

## Original article

Association between exposure to farming, allergies and genetic variation in *CARD4/NOD1*

**Background:** Caspase recruitment domain protein (CARD) 4 has been recently identified as an intracellular pattern recognition receptor that interacts with muropeptides found in common Gram-negative bacteria. We therefore aimed to explore whether the previously observed inverse association between exposure to microbial products and asthma and allergies in childhood is modified by genetic variation in *CARD4*.

**Methods:** We genotyped 668 children [mean age 9.3 (SD 1.5) years] enrolled in the cross-sectional ALEX study for seven haplotype tagging single nucleotide polymorphisms in *CARD4*. We studied the association of asthma, hay fever and allergen-specific serum immunoglobulin E with exposure to a farming environment and with levels of endotoxin and muramic acid measured in house dust samples. We tested whether these associations differed between the genotypes of the polymorphisms under study.

**Results:** A strong protective effect of a farming environment on allergies was only found in children homozygous for the T allele in *CARD4*–21596, but not in children carrying the minor allele (C). Among the former, farmers' children had a significantly lower frequency of sensitization against pollen (5.8%), hay fever (1.7%) and atopic asthma symptoms (1.7%) compared with children not living on a farm (19.4%, 13.0% and 7.6%,  $P < 0.01$ ,  $< 0.01$  and  $< 0.05$ , respectively). Conversely, no significant difference in prevalence of these phenotypes by farming status was found among children with a C allele in *CARD4*–21596 (14.3%, 7.1% and 8.0% vs 16.5%, 9.0% and 5.7%, respectively).

**Conclusion:** Polymorphisms in *CARD4* significantly modify the protective effect of exposure to a farming environment.

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Over the last years results from cross-sectional surveys and prospective cohort studies have supported the hypothesis that exposure to microbial products in early childhood protects against the development of asthma and allergies (1–4). Several population-based investigations have shown an inverse association between levels of endotoxin measured in house dust, and both concentrations of specific serum immunoglobulin (Ig) E against common aeroallergens and their related phenotypes, hay fever and atopic asthma (5–7). We have recently described muramic acid as another microbial molecule in house dust that is inversely associated with asthma symptoms in school-children (8). Experimental studies support these epidemiological findings: cell stimulation *in vitro* using microbial products favours the release of cytokines of the T-helper type (Th) 1 and inhibits the production of Th 2 cytokines, which are important

mediators of asthma and allergies (9, 10). In murine models of allergic sensitization, high exposure to lipopolysaccharide (LPS), the endotoxin from the outer membrane of the cell wall of Gram-negative bacteria, prevents the production of specific IgE, whereas low exposure does not (11–14).

Support for the concept that gene–environment interactions play a central role in the pathogenesis of asthma and allergies comes from studies in which both environmental exposures and polymorphisms in genes that encode for proteins that interact with these exposures were assessed. For example, in two independent populations, a polymorphism in *toll-like (TLR) 4* (Asp299Gly) that disrupts TLR4-mediated LPS signalling (15) was associated with less bronchial responsiveness and fewer allergies, respectively, but only in subjects heavily exposed to endotoxin (16, 17). CD14 is another pattern

recognition receptor that interacts with LPS. A functional polymorphism in the promoter region of *CD14* (*CD14*/–260) influences total and specific serum IgE levels (18–21), and recent findings suggest that the association between this *CD14* polymorphism and markers of atopy is modified by exposure to microbial products (22, 23).

Recently, caspase recruitment domain protein (CARD) 4, also known as NOD1, has been identified as a cytosolic pattern recognition receptor that interacts with a component of Gram-negative bacteria other than LPS; CARD4 is expressed in a variety of tissues and interacts in a highly specific way with diaminopimelic acid containing muropeptides (24, 25). These muropeptides are components of the thin layer of peptidoglycan in the cell wall of common Gram-negative bacteria, and their interaction with CARD4 leads to NF- $\kappa$ B activation and is followed by the release of pro-inflammatory cytokines (24–26). We therefore aimed to explore whether the previously observed inverse association between exposure to microbial products and allergies in childhood is modified by genetic variation in *CARD4*.

## Methods

### Study population

The study population included 668 children from the cross-sectional ALEX study, as previously described in detail (3). In brief, a self-administered questionnaire on respiratory and allergic diseases was filled in by the parents of children living on a farm and of children living in the same rural area but not raised on a farm. Farmers' children were defined as those whose parents answered 'yes' to the question 'do you live on a farm?'.

Participating children and their parents were asked for consent to take a blood sample and to collect dust from the child's mattress (5). Dust samples were used to measure endotoxin in a central laboratory (Munich, Germany) using a kinetic Limulus assay (Kinetic-QCL; BioWhittaker Inc., Walkersville, MD) (27). High exposure to endotoxin was defined in children with endotoxin concentrations above the median of the study group (27.4 EU/mg dust) and compared with those below the median. Samples for which mattress dust was still available after measurement of endotoxin were sent to a different laboratory (Borstel, Germany) to measure the amount of muramic acid by combined gas-liquid chromatography/mass spectrometry (8). Muramic acid concentrations were divided into tertiles (<92, 92–146, >146 ng/mg dust) in accordance with previous reports in this population (8). The analyses in this study were restricted to children from Austria and Germany, as DNA samples from Swiss children were not available.

### Measurement of serum IgE

Total serum IgE levels and those of specific IgE against timothy grass, birch pollen, house dust mite (*Dermatophagoides pteronyssinus*), and cat dander in serum were measured in a central laboratory (University Children's Hospital Berlin 'Charité') using commercially available kits (CAP; Pharmacia, Uppsala, Sweden). Atopic sensitization was defined in children with specific IgE  $\geq 3.5$  IU/ml serum to any of the allergens tested, in accordance with previous reports in this population (3, 5, 17).

### Phenotypes

Asthma was defined as a doctor's diagnosis of asthma or recurrent asthmatic, obstructive, or spastic bronchitis. Atopic asthma was defined when the child had additionally specific IgE  $\geq 0.35$  IU/ml serum to at least one of the aeroallergens tested. Children whose parents reported 'wheezing or whistling in the chest in the last 12 months' were considered as having wheeze in the last 12 months, and wheezy children with specific IgE  $\geq 0.35$  IU/ml were classified as having atopic wheeze. Hay fever was defined in children whose parents reported a doctor's diagnosis of hay fever in their child. Children were classified as having allergic rhinoconjunctivitis in the last 12 months if there was a positive response to the question 'in the last 12 months, has your child had problems with sneezing or a runny or blocked nose without a cold accompanied by itchy-watery eyes?'.

### Genotyping

*CARD4* has been screened for single nucleotide polymorphisms (SNPs) in Caucasian subjects (<http://innateimmunity.net>). Haplotype tagging SNPs closely linked ( $r^2 > 0.8$ ) with known polymorphisms and with a minor allele frequency of  $> 10\%$  were chosen for this study using the algorithm implemented in *ldselect* (28). This algorithm is based on linkage disequilibrium. Statistical power to detect unassayed, disease-associated polymorphisms depends on the correlation ( $r^2$ ) between the unassayed site and the assayed site. The SNPs chosen for this study were *CARD4*/–21596 (rs2075817), *CARD4*/–3038 (rs2906766), *CARD4*/–2914 (rs2975632), *CARD4*/+1915 (rs2075819), *CARD4*/+5227 (rs2075821), *CARD4*/+10797 (rs2907749) and *CARD4*/+28354 (rs5743369). Genomic DNA was then assayed by 5' exonuclease reaction (Taqman®; Applied Biosystems, Foster City, CA). Primer and probe sequences used and further details of the genotyping method are available upon request.

### Statistical analyses

Genotype frequencies were compared between farmers' children and nonfarmers' children. According to the Hardy–Weinberg principle, the expected genotype distribution in a random mating population and in the absence of factors that change allele frequencies is  $p^2$ ,  $2pq$ ,  $q^2$  with  $p$  being the proportion of allele 1 and  $q$  the proportion of allele 2 in the population. We therefore compared the observed genotype frequencies in farmers' and in nonfarmers' children to a population in Hardy–Weinberg equilibrium using a goodness of fit test.

Measures of pairwise linkage disequilibrium between the *CARD4* SNPs were estimated and visualized using Haploview (29). This statistical software for genetic analyses uses a two marker expectation–maximization algorithm to estimate the maximum-likelihood values of the four gamete frequencies, from which the calculation of measures of allelic association derives.

As the proportion of children homozygous for the minor allele for any of the *CARD4* SNPs was very small, these children were combined with heterozygotes for further analyses. Total IgE values were log transformed and analysed using analysis of variance. The proportions of children with asthma, hay fever and atopic sensitization were compared between farmers' and nonfarmers' children within the genotypes for the *CARD4* polymorphisms using the chi-squared test and the Fisher's exact test, respectively. Mantel Haenszel odds ratios for the association between farming and phenotype were computed and tested for homogeneity across genotypes. When a univariate test was found to be suggestive ( $P < 0.2$ ) of an association, a logistic regression model was used to control for

the following potential confounders: study area (Austria, Germany), age in years (continuous), gender, maternal education (low, medium, high), exposure to stables and/or farm milk in the first year of life (yes, no). When using logistic regression models, the log likelihood ratio test was applied to test for interaction between exposure and genotypes. The role of exposure to endotoxin and to levels of muramic acid concentrations in the association between *CARD4* genotypes and asthma and allergies was assessed in a similar manner. All analyses were performed using Stata/SE 8.0 (Stata Corporation, College Station, TX).

This study was approved by the Institutional Review Board of the University of Arizona, by the Human Subject Committees of the Children's Hospital, University of Munich, Germany, and by the Children's Hospital, Salzburg, Austria. The nature and possible consequences of the studies were explained to the parents of the children involved, who signed informed consents for this study.

## Results

The number of participants in this study are shown in Fig. 1. For 668 of 684 children sufficient DNA was available for genotyping haplotype tagging SNPs in *CARD4*. Genotype frequencies for these polymorphisms in farmers' and in nonfarmers' children are given in Table 1. Residual linkage disequilibrium was found between the *CARD4* SNPs genotyped for this study (Fig. 2).

Characteristics of the study population and the frequency of the phenotypes under study in farmers' and in nonfarmers' children are given in Table 2. As reported elsewhere (3), farmers' children had less asthma symptoms, less hay fever, and less atopic sensitization against pollen compared with the children living in the same rural area but not living on a farm. There was, however, significant heterogeneity in the association of exposure to a farming environment and allergies across the *CARD4*/–21596 polymorphisms (Table 3). The odds ratios for

Table 1. Genotype frequencies of *CARD4* polymorphisms in farmers' children ( $n = 255$ ) and in nonfarmers' children ( $n = 413$ )

| Locus                | rs number | Genotype | Nonfarmers' children, $n$ (%) | Farmers' children, $n$ (%) |
|----------------------|-----------|----------|-------------------------------|----------------------------|
| <i>CARD4</i> /–21596 | rs2075817 | Total    | 399 (100)                     | 247 (100)                  |
|                      |           | TT       | 186 (47)                      | 121 (49)                   |
|                      |           | CT       | 182 (46)                      | 110 (45)                   |
|                      |           | CC       | 31 (8)                        | 16 (6)                     |
|                      |           |          | $P = 0.137^*$                 | $P = 0.171^*$              |
| <i>CARD4</i> /–3038  | rs2906766 | Total    | 400 (100)                     | 242 (100)                  |
|                      |           | AA       | 168 (42.0)                    | 117 (48.4)                 |
|                      |           | AG       | 192 (48.0)                    | 107 (44.2)                 |
|                      |           | GG       | 40 (10.0)                     | 18 (7.4)                   |
| <i>CARD4</i> /–2914  | rs2975632 | Total    | 397 (100)                     | 247 (100)                  |
|                      |           | TT       | 264 (67)                      | 159 (64)                   |
|                      |           | CT       | 123 (31)                      | 78 (32)                    |
|                      |           | CC       | 10 (3)                        | 10 (4)                     |
|                      |           |          | $P = 0.328^*$                 | $P = 0.911^*$              |
| <i>CARD4</i> /+1915  | rs2075819 | Total    | 403 (100)                     | 247 (100)                  |
|                      |           | AA       | 199 (49.4)                    | 143 (57.9)                 |
|                      |           | AG       | 179 (44.4)                    | 88 (35.6)                  |
|                      |           | GG       | 25 (6.2)                      | 16 (6.5)                   |
| <i>CARD4</i> /+5227  | rs2075821 | Total    | 402 (100)                     | 246 (100)                  |
|                      |           | GG       | 190 (47)                      | 143 (58)                   |
|                      |           | AG       | 189 (47)                      | 83 (34)                    |
|                      |           | AA       | 23 (6)                        | 20 (8)                     |
|                      |           |          | $P = 0.006^*$                 | $P = 0.116^*$              |
| <i>CARD4</i> /+10797 | rs2907749 | Total    | 399 (100)                     | 244 (100)                  |
|                      |           | TT       | 208 (52)                      | 133 (55)                   |
|                      |           | CT       | 169 (42)                      | 96 (39)                    |
|                      |           | CC       | 22 (6)                        | 15 (6)                     |
| <i>CARD4</i> /+28354 | rs5743369 | Total    | 395 (100)                     | 243 (100)                  |
|                      |           | AA       | 276 (70)                      | 191 (79)                   |
|                      |           | AG       | 110 (28)                      | 48 (20)                    |
|                      |           | GG       | 9 (2)                         | 4 (2)                      |
|                      |           |          | $P = 0.612^*$                 | $P = 0.626^*$              |

\* $P$ -value derived from the goodness of fit test (1 d.f.) for Hardy–Weinberg equilibrium.

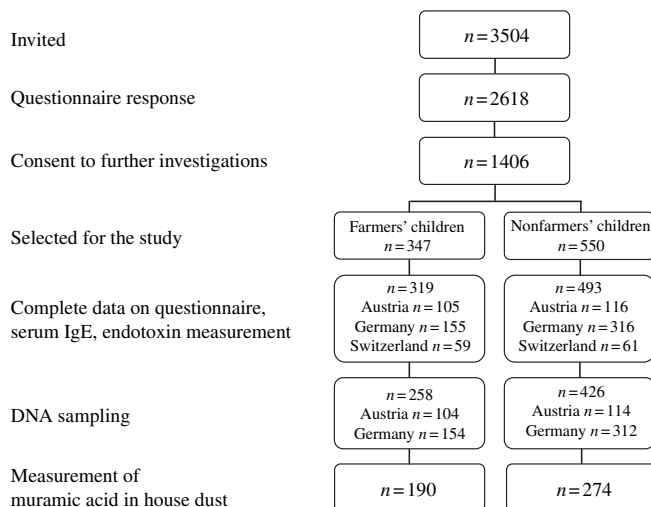


Figure 1. Number of children participating in the genetic part of the ALEX Study.

the association between farming status and phenotype were significantly lower in children with genotype TT in *CARD4*/–21596 for specific IgE against pollen and cat dander, hay fever and atopic wheeze in the last 12 months, compared with children with a C allele in this polymorphism (Table 3). No significant interaction between farming status and genotypes of *CARD4*/–21596 was found for asthma, although the trends observed for the atopic form of asthma were very similar to those for atopic wheeze (Table 3). There was no association between total serum IgE and either exposure to farming or *CARD4*/–21596 polymorphisms; farmers' children with the TT genotype had a geometric mean (95% CI) of total serum IgE of 65.4 (51.2–83.6) IU/ml compared with 67.8 (54.8–83.9) IU/ml in nonfarmers' children. Among children with at least one C allele in *CARD4*/–21596, the geometric mean (95% CI) of total serum IgE

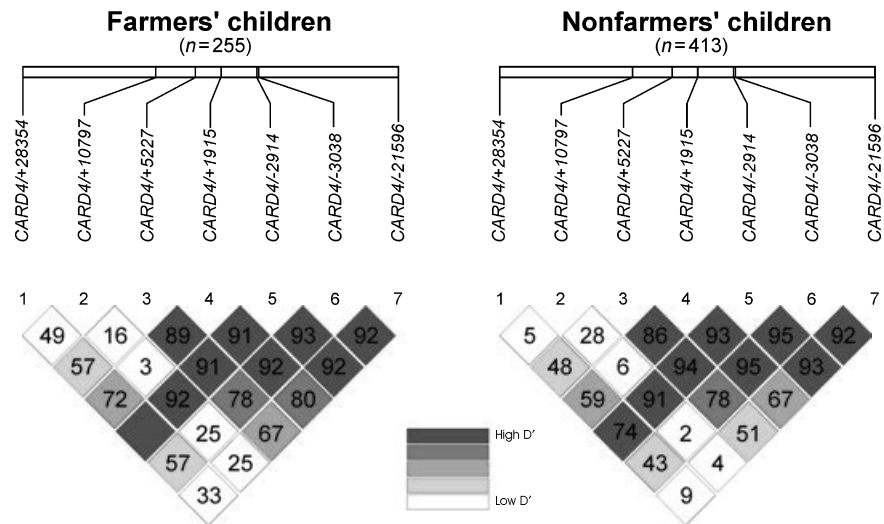


Figure 2. Measure of pairwise linkage disequilibrium ( $D'$ ) between the *CARD4* single nucleotide polymorphisms in farmers' children ( $n = 255$ ) and in nonfarmers' children ( $n = 413$ ).

Table 2. Characteristics of the study population

|                                                                 | Nonfarmers' children ( $n = 413$ ) | Farmers' children ( $n = 255$ ) | $\chi^2$ -test ( $P$ -value) |
|-----------------------------------------------------------------|------------------------------------|---------------------------------|------------------------------|
| Boys % ( $n$ )                                                  | 50.4 (208)                         | 54.9 (140)                      | 0.254                        |
| Mean age in years (SD)                                          | 9.3 (1.48)                         | 9.3 (1.47)                      | 0.926*                       |
| Study area is Germany % ( $n$ )                                 | 72.4 (299)                         | 59.2 (151)                      | <0.001                       |
| Maternal education % ( $n$ )                                    |                                    |                                 |                              |
| Low                                                             | 35.6 (143)                         | 41.4 (103)                      |                              |
| Medium                                                          | 44.8 (180)                         | 45.9 (114)                      |                              |
| High                                                            | 19.7 (79)                          | 12.9 (32)                       | 0.062                        |
| Exposure to stables and/or farm milk 1st year of life % ( $n$ ) | 42.5 (175)                         | 85.5 (213)                      | <0.001                       |
| Endotoxin (EU/mg) in mattress dust, geometric mean (95% CI)     | 23.1 (21.7–24.5)                   | 35.1 (32.9–37.6)                | <0.001*                      |
| Muramic acid (ng/mg) in mattress dust, geometric mean (95% CI)  | 95.7 (89.1–102.8)                  | 144.4 (132.3–157.7)             | <0.001*                      |
| Phenotypes in % ( $n$ )†                                        |                                    |                                 |                              |
| Asthma                                                          | 9.4 (39)                           | 5.5 (14)                        | 0.066                        |
| Atopic asthma                                                   | 6.7 (27)                           | 3.6 (9)                         | 0.089                        |
| Wheeze in the last 12 months                                    | 13.1 (53)                          | 6.3 (16)                        | 0.006                        |
| Atopic wheeze in the last 12 months                             | 6.6 (25)                           | 4.8 (12)                        | 0.347                        |
| Hay fever                                                       | 10.8 (44)                          | 4.3 (11)                        | 0.003                        |
| Allergic rhinoconjunctivitis in the last 12 months              | 12.1 (49)                          | 5.6 (14)                        | 0.005                        |
| Specific IgE $\geq 3.5$ IU/ml % ( $n$ )                         |                                    |                                 |                              |
| Pollen (grass and/or birch)                                     | 17.5 (72)                          | 10.6 (27)                       | 0.015                        |
| House dust mite                                                 | 9.4 (39)                           | 11.8 (30)                       | 0.338                        |
| Cat dander                                                      | 3.4 (14)                           | 1.6 (4)                         | 0.158                        |
| Total serum IgE (IU/ml), geometric mean (95% CI)                | 69.2 (59.9–80.0)                   | 71.5 (60.3–84.8)                | 0.776*                       |

\* $t$ -Test on equality of means.

†For definition of phenotypes see Methods.

was 76.5 (60.2–97.2) IU/ml in farmers' children and 69.6 (56.9–85.2) IU/ml in nonfarmers' children.

We next tested whether the differential effects of exposure to a farming environment on the *CARD4*/–21596 genotypes were explained by levels of endotoxin or muramic acid measured in mattress dust samples. In a logistic regression model with endotoxin and muramic acid as the covariate, respectively, exposure to a farming environment remained significantly and inversely associ-

ated with sensitization against pollen, hay fever and atopic wheeze in the last 12 months in carriers of the genotype TT, and remained not significantly associated in children with a C allele in *CARD4*/–21596 (data not shown).

Neither tertiles of muramic acid concentrations nor muramic acid used as a continuous variable did modify the association between *CARD4*/–21596 genotypes and allergies, but levels of endotoxin did. A protective effect

Table 3. Frequency (%) of specific serum IgE to aeroallergens, asthma and allergies in *CARD4*–21596 polymorphisms in farmers' and in nonfarmers' children, and odds ratios (OR), (95% CI) for the association between farming status and phenotype in *CARD4*–21596 variants (CC/CT *n* = 47/292, TT *n* = 307)

| <i>CARD4</i> –21596                   | Nonfarmers<br>( <i>n</i> = 399) | Farmers<br>( <i>n</i> = 247) | OR (95% CI)      | <i>P</i> -value† |
|---------------------------------------|---------------------------------|------------------------------|------------------|------------------|
| <i>Specific IgE ≥ 3.5 IU/ml</i>       |                                 |                              |                  |                  |
| Pollen (grass and/or birch)           |                                 |                              |                  |                  |
| CC/CT                                 | 16.5                            | 14.3                         | 0.84 (0.43–1.62) | 0.025            |
| TT                                    | 19.4                            | 5.8***                       | 0.26 (0.09–0.61) |                  |
| House dust mite                       |                                 |                              |                  |                  |
| CC/CT                                 | 7.5                             | 14.3**                       | 2.05 (0.94–4.48) | 0.092            |
| TT                                    | 10.8                            | 9.1                          | 0.83 (0.35–1.90) |                  |
| Cat dander                            |                                 |                              |                  |                  |
| CC/CT                                 | 1.9                             | 2.4                          | 1.27 (0.18–7.66) | 0.023            |
| TT                                    | 4.8                             | 0.0**                        | 0.00 (0.00–0.63) |                  |
| <i>Allergic diseases</i>              |                                 |                              |                  |                  |
| Asthma                                |                                 |                              |                  |                  |
| CC/CT                                 | 7.5                             | 5.6                          | 0.72 (0.24–1.93) | 0.533            |
| TT                                    | 11.3                            | 5.8                          | 0.48 (0.17–1.23) |                  |
| Atopic asthma‡                        |                                 |                              |                  |                  |
| CC/CT                                 | 4.8                             | 4.8                          | 0.99 (0.29–3.11) | 0.136            |
| TT                                    | 8.3                             | 2.6**                        | 0.29 (0.05–1.06) |                  |
| Hay fever                             |                                 |                              |                  |                  |
| CC/CT                                 | 9.0                             | 7.1                          | 0.77 (0.30–1.87) | 0.020            |
| TT                                    | 13.0                            | 1.7***                       | 0.11 (0.01–0.47) |                  |
| <i>Symptoms in the last 12 months</i> |                                 |                              |                  |                  |
| Wheeze                                |                                 |                              |                  |                  |
| CC/CT                                 | 13.3                            | 8.7                          | 0.62 (0.27–1.35) | 0.151            |
| TT                                    | 12.7                            | 3.4                          | 0.24 (0.06–0.73) |                  |
| Atopic wheeze‡                        |                                 |                              |                  |                  |
| CC/CT                                 | 5.7                             | 8.0                          | 1.44 (0.53–3.86) | 0.029            |
| TT                                    | 7.6                             | 1.7**                        | 0.21 (0.05–0.95) |                  |
| Allergic rhinoconjunctivitis          |                                 |                              |                  |                  |
| CC/CT                                 | 12.0                            | 8.0                          | 0.64 (0.24–1.44) | 0.164            |
| TT                                    | 12.2                            | 3.3***                       | 0.25 (0.06–0.76) |                  |

\*0.05 ≤ *P* < 0.10, \*\* 0.01 ≤ *P* < 0.05, \*\*\* *P* < 0.01 compared with nonfarmers' children.

†Mantel–Haenszel test for homogeneity across strata.

‡See Methods.

of high exposure to endotoxin was only seen in children with genotype TT with respect to sensitization against pollen, but not in those carrying a C allele (Table 4). Similarly, children with genotype TT had a lower estimate of the odds ratio for the association between endotoxin and hay fever than those with a C allele, although the associations were not significantly different at the predefined  $\alpha$ -level of 0.05. When using endotoxin ( $\log_{10}$  transformed) as a continuous variable, *P*-values for interaction between endotoxin and *CARD4*–21596 genotypes remained highly significant for sensitization against pollen (*P* < 0.0005) and were significant for hay fever (*P* < 0.05). No effect modification was seen for sensitization against house dust mite and cat and clinical phenotypes triggered by other allergens besides those derived from pollen, i.e. asthma, wheeze in the last 12 months, and allergic rhinoconjunctivitis during the previous year. Interactions remained nonsignificant for

Table 4. Frequency (%) of specific serum IgE to aeroallergens, asthma and allergies in *CARD4*–21596 polymorphisms in children exposed below and above 50th percentile (27.4 EU/mg mattress dust) of endotoxin, and adjusted odds ratios† (OR) for the association between endotoxin and the phenotypes in *CARD4*–21596 variants (CC/CT *n* = 47/292, TT *n* = 307)

| <i>CARD4</i> –21596                   | <50th percentile | ≥50th percentile | aOR (95% CI)†    | <i>P</i> -value‡ |
|---------------------------------------|------------------|------------------|------------------|------------------|
| <i>Specific IgE ≥ 3.5 IU/ml</i>       |                  |                  |                  |                  |
| Pollen (grass and/or birch)           |                  |                  |                  |                  |
| CC/CT                                 | 15.4             | 16.0             | 1.11 (0.60–2.04) | 0.003            |
| TT                                    | 21.6             | 6.5***           | 0.32 (0.15–0.70) |                  |
| House dust mite                       |                  |                  |                  |                  |
| CC/CT                                 | 10.0             | 10.1             | 0.79 (0.37–1.68) | 0.868            |
| TT                                    | 10.5             | 9.7              | 0.97 (0.45–2.11) |                  |
| Cat dander                            |                  |                  |                  |                  |
| CC/CT                                 | 3.5              | 0.6*             | 0.13 (0.01–1.14) | 0.078            |
| TT                                    | 2.6              | 3.3              | 2.10 (0.55–8.11) |                  |
| <i>Allergic diseases</i>              |                  |                  |                  |                  |
| Asthma                                |                  |                  |                  |                  |
| CC/CT                                 | 8.2              | 5.3              | 0.66 (0.27–1.63) | 0.432            |
| TT                                    | 9.2              | 9.1              | 1.22 (0.54–2.75) |                  |
| Atopic asthma§                        |                  |                  |                  |                  |
| CC/CT                                 | 6.0              | 3.6              | 0.56 (0.19–1.65) | 0.658            |
| TT                                    | 6.7              | 5.4              | 1.09 (0.40–2.96) |                  |
| Hay fever                             |                  |                  |                  |                  |
| CC/CT                                 | 9.5              | 7.2              | 0.78 (0.34–1.76) | 0.096            |
| TT                                    | 13.2             | 3.9***           | 0.39 (0.15–1.02) |                  |
| <i>Symptoms in the last 12 months</i> |                  |                  |                  |                  |
| Wheeze                                |                  |                  |                  |                  |
| CC/CT                                 | 12.4             | 10.8             | 0.97 (0.46–1.95) | 0.633            |
| TT                                    | 8.7              | 9.3              | 1.59 (0.70–3.63) |                  |
| Atopic wheeze§                        |                  |                  |                  |                  |
| CC/CT                                 | 6.3              | 6.9              | 0.98 (0.39–2.48) | 0.768            |
| TT                                    | 5.5              | 4.9              | 1.32 (0.45–3.89) |                  |
| Allergic rhinoconjunctivitis          |                  |                  |                  |                  |
| CC/CT                                 | 10.7             | 10.3             | 1.10 (0.53–2.28) | 0.369            |
| TT                                    | 10.7             | 6.6              | 0.80 (0.34–1.90) |                  |

\*0.05 ≤ *P* < 0.10, \*\* 0.01 ≤ *P* < 0.05, \*\*\* *P* < 0.01.

†Odds ratios adjusted for farming status. Reference: children exposed <50th percentile of endotoxin.

‡Likelihood ratio test for improvement of goodness of fit by including the interaction term *CARD4*–21596\*endotoxin into the logistic regression model.

§See Methods.

these phenotypes when using endotoxin as a continuous variable.

None of the other *CARD4* SNPs studied modified the association between any of the exposures under study and the likelihood of having asthma or allergic phenotypes (data not shown).

## Discussion

In this study we found that the previously reported inverse association between exposure to a farming environment and asthma and allergies is significantly modified by a very common polymorphism in *CARD4* (*CARD4*–21596). The difference between farmers' children and nonfarmers' children in the frequency of hay fever, atopic asthma

symptoms, and specific serum IgE against pollen and cat dander was significantly larger in children homozygous for the wild type allele (*CARD4*/–21596 *T*) compared with carriers of the minor allele (*C*). The results of this study are in line with the current concept in the pathogenesis of asthma and allergies that proposes that the onset of the disease and its clinical course are determined by gene–environment interactions. The results particularly support the hypothesis that exposure to microbial products in childhood modifies the frequency of allergies in susceptible individuals (30), as *CARD4* is a pattern recognition receptor that interacts with molecules found in common Gram-negative bacteria.

A recent study showed associations between polymorphisms in *CARD4* and asthma and total IgE levels, respectively, and part of their population is similar to ours with respect to age and presumably their genetic background (31). The study group found two polymorphisms (rs2975632 and rs2075822) associated with total IgE levels and one polymorphism (rs3020207) associated with asthma, and these findings allow for a comparison of their results with ours. *CARD4*/–2914 (rs2975632), which was also used as a haplotype tagging SNP for rs2075822 in our population (using  $r^2 \geq 0.8$ ), was not associated with total IgE, either in farmers' or in nonfarmers' children. The polymorphism with the minor allele being associated with a higher risk of asthma in the study of Hysi et al. (31) is in high linkage disequilibrium ( $r^2 > 0.9$ ) with *CARD4*/+1915 (rs2075819) genotyped in our population. There was a trend towards a lower frequency of asthma in carriers of the minor allele among our nonfarmers' children (*CARD4*/+1915 AA 12.6%, AG/GG 6.9%,  $P = 0.053$ ), but no association with wheeze in the last 12 months (*CARD4*/+1915 AA 11.3%, AG/GG 15.4%,  $P = 0.23$ ) or any of the other phenotypes under study. Apart from less statistical power in our study due to the smaller sample size and especially fewer cases, other methodological factors such as differences in the definition of cases and controls and variation in environmental characteristics of the area from which the study populations were drawn, may contribute to the heterogeneity of the findings. The latter may be particularly important in studies of genetic variation in genes that encode for innate immunity receptors.

To our knowledge, this study is the first in which environmental exposures and *CARD4* polymorphisms were assessed concurrently. There are, however, several limitations that need to be addressed. Measurements of microbial molecules in house dust and health assessment were done cross-sectionally at school-age. Whereas exposure to a farming environment is fairly constant through childhood in our populations, we have no information on exposure to endotoxin and muramic acid in house dust in different genotypes before the onset of allergies, and possible changes of exposure over time. Further, the sample size of our study was also too small to estimate genotypic effects on asthma and allergies in

children homozygous for the minor alleles in *CARD4* SNPs ( $\leq 10\%$ ). Replication of the gene–environment interaction observed in our study is clearly needed.

Stimulation of innate immunity receptors may promote the development of T regulatory cells and by that protect or lead away from allergies (32). The underlying molecular mechanisms that explain the observed associations in our study population are not known. The *CARD4*/–21596 polymorphism is located in intron1 but it is also in high linkage disequilibrium with other known polymorphisms in the promoter region (<http://innateimmunity.net>), and could as well be in linkage disequilibrium with other variations in regions not sequenced yet. It has been shown that *CARD4* gene and protein expression is regulated by interferon- $\gamma$ , which activates transcription through binding to interferon regulatory factor-1 binding motifs in the promoter of *CARD4* (33). Polymorphisms in this region may lead to differences in the availability of the *CARD4* protein by either enhanced or diminished transcription rates. Other possibilities remain open, as no functional studies on *CARD4*/–21596 are available.

Measurement of endotoxin is widely used as a surrogate for exposure to LPS. Although *CARD4* is not a pattern-recognition receptor for this molecule, LPS indicates the presence of Gram-negative bacteria. This may explain the finding of a protective effect of endotoxin on sensitization against pollen and hay fever among carriers of the same genotype of *CARD4*/–21596 as seen for farming exposure. Although this effect of endotoxin was independent of farming status, the protective effect of exposure to a farming environment was stronger and consistently seen for all specific IgE-related phenotypes in children with *CARD4*/–21596 *TT* compared with those carrying the *C* allele. There are several possible explanations for this finding. First, measurement of endotoxin in mattress samples may only moderately or disproportionately correlate with the amount of diaminopimelic acid containing muropeptides, the ligands for *CARD4*, to which children are exposed. Secondly, epidemiological studies in which endotoxin in house dust was measured suggest that exposure to naturally occurring endotoxin in early life particularly protects against specific IgE to pollen and phenotypes triggered by pollen exposure, i.e. hay fever and, less specifically, the atopic form of asthma (5–7). Of special interest in this context are results from animal models suggesting an additional role for *CARD4*, besides that of sensing diaminopimelic acid. In mice, the interaction between *CARD4* and diaminopimelic acid increases the secretion of pro-inflammatory cytokines induced by LPS stimulation (24). It is therefore tempting to speculate that carriers of the *TT* genotype in *CARD4*/–21596 are strongly protected against the development of pollen-specific IgE and its related phenotypes by an amplified immune response to diaminopimelic acid conferred by high levels of endotoxin. There is one more possibility that could lead to a stronger protective effect

of living on a farm than levels of endotoxin in children with *CARD4*/–21596 *TT*. Living on an animal farm represents exposure to a variety of microbial products (8, 34–36), and this diversity of microbial molecules presented to innate immunity receptors may not only considerably differ from that of a nonfarming environment but also elicit stronger immune responses.

There was no interaction between levels of muramic acid, genetic variation in *CARD4*/–21596, and the phenotypes under study. This result is not unexpected. Muramic acid is a component of peptidoglycan, which is the major constituent (up to 90%) of the cell wall of Gram-positive bacteria, and is present in lower quantities (about 10%) in Gram-negative bacteria. Apart from this difference, muramic acid from Gram-positive bacteria commonly contains the amino acid lysine at a certain location of the peptide, whereas diaminopimelic acid is found in most Gram-negative bacteria. Only the diaminopimelic acid containing mureopeptide is detected by *CARD4*.

The findings in our study strengthen the hypothesis that, in susceptible individuals, natural exposure to molecules from common Gram-negative bacteria is strongly associated with allergies, but not with all forms of asthma. The protective effects of exposure to a farming environment on the development of other forms of asthma, such as nonatopic asthma, is therefore likely to be conferred by genetic and environmental factors other than those affecting specific IgE and IgE-mediated phenotypes. In the same population as investigated in this study, we have recently reported that genetic variation in *TLR4*, which encodes for part of the receptor

complex for LPS, is associated with less atopy in children heavily exposed to endotoxin (17). Because the minor allele frequency of the *TLR4* polymorphism was low (<10%), we concluded that this genetic variation explained only part of the observed inverse association between allergies and endotoxin (5). In this study we found that a very common polymorphism (minor allele frequency 30%) in *CARD4* significantly contributes to the lower frequency of allergies in children heavily exposed to microbial products. In this population, genetic variation in both *CARD4* and *TLR4* may therefore represent susceptibility loci that no longer confer protection in an environment in which such exposure is lacking.

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### References

- Ernst P, Cormier Y. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Respir Crit Care Med* 2000;**161**:1563–1566.
- Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *N Engl J Med* 2000;**343**:538–543.
- Riedler J, Braun-Fahrlander CH, Eder W, Schreuer M, Waser M, Maisch S et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;**358**:1129–1133.
- Matricardi PM, Rosmini F, Panetta V, Ferrigno L, Bonini S. Hay fever and asthma in relation to markers of infection in the United States. *J Allergy Clin Immunol* 2002;**110**:381–387.
- Braun-Fahrlander CH, Riedler J, Herz U, Eder W, Waser M, Grize L et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;**347**:869–877.
- Bottcher MF, Bjorksten B, Gustafson S, Voor T, Jenmalm MC. Endotoxin levels in Estonian and Swedish house dust and atopy in infancy. *Clin Exp Allergy* 2003;**33**:295–300.
- Litonjua AA, Milton DK, Celedon JC, Ryan L, Weiss ST, Gold DR. A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. *J Allergy Clin Immunol* 2002;**110**:736–742.
- Van Strien RT, Engel R, Holst O, Bufer A, Eder W, Waser M et al. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004;**113**:860–867.
- Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell* 1994;**76**:241–251.
- O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;**8**:275–283.
- Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. *Am J Respir Cell Mol Biol* 2000;**22**:604–612.
- Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;**196**:1645–1651.
- Gerhold K, Blümchen K, Franke A, Stock P, Hamelmann E. Exposure to endotoxin and allergen in early life and its effect on allergen sensitization in mice. *J Allergy Clin Immunol* 2003;**112**:389–396.

14. Velasco G, Campo M, Manrique OJ, Bellou A, He H, Arestides RS et al. Toll-like receptor 4 or 2 agonists decrease allergic inflammation. *Am J Respir Cell Mol Biol* 2005;**32**:218–224.
15. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;**25**:187–191.
16. Werner M, Topp R, Wimmer K, Richter K, Bischof W, Wjst M et al. TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol* 2003;**112**:1–8.
17. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol* 2004;**113**:482–488.
18. Baldini M, Lohman C, Halonen M, Erickson RP, Holt PG, Martinez FD. A polymorphism in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am J Respir Cell Mol Biol* 1999;**20**:976–983.
19. Gao PS, Mao XQ, Baldini M, Roberts MH, Adra CN, Shirakawa T et al. Serum total IgE levels and CD14 on chromosome 5q31. *Clin Genet* 1999;**56**:164–165.
20. Koppelman GH, Reijmerink NE, Stine OC, Howard TD, Whittaker PA, Meyers DA et al. Association of a promoter polymorphism of the CD14 gene and atopy. *Am J Respir Crit Care Med* 2001;**163**:965–969.
21. LeVan TD, Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD et al. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *J Immunol* 2002;**167**:5838–5844.
22. Gern JE, Reardon CL, Hoffjan S, Nicolae D, Li Z, Roberg KA et al. Effects of dog ownership and genotype on immune development and atopy in infancy. *J Allergy Clin Immunol* 2004;**113**:307–314.
23. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C et al. Opposite effects of *CD14*/-260 on serum IgE levels in children raised in different environments. *J Allergy Clin Immunol* 2005;**116**:601–607.
24. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Natur Immunol* 2003;**4**:702–707.
25. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 2003;**300**:1584–1587.
26. Bertin J, Nir WJ, Fischer CM, Tayber OV, Errada PR, Grant JR et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J Biol Chem* 1999;**274**:12955–12958.
27. Hollander A, Heederik D, Versloot P, Douwes J. Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. *Am Ind Hyg Assoc J* 1993;**54**:647–653.
28. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 2004;**74**:106–120.
29. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotypes maps. *Bioinformatics* 2005;**21**:263–265.
30. Martinez FD, Holt P. Role of microbial burden in aetiology of allergy and asthma. *Lancet* 1999;**354**S2:12–15.
31. Hysi P, Kabesch M, Moffatt MF, Schedel M, Carr D, Zhang Y et al. NOD1 variation, immunoglobulin E and asthma. *Hum Mol Genet* 2005;**14**:935–941.
32. Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;**112**:480–487.
33. Hisamatsu T, Suzuki M, Podolsky DK. Interferon- $\gamma$  augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells. *J Biol Chem* 2003;**278**:32962–32968.
34. Dutkiewicz J. Exposure to dust-borne bacteria in agriculture. I. Environmental studies. *Arch Environ Health* 1978;**33**:250–259.
35. Von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000;**30**:1230–1234.
36. Waser M, Schierl R, von Mutius E, Maisch S, Carr D, Riedler J et al. Determinants of endotoxin levels in living environments of farmers' children and their peers from rural areas. *Clin Exp Allergy* 2004;**34**:389–397.