95% CI (0.95-3.82).

When the same analyses for *CD14*/-260 were run separately for farmers with and without farm childhood, we found an OR of 0.21 in farmers who were born and raised on a farm (additive genetic model for the T allele; 95%CI 0.06-0.73) compared to an OR of 0.45 in farmers without farm childhood (95%CI 0.20-1.03). Accordingly, stratified analysis for *CD14*/-651 showed a stronger positive association in farmers with farm childhood (OR 7.10; 95%CI 1.55-32.59), compared to farmers without farm childhood (OR 2.43; 95%CI 1.01-5.83). Although we did not find a significant interaction between *CD14* genotypes and farm childhood, these ORs are suggestive of a stronger effect on atopy of *CD14* genotypes in farmers who were born and raised on a farm.

Table 3 General characteristics and *CD14*, *TLR4*, and *TLR2* genotypes among 188 young Danish farmers by asthma status.

	Control subjects (n = 88)	Asthma cases (n = 100)	*P*
Male	79 (89.8)	85 (85.0)	0.3
Current smokers	23 (26.1)	57 (57.0)	<0.001
Born and raised on a farm [†]	39 (47.6)	24 (29.6)	0.02
≥1 positive SPT, common allergens (atopy)	17 (19.3)	17 (17.0)	0.7
≥1 positive SPT, farm-related allergens	16 (18.2)	19 (19.0)	0.9
Genotype [‡]			
CD14/-651 (rs5744455)			0.7
CC	52 (59.1)	53 (53.0)	
CT	33 (37.5)	43 (43.0)	
TT	3 (3.4)	4 (4.0)	
CD14/-260 (rs2569190)			0.7
CC	28 (29.6)	34 (34.0)	
CT	47 (53.4)	47 (47.0)	
TT	15 (17.1)	19 (19.0)	
CD14/+1342 (rs2563298)			0.6
CC	40 (45.5)	52 (52.0)	
CA	38 (43.2)	37 (37.0)	
AA	10 (11.4)	11 (11.0)	
TLR4 Asp299Gly (rs4986790)			0.8
AA	78 (89.7)	91 (91.0)	
AG	9 (10.3)	8 (8.0)	
GG	0 (0.0)	1 (1.0)	
TLR4 Thr399Ile (rs4986791)			0.2
CC	77 (88.5)	93 (93.0)	
CT	10 (11.5)	6 (6.0)	
TT	0 (0.0)	1 (1.0)	
TLR2/-16934 (rs4696480)			0.3
AA	24 (27.6)	22 (22.0)	
AT	41 (47.1)	58 (58.0)	
TT	22 (25.3)	20 (20.0)	
TLR2 Pro631His (rs5743704)			0.3
CC	76 (87.4)	92 (92.0)	
CA	11 (12.6)	8 (8.0)	
TLR2 Arg753Gln (rs5743708)			0.2
CC	80 (92.0)	97 (97.0)	
CT	7 (8.0)	3 (3.0)	

Data are presented as numbers with percentages in parentheses.
[†] Information on farm childhood is available for 82 control subjects and 81 asthma cases.
[‡] Information on *TLR4* and *TLR2* genotype is available for 87 control subjects.

Table 4 Associations of the *CD14*/-260 and -651 promoter polymorphisms and atopy (a positive SPT to one or more common allergens).

	SPT– n (%)	SPT+ n (%)	OR (95% CI)	*P*	Adjusted OR (95% CI)[†]	*P*
CD14/-260						
CC	43 (27.9)	17 (50.0)	1.0		1.0	
CT	78 (50.7)	16 (47.1)	0.51 (0.24-1.12)	0.09	0.42 (0.17-1.02)	0.05
TT	33 (21.4)	1 (2.9)	0.08 (0.01-0.60)	0.01	0.08 (0.01-0.63)	0.02
T allele [‡]			0.39 (0.21-0.72)	0.003	0.35 (0.17-0.69)	0.003
CD14/-651						
CC	92 (59.7)	13 (38.2)	1.0		1.0	
CT	59 (38.3)	17 (50.0)	2.07 (0.93-4.59)	0.07	3.51 (1.38-8.94)	0.009
TT	3 (2.0)	4 (11.8)	9.63 (1.93-48.21)	0.006	11.13 (1.87-66.19)	0.008
T allele [‡]			2.53 (1.33-4.80)	0.005	3.42 (1.63-7.17)	0.001

[†] Odds Ratio adjusted for farm childhood, smoking habits, and case-control status. Information on farm childhood is available for 136/154 non-atopic subjects and 27/34 atopic subjects.
[‡] In the additive genetic model, the genotype was categorized into a 3-level variable for the number of minor alleles (0,1,2).

Table 5 *CD14* haplotype frequencies in 34 atopic and 154 non-atopic farmers and association of *CD14* haplotypes with atopy.

Haplotype	-651	-260	+1342	Haplotype frequency, n (%) SPT –	SPT +	OR (95% CI) [†]	*P*
1	C	T	C	144 (47)	18 (26)	1.0 (reference)	
2	C	C	A	93 (30)	24 (35)	2.24 (1.14-4.41)	0.02
3	T	C	C	65 (21)	25 (37)	4.03 (1.83-8.88)	<0.001
4	C	C	C	6 (2)	1 (1)	1.14 (0.15-9.01)	0.90

[†] Calculations are based on the number of copies of the haplotype (0,1,2), assuming additivity on the logit scale. ORs estimate the risk of atopy for carriers of one copy of the haplotype as compared with two copies of the reference haplotype.[36]

Separate analyses for asthma cases and control subjects showed a somewhat stronger effect of the *CD14*/-260 T allele in cases (OR 0.36; 95%CI 0.06-0.73) compared with controls (OR 0.43; 95%CI 0.18-1.02), whereas for *CD14*/-651, the effect was more pronounced in controls (OR 4.20; 95%CI 1.57-11.21) compared with cases (OR 1.62; 95% CI 0.67-3.91), however, no significant interactions were found between *CD14* genotypes and case-control status. *TLR4* and *TLR2* genotypes were not associated with atopy (data not shown).

Haplotypes were constructed to evaluate the respective contribution of the three *CD14* SNPs on atopy. Four haplotypes were obtained for the *CD14* gene, of which three were common and one was found in only six subjects (Table 5). The risk of atopy was significantly higher in carriers of haplotype 2 and 3, as compared to haplotype 1, which contained *CD14*/-260T and *CD14*/-651C. Haplotype 3, which contained *CD14*/-651T, was associated with the highest risk of atopy.

Discussion

In this nested case-control study, we found that the *CD14*/-260 T allele was significantly associated with reduced prevalence of atopy, whereas the *CD14*/-651 T allele was associated with increased atopy. Results of the haplotype analysis confirmed the role of *CD14*/-260 and *CD14*/-651 on atopy in our study population. The two SNPs were not in strong linkage disequilibrium, and they were independently associated with atopy in a multiple regression analysis. Unlike *CD14*/-260, studies taking into account *CD14*/-651 are scarce and haplotypes including both SNPs have not been reported before as a risk factor for atopy.

We did not find associations between *TLR2*, *TLR4*, or *CD14* genotypes and new-onset asthma in young Danish farmers. Because of the limited power of our relatively small study, we cannot exclude an effect on asthma for the less-common *TLR* polymorphisms. However, for the common *CD14* polymorphisms and *TLR2*/-16934 we did not observe any trend suggestive of an association with incident asthma. The majority of new asthma cases had a non-atopic phenotype (83%). Non-atopic asthma is common among farmers, and has been associated with increased exposure to microbial agents.[8] Interestingly, LeVan et al. have shown associations between *CD14* promoter polymorphisms and increased wheeze and decreased pulmonary function in a cross-sectional study among non-smoking, male farmers, supporting the hypothesis that higher levels of sCD14 may lead to increased susceptibility for endotoxin-induced airway inflammation.[37] The longitudinal design of our study might be one of the reasons as to why we did not find a relationship with the studied SNPs, as new-onset asthma in the cohort of young farmers might include less-severe phenotypes. All subjects were tested for bronchial hyperresponsiveness (BHR) and lung function, and a strong association was found between BHR and case status (OR = 10.8).[31] The present definition of asthma, however, has been made from several symptoms without reference to lung function data or atopy. Recently, it has been discussed in two large population studies that by using asthma definitions that require positive answers to several asthma symptom questions, one increases the

precision of the asthma diagnosis.[38, 39] Thus, we believe our symptom diagnosis of asthma was suitable for the analysis.

Because of the large number of SNPs currently identified and the absence of knowledge about the functional consequences of most of them, our approach was to study supposedly functional SNPs or at least SNPs that have been extensively studied. We covered the most well-known SNPs in the studied genes, but we were not able to detect possible associations between new-onset asthma and other SNPs in the *TLR* and *CD14* genes that were not included in the present study. Moreover, interactions with other, unstudied genes are also possible in complex diseases such as asthma and allergy. Relevance of SNPs - especially from a pathological point of view - is a general challenge in genetic research at this moment - and not easy to resolve.

The inverse association between the *CD14*/-260T allele and atopy was stronger for common inhalant allergens than for farm-related allergens, perhaps because protection against sensitization through increased susceptibility to endotoxin does not outbalance the burden of farm allergens. Previous studies have reported lower levels of IgE or a lower number of total positive SPT in skin test-positive children and adults carrying the *CD14*/-260 T allele.[17, 18, 20] Therefore, it has been speculated that the *CD14*/-260 polymorphism does not modulate the susceptibility to become sensitized, but rather leads to a more severe atopic phenotype.[18] On the other hand, a recent study among 600 French adults participating in the European Community Respiratory Health Survey (ECRHS)-II showed a lower risk for atopic sensitization and nasal allergies in carriers of the *CD14*/-260 T allele. Interestingly, these associations were more pronounced in individuals who lived on a farm during the first year of life compared to individuals who never lived on a farm.[21] Results of our study among farmers also suggest a stronger effect in farmers who were born and raised on a farm. This might indicate an interaction between genetic factors and timing of exposure, although the lack of power prevents us from drawing any conclusions on whether long-term farming exposures modify the effect of the T allele. Gene-environment interactions arguably play an important role in the development of complex diseases like allergy and asthma. Especially a role for microbial exposure has been described. Most participants in the present study were livestock farmers, who are known to be highly exposed to a wide range of micro-organisms and PAMPs, with average endotoxin exposure levels around 500-2000 EU/m^3.[8, 40, 41] In a few other cross-sectional studies, endotoxin load (EU/m^2) in mattress dust[42] or living room floor dust,[26, 43] or endotoxin level (EU/mg) in living room floor dust[44] were investigated as an interactive factor in the relationship between *CD14*/-260 genotype and asthma or atopy. In contrast with the results of our study, less atopic

sensitization, lower serum IgE levels, or less asthma were observed in subjects with the CC genotype compared to TT homozygotes, but only at elevated endotoxin exposure levels.[26, 42-44] Conversely, at lower endotoxin exposure levels, the T allele was associated with lower serum IgE levels and less asthma.[43, 44] Although these results contradict the findings of our study, it should be noted that seemingly incompatible results in genetic association studies might at least partly be explained by differences in the composition of the study population (age, ethnicity), and timing of exposure. In addition, levels of inhalable endotoxin during agricultural activities arguably exceed those measured in floor or mattress dust.

Thus, the key to the association between innate immunity genes and atopy might lie in complex interactions with environmental exposures. Vercelli proposed the 'endotoxin switch hypothesis' as an explanation for the heterogeneous results among different studies.[45] According to this concept, which is intriguing, but to date now not substantiated by any empirical evidence, each polymorphic allele would have its own multimodal response curve, representing the association between microbial exposure and immune response. At a certain exposure level, a switch from Th2 to Th1 responses might occur, which would occur at lower endotoxin levels for the *CD14/-260* T allele. At higher endotoxin levels, a second switch would occur, and it can be conceived that our results fit in such a model. Nevertheless, many questions remain unsolved, which illustrates the need for large-scale studies that include detailed microbial exposure data, taking into account both childhood and current exposures.

In conclusion, the *CD14/-651* and *CD14/-260* promoter polymorphisms are associated with the prevalence of atopy in young adult farmers. Effects appeared to be stronger in farmers who were born and raised on a farm, although interactions between *CD14* genotype and long-term farming exposures were not significant. No further associations between *CD14*, *TLR2*, or *TLR4* genotypes and atopy or new-onset asthma were found.

Acknowledgments

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Chapter 8

CD14 and Toll-like receptor gene polymorphisms, country living during childhood, and asthma in adults of the French EGEA study

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Abstract

Background: It has been shown that country living protects against asthma, which may be explained by microbial exposures.

Objective: To study whether single nucleotide polymorphisms (SNPs) in *CD14* and Toll-like receptor (*TLR*) *2, TLR4* and *TLR9* genes are associated with asthma in adults, and whether these SNPs modify associations between country living and asthma.

Methods: Twenty-five SNPs in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes were genotyped in adult subjects from the French Epidemiological study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA). We conducted a case-control analysis on unrelated subjects (239 asthmatics and 596 non asthmatics), and a family based association test (FBAT) in 192 families ascertained through asthmatic probands.

Results: The *TLR2/+596 C* allele was associated with an increased risk for asthma in both case-control and family-based analyses (under a dominant model, OR [95%CI]=1.91 [1.34-2.72], *P*=.0003; z statistics from FBAT=2.48, *P*=.01). In skin prick test (SPT) positive subjects, the *CD14/-260 C* allele was negatively associated with asthma (additive model, OR[95%CI]=0.66 [0.48-0.91]). Significant gene-environment interactions between variation in *CD14* and *TLR* genes and country living during childhood were found for ten SNPs. In SPT positive subjects carrying *CD14/-260 CC*, country living protected against asthma (OR=0.32 [0.12-0.85]), whereas country living was not associated with asthma in atopics carrying *CD14/-260 T* (OR=1.11 [0.65-1.90]) (gene-environment interaction *P*<.05).

Conclusion: *TLR2* and *CD14* SNPs were associated with asthma in adults. In addition, *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs may modify associations between country living and asthma.

Introduction

Epidemiological studies have shown a lower prevalence of asthma and allergic sensitization in children and adults who lived on a farm or in a rural area during childhood.[1-6] According to the hygiene hypothesis, the development of allergic disease may be influenced by bacterial and viral infections, and environmental exposure to noninfectious microbial agents such as endotoxin – which are abundantly present in the farm environment.[7] Both exposure to environmental factors and genetic susceptibility play a role in the occurrence of asthma and allergy. Therefore, genes dependent of the innate immunity pathway are obvious candidates for the understanding of the protective effects of exposure to microbial agents on allergy and asthma. Indeed, several single nucleotide polymorphisms (SNPs) in genes encoding pattern recognition receptors such as CD14 and Toll-like receptors (TLR) have been associated with atopic sensitization and asthma.[8-12] The first report by Baldini et al. [8] on the functional[13] *CD14 C -159 T* SNP (*C -260 T* when counting from the translation start site) has shown increased levels of circulating soluble CD14 (sCD14) in TT homozygotes, and a lower number of positive skin prick tests in atopic children carrying this genotype. Although subsequent studies have often replicated the association between *CD14/-260* T and increased sCD14, results on atopic sensitization have been conflicting and results for asthma were mainly negative.[14-19] Several studies have suggested that gene-environment interactions play an important role, since associations between *CD14/-260* genotype and allergy and asthma-related outcomes have been shown to depend on the level of microbial exposures.[20-25] A few studies have also shown gene-environment interactions in relation to SNPs in *TLR2* and *TLR4* genes, and other SNPs in the *CD14* gene *(-1721* showing a high linkage disequilibrium with *CD14/-260*).[9, 26, 27]

In the French Epidemiological study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA) a lower prevalence of asthma has been shown in adults who lived in the country during childhood.[3] The purpose of the present study was to investigate whether SNPs in the candidate genes *CD14*, *TLR2*, *TLR4*, and *TLR9* are associated with asthma in adults from the EGEA study by conducting a case-control analysis on unrelated subjects and a family based association test. We further investigated whether these SNPs modify associations between living in the country and asthma. Studies in children and adults have shown that exposure to endotoxin and other microbial agents may protect against asthma or wheeze in atopic subjects, whereas increased exposure may be a risk factor for nonatopic asthma or wheeze.[28-30] Therefore, the role of atopy was evaluated by conducting separate analyses for atopic and nonatopic subjects.

Methods

Study population and design

The design of EGEA combines a case-control study and a family study of adult and childhood asthma. The design, ascertainment of asthmatic cases, and descriptive characteristics have been described earlier.[31-34] Briefly, asthmatic patients (age 7-70 years) were recruited from six chest clinics in five French cities. Family members of asthmatic probands were included, either by including the proband's parents and siblings, or by including the proband's spouse and children. Controls were selected from electoral rolls, a surgery department, and a social security check-up center. Information on country living was only available for adults. In the present study, a case-control analysis was conducted in 825 subjects from the "founder" generation, i.e. adult asthmatic probands and their spouses, parents of asthmatic probands from families where the proband is offspring, and controls from the same generation. By selecting these subjects, a population of genetically unrelated adults from the same generation was obtained. In addition, a family based analysis was conducted in 708 adult subjects from 192 families, comprising 378 parents who were included in the case-control analysis as well, and 330 adult offspring subjects who were not included in the analysis among unrelated subjects. The study protocol was approved by the institutional ethics committee and all participants gave written informed consent.

Asthma and atopy

Asthma in relatives of probands was defined as a positive answer to the question "Have you ever had attacks of breathlessness at rest with wheezing?" or "Have you ever had asthma attacks?".[32] Atopy was defined as a positive skin prick test (SPT) to one of the 11 common allergens tested.[35]

Environmental exposure

Country living during childhood was defined as a positive answer to the question "Have you ever lived in the country for at least one year?", and being 16 years of age or younger when beginning to live in the country. Country living was not taken into account during recruitment, as this was not considered of importance when the study was designed in 1990/1991. As described earlier, the area of residence for asthmatic probands was broader than for most controls, reflecting the area of attraction of the chest clinics, and significantly more asthmatic cases were living in the country than controls.[3] Analyses that included country living were therefore conducted in asthmatic probands and relatives only, excluding controls (n=117).

Genotyping

Candidate genes considered were *CD14* (5 SNPs), *TLR2* (5 SNPs), *TLR4* (10 SNPs) and *TLR9* (5 SNPs). Genotyping was performed using either Taqman Probes (Applied Biosystems, Foster City, CA) on an AB17900HT Sequence Detection System or 1536-plex Illumina Golden Gate assay (Illumina, San Diego, CA) at the Centre National de Génotypage (CNG, Evry, France). Identification numbers, position, and minor allele frequencies of studied SNPs are shown in Table A1 in the Appendix. All SNPs were in Hardy-Weinberg equilibrium ($P>.05$). Tables A2-A5 in the Appendix show LD between polymorphisms for the four genes under study.

Statistical analysis

Association between phenotypes and polymorphisms of *CD14*, *TLR2*, *TLR4* and *TLR9* genes was assessed using two complementary methods: 1) a logistic regression model taking into account familial dependence of the observations was applied to adults who were genetically unrelated but sharing the same household; 2) a family-based method was performed in an adult offspring sample.

For the unrelated adult sample, odds ratios (ORs) for associations between genotype and asthma were calculated by generalized estimating equations (GEE model; Proc GENMOD using SAS statistical software) to adjust for dependence between subjects sharing the same household (asthmatic probands and their spouses, and mothers and fathers of asthmatics). ORs were adjusted for age and sex. The effect of each SNP on disease was tested under a general genetic model (2 degrees of freedom test) and ORs were calculated using homozygotes for the most frequent allele as reference group. In addition, dominant, recessive, and additive genetic models were considered and the best-fitting model was selected. We tested the null hypothesis that effects of genotype and country living on asthma were independent by including an interaction term in the model (gene-environment interaction) and testing the significance (GEE model, Z-score). Similarly, we tested for statistical interaction between genotypes (gene-gene interaction). A large number of SNPs were tested (n=25), and therefore False Discovery Rate (FDR) adjusted P values were calculated to take multiple comparisons into account.[36]

A family-based association test, as implemented in FBAT[37] was performed to test for association between asthma and genetic polymorphisms in the adult offspring sample. This approach tests for the association between the phenotype and excess transmission of a specific allele from parent to offspring. The advantage of this approach is that population stratification is avoided. We used the –o option in FBAT program allowing to take into account affected and unaffected offspring in the

analysis. Association was initially tested under a general model and subsequently under additive, dominant and recessive models.

Results

Characteristics of nonasthmatic (n=586) and asthmatic (n=239) subjects are shown in Table 1. Living in the country during childhood was significantly associated with a lower prevalence of asthma with an OR of 0.67 (0.48-0.92), which confirmed the earlier report in a different subpopulation of the EGEA study.[3] Adjusting for age, sex, and smoking habits did not change the association.

Table 1 Characteristics of the study population.

	Nonasthmatics	Asthmatics	*P*
n	586	239	
Age, y, mean ± sd	47.1 ± 8.0	46.0 ± 8.8	.07
Males, n (%)	293 (50.0)	123 (51.5)	.70
Smoking habits			
Never smokers, n (%)	258 (44.3)	104 (43.9)	.01
Exsmokers, n (%)	178 (30.5)	93 (39.2)	
Current smokers, n (%)	147 (25.2)	40 (16.9)	
Living in the country during childhood, n (%)[*]	254 (55.0)	105 (44.9)	.01
Atopy, n (%)	182 (31.8)	148 (64.4)	<.0001

[*] controls (n=117) excluded.

Association between CD14, TLR2, TLR4, and TLR9 genetic polymorphisms and asthma

The genotypic distribution of *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and the associations between these polymorphisms and asthma in the 825 unrelated subjects are shown in Tables A6-A9 in the Appendix. Carrying the *TLR2/+596 C* allele was associated with an increased risk for asthma (OR (95%CI) = 1.91 (1.34-2.72); *P*=.0003, *P*-value adjusted for multiple comparisons =.008). Two other *TLR2* SNPs, *TLR2/-24438* and rs2289318, and two *TLR9* SNPs, rs353547 and *TLR9/+2848*, were also associated with asthma, but not significantly after adjusting for multiple comparisons. Since low LD was observed among the three *TLR2* SNPs on the one hand (LD coefficient $r^2 \leq 0.34$; Table A3) and between the two *TLR9* SNPs on the other hand (LD coefficient $r^2 = 0.53$; Table A5), we conducted two separate

multivariate-SNP analyses by including these polymorphisms in the regression model. *TLR2/+596* was the only SNP significantly associated with asthma in the model testing for three *TLR2* markers. Estimates for each *TLR9* SNP in the multiple regression model were similar to those in the univariate model. However, none of them was significant after mutual adjustment. Associations between genotype and asthma were not different for men and women (interaction *P*>.10 for all SNPs). Similar results were obtained when controls (n=117) were not included (data not shown).

The positive association between the *TLR2/+596 C* allele and asthma under a dominant model was confirmed by a family-based association test (Z=2.48; *P*=.01, FBAT). For other SNPs no significant association was found using FBAT (*P*>.05 ; data not shown).

In the 825 genetically unrelated subjects, the influence of the *TLR2/+596* polymorphism on associations between other SNPs and asthma (i.e. gene-gene interactions) was assessed. For three SNPs (*CD14/+1188* and two *TLR9* SNPs), a significant interaction with *TLR2/+596* genotype was found (Table 2), showing significant associations between these SNPs and asthma in *TLR2/+596 TT* homozygotes, but ORs around 1 in subjects carrying *TLR2/+596 C*.

Table 2 Associations between *CD14* and *TLR9* SNPs and asthma for SNPs showing significant gene-gene interactions with *TLR2/+596*.

SNP	Model	Asthma (OR (95% CI))		P interaction	
		TLR2/+596 TT **subjects**	*TLR2/+596 CT/CC* **subjects**	**Not adjusted**	**FDR adjusted**
CD14/+1188	CG+GG vs. CC (ref)	0.30 (0.11-0.81)	0.93 (0.60-1.45)	.03	.16
TLR9 rs352143	AG+GG vs. AA (ref)	2.31 (1.24-4.30)	0.95 (0.66-1.38)	.02	.15
TLR9 rs352163	T allele [¶]	0.48 (0.30-0.74)	1.04 (0.80-1.35)	.002	.04

[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).
Ref: reference genotype coded as 0.

Separate analyses were conducted for atopic and non-atopic subjects. Associations between genotype and asthma outcomes for 11 SNPs associated with at least one of the asthma outcomes are presented in Table 3 (unadjusted *P*<.05 for 11 SNPs, FDR-adjusted *P*<.05 for 6 SNPs). Carrying the *TLR2/+596 C* allele was a significant risk factor for asthma in atopic subjects (OR 2.39, *P*=.0006), whereas a positive but not significant association was found in non-atopic subjects (OR 1.55, *P*=.13). The *CD14/-260 C* allele protected against asthma in atopic subjects with an OR of 0.66 (0.48-0.91). Four other *CD14* SNPs were also significantly associated with a lower

risk of asthma in atopic subjects, and for these SNPs, associations differed significantly from those observed in non-atopic subjects, who had ORs around 1 (gene-atopy interaction $P<.05$). Atopic subjects carrying a protective allele for each *CD14* SNP had significantly less asthma compared with subjects carrying none of the protective alleles, with an OR of 0.34 (0.18-0.67). Carrying a protective allele for one to four *CD14* SNPs showed an OR of 0.64 (0.37-1.16) compared with subjects carrying none. Multiple regression models including more than one *CD14* SNP showed independent effects that were similar in size for each different SNP, thus the effect was not dominated by one particular SNP.

Table 3 Associations between *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and asthma for SNPs significantly associated with at least one of the asthma outcomes.

| Gene | SNP | Model | OR (95% CI) | | |
			Asthma	Asthma in atopic subjects	Asthma in nonatopic subjects
CD14	*-260*	C allele [¶]	0.81 (0.65-1.01)	**0.66 (0.48-0.91)**[**]	0.93 (0.65-1.33)
	+1188	CG+GG vs. CC (ref)	0.72 (0.49-1.06)	**0.44 (0.25-0.78)**[**]	1.00 (0.56-1.82)
	+1341	AC+AA vs. CC (ref)	0.77 (0.56-1.06)	**0.50 (0.31-0.81)**[**]	1.02 (0.62-1.69)
	rs778584	T allele [¶]	0.86 (0.69-1.06)	**0.62 (0.46-0.85)**[**]	1.09 (0.77-1.52)
	rs778583	CT+TT vs. CC (ref)	0.80 (0.59-1.09)	**0.47 (0.29-0.75)**[**]	1.11 (0.69-1.80)
TLR2	*-24438*	GG vs. AA+AG (ref)	**0.57 (0.36-0.90)**	**0.47 (0.25-0.89)**[*]	0.66 (0.32-1.36)
	+596	CT+CC vs. TT (ref)	**1.91 (1.34-2.72)**[**]	**2.39 (1.45-3.94)**[**]	1.55 (0.88-2.73)
	+1349	C allele [¶]	1.30 (0.90-1.88)	1.04 (0.55-1.98)	**1.92 (1.17-3.15)**
	rs2289318	CG+GG vs. CC (ref)	**1.40 (1.01-1.94)**	**1.61 (1.01-2.56)**	0.83 (0.48-1.43)
TLR9	rs353547	AG+AA vs. GG (ref)	**1.43 (1.03-1.99)**	1.27 (0.79-2.06)	1.43 (0.86-2.38)
	+2848	AA vs. GG+AG (ref)	**0.65 (0.43-0.99)**	0.62 (0.34-1.13)	0.77 (0.40-1.46)

Bold: $P<.05$, not adjusted for multiple comparisons; [*] $P<.10$, FDR adjusted P-value; [**] $P<.05$, FDR adjusted P-value.
[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).
Ref: reference genotype coded as 0.

Role of CD14, TLR2, TLR4, and TLR9 genetic polymorphisms in the associations between living in the country and asthma
Gene-environment interactions between 10 SNPs and living in the country during childhood were found for at least one of the asthma outcomes (unadjusted P-value for interaction <.05 for 10 SNPs, FDR-adjusted $P<.10$ for 6 SNPs; Table 4).

Table 4 Gene-environment interactions between *CD14*, *TLR2*, *TLR4*, and *TLR9* SNPs and country living during childhood in asthma outcomes, shown as associations between country living and asthma, stratified by genotype.

Gene	SNP	Genotype	Living in the country during childhood, OR (95% CI)		
			Asthma	**Asthma in atopic subjects**	**Asthma in nonatopic subjects**
CD14	*-260*	TT+CT	0.66 (0.45-0.97)	**1.11 (0.65-1.90)**[*]	0.41 (0.22-0.79)
		CC	0.63 (0.32-1.24)	**0.32 (0.12-0.85)**	1.33 (0.46-3.84)
	+1341	CC	**0.92 (0.59-1.44)**	**1.59 (0.81-3.13)**[*]	0.75 (0.36-1.55)
		AC+AA	**0.44 (0.26-0.73)**	**0.48 (0.23-0.99)**	0.42 (0.18-1.00)
	rs778584	CC+CT	0.64 (0.44-0.94)	**1.07 (0.63-1.82)**[*]	**0.42 (0.22-0.78)**
		TT	0.72 (0.38-1.40)	**0.32 (0.12-0.84)**	**1.41 (0.51-3.87)**
	rs778583	CC	0.89 (0.58-1.37)	**1.50 (0.77-2.95)**[*]	0.76 (0.37-1.55)
		CT+TT	0.47 (0.28-0.76)	**0.47 (0.23-0.94)**	0.48 (0.22-1.05)
TLR2	*-24438*	AA+AG	0.63 (0.45-0.90)	0.87 (0.54-1.42)	**0.47 (0.26-0.85)**
		GG	0.94 (0.38-2.33)	0.44 (0.12-1.62)	**3.57 (0.58-21.8)**
	+596	TT	0.66 (0.35-1.27)	0.53 (0.20-1.35)	**1.36 (0.48-3.88)**
		CT+CC	0.65 (0.43-0.97)	0.91 (0.52-1.59)	**0.40 (0.20-0.78)**
	+1349	TT	0.68 (0.47-0.96)	0.78 (0.48-1.27)	**0.71 (0.39-1.30)**
		CT+CC	0.57 (0.26-1.27)	0.91 (0.26-3.22)	**0.23 (0.07-0.76)**
TLR4	rs11536889	GG	**0.79 (0.54-1.15)**	**1.17 (0.67-2.05)**[*]	0.70 (0.37-1.32)
		CG+CC	**0.43 (0.23-0.80)**	**0.36 (0.14-0.91)**	0.36 (0.13-0.99)
	rs7045953	AA	**0.53 (0.37-0.78)**	0.66 (0.38-1.13)	0.46 (0.25-0.87)
		AG+GG	**1.14 (0.62-2.11)**	1.34 (0.54-3.33)	1.16 (0.43-3.10)
TLR9	rs353547	GG+AG	0.72 (0.50-1.03)	**1.11 (0.67-1.86)**[*]	0.53 (0.29-0.95)
		AA	0.41 (0.18-0.96)	**0.18 (0.05-0.63)**	1.66 (0.40-6.92)

Bold: interaction *P*<0.05, not adjusted for multiple comparisons; [*] interaction *P*<0.10, FDR adjusted *P*-value.

In atopic subjects with *CD14/-260 CC* genotype, country living protected against asthma (OR 0.32), whereas country living was not associated with asthma in atopics carrying the *CD14/-260 T* allele (OR 1.11). Conversely, in non-atopic subjects carrying *CD14/-260 CC*, country living was not associated with asthma (OR 1.33), whereas country living was associated with a lower risk of asthma in non-atopic subjects carrying the *CD14/-260 T* allele (OR 0.41). For this SNP, a significant second-order interaction term (gene x environment x atopy) was found (*P*=.005).

In atopics, gene-environment interactions were found as well for three other *CD14* SNPs, one *TLR4* SNP, and one *TLR9* SNP (all FDR-adjusted *P*-values for interaction were borderline significant). In non-atopic subjects, gene-environment interactions were found for four SNPs, including *TLR2/+596*. In non-atopic carriers of the

TLR2/+596 C allele, country life protected against asthma. Alternatively, when the results were stratified for living in the country, carrying at least one *TLR2/+596 C* allele (CC/CT vs. TT) was a risk factor for asthma in non-atopic subjects who had not grown up in the country with an OR of 2.94 (1.16-7.41), but not in those who had grown up in the country (OR 0.77 (0.35-1.70)).

Discussion

In the present study, an association was found between the *TLR2/+596* polymorphism and asthma among French adults from the EGEA study. The well-studied *CD14/-260* SNP was associated with asthma in SPT positive subjects – once again confirming the importance of this SNP in the occurrence of allergy and asthma related phenotypes. Gene-environment interactions were found between ten SNPs in candidate genes *CD14*, *TLR4*, *TLR2*, and *TLR9* and living in the country during childhood, which was presumed to represent higher exposures to various microbial agents. Main effects and gene-environment interactions were stronger in atopic subjects than in non-atopic subjects. Significant interactions between *CD14* SNPs and atopy were found in the association with asthma, moreover for *CD14/-260*, a significant interaction between genotype, atopy, and country living was found.

Eder et al. found that a SNP in *TLR2* (*A -16934 T*) was associated with asthma, atopy, and hay fever in children of farmers.[9] On the other hand, two studies investigating genetic variation in *TLR2* reported no association with childhood-onset atopic asthma in a Japanese population,[38] and no association with atopy or new-onset asthma in young Danish farmers.[23] Findings of the present study for *TLR2/+596* were consistent in case-control and family-based analyses. In the case-control analysis only the parental generation was included, whereas in the family-based analysis, genotype and asthma outcome of offspring determined the magnitude of the test statistic. Thus, results of the two different analyses were based on a different population within the EGEA study, which substantiates findings on *TLR2*. Moreover, although we tested a large number of SNPs in four candidate genes which increases the risk for false positive results, the findings on *TLR2/+596* were still statistically significant after adjusting for multiple comparisons. Further analyses of different asthma outcomes showed that *TLR2/+596* was in particular associated with asthma in SPT positive subjects, whereas in non-atopic subjects, a significant interaction with childhood environment was found, showing that the *TLR2/+596* polymorphism was only a risk factor for asthma in non-atopic subjects who had not grown up in the country. Thus, variation in *TLR2* appears to be

associated with asthma, but environmental exposure, asthma subphenotype, and also interactions with other genes such as *CD14* and *TLR9* were shown to play a role.

TLR9 is a receptor for bacterial CpG DNA motifs, which have been found in increased levels in farm barn dust and dust from rural homes.[39] Only few studies have investigated *TLR9* SNPs in relation to allergy or asthma, showing inconsistent results.[11, 38, 40, 41] In the present study, only minor associations with asthma were found for *TLR9* SNPs when other factors were not taken into account. However, significant gene-gene interactions with the *TLR2/+596* SNP were found, showing effects of two *TLR9* SNPs on asthma in *TLR2/+596 TT* subjects. Another *TLR9* SNP was associated with asthma in atopic subjects, but only when childhood environment was taken into account.

In contrast to *TLR2* and *TLR9*, which have not been studied widely in relation to asthma or allergic outcomes, *CD14* is one of the best established susceptibility genes for asthma-related phenotypes such as allergic sensitization and total IgE.[42] Studies investigating asthma in relation to *CD14/-260* have often been negative,[14-16, 23, 24] whereas either the C allele or T allele were alternately found to be a risk factor for allergic sensitization, number of positive SPT, or increased total IgE.[8, 17, 18, 23, 24, 43, 44] In the present study, *CD14* polymorphisms protected against asthma, but only in atopic subjects, showing significant gene-atopy interactions.

Inverse associations between house dust endotoxin and allergic sensitization,[20, 22] total serum IgE,[21] and asthma[25] have been shown to be strongest in *CD14/-260 CC* homozygotes. A similar and significant gene-environment interaction was observed in the present study for the inverse relationship between a rural childhood and asthma in atopic adults. This apparent replication of a gene-environment is remarkable, given the different phenotypes, age of study subjects, timing of exposure, study region, and exposure definitions across these studies. Conversely, in three other studies, the *CD14/-260 TT* genotype protected against atopic dermatitis and atopy, especially in children with a dog in the home,[45] and in adults or young farmers who lived on a farm during childhood.[23, 24] Gene-environment interactions are therefore likely to depend on qualitative and quantitative characteristics of environmental exposure, but also on phenotype, interactions with other genes, and on the age of study subjects.[42, 43] The importance of atopy as an effect modifier in the present study underlines the complex interrelationships between environmental exposures, genetic variation in *CD14*, and asthma.

The fact that interactions were found between a rural childhood and polymorphisms in genes encoding four different pattern recognition receptors that become activated by a wide array of ligands, suggests that the protective effect of living in the country

is – at least partly – a result of increased microbial exposures. In the present study, living in the country was used as a proxy for high exposure to microbial agents during childhood, since measured domestic endotoxin levels or more specific information regarding farm (animal) exposures was not available. Nevertheless, in the EGEA study it has been demonstrated that several lifestyle factors differed substantially in subjects who ever lived in rural communities and subjects who had always lived in urban regions. For instance, rural life was associated with factors such as using wood for heating, household size, and the presence of pets.[3] Considering the fact that most of the present study subjects were born in the 1940s/50s, it is likely that other relevant factors such as raw milk consumption, and exposure to stables and livestock were relatively common among those living in the country during their childhood.

In conclusion, *TLR2* and *CD14* are important genes for asthma in adults. Variation in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes modified associations between country living during childhood and asthma, in particular for asthma in atopic subjects.

Acknowledgments

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Appendix

Table A1 SNP identification numbers, position, and minor allele frequencies.

dbSNP ID	Gene	Genomic location	Position from translation start site	Alleles	Minor allele frequency	N
rs2569190	*CD14*	5 :139993100	-260	T/C	0.50	777
rs4914	*CD14*	5 :139991652	+1188	C/G	0.12	783
rs2563298	*CD14*	5 :139991499	+1341	C/A	0.27	781
rs778584	*CD14*	5 :139985396		C/T	0.49	819
rs778583	*CD14*	5 :139985294		C/T	0.28	822
rs13150331	*TLR2*	4 :154819072	-24438	A/G	0.40	817
rs3804099	*TLR2*	4 :154844106	+596	T/C	0.43	788
rs3804100	*TLR2*	4 :154844859	+1349	T/C	0.08	822
rs5743700	*TLR2*	4 :154845132	+1622	C/T	0.04	786
rs2289318	*TLR2, RNF175*	4 :154853184		C/G	0.17	822
rs10759930	*TLR4*	9 :119501442		C/T	0.41	822
rs2737191	*TLR4*	9 :119502536		A/G	0.27	821
rs4986791	*TLR4*	9 :119515423	+1196, Thr399Ile	C/T	0.06	792
rs7869402	*TLR4*	9 :119517853		C/T	0.02	790
rs11536889	*TLR4*	9 :119517952		G/C	0.17	820
rs1927906	*TLR4*	9 :119519936		A/G	0.09	821
rs1554973	*TLR4*	9 :119520633		T/C	0.24	817
rs913930	*TLR4*	9 :119523830		T/C	0.34	820
rs1927905	*TLR4*	9 :119525129		A/G	0.06	822
rs7045953	*TLR4*	9 :119525616		A/G	0.15	821
rs353547	*TLR9, PTK9L*	3 :52243906		G/A	0.39	821
rs11717574	*TLR9, PTK9L*	3 :52243286		T/C	0.15	815
rs352143	*TLR9, PTK9L*	3 :52239947		A/G	0.21	822
rs352140	*TLR9*	3 :52231737	+2848	G/A	0.47	778
rs352163	*TLR9, ALAS1*	3 :52222150		C/T	0.47	817

Table A2 Linkage disequilibrium (r^2) between *CD14* SNPs.

	+1188	+1341	rs778584	rs778583
-260	0.14	0.37	0.95	0.37
+1188		0.37	0.14	0.35
+1341			0.38	0.98
rs778584				0.39
rs778583				

Table A3 Linkage disequilibrium (r^2) between *TLR2* SNPs.

	+596	+1349	+1622	rs2289318
-24438	0.34	0.05	0.03	0.05
+596		0.11	0.06	0.26
+1349			0	0.02
+1622				0.23
rs2289318				

Table A4 Linkage disequilibrium (r^2) between *TLR4* SNPs.

	rs2737191	Thr399Ile	rs7869402	rs11536889	rs1927906	rs1554973	rs913930	rs1927905	rs7045953
rs10759930	0.26	0.05	0.02	0.26	0.07	0.21	0.34	0.04	0.12
rs2737191		0.03	0.01	0.06	0.04	0.11	0.65	0.02	0.06
Thr399Ile			0	0.01	0.70	0.22	0.03	0	0.01
rs7869402				0.01	0.25	0.08	0.01	0	0
rs11536889					0.02	0.07	0.11	0.01	0.04
rs1927906						0.31	0.05	0	0.01
rs1554973							0.16	0.21	0.57
rs913930								0.03	0.09
rs1927905									0.37
rs7045953									

Table A5 Linkage disequilibrium (r^2) between *TLR9* SNPs.

	rs11717574	rs352143	+2848	rs352163
rs353547	0.11	0.01	0.53	0.45
rs11717574		0.65	0.13	0.13
rs352143			0.21	0.21
+2848				0.89
rs352163				

Table A6 Association between *CD14* genotype and asthma.

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
CD14/-260			
TT	133 (24.0)	67 (30.0)	1.00
CT	276 (49.8)	107 (48.0)	0.75 (0.51-1.10)
CC	145 (26.2)	49 (22.0)	0.66 (0.43-1.02)
C allele [¶]			0.81 (0.65-1.01)
CD14/+1188			
CC	428 (76.3)	181 (81.5)	1.00
CG	122 (21.8)	37 (16.7)	0.71 (0.48-1.06)
GG	11 (2.0)	4 (1.8)	0.85 (0.27-2.69)
CG+GG vs. CC (ref)			0.72 (0.49-1.06)
CD14/+1341			
CC	292 (52.4)	131 (58.5)	1.00
AC	214 (38.4)	76 (33.9)	0.78 (0.56-1.09)
AA	51 (9.2)	17 (7.6)	0.74 (0.42-1.30)
AC+AA vs. CC (ref)			0.77 (0.56-1.06)
rs778584			
CC	146 (25.1)	69 (29.0)	1.00
CT	286 (49.2)	117 (49.2)	0.85 (0.59-1.23)
TT	149 (25.7)	52 (21.9)	0.73 (0.48-1.12)
T allele [¶]			0.86 (0.69-1.06)
rs778583			
CC	303 (52.0)	137 (57.3)	1.00
CT	226 (38.8)	85 (35.6)	0.82 (0.60-1.14)
TT	54 (9.3)	17 (7.1)	0.70 (0.39-1.22)
CT+TT vs. CC (ref)			0.80 (0.59-1.09)

[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).
Ref: reference genotype coded as 0.

Table A7 Association between *TLR2* genotype and asthma.

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
TLR2/-24438			
AA	192 (33.2)	94 (39.3)	1.00
AG	286 (49.5)	120 (50.2)	0.86 (0.62-1.21)
GG	100 (17.3)	25 (10.5)	**0.52 (0.31-0.86)**
GG vs. AA+AG (ref)			**0.57 (0.36-0.90)**
TLR2/+596			
TT	207 (36.6)	52 (23.3)	1.00
CT	259 (45.8)	122 (54.7)	**1.88 (1.30-2.71)**
CC	99 (17.5)	49 (22.0)	**1.99 (1.24-3.19)**
CT+CC vs. TT (ref)			**1.91 (1.34-2.72)**
TLR2/+1349			
TT	502 (86.1)	199 (83.3)	1.00
CT	77 (13.2)	36 (15.1)	1.20 (0.79-1.85)
CC	4 (0.7)	4 (1.7)	2.58 (0.64-10.39)
C allele [¶]			1.30 (0.90-1.88)
TLR2/+1622			
CC	515 (91.3)	201 (90.5)	1.00
CT	49 (8.7)	21 (9.5)	1.10 (0.64-1.92)
rs2289318			
CC	419 (71.9)	154 (64.4)	1.00
CG	145 (24.9)	81 (33.9)	**1.51 (1.08-2.10)**
GG	19 (3.3)	4 (1.7)	0.57 (0.19-1.72)
CG+GG vs. CC (ref)			**1.40 (1.01-1.94)**

Bold: *P*<.05.
[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).
Ref: reference genotype coded as 0.

Table A8 Association between *TLR4* genotype and asthma.

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
rs10759930			
CC	192 (32.9)	88 (36.8)	1.00
CT	305 (52.3)	107 (44.8)	0.76 (0.55-1.06)
TT	86 (14.8)	44 (18.4)	1.14 (0.73-1.78)
TT vs. CC+CT (ref)			1.34 (0.88-2.03)
rs2737191			
AA	299 (51.4)	123 (51.5)	1.00
AG	248 (42.6)	101 (42.3)	0.99 (0.73-1.34)
GG	35 (6.0)	15 (6.3)	1.00 (0.52-1.89)
AG+GG vs. AA (ref)			0.99 (0.74-1.32)
TLR4 Thr399Ile			
CC	492 (86.6)	198 (88.4)	1.00
CT	76 (13.4)	26 (11.6)	0.85 (0.52-1.39)
rs7869402			
CC	544 (96.3)	211 (93.8)	1.00
CT	21 (3.7)	13 (5.8)	1.56 (0.78-3.12)
TT	0 (0.0)	1 (0.4)	n.e.
rs11536889			
GG	385 (66.2)	174 (73.1)	1.00
CG	183 (31.4)	55 (23.1)	**0.69 (0.48-0.99)**
CC	14 (2.4)	9 (3.8)	1.63 (0.64-4.11)
CG+CC vs. GG (ref)			0.72 (0.51-1.00)
rs1927906			
AA	482 (82.7)	196 (82.4)	1.00
AG	99 (17.0)	39 (16.4)	0.97 (0.65-1.47)
GG	2 (0.3)	3 (1.3)	3.33 (0.58-19.08)
AG+GG vs. AA (ref)			1.03 (0.69-1.53)
rs1554973			
TT	332 (57.4)	140 (58.6)	1.00
CT	219 (37.9)	83 (34.7)	0.92 (0.67-1.26)
CC	27 (4.7)	16 (6.7)	1.39 (0.75-2.59)
CC vs. TT+CT (ref)			1.44 (0.79-2.64)
rs913930			
TT	252 (43.3)	99 (41.6)	1.00
CT	270 (46.4)	113 (47.5)	1.06 (0.78-1.45)
CC	60 (10.3)	26 (10.9)	1.07 (0.64-1.78)
CT+CC vs. TT (ref)			1.06 (0.79-1.43)
rs1927905			
AA	513 (88.0)	208 (87.0)	1.00
AG	69 (11.8)	29 (12.1)	1.06 (0.67-1.66)
GG	1 (0.2)	2 (0.8)	4.62 (0.44-49.07)
AG+GG vs. AA (ref)			1.11 (0.72-1.72)
rs7045953			
AA	415 (71.3)	174 (72.8)	1.00
AG	157 (27.0)	60 (25.1)	0.92 (0.66-1.29)
GG	10 (1.7)	5 (2.1)	1.21 (0.45-3.21)
GG vs. AA+AG (ref)			1.23 (0.47-3.26)

Bold: *P*<.05.
Ref: reference genotype coded as 0.
n.e. not estimable.

Table A9 Association between *TLR9* genotype and asthma.

SNP	Nonasthmatics, n (%)	Asthmatics, n (%)	OR (95%CI)
rs353547			
GG	233 (40.0)	76 (31.9)	1.00
AG	263 (45.1)	124 (52.1)	**1.46 (1.04-2.07)**
AA	87 (14.9)	38 (16.0)	1.35 (0.85-2.15)
AG+AA vs. GG (ref)			**1.43 (1.03-1.99)**
rs11717574			
TT	419 (72.5)	171 (72.2)	1.00
CT	140 (24.2)	62 (26.2)	1.08 (0.77-1.53)
CC	19 (3.3)	4 (1.7)	0.52 (0.17-1.55)
CC vs. TT+CT (ref)			0.51 (0.17-1.51)
rs352143			
AA	370 (63.5)	144 (60.3)	1.00
AG	180 (30.9)	83 (34.7)	1.19 (0.87-1.64)
GG	33 (5.7)	12 (5.0)	0.92 (0.47-1.83)
AG+GG vs. AA (ref)			1.15 (0.85-1.55)
TLR9/+2848			
GG	154 (27.6)	65 (29.6)	1.00
AG	275 (49.3)	118 (53.6)	1.00 (0.70-1.44)
AA	129 (23.1)	37 (16.8)	0.65 (0.40-1.07)
AA vs. GG+AG (ref)			**0.65 (0.43-0.99)**
rs352163			
CC	159 (27.4)	67 (28.3)	1.00
CT	286 (49.3)	128 (54.0)	1.06 (0.75-1.50)
TT	135 (23.3)	42 (17.7)	0.73 (0.46-1.16)
T allele [¶]			0.87 (0.70-1.09)

Bold: *P*<.05.
[¶] Additive genetic model, the genotype was categorized into a three level variable for the number of minor alleles (0,1,2).
Ref: reference genotype coded as 0.

Chapter 9

General Discussion

The main aim of this thesis was to investigate associations between endotoxin exposure and the occurrence of respiratory and allergic outcomes in adults, and to evaluate susceptibility factors acting as effect modifiers in associations between exposure and health outcomes. In farmers and workers in agricultural processing industries, significant and positive exposure-response relationships were found between endotoxin exposure and asthma-related outcomes, which were characterized by a predominantly non-atopic phenotype. In contrast, inverse associations between occupational endotoxin exposure and hay fever and atopic sensitization were found. An important role for individual susceptibility to the adverse effects of endotoxin was implied by the finding that associations between occupational endotoxin exposure and respiratory effects were only observed in the subgroup of subjects with above median cytokine response by *ex vivo* LPS-stimulated blood monocytes. In this chapter, some aspects of the influence of susceptibility factors in associations between occupational exposure to endotoxin and respiratory health effects will be evaluated in greater detail.

Individual susceptibility

The concept that individual factors modify the impact of environmental exposures on disease dates back to the ancient Greek Hippocratic school around 400 B.C.[1, 2] In the treatise "On Airs, Waters, and Places", Hippocrates describes how certain environmental conditions are connected with diseases in distinct subgroups: " ... after such a spring … acute fevers will attack all, but especially those of a phlegmatic constitution. Dysenteries are also likely to occur to women and those of a very humid temperament."[1] In the late 19th century, however, attention shifted towards specific pathogenic agents and the pursuit of universally valid cause-effect hypotheses.[2, 3] This reductionist approach to understand relations between exposures and disease fails to explain the variation in disease risk among subgroups within a population with similar conditions of exposure. Genetic and acquired host factors may influence the ability to resist a potentially harmful agent. As a consequence, individuals may differ with respect to their susceptibility for a disease given a certain level of exposure. With regard to occupational endotoxin exposure, clearly not all workers develop the same physiologic response under similar circumstances of exposure. As has been shown in this thesis, the shape and slope of dose-response relationships may differ between particular subgroups of individuals. The associations differed between subjects who had grown up in a different environment (Chapter 4), between current smokers and non-smokers, between atopic and non-atopic individuals (exhaled nitric oxide, Chapter 5), and between subjects with a different (*ex vivo*) inflammatory

response to LPS (Chapter 6). The scientific evidence regarding a number of potential susceptibility factors will be further discussed, and suggestions for future research will be outlined.

Atopy

Endotoxin-induced respiratory health effects are mediated through non-allergic mechanisms.[4, 5] However, atopic status, which is generally defined by a positive skin prick test or elevated specific serum IgE to one or more common allergens, may be a potential effect modifier for associations between occupational endotoxin exposure and respiratory disorders.

Table 1 gives an overview of studies showing separate analyses for atopic, or atopic asthmatic, and non-atopic subjects. In some experimental endotoxin provocation studies, atopic asthma or airway hyper-responsiveness (AHR) appeared to increase sensitivity to acute endotoxin exposure,[6, 7, 10, 13] but no marked differences in responsiveness between healthy non-atopic and atopic subjects were found.[8, 9] Michel et al.[12] found even stronger systemic and local effects of endotoxin inhalation in non-atopic than in atopic subjects. Thus, evidence from experimental exposure studies implies that asthma and AHR, but not atopy in itself, increase sensitivity for endotoxin inhalation.

In a Dutch population-based study, only atopic subjects had an increased risk of asthma if occupationally exposed to organic dust.[15] However, there is no compelling evidence from epidemiological studies with measured exposure data that atopy increases susceptibility to endotoxin-related respiratory effects. On the contrary, in Norwegian farmers an *inverse* association between endotoxin exposure and asthma was found in atopic subjects, whereas endotoxin and asthma were positively associated in non-atopic farmers.[16] These opposite relationships were attributed to inhibition of Th_2 driven immune responses leading to less atopic asthma, and induction of non-atopic asthma through non-IgE mediated inflammation.[16] However, this did not satisfactorily explain why a protective effect of endotoxin exposure on asthma was found in the subgroup of atopics, but not on atopy *per se*.

In Dutch pig farmers, endotoxin exposure appeared to be a protective factor for atopy.[17] Paradoxically, endotoxin exposure was also associated with an increased risk of asthma-related effects, especially in atopic subjects.[17, 18] A study in the potato processing industry found similar relationships between occupational endotoxin exposure and respiratory symptoms and across-shift peak flow changes for atopic and non-atopic workers.[14]

Table 1 Studies investigating atopy and/or asthma as a susceptibility factor for inflammatory or respiratory effects induced by endotoxin exposure.

Population	Exposure	Main results	Reference
Experimental challenge studies			
6 normal subjects, 8 asthmatics	LPS inhalation, 22.2 µg	FEV_1 decrease and increased AHR in asthmatics but not in normal subjects	Michel et al. 1989[6]
6 non-atopic asthmatics, 10 atopic asthmatics	LPS inhalation, 20 µg	FEV_1 decrease is associated with baseline AHR, but not with atopy	Michel et al. 1992[7]
10 normal subjects, 10 atopic non-asthmatics	Corn dust extract inhalation, 0.4 µg LPS/kg	FEV_1 decrease and increase in BAL PMN, TNFα, IL1β, IL6, and IL8, no differences between atopic and non-atopic subjects	Blaski et al. 1996[8]
11 normal subjects, 7 atopic non-asthmatics, 8 atopic asthmatics	LPS inhalation, 60 µg	Increase in sputum PMN and IL8 in normal and atopic asthmatic subjects, but no changes in atopic non-asthmatics	Nightingale et al. 1998[9]
6 normal subjects, 10 atopic asthmatics	nasal challenge, 1 µg LPS	Eosinophil influx in nasal airways, only in atopic asthmatics	Peden et al. 1999[10]
8 normal subjects, 10 atopic asthmatics	LPS inhalation, 5 µg	Increase in sputum PMN in normal and atopic asthmatic subjects	Alexis et al. 2001[11]
6 normal subjects, 9 atopic non-asthmatics	LPS inhalation, 0.5 - 5 - 50 µg	FEV_1 decrease and rise in body temperaure, blood PMN and LBP are larger in normal than in atopic subjects	Michel et al. 2001[12]
6 normal subjects, 8 asthmatics	Grain dust aerosol inhalation, 30 min, 4 µg LPS/m^3	FEV_1 decrease and increased AHR in asthmatics but not in normal subjects	Sigurdarson et al. 2004[13]
Observational studies			
76 non-atopic and 17 atopic potato processing workers	Airborne endotoxin, GM 534 EU/m^3	Across-shift peak-flow decline and increased respiratory symptoms, exposure-response relationships not different in normal and atopic subjects	Zock et al. 1999[14]
1 271 non-atopic and 635 atopic subjects, general population	Job with organic dust exposure	Increased risk for current asthma, only in atopic subjects	De Meer et al. 2004[15]
1 350 non-atopic and 264 atopic farmers	Airborne endotoxin, annual GM 28x10^3 EU/m^3	Positive association with asthma (current or ever diagnosed) in non-atopic farmers, but an inverse association with asthma in atopic farmers	Eduard et al. 2004[16]
134 non-atopic and 28 atopic pig farmers	Airborne endotoxin, median 1010 EU/m^3	Positive associations with respiratory symptoms and AHR and a lower FEV_1, stronger effects in atopic farmers	Portengen et al. 2005[17]

AHR: airway hyperresponsiveness; BAL: bronchoalveolar lavage; LBP: LPS-binding protein; PMN: Polymorphonuclear neutrohils.

Atopy or self-reported allergy were also no effect modifiers of associations between endotoxin exposure and respiratory symptoms or AHR in the study presented in Chapters 3 and 4 of this thesis. Only a small fraction of wheeze and AHR was attributable to atopy, and occupational endotoxin exposure was independently associated with a lower prevalence of atopy and hay fever.

Thus, evidence from experimental and observational studies is inconclusive with respect to the role of atopy as a susceptibility factor, and correlations with asthma and AHR complicate the interpretation. Most studies in occupational settings have relatively consistently shown an increased risk to develop endotoxin-associated respiratory disease both for atopic and non-atopic subjects. A recent epidemiological study[17] and the study described in Chapter 4 considered atopy as an outcome variable. In accordance with the hypothesis that endotoxin may suppress TH_2 responses, inverse associations with endotoxin exposure were found. However, selection effects may play a role as well, as atopic subjects may be less likely to be hired or to stay in a job with high endotoxin exposure. Longitudinal studies over an extended period of time are needed to assess whether healthy worker selection effects differ in magnitude between atopic and non-atopic workers, as this would lead to biased associations between endotoxin exposure and atopy.

Cytokine responsiveness

Kline et al.[19] found that *ex vivo* LPS-stimulated peripheral blood monocytes from subjects hyporesponsive to inhaled endotoxin released less IL6 and IL8 than cells from sensitive subjects. These findings indicated a possible underlying mechanism of inter-individual differences in susceptibility to endotoxin-induced health effects. We tested the hypothesis that cytokine responsiveness in a whole blood assay (WBA) is associated with susceptibility to occupational endotoxin-induced respiratory health effects. As shown in Chapter 6, statistically significant positive exposure-response relationships between endotoxin exposure and respiratory effects were only observed in the subgroup of subjects with above median IL1β, TNFα, and IL10 response by *ex vivo* LPS-stimulated blood monocytes. In this study, we only focused on health effects *positively* associated with endotoxin exposure. However, it would also be worthwhile to investigate whether cytokine responsiveness influences the *inverse* associations between endotoxin exposure and atopy or allergic symptoms. In line with the theory that endotoxin-induced TH_2 suppression is pivotal in the reduced risk of atopy, it may be more relevant to investigate cytokines produced by T cells stimulated with a T cell mitogen such as concanavalin A. Individual patterns of TH_1/TH_2 and regulatory T cell cytokine production could provide valuable information

regarding underlying mechanims of the lowered risk of atopy among endotoxin exposed workers.[20]

As we argued in Chapter 6, it would also be of interest to assess the predictive value of cytokine responsiveness longitudinally. Follow-up of an exposed population would show whether the rate of lung function decline or incidence of respiratory symptoms is different among subgroups of cytokine responders. Moreover, a longitudinal study could determine the reproducibility of cytokine responsiveness over a longer period of time, and investigate whether endotoxin exposure influences cytokine responses over time.

Gender

Gender differences in airway behavior occur throughout human life and are related to biological as well as sociocultural factors.[21] As a consequence, it is plausible that sensitivity to endotoxin also differs between male and female workers. Gender-related variability in susceptibility to occupational endotoxin exposure has received little attention. Women are likely to be employed in lower exposed jobs than men, and some occupations with endotoxin exposure such as swine farming and waste treatment are almost entirely dominated by men. Inevitably, in some studies only men were recruited, and studies focusing specifically on female workers, for instance in the textile industry, are exceptions.[22]

When both women and men are included, gender is usually considered as a confounder. Gender (male and female) was inconsistently identified as a risk factor for respiratory disorders in a review of studies among farming populations,[23] which in itself does not indicate a difference in sensitivity to farm exposures. A recent Canadian study showed that the risks of respiratory symptoms were somewhat greater in female swine workers than in male workers, but the authors did not explicitly test for interaction between sex and farm work.[24] In an experimental LPS provocation study, LPS-sensitive subjects were more commonly female and hyporesponsive subjects were more often male.[19] A longitudinal study that performed separate analyses for Chinese male and female cotton workers, showed that both lost pulmonary function at a faster rate than unexposed workers.[25] In the study described in Chapter 3, women were on average lower exposed than men (GM (GSD) 191 (4.2) vs. 284 (4.4) EU/m^3), but a wide range of endotoxin exposures was found for both groups. In Chapter 3, we did not present stratified exposure-response analyses for men and women, but wheeze and hay fever risk estimates were similar for male and female workers (ORs 1.42 and 1.35, and 0.59 and 0.67, respectively).

In epidemiological studies, gender-related susceptibility differences can be obscured by selection effects. Differences between men and women in "healthy hire effects" and "healthy worker survivor effects" have been reported,[26] and it can be envisaged that selection effects in women may play a greater role in typically male-dominated workplaces.

Tobacco smoking and other lifestyle factors

Smoking is undeniably an important determinant of lung function decline and chronic respiratory disease. Moreover, smoking can suppress or enhance inflammatory responses.[27] The concentration of nitric oxide in exhaled air, for instance, is suppressed by smoking,[28] which probably affected exposure-response relationships between endotoxin exposure and exhaled NO in current smokers (Chapter 5). Most studies on occupational endotoxin exposure have regarded smoking as a potential confounder. In a number of studies separate analyses for smokers and non-smokers have been conducted and these studies have mostly shown additive effects of smoking and organic dust exposure, i.e. smokers did not appear to be at higher risk than non-smokers to develop endotoxin-induced respiratory illness.[23, 29] Christiani et al.,[25] however, showed that cotton dust exposure-associated lung function decline was restricted to smokers. Chen et al.[30], also showed a positive interactive effect of grain dust exposure and smoking on lung function and chronic bronchitis, but only in female farmers.

Other lifestyle factors like diet may also interfere with the response to endotoxin exposure, but evidence is scarce. In swine workers and unexposed controls, different correlations were found between girth circumference and serum levels of markers of subclinical inflammation, which suggested an interaction between exposure and obesity.[31] Obesity is related to respiratory illness, and the prevalence of overweight and obesity is increasing worldwide.[32] Thus, this characteristic might deserve more attention in future studies.

Genetic factors

To date, only a few studies have investigated associations between candidate gene polymorphisms and health outcomes in endotoxin-exposed occupational populations (summarized in Table 2). Four studies investigated α-1-antitrypsin variants that may lead to a moderate deficiency of plasma α-1-antitrypsin and an impaired protection against proteases.[33-36] It was hypothesized that subjects carrying a deficiency variant would be more susceptible to organic dust-induced inflammation and therefore be at

increased risk to develop chronic obstructive pulmonary disease. Sigsgaard et al.[36] found a significant association between the Pi-MZ phenotype and bronchial hyper-responsiveness in young farmers, but not in control subjects, which suggested a gene-environment interaction between α-1-antitrypsin and organic dust exposure.

Two recent studies have investigated single nucleotide polymorphisms (SNPs) in endotoxin receptors *CD14* and *TLR4*.[38, 39] A longitudinal study on endotoxin-related accelerated lung function decline in Chinese cotton workers investigated SNPs that result in decreased activity of microsomal epoxide hydrolase, which plays an important role in the defense against oxidative stress,[37] and SNPs in the gene encoding TNFα, a cytokine with a pivotal role in endotoxin-induced inflammation.[40] In general, only modest effects of candidate gene polymorphisms on susceptibility to endotoxin-associated outcomes have been observed.

Asthma and asthma-related traits behave as typical complex diseases, that is, multiple genetic polymorphisms with moderate and even small effects may be capable of causing illness when acting together in the presence of relevant environmental factors.[41] Thus far, studies in endotoxin-exposed occupational populations have each investigated only a few SNPs in one or two genes, whereas >30 susceptibility genes for asthma have already been identified as robust candidates.[41] The list of candidates keeps growing longer, for instance through discovery of new genes by genome wide association studies.[42] The recent advance in genotyping technology has resulted in easy access to a large amount of genetic data. Consequently, selection of the most relevant SNPs, the issue of multiple comparisons and study sample size are major challenges of genetic research on complex disorders. Intensive international collaboration, for instance within the EU supported Gabriel project,[43] will be of great importance to overcome at least some of these difficulties.

The studies shown in Table 2 defined organic dust or endotoxin exposure on the basis of occupation (exposed or non-exposed). There is a clear need to increase efforts to improve the exposure assessment component of these gene-environment studies to see whether the shape and slope of exposure-response relationships differ according to genotype, as has been shown in studies in domestic settings investigating *CD14*/-159 C/T as an effect modifier of associations between house dust endotoxin and allergic outcomes.[44, 45] *CD14*, *TLR4*, and other genes encoding proteins involved in LPS signaling pathways such as *MD2* and *MyD88* would be obvious candidate genes to include in a study on genetic susceptibility to the respiratory health effects of occupational endotoxin exposure. Moreover, genes encoding receptors for other microbial agents than LPS, such as *TLR2* for Gram-positive microorganisms, mycobacteria and yeast, and *TLR9* for CpG DNA motifs,

should not be overlooked as endotoxin exposed workers are commonly exposed to a mixed bioaerosol which may also contain other bacterial and fungal pro-inflammatory agents than endotoxin.

Table 2 Genetic polymorphisms associated with endotoxin exposure-related health outcomes in occupational populations.

Population	Gene	Main results	Reference
1 138 male grain and sawmill workers, Canada	*AAT*	No association between Pi phenotypes and lung function or respiratory symptoms	Chan-Yeung et al. 1978[33]
28 male grain workers with the Pi-MZ phenotype, 28 matched workers with the normal Pi-M variant, Canada	*AAT*	Pi-MZ variant is associated with a lower lung function	Horne et al. 1986[34]
206 cotton workers, Denmark	*AAT*	Pi-MZ variant is associated with byssinosis	Sigsgaard et al. 1994[35]
1 835 farming students and 369 rural controls, Denmark	*AAT*	Pi-MZ and rare Pi-alleles are associated with bronchial hyperresponsiveness in young farmers, but not in rural controls	Sigsgaard et al. 2000[36]
267 cotton workers and 234 non-exposed workers, China	*mEH*	Tyr113His and His139Arg are associated with accelerated lung function decline in endotoxin exposed workers	Hang et al. 2005[37]
97 non-smoking, male farmers, Iowa	*CD14*	*CD14*/-1619 and *CD14*/-159 are associated with FEV_1 and wheeze	LeVan et al. 2005[38]
146 endotoxin exposed workers and 53 non-exposed workers, Sweden	*TLR4*	*TLR4*/+896 is associated with serum ECP and IL6 in exposed workers	Rylander and Michel 2005[39]
263 cotton workers and 230 non-exposed workers, China	*TNF*, *LTA*	*TNF/-308* and *LTA/+252* are associated with accelerated lung function decline in endotoxin exposed workers	Zhang et al. 2007[40]

AAT (*SERPINA1*): α-1-antitrypsin; *mEH*: microsomal epoxide hydrolase; *TLR4*: Toll-like receptor 4; *TNF*: Tumor necrosis factor; *LTA*: lymphotoxin alpha or *TNF* beta.

A number of studies have shown relationships between polymorphisms in genes encoding cytokines or CD14 and LPS-stimulated cytokine production in a WBA.[46-48] However, the ability to release cytokines can also be influenced by environmental determinants. Thus, combining cytokine responsiveness and genetic factors might provide more insights into the mechanisms underlying susceptibility to endotoxin exposure-related health effects.

Concluding remarks

There is abundant evidence that occupational exposure to high levels of endotoxin can lead to adverse respiratory effects. However, some people are more susceptible to these effects than others. Hence, identification of subgroups on the basis of relevant host factors may reveal steep exposure-response relationships that would be blunted in the whole population. Studying susceptibility factors could, therefore, lead to a better understanding of the underlying mechanisms of endotoxin exposure-associated disease. Large, well-characterized studies are needed to study the impact of susceptibility factors, especially in the study of multiple genetic factors.

From a practical point of view, an important aim of identifying genetic and acquired susceptibility factors would be a better targeted protection of exposed workers. The primary emphasis of occupational health regulations should be the reduction of endotoxin exposure levels, and importantly, regulations should not only focus on the average worker but also provide sufficient protection for the most susceptible individuals in any given context.[2] Another approach to protect workers against endotoxin exposure-related illness would be the use of screening for (genetic) susceptibility factors to identify high risk subgroups in the population. Screening, however, is surrounded by many scientific, social, ethical and legal issues. So far, no relevant high-penetrant genes or other robust susceptibility factors have been identified which can be used for that purpose. Thus, at this stage, there is no scientific evidence that supports screening as a reasonable approach to prevention.[49, 50]

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Summary

Endotoxins are cell wall components of Gram-negative bacteria that occur commonly on plants and plant products and as gut commensals. A large variability in airborne endotoxin exposure levels has been measured in a range of agricultural and other occupational environments. Inhalation of endotoxins may cause respiratory and inflammatory effects characterized by increased levels of infiltrating neutrophils and production of pro-inflammatory cytokines. Epidemiological studies in occupational populations have shown exposure-response relationships between endotoxin exposure and an accelerated lung function decline, respiratory symptoms, and airway hyper-responsiveness. Workers chronically exposed to high levels of endotoxin may be at risk to develop asthma and chronic obstructive pulmonary disease. Conversely, several studies have shown inverse associations between domestic endotoxin exposure and atopy and atopic asthma in children from farming families, but also in children living in urban areas. A number of studies has shown that childhood farm exposures continue to protect against atopic sensitization into adulthood, and recent studies suggested that endotoxin exposure during adulthood still protect against atopy and atopic asthma. Evidently, not all endotoxin-exposed workers develop respiratory or inflammatory responses, even at very high exposure levels. Genetic and non-genetic susceptibility factors may interact with endotoxin exposure, resulting in inter-individual heterogeneity in type and magnitude of responses within groups of individuals with apparently identical exposures.

The main aim of this thesis was to investigate associations between endotoxin exposure and the occurrence of respiratory and allergic effects in adults with sometimes extremely high work-related exposure. Cytokine responsiveness in an *ex vivo* whole blood assay and genetic variation in innate immunity genes were evaluated as susceptibility factors in associations between exposure and respiratory and allergic outcomes.

Instead of actually measured endotoxin levels, many studies, including the study presented in **Chapter 2**, have used contact with farm animals as a proxy for increased endotoxin exposure. Chapter 2 describes a questionnaire survey in 1205 conventional and 593 organic farmers. The aim of this study was to compare the prevalence of hay fever and asthma-like symptoms in organic and conventional

farmers and to assess associations between current and childhood farm exposures and respiratory health effects. We found that organic farmers reported less wheezing with shortness of breath and slightly more hay fever than conventional farmers. However, organic farming was not an independent determinant of hay fever when adjusted for farming practices and potential confounders. Livestock farmers who had grown up on a farm had a threefold lower prevalence of hay fever than crop farmers without a farm childhood with an odds ratio (OR) and 95% confidence interval (CI) of 0.3 (0.1 - 0.5). Both crop farmers who grew up on a farm and livestock farmers who did not grow up on a farm had a reduced prevalence compared with crop farmers without a farm childhood, although less pronounced and not statistically significant. Use of disinfectants containing quaternary ammonium compounds was positively related to hay fever with an OR of 2.1 (1.0 - 4.4). No effects of farming practices were found for asthma. A lower risk of asthma and asthma-like symptoms such as wheeze was found in farmers compared with a general Dutch population sample from the European Community Respiratory Health Survey (ECRHS). This study adds to the evidence that a farm childhood in combination with current livestock farming protects against allergic disorders, which was found both for organic and conventional farmers.

Chapter 3 to Chapter 6 describe a study in Dutch farmers and agricultural industry workers among whom a total of 249 full-shift personal inhalable dust samples were collected for endotoxin exposure measurements. A wide range of endotoxin levels were found (~10 to 10,000 EU/m^3) ranging from slightly elevated levels close to background to extremely high levels usually not encountered in the home and general environment. A job-exposure matrix was constructed to assign endotoxin exposure levels to all participants in the study. The objective of the study described in **Chapter 3** was to investigate exposure-response relationships between occupational endotoxin exposure and allergic and respiratory symptoms, taking into account farming exposures during childhood. Associations between endotoxin exposure and questionnaire data (n=877) on symptoms were studied by multiple logistic regression. Adjusted ORs for an interquartile range increase of the endotoxin level were significantly elevated for respiratory symptoms such as wheezing (OR (95%CI) 1.41 (1.16-1.72)), wheezing with shortness of breath (1.50 (1.18-1.90)) and daily cough (1.29 (1.03-1.62)). In contrast, endotoxin was strongly associated with a decreased prevalence of hay fever (0.62 (0.49-0.78)). Workers who had grown up on a farm had a lower prevalence of hay fever, but no evidence was found of effect modification by farm childhood.

In **Chapter 4**, we investigated associations between occupational endotoxin exposure and objectively measured outcomes, i.e. atopy and bronchial hyperresponsiveness to methacholine (BHR). In addition, the role of atopy in endotoxin-related respiratory effects was explored. Atopy was assessed as specific serum IgE to common inhalant allergens in a sample of 427 farmers and agricultural industry workers. BHR was determined in 113 subjects. Agricultural workers with a farm childhood were less often atopic, and had less often specific IgE to grass pollen than workers without a farm childhood. Endotoxin exposure was inversely associated with atopy (OR for an interquartile range increase of the endotoxin level 0.56 (0.42-0.74)) and IgE to grass pollen (OR 0.52 (0.37-0.73)), but only in workers who had not grown up on a farm. It is unlikely that this is a result of healthy worker selection, as significant associations between endotoxin and atopy or IgE to grass pollen were found regardless of reported allergic symptoms. As in the whole population, endotoxin exposure was positively associated with wheeze (OR 1.30 (1.01-1.67)) and also with BHR (OR 1.55 (1.03-2.35)), irrespective of growing up on a farm or atopy. While both wheeze and BHR were significantly associated with atopy, only 14.7% and 26.4% of their prevalences could be attributed to atopy.

In **Chapter 5**, we investigated the role of mucosal nitric oxide (NO) production in endotoxin-induced airway inflammation. The fractional concentration of exhaled NO (FE_{NO}), a marker of airway inflammation, was measured online in 425 farmers and agricultural industry workers. A significant positive exposure-response relationship was found between endotoxin exposure and FE_{NO}, but only in non-atopic, non-smoking subjects (*P*=0.001). FE_{NO} was significantly associated with current wheeze and other asthma-like symptoms irrespective of atopy and current smoking. Associations between endotoxin exposure and symptoms changed slightly after adjusting for FE_{NO}, thus the role of FE_{NO} as an intermediate factor between endotoxin exposure and airway symptoms appeared to be limited.

We hypothesized that the presence of respiratory symptoms and FEV_1 reductions due to occupational endotoxin exposure may partly be attributed to cytokine responsiveness of inflammatory cells. The objective of **Chapter 6** was to investigate associations between LPS-induced cytokine release from blood leukocytes and respiratory effects in endotoxin-exposed workers, and to study whether the cytokine response modifies associations between endotoxin exposure and respiratory outcomes. Lung function was measured in a sample of 412 farmers and agricultural industry workers, and TNFα, IL1β, and IL10 release was measured in *ex vivo* LPS-stimulated whole blood. For each cytokine, subjects were dichotomized into low and high responders according to median cytokine concentrations. High IL10 responders had a higher prevalence of airway symptoms than low IL10 responders (ORs

between 2.03 and 5.10, $P<0.05$). The TNFα response was also positively, but not significantly, associated with symptom prevalence, whereas no relationship was found between the IL1β response and symptoms. For all three cytokines, subjects with responses above the median showed significant positive dose-response relationships between endotoxin exposure and asthma symptoms, and significant associations between endotoxin exposure and a lower FEV_1 ($P<0.05$). In contrast, exposure-response relationships were clearly attenuated and statistically non-significant for low responders. The TNFα response was a significant effect modifier for the association between endotoxin exposure and wheeze (interaction $P<0.05$). Thus, susceptibility to endotoxin exposure-related respiratory disorders was associated with *ex vivo* cytokine responses to LPS.

Chapter 7 and 8 describe two studies on genetic factors and gene-environment interactions in asthma and allergy. Both studies investigated the hypothesis that single nucleotide polymorphisms (SNPs) in innate immunity genes such as *CD14* and Toll-like receptors 2 and 4 (*TLR2* and *TLR4*) may modulate the response to environmental exposure to microbial agents such as endotoxin, and thus influence susceptibility to asthma and atopy.

Chapter 7 describes a nested case-control study that was conducted within a cohort of 1901 young Danish farmers. A total of 100 new-onset asthma cases and 88 control subjects were genotyped for three *CD14* SNPs, three *TLR2* SNPs, and two *TLR4* SNPs. Atopy at baseline (defined as a positive skin prick test (SPT) to one or more common inhalant allergens) was found in 17 asthma cases (17.0%) and in 17 controls (19.3%). The *CD14*/-260T allele was significantly associated with less atopy with an OR of 0.39 (0.21-0.72; additive genetic model), whereas the *CD14*/-651T allele was positively associated with atopy (OR 2.53 (1.33-4.80; additive genetic model)). Similar results were obtained by haplotype analysis. Stratified analysis by farm childhood showed stronger effects of both *CD14* SNPs on atopy among farmers who were born and raised on a farm, although no significant interaction was found. No associations between *CD14*, *TLR2*, or *TLR4* genotypes and new-onset asthma were found.

In adults, it has been shown that country living during childhood protects against asthma, which may be explained by microbial exposures. In **Chapter 8**, we investigated whether SNPs in *CD14*, and *TLR2*, *TLR4* and *TLR9* genes are associated with asthma in adults, and whether these SNPs modify associations between country living and asthma. Twenty-five SNPs in *CD14*, *TLR2*, *TLR4*, and *TLR9* genes were genotyped in adult subjects from the French Epidemiological study on the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and

Atopy (EGEA). We conducted a case-control analysis on unrelated subjects (239 asthmatics and 596 non asthmatics), and a family based association test (FBAT) in 192 families ascertained through asthmatic probands. The *TLR2/+596C* allele was associated with an increased risk for asthma in both case-control and family-based analyses (under a dominant model, OR 1.91 (1.34-2.72), P=0.0003; Z statistics from FBAT=2.48, P=0.01). In SPT positive subjects, the *CD14/-260C* allele was negatively associated with asthma (additive model, OR 0.66 (0.48-0.91)). Significant gene-environment interactions between variation in *CD14* and *TLR* genes and country living during childhood were found for ten SNPs. In SPT positive subjects carrying *CD14/-260CC*, country living protected against asthma (OR 0.32 (0.12-0.85)), whereas country living was not associated with asthma in atopics carrying *CD14/-260T* (OR 1.11 (0.65-1.90); gene-environment interaction P<0.05).

To summarize, in farmers and workers in agricultural processing industries, significant and positive exposure-response relationships were found between endotoxin exposure and asthma-related outcomes, which were characterized by a predominantly non-atopic phenotype. In contrast, inverse associations between occupational endotoxin exposure and hay fever and atopic sensitization were found. An important role for individual susceptibility to the adverse effects of endotoxin was implied by the finding that associations between occupational endotoxin exposure and respiratory effects were stronger in the subgroup of subjects with higher cytokine responses of LPS-stimulated blood monocytes. A nested case-control study in young farmers, and a case-control and family-based study in adults suggested gene-environment interactions between genes involved in innate immunity responses and occupational and environmental exposure to endotoxin or other microbial agents, in their associations with asthma or atopy. Although endotoxin might protect against allergies, this thesis emphasizes the need to lower endotoxin exposure levels in the agricultural environment to protect workers against chronic respiratory health effects. Regulations should not only focus on the average worker but also provide sufficient protection for the most susceptible individuals in any given context.

Samenvatting

Endotoxines zijn bestanddelen van de celwand van Gram-negatieve bacteriën die algemeen voorkomen op planten en plantaardige producten en als commensale darmbacteriën. Op verschillende plaatsen in de werkomgeving, met name in de landbouw, is sprake van een verhoogde luchtwegblootstelling aan endotoxine, met een zeer grote variatie in het niveau van blootstelling. Het inademen van stofdeeltjes of aërosolen die endotoxine bevatten kan leiden tot ontstekingsreacties en luchtwegaandoeningen, die gekenmerkt worden door infiltratie van neutrofiele granulocyten en de productie van pro-inflammatoire cytokines. Epidemiologisch onderzoek heeft blootstelling-respons relaties aangetoond tussen werkgerelateerde endotoxineblootstelling en een versnelde afname van de longfunctie, luchtwegklachten en bronchiale hyper-reactiviteit. Werknemers die langdurig blootstaan aan hoge endotoxineniveaus hebben een verhoogd risico op astma en chronische obstructieve longaandoeningen. Anderzijds heeft een aantal onderzoeken juist omgekeerde verbanden aangetoond tussen endotoxineconcentraties in huisstof en atopie en allergische astma bij kinderen uit boerengezinnen, maar ook bij kinderen uit een stedelijke omgeving. Volgens weer andere onderzoeksresultaten zijn ook volwassenen die op een boerderij zijn opgegroeid nog steeds aantoonbaar minder vaak atopisch dan leeftijdsgenoten die niet uit een boerengezin afkomstig zijn. Een mogelijke verklaring hiervoor is dat de endotoxineconcentraties in huisstof vaak hoger zijn bij boerengezinnen. Recent onderzoek heeft gesuggereerd dat endotoxineblootstelling op latere leeftijd nog steeds bescherming biedt tegen atopie en allergische astma. Niet alle aan endotoxine blootgestelde werknemers ontwikkelen luchtwegklachten of meetbare ontstekingsreacties, zelfs bij zeer hoge blootstellingsniveaus. Aangeboren en verworven kenmerken kunnen de gevoeligheid voor endotoxineblootstelling beïnvloeden wat een aanzienlijke heterogeniteit in gezondheidseffecten kan veroorzaken binnen een groep werknemers met vergelijkbare endotoxineblootstelling.

Het belangrijkste doel van dit proefschrift was het onderzoeken van relaties tussen endotoxineblootstelling en effecten op de luchtwegen en allergie bij volwassenen met een soms zeer hoge werkgerelateerde blootstelling aan endotoxine. Daarnaast werd onderzocht of 1) de capaciteit van *ex vivo* gestimuleerde witte bloedcellen om cytokines te produceren en 2) variatie in genen die een rol spelen in de aangeboren

immuniteit van invloed waren op verbanden tussen blootstelling en effecten op de luchtwegen.

In plaats van gemeten endotoxineblootstellingsniveaus hebben veel studies, waaronder het onderzoek beschreven in **Hoofdstuk 2**, het houden van vee gebruikt als maat voor verhoogde endotoxineblootstelling. Hoofdstuk 2 beschrijft een vragenlijstonderzoek onder 1.205 conventionele en 593 biologische boeren. Het doel van deze studie was het vergelijken van de prevalentie van hooikoorts en astmasymptomen bij conventionele en biologische boeren en het onderzoeken van verbanden tussen het opgroeien op een boerderij, agrarisch werk als volwassene en luchtwegsymptomen. Biologische boeren bleken minder vaak last te hebben van een piepende ademhaling ('piepen') met kortademigheid en hadden iets vaker hooikoorts dan conventionele boeren. Een biologische bedrijfsvoering bleek echter geen onafhankelijke determinant voor hooikoorts te zijn wanneer dit verband gecorrigeerd werd voor andere variabelen. Veehouders die op een boerderij waren opgegroeid hadden een drie keer lagere hooikoortsprevalentie dan akker- en tuinbouwers die als kind niet op een boerderij woonden met een odds ratio (OR) en 95% betrouwbaarheidsinterval (BI) van 0,3 (0,1-0,5). Akker- en tuinbouwers die als kind op een boerderij woonden en veehouders die als kind niet op een boerderij woonden hadden een verlaagde hooikoortsprevalentie ten opzichte van akker- en tuinbouwers die als kind niet op een boerderij woonden, maar deze verschillen waren niet statistisch significant. Het gebruik van desinfecterende middelen die quaternaire ammoniumverbindingen bevatten was positief geassocieerd met hooikoorts (OR 2,1 (1,0-4,4)). Opgroeien op een boerderij en werkgerelateerde variabelen waren niet geassocieerd met astma. Boeren hadden een lager risico op astma en astmasymptomen zoals piepen vergeleken met een steekproef uit de algemene Nederlandse bevolking van het Europese Luchtwegonderzoek (ECRHS). Deze studie laat zien dat de combinatie van opgroeien op een boerderij en werk als veehouder op volwassen leeftijd zowel bij biologische als conventionele boeren bescherming lijkt te bieden tegen allergie.

Hoofdstuk 3 tot en met Hoofdstuk 6 beschrijven een onderzoek onder Nederlandse boeren en werknemers in de agrarische industrie (bloembollen-, diervoeder-, uien-, en zaaizaadverwerkende industrie). Onder deze populatie zijn in totaal 249 persoonlijke inhaleerbaar-stofmonsters verzameld gedurende de werkdag. In de stofmonsters werd het endotoxinegehalte bepaald, waarbij sterk uiteenlopende endotoxineblootstellingsniveaus werden gemeten (~10 tot 10.000 EU/m^3). De niveaus bij de minst blootgestelde werknemers waren maar zeer licht verhoogd in vergelijking met achtergrondwaardes, terwijl andere werknemers zeer hoog

blootgesteld waren met niveaus die buiten de werkomgeving normaalgesproken nooit worden gevonden. De meetgegevens zijn bewerkt tot een "job-exposure matrix". Met deze matrix werden endotoxineblootstellingsniveaus voor alle deelnemers aan het onderzoek vastgesteld. Het doel van de studie beschreven in **Hoofdstuk 3** was het onderzoeken van blootstelling-respons relaties tussen werkgerelateerde endotoxineblootstelling en allergie- en luchtwegklachten, waarbij er rekening mee werd gehouden of deelnemers al dan niet op een boerderij opgegroeid waren. Verbanden tussen endotoxineblootstelling en vragenlijstgegevens over symptomen van 877 deelnemers werden geanalyseerd met behulp van meervoudige logistische regressie. Gecorrigeerde Odds Ratio's (ORs) voor een interkwartiel range toename in de hoogte van endotoxineblootstelling waren significant verhoogd voor luchtwegsymptomen zoals piepen (OR (95%BI) 1,41 (1,16-1,72)), piepen met kortademigheid (OR 1,50 (1,18-1,90)) en dagelijks hoesten (OR 1,29 (1,03-1,62)). Aan de andere kant was endotoxineblootstelling juist geassocieerd met een lagere hooikoortsprevalentie (OR 0,62 (0,49-0,78)). Mensen die op een boerderij waren opgegroeid hadden een lagere hooikoortsprevalentie, maar de eerder genoemde verbanden tussen endotoxineblootstelling, luchtwegklachten en hooikoorts weken niet af bij mensen die op een boerderij waren opgegroeid.

In **Hoofdstuk 4** werden verbanden geanalyseerd tussen endotoxineblootstelling en objectief gemeten gezondheidseffecten, namelijk atopie en bronchiale hyper-reactiviteit voor methacholine (BHR). Daarnaast werd onderzocht of atopie een rol speelt bij het verkrijgen van endotoxine-gerelateerde effecten op de luchtwegen. Atopie werd bij 427 boeren en werknemers in de agrarische industrie bepaald op basis van specifiek serum IgE tegen veel voorkomende inhalatie-allergenen. BHR werd bij 113 deelnemers bepaald. Mensen die op een boerderij waren opgegroeid waren minder vaak atopisch en hadden minder vaak specifiek IgE tegen graspollen dan mensen die niet op een boerderij waren opgegroeid. Er was een omgekeerd verband tussen endotoxineblootstelling en atopie (OR voor een interkwartiel range toename in de hoogte van endotoxineblootstelling 0,56 (0,42-0,74)) en IgE tegen graspollen (OR 0,52 (0,37-0,73)), maar dit verband werd alleen gevonden bij mensen die *niet* op een boerderij waren opgegroeid. Het is onwaarschijnlijk dat deze omgekeerde associatie vooral een resultaat is van "healthy worker" selectie, aangezien een significant verband tussen endotoxineblootstelling en atopie en IgE tegen graspollen ook werd gevonden bij werknemers die geen allergische klachten hadden. Net als in de gehele (vragenlijst)populatie was endotoxineblootstelling positief geassocieerd met piepen op de borst (OR 1,30 (1,01-1,67)), en daarnaast ook met de BHR (1,55 (1,03-2,35)), ongeacht of deelnemers op een boerderij opgegroeid waren. Hoewel piepen en BHR significant geassocieerd waren met

atopie, was slechts 14,7% en 26,4% respectievelijk van de prevalentie toe te schrijven aan atopie. Verbanden tussen endotoxineblootstelling en luchtwegeffecten werden bij zowel atopische als niet-atopische deelnemers gevonden.

Stikstofoxide (NO) wordt door de luchtwegen geproduceerd, en de fractie uitgeademde NO (FE_{NO}) kan worden gemeten als marker van luchtweginflammatie. In **Hoofdstuk 5** werd het verband tussen endotoxineblootstelling en FE_{NO} onderzocht. FE_{NO} werd online bepaald bij 425 boeren en werknemers in de agrarische industrie. Een significante, positieve blootstelling-respons relatie tussen endotoxineblootstelling en FE_{NO} werd gevonden, echter alleen bij niet-atopische niet-rokers (P=0,001). FE_{NO} was significant geassocieerd met piepen en andere astmasymptomen, ongeacht atopie en rookgewoonten. Relaties tussen endotoxineblootstelling en symptomen veranderden nauwelijks wanneer gecorrigeerd werd voor FE_{NO}, wat erop wijst dat de rol van FE_{NO} als intermediaire factor tussen endotoxineblootstelling en luchtwegsymptomen beperkt lijkt te zijn.

Een hoge blootstelling aan endotoxines kan leiden tot luchtwegklachten en luchtwegobstructie, maar niet iedereen is hier even gevoelig voor. In **Hoofdstuk 6** werd onderzocht of de aanwezigheid van luchtwegproblemen voor een deel toe te schrijven is aan het vermogen van ontstekingscellen om cytokines te produceren wanneer ze gestimuleerd worden met lipopolysaccharide (LPS, gezuiverd endotoxine). Bij 412 boeren en werknemers in de agrarische industrie werd de longfunctie bepaald en TNFα, IL1β, en IL10 productie door perifere bloedcellen werd gemeten in *ex vivo* LPS-gestimuleerde bloedmonsters. Voor elke cytokine werd de mediane concentratie gebruikt als afkappunt om deelnemers onder te verdelen in een groep "lage responders" en een groep "hoge responders". Hoge IL10 responders hadden een hogere prevalentie luchtwegklachten dan lage IL10 responders (ORs tussen 2,03 and 5,10; P<0,05). De ex vivo TNFα respons was ook positief geassocieerd met de aanwezigheid van symptomen, maar dit verband was niet statistisch significant. Er was geen verband tussen IL1β respons en symptomen. Voor elk van de drie cytokines bleek dat onder hoge responders positieve en significante verbanden aanwezig waren tussen endotoxineblootstelling en astmasymptomen, en tussen endotoxineblootstelling en luchtwegobstructie (een lagere FEV1; P<0,05), terwijl bij lage responders slechts zwakke, statistisch niet-significante verbanden werden gevonden. Het verband tussen endotoxineblootstelling en piepen was statistisch significant verschillend bij lage en hoge TNFα responders (interactie P<0,05). Met deze resultaten heeft dit onderzoek sterke aanwijzingen gegeven voor het bestaan van verschillen in individuele gevoeligheid voor de effecten van endotoxineblootstelling op de luchtwegen. Deze

bleek geassocieerd te zijn met de *ex vivo* cytokine respons van LPS-gestimuleerde ontstekingscellen.

Hoofdstuk 7 en 8 beschrijven twee onderzoeken naar genetische factoren en gen-omgeving-interacties die een rol kunnen spelen bij astma en allergie. In allebei de studies werd onderzocht of "single nucleotide polymorphisms (SNPs)" in genen van het aangeboren immuunsysteem zoals *CD14* en Toll-like receptor 2 en 4 (*TLR2* en *TLR4*) zouden kunnen leiden tot een afwijkende respons op microbiële blootstellingen, wat tot uiting zou kunnen komen in de gevoeligheid voor astma en atopie.

In **Hoofdstuk 7** wordt een patiënt-controle-onderzoek beschreven dat werd uitgevoerd binnen een cohort van 1.901 jonge Deense boeren. Bij 100 boeren met recent ontstane astma en 88 boeren zonder astma werd het genotype bepaald voor drie *CD14* SNPs, drie *TLR2* SNPs en twee *TLR4* SNPs. Aan het begin van de studie waren huidpriktesten uitgevoerd, en atopie werd gedefinieerd als een positieve huidpriktest tegen ten minste een van de veel voorkomende inhalatie-allergenen. Zowel bij de boeren met astma als bij de controles waren 17 deelnemers atopisch (respectievelijk 17,0% en 19,3%). Het *CD14*/-260T allel was significant geassocieerd met een lagere atopieprevalentie met een OR van 0,39 (0,21-0,72; additief genetisch model), terwijl het *CD14*/-651T allel juist positief geassocieerd was met atopie (OR 2,53 (1,33-4,80; additief genetisch model)). Deze resultaten werden bevestigd door haplotype-analyse. Gestratificeerde analyse voor opgroeien op een boerderij liet zien dat de effecten van beide *CD14* SNPs op atopie sterker waren bij boeren die op een boerderij geboren en opgegroeid waren, hoewel er geen statistisch significante interactie was. Er werden geen verbanden gevonden tussen *CD14*, *TLR2* of *TLR4* genotypes en nieuw-ontstane astma.

Het is eerder aangetoond dat volwassenen een lagere astma prevalentie hebben wanneer ze als kind in een landelijk gebied woonden. Een mogelijke verklaring voor deze observatie zou kunnen zijn dat deze mensen als kind een hogere blootstelling hadden aan microbiële agentia. In **Hoofdstuk 8** werd de relatie tussen SNPs in *CD14*, *TLR2*, *TLR4* en *TLR9* genen en astma bij volwassenen onderzocht, waarbij rekening werd gehouden of ze als kind in een landelijk gebied woonden. Bij volwassen deelnemers aan de Franse epidemiologische studie naar de genetica en omgevingsfactoren van astma, bronchiale hyper-reactiviteit en atopie (EGEA) werd het genotype bepaald van 25 SNPs in *CD14*, *TLR2*, *TLR4* en *TLR9*. Een patiënt-controle-onderzoek werd uitgevoerd onder niet-verwante deelnemers (239 astmapatiënten en 596 personen zonder astma), en een associatietest onder familieleden (FBAT) werd uitgevoerd bij 192 families die via een astmapatiënt waren geselecteerd. Het *TLR2*/+596C allel was geassocieerd met een verhoogd risico op

astma, zowel in het patiënt-controle onderzoek als in de analyses onder familieleden (onder een dominant genetisch model OR 1,91 (1,34-2,72), *P*=0,0003; Z-waarde FBAT=2,48, *P*=0,01). In atopische deelnemers (atopie was vastgesteld via een positieve huidpriktest) bleek het *CD14/-260C* allel negatief geassocieerd te zijn met astma (additief genetisch model, OR 0,66 (0,48-0,91)). Significante gen-omgeving interacties tussen variatie in *CD14* en *TLR* genen en het opgroeien in een landelijk gebied werden aangetoond voor tien SNPs. In atopische deelnemers met het *CD14/-260CC* genotype bleek het opgroeien in een landelijk gebied te beschermen tegen astma (OR 0,32 (0,12-0,85)), terwijl er geen verband was tussen het opgroeien in een landelijk gebied en astma bij atopische dragers van het *CD14/-260T* allel (OR 1,11 (0,65-1,90); gen-omgeving interactie *P*<0,05).

Samengevat laten de resultaten beschreven in dit proefschrift zien dat de endotoxineblootstelling bij boeren en werknemers in de agrarische industrie een significant dosis-afhankelijk verband vertoont met een verhoogd risico op astma-gerelateerde luchtwegproblemen, die voornamelijk worden gekarakteriseerd door een niet-atopisch fenotype. Aan de andere kant werden juist omgekeerde verbanden gevonden tussen de mate van werkgerelateerde endotoxineblootstelling en het voorkomen van hooikoorts en atopische sensibilisatie. Individuele gevoeligheid voor de negatieve gevolgen van blootstelling aan endotoxines bleek een belangrijke rol te spelen: in de groep deelnemers van wie de LPS-gestimuleerde bloed monocyten een hogere cytokine respons vertoonden waren de verbanden tussen endotoxineblootstelling en effecten op de luchtwegen veel sterker dan bij mensen met een lage cytokine respons. Een patiënt-controle onderzoek onder jonge boeren, en een patiënt-controle-onderzoek en een familieonderzoek onder volwassenen naar astma en atopie suggereerden gen-omgeving interacties tussen genen die een rol spelen in de aangeboren immuniteit en blootstelling aan endotoxine of andere microbiële agentia in de werk- of leefomgeving. Hoewel endotoxine – ook op latere leeftijd – bescherming zou kunnen bieden tegen allergieën, benadrukt dit proefschrift het belang van de reductie van endotoxineblootstellingsniveaus in de landbouw en agrarische industrie om werknemers beter te beschermen tegen chronische luchtwegaandoeningen. Maatregelen en grenswaardes zouden daarbij niet alleen op de gemiddelde werknemer gericht moeten zijn, maar ook - indien enigszins mogelijk - onder alle omstandigheden voldoende bescherming moeten bieden aan de meest gevoelige individuen.

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Curriculum Vitae

Lidwien Smit was born in Gorinchem, The Netherlands on December 14, 1976. She grew up in Utrecht, where she graduated from secondary school at the Christelijk Gymnasium in 1995. She received her MSc degree (cum laude) in Environmental and Occupational Health from Wageningen University, The Netherlands in 2001. As part of her study, she spent one year at the International Water Management Institute (IWMI) in Colombo, Sri Lanka, where she was involved in an epidemiological research project on occupational pesticide exposure. After graduation, she rejoined IWMI, and continued to work in the area of pesticide poisoning. In 2003, she returned to The Netherlands and started working at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University on the research described in this thesis. In 2007, she spent three months at the Epidemiology and Biostatistics group of the Institut National de la Santé et de la Recherche Médicale (INSERM) in Paris/Villejuif, France, where she conducted a study on gene-environment interactions in asthma which is included in this thesis. She was awarded a 1-year EAACI/GA²LEN fellowship for post-doctoral research on gene-environment interactions in asthma and allergy, which she will conduct at INSERM as of September 2008.

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"Why don't you write books people can read?" - Nora Joyce to her husband James

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