Toll-like receptors and microbial exposure: gene-gene and gene-environment interaction in the development of atopy

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Abstract

Background: Environmental and genetic factors contribute to atopy development. High microbial exposure may confer a protective effect on atopy. Toll-like receptors (TLRs) bind microbial products and are important in activating the immune system.

Objective: To assess whether interactions between microbial exposures and genes encoding TLRs (related) genes result in atopy. Methods: Genes, environmental factors and gene-environment interactions of 66 SNPs of 12 genes (TLR 1-6 and 9-10, CD14, MD2, LBP and Dectin-1) and six proxy parameters of microbial exposure (i.e. sibship size, pets, day-care and tobacco smoke exposure) were analysed for association with atopic phenotypes in 3,062 Dutch children (The Allergenic study).

Results: The presence of >2 older siblings increased the risk to develop high total IgE levels at different ages. This risk increased further in children aged 1-2 years carrying the minor allele of TLR6 SNP rs1039559. Furthermore, novel two and three factor gene-gene and gene-environment interactions were found (e.g between sibship size, day-care and LBP SNP rs2232596) Conclusion: Larger sibship size is associated with increased total IgE levels. Furthermore, complex 2 and 3 factor interactions exist between genes and the environment. The TLRs and related genes interact with proxy parameters of high microbial exposure in atopy development.
Introduction

Atopy is caused by an interaction between environmental factors and genes. Exposure to microbial products and infections may protect from the development of atopy (reviewed in 1). It is not exactly known which (combinations of) microbial product(s) account for this protective effect, but several proxy measurements of high microbial exposure or infections (e.g. day-care, sibship size, farm and raw milk exposure) showed consistent protective effects on the development of atopic diseases. 1-3

Microbial products are recognized and bound by Toll like receptors (TLRs) which are germline encoded receptors that are widely expressed, e.g. by antigen presenting cells such as macrophages and dendritic cells as well as T-regulatory cells and epithelial cells. 4 These receptors form either homo- or heterodimers in order to bind diverse microbial products. 5 Each Toll like receptor can bind numerous pathogen associated moieties, e.g. TLR4 can bind Lipopolysaccharide (LPS) from gram negative bacteria, but can also bind gram positive bacteria and RSV. 6 TLR activation stimulates T-regulatory cells and/or skews the Th-1/Th-2 balance towards Th-1. 6-8

Evidence for (single) gene- and gene-environment interaction between these TLRs, related genes (such as CD14) and proxy measurements of infection and high microbial exposure in the development of atopy has been shown. 9-12 However, aside from single gene and gene-environment interactions, higher order interactions may also exist between genes and environmental factors that play a role in the development of atopy. For example, two environmental factors may act in concert with a gene or with multiple genes.

Our aim was to identify complex gene-environment interactions important in the development of atopic phenotypes, using TLR and TLR related genes and proxy measurements of infection and high microbial exposure in the Allergenic study (composed of three Dutch birth cohorts KOALA, PIAMA, and PREVASC). In these three cohorts, we previously indentified consistent genetic associations as well as consistent gene by environment interactions. 12 We used the following atopy phenotypes. 1) Total IgE at 1-2 years; 2) Total IgE at 6-8 years; 3) Specific IgE to indoor allergens at 6-8 years. For this purpose, we used the unbiased data mining approach known as Multifactor Dimensionality Reduction (MDR), that is designed to
translate high-dimensional data into a single dimension, 13 in order to detect relevant interactions.

**Subjects and methods**

**Study populations**

The Allergenic study (i.e. PIAMA, PREVASC and KOALA) 12 is a combination of three Dutch birth cohorts with similar design. Local medical ethics committees of participating institutes approved of the study. All parents provided written informed consent.

**IgE measurements**

Total IgE levels were determined in capillary or venous blood collected at age 1, 4 and 8 years in PIAMA, age 1, 2, 4 and 6 in PREVASC, and age 1 and 2 in KOALA (Sanquin Research, Amsterdam) and measured as described previously. 12 Cases and controls were defined as children with a serum IgE level in the highest (cases) and the lowest (controls) tertile respectively, as estimated at each age in boys and girls separately. We choose to analyse both extremes of the age range and clustered measurements from ages 1 and 2 years, and 6 and 8 years. In the subgroup of children with IgE measurements available at both 1 and 2 years, the highest and lowest tertiles were determined from the mean of the two measurements.

Specific IgE for indoor allergens was also clustered at age 6-8 years. Positive specific IgE was defined as specific IgE >0.35 IU/ml for house dust mite, dog or cat (indoor allergens) at age 6 (PREVASC) or 8 (PIAMA).

**SNP selection and genotyping**

We selected haplotype tagging Single Nucleotide Polymorphisms and SNPs of special interest due to previous association or functionality (table 2 online repository). SNPs of TLR genes (TLR1-6, 9 and 10) and accessory genes (CD14, MD2 (Huge name: LY96), LBP and DECTIN-1), (see figure 1 and table 1 in the online repository). These TLR genes were selected based on their function to bind diverse microbial
products, and/or previously known associations with atopic phenotypes. The accessory proteins that were selected are crucial in binding these microbial products to the TLRs. The ligands of these genes are described in table 1, in the online repository. Haplotype tagging (ht) SNPs (based on $r^2 > 0.80$) were selected from the HapMap database $^{14}$ or from the Innate Immunity website $^{15}$ depending on the largest number of SNPs with a minor allele frequency > 0.1 available in each database. Additionally, the biomedical literature was screened for SNPs with known functional impact or association with asthma or atopy and these SNPs were forced into the haplotype selection.

Genomic DNA was extracted from buccal swabs or blood using standard methods. $^{16}$ DNA was amplified by using REPLI-g UltraFast technology (Qiagen™). Genotyping was performed by Competitive Allele-Specific PCR using KASPar™ genotyping chemistry, performed under contract by K-Biosciences. Quality of genotype data was verified as described previously. $^{12}$

**Environmental exposures**

The following environmental exposures were selected as proxy measurements of high microbial exposure or infections: 1) Siblings (number of older siblings present at birth; 0; 1; 2 or more); 2) Day-care (day-care in first year of life; 4 hours or more per week versus no day-care); 3) Dog present in first year of life; 4) Cat present in first year of life; 5) Intrauterine smoke exposure in the last three months of pregnancy; 6) Environmental tobacco smoke exposure at home in the first year of life (tobacco smoke contains high levels of endotoxin $^{17}$).

**Statistical methods**

All SNPs were analysed for Hardy-Weinberg equilibrium (HWE) using $\chi^2$ statistics ($p>0.01$). Children with >10% missing genotype data were excluded.

Gene-gene and gene-environment interactions were analysed using MDR (version 1.0.0) as described in the online repository and used by us previously. $^{18-20}$

The haplotype tagging SNPs, environmental exposures and gender were included in the MDR analysis. Best models were computed for 1, 2 and 3 factors. Since we are specifically interested in gene by environment interactions we also used the landscape option for the event that an environmental factor would not have been found in the “best model”. Landscape arranges all possible combinations from best
model to “worst” model. The best model including an environmental factor was selected and tested for significance by logistic regression. Logistic regression analyses were also performed to interpret the significant single factor and 2-factor interaction results from MDR analysis. In the logistic regression analyses an interaction term of the two variables were included. Graphical displays were used to interpret the 3-factor interactions. The single SNP associations and association between total IgE and sibship size was analysed using analysis of variance (ANOVA) and linear regression. The linear regression was corrected for atopic status of the parents, smoke exposure, pet exposure, gender, breastfeeding and day-care attendance. The ANOVA and linear regression analyses were performed using SPSS 14.0. The Graphs displayed were created by Graph path prism.

Results

Characteristics of the study population are shown in Table 1. The 12 genes were tagged by 66 haplotype tagging SNPs (including 14 forcedly included SNPs of special interest). 2 SNPs deviated from HWE and were not included in the MDR analysis (see the online repository table 2). The genotype completion rate ranged from 94.4 to 99.0%.

Single SNP analysis
Table 3 in the online repository shows the association between single SNPs and total and specific IgE.

MDR analysis
Total IgE age 1-2 years
Of all genetic and environmental factors, the environmental factor ‘siblings’ was selected by MDR as best predictor in the 1-factor model ($P_{perm} = 0.004$ (p-value after 1000x permutations of the data); table 2. Children having 2 or more older siblings had an increased risk for high IgE at age 1-2 compared to 1 or no siblings after birth.

Splitting the data into the three different cohorts showed a significantly higher mean total IgE in children with two or more older siblings in the different cohorts, at the different ages (for KOALA at age 1 and 2, for PIAMA and PREVASC separately
at age 4 and for PIAMA at age 8 years (figure 2). For PIAMA at age 1 and PREVASC at age 1, 2 and 6 there was a similar trend, however this was not significant. (figure 2)

Siblings and TLR6 (rs1039559) were selected as best 2-factor model by MDR ($P_{perm}=0.04$). The rs1039559 SNP was not associated with development of high IgE in children having no or 1 sibling. In contrast, the risk of developing high total IgE for children with more than 1 sibling increased when they carried the minor allele of rs1039559 (table 2 and figure 3).

Day-care, siblings and LBP (rs2232596) were selected as the best 3–factor model ($P_{perm}=0.007$). Day-care protected for the development of high total IgE at age 1-2 year, especially in children heterozygous for rs2232596, but not in children with more than 1 sibling (figure 4).

Total IgE at 6-8 years

In the 1-factor model, LBP (rs745144) was selected by MDR as best predictor. However this was not significant ($P_{perm}=0.13$, table 2).

TLR10 (rs11096957) and MD2 (rs7838114) were selected as best 2–factor model ($P_{perm}=0.03$, table 2). Especially children carrying the minor allele of rs7838114 and the wild type of rs11096957 were protected for the development of high IgE at age 6-8 years. Siblings and rs11096957 (TLR10) emerged as the best predictive gene-environment interaction, however this was not significant.

Finally, in the best 3-factor model, siblings, CD14 (rs2915863) and LBP (rs745144) were selected ($P_{perm}=0.03$) as the best predictors (table 2).

Specific IgE to Indoor allergens 6-8 years

TLR1 (rs5743604) was selected by MDR as the best predictor in the 1-factor model ($P_{perm}=0.04$). Carriers of the minor allele were protected from the development of specific IgE to indoor allergens in a dominant model (table 2).

Two TLR6 SNPs (rs5743798 and rs5743810) were selected in the best 2-factor model, but this was not significant ($P_{perm}=0.08$, table 2). Siblings and rs11096957 (TLR10) emerged as the best gene-environment interaction, but this was not significant.
TLR1 (rs5743594), LBP (rs6025083) and TLR5 (rs851186) were selected by MDR as best 3-factor model (P_{perm}=0.02), which indicated a complex three-way gene-gene interaction (table 2).

Discussion

This is the first study that identifies genes, environmental factors and gene-environment interactions of Toll like receptors and their related genes with environmental factors that are associated with increased microbial exposure and infections using the unbiased data mining approach MDR.

We identified a significant association between having 2 or more older siblings and increased total serum IgE levels. This was observed at four different ages in all 3 birth cohorts (figure 2). Furthermore this association remained significant after adjusting for multiple confounders. This was an unexpected observation, since the sibling effect has been reported to decrease the risk of atopy development, especially when atopy was defined as specific IgE and / or skin test positivity. This sibling effect on atopy was first described by Strachan who showed a protective effect of larger sibship size on the development of hay fever. 21 Hereafter, several studies have replicated this sibling effect with atopic phenotypes such as specific IgE, hay fever, rhinitis and positive skin prick tests. 22-25 Controversially, there have only been few studies reporting associations between larger sibship size and decreased total IgE. For total IgE levels at birth, one study showed an association between larger sibship size and decreased cord blood total IgE levels. 23 However, other studies, including one in one of our own birth cohorts, did not replicate this protective effect on cord blood IgE levels. 26,27 Furthermore, no evidence for association of larger sibship size on decreased total IgE levels at older ages have been reported. 22,28 and specific IgE levels. To our knowledge, we are the first to show an association between larger sibship size and increased total IgE levels. The biological mechanism behind this finding is unclear. Our findings should be interpreted with caution and replicated studies are essential to generalise this finding. Further studies should be performed on the effect of sibship size on specific atopic variables and include a comparison of total serum IgE.

We additionally showed that gene-environment interaction exists between a SNP located in TLR6 and sibship size with respect to the development of total IgE at age 1-2 years. TLR6 forms a heterodimer with TLR2 and TLR2/6 heterodimers.
recognize lipopeptides, often found in the upper respiratory tract. A recent study by Kormann in a German population was the first to show a strong protective effect of a SNP located in TLR6 (rs5743789) on atopic asthma. Furthermore this SNP was associated with increased mRNA expression, and carriers of the rare allele of this TLR6 SNP showed increased Th-1 cytokine expression, and reduced Th-2 cytokine production after stimulation with the TLR6 ligand. In our data a gene-environment interaction became apparent with a SNP (rs1039559) not genotyped by Kormann et al. We showed an increased risk for high total IgE in children carrying the minor allele of rs1039559 and having 2 or more older siblings. Since this SNP was not investigated by Kormann et al, it is difficult to compare our results. However, our data lend further support to the role of TLR6 in atopic diseases and indicates that this might act through a gene-environment interaction.

The MDR approach revealed several novel significant gene-environment interactions, such as the interaction between day-care, sibship size and LBP (rs2232596). In our data day-care protects for the development of high total IgE levels at age 1-2 year, predominantly in children heterozygous for rs2232596, but not in children with more than 1 sibling. The protective effect of day-care on total IgE development has been shown before. We confirm and extend this observation by showing that the “day-care” effect also depends on genetic and an additional environmental factor, namely, sibship size. The intriguing finding that day-care decreases, but sibship size increases the risk of developing high total IgE levels might be explained by an even higher microbial exposure resulting from day-care than from increased sibship size. However, the exact mechanism behind this difference requires further study.

The SNP that emerged in the complex 3 factor gene-environment interaction for total IgE at age 1-2 years is located in the Lipopolysaccharide Binding Protein (LBP) gene. SNPs located in this gene were present in the best multifactor models of all analysed phenotypes. LBP is an extracellular protein that binds LPS and is essential for binding LPS to the CD14-TLR4-MD2 (LY96) complex. To our knowledge, no associations with SNPs located in LBP and atopic phenotypes have been published before. Michel et al showed that a rise in serum LBP after LPS inhalation by healthy human individuals was inversely associated with atopic status (defined as high total IgE and skin test positivity). We hypothesize that SNPs located in LBP alter its expression. These SNPs may lead to a decrease in soluble
LBP and therefore a decreased binding of LPS to the TLR4 complex, thereby possibly increasing the risk of developing atopy.

We have analysed several proxy measurements of high environmental microbial exposures. There is convincing evidence that exposure to such environments have a protective effect on the development of atopy. The “sibling effect” and “day-care” effect are both thought to act through increased microbial exposure which will trigger the immune system and preferentially activate Th-1 instead of Th-2 development. Interestingly, we show that these effects are also dependent on genetic factors and may differ between total and specific IgE levels. Furthermore these interactions may be age dependent, since we discovered different interactions at age 1-2 than at age 6-8 years.

We and others have previously published that gene-environment interaction between dog exposure and CD14 genotype plays a role in the development of sensitisation and total IgE levels at different ages. This interaction did not emerged as the “best model” in this study. This confirms that multiple different complex gene-environment interactions play a role in atopy development, and it is important to realize that MDR identifies the models that describe the data best, but that it does not exclude other models.

To date it is not exactly known which microbial exposures account for the protective effect. Interestingly, our data indicate that higher order interactions between environmental factors exist, which adds to the complexity of gene by environment interaction in atopy. We chose to analyse proxy measurements that were available for all three birth cohorts in order to reduce the chance of “missing” important microbial exposures, especially since, to date, it is not known which (combination of) exposures are important. In addition, direct measurements of LPS in house dust or other factors were only available for a subset of children in this study.

The MDR approach made the analysis of complex gene-gene and gene-environment interaction feasible since it is designed to compress multi-dimensional genetic data into a single dimension. This is a promising method to analyse complex interactions in large datasets. Furthermore MDR selects SNPs in an unbiased way, and it is thus possible to analyse a biological pathway without prior knowledge of the combination of genes and environmental factors which could be important in the development of atopy. Although many of our findings were
significant after permutation testing, we would like to stress that our findings need to be replicated in other studies.

This study has some limitations. First of all three different birth cohorts (PIAMA, PREVASC and KOALA) were pooled for the analysis. We reported previously 12 Nevertheless, bias resulting from to the presence of three populations can not be ruled out completely. Secondly, we selected a subset of genes that felt to be important to the development of atopy. We acknowledge, however, that new insights might point to the importance of other genes such as those for $\text{TLR7}$ and $\text{TLR8}$ in future analysis. It would also have been of considerable interest to analyse the same SNPs studies by Kormann et. al.29. However, this information was not available at the beginning of this study. Moreover, rs5743789 is not available to date in Hapmap. We do acknowledge that replication of these results is crucial, particularly as we were unable to perform an independent replication study. We choose instead to analyse the entire cohort to optimize our statistical power. Furthermore, in MDR analyses, the presence of 'highly' significant main effect SNPs could push other SNPs towards an interaction result. Therefore, we also used logistic regression to confirm the two factor MDR analysis. Finally, the best evidence for a protective effect of high microbial exposure on the development of atopy comes from studies that analyse the protective effect of farm life in Central Europe. 1 Our study did not include enough children living on a farm to investigate this parameter in relation to atopy development.

In conclusion we report evidence that the presence of older siblings, irrespective of genotype, increases the risk of having high total IgE levels at different ages and within three different cohorts. Furthermore, complex 2 and 3 factor interactions exist between genes and the environment. The TLRs and related genes interact with environmental factors associated with proxy parameters of infection or high microbial exposure and in this way contribute to atopy development. Future studies should be done to replicate these findings, and functional studies could be useful to investigate the exact biological mechanism of these gene-environment interactions.
Acknowledgements

The authors would like to thank the children and parents of the PIAMA, PREVASC and KOALA study for their participation. In addition we acknowledge the field workers, secretaries and scientific collaborators dedicated to the PIAMA, PREVASC and KOALA cohorts. Further more we would like to thank Professor du Bois for his grammatical advices.
### Table 1
Characteristics of participating children in the Allergenic study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PIAMA</th>
<th>PREVASC</th>
<th>KOALA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Participants in genetic study, number (% from total cohort)</strong></td>
<td>1,037 (25.0)</td>
<td>374 (49.8)</td>
<td>1,651 (58.1)</td>
</tr>
<tr>
<td><strong>Ethnicity (% Dutch origin)</strong></td>
<td>95.1</td>
<td>95.7</td>
<td>95.2</td>
</tr>
<tr>
<td><strong>Boys (%)</strong></td>
<td>51.2</td>
<td>49.2</td>
<td>50.6</td>
</tr>
<tr>
<td><strong>Family history (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atopy mother</td>
<td>66.5</td>
<td>51.4</td>
<td>33.3</td>
</tr>
<tr>
<td>Asthma mother</td>
<td>16.7</td>
<td>31.4</td>
<td>8.8</td>
</tr>
<tr>
<td>Atopy father</td>
<td>31.6</td>
<td>47.7</td>
<td>36.4</td>
</tr>
<tr>
<td>Asthma father</td>
<td>7.4</td>
<td>21.9</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>Environmental exposures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother smoking last trimester pregnancy (%)</td>
<td>10.8</td>
<td>9.1</td>
<td>4.5</td>
</tr>
<tr>
<td>ETS at home first year (%)</td>
<td>23.2</td>
<td>21.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Pet (dog and/or cat) first year (%)</td>
<td>39.1</td>
<td>33.3</td>
<td>39.8</td>
</tr>
<tr>
<td>Dog first year (%)</td>
<td>14.2</td>
<td>22.9</td>
<td>19.4</td>
</tr>
<tr>
<td>Cat first year (%)</td>
<td>28.7</td>
<td>12.9</td>
<td>24.5</td>
</tr>
<tr>
<td>Presence older siblings at birth 0 (%)</td>
<td>51.5</td>
<td>40.1</td>
<td>41.9</td>
</tr>
<tr>
<td>1</td>
<td>34.8</td>
<td>42.7</td>
<td>40.9</td>
</tr>
<tr>
<td>≥ 2</td>
<td>13.7</td>
<td>17.2</td>
<td>15.2</td>
</tr>
<tr>
<td><strong>Total IgE, geometric mean (SD), IU/ml / n</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age 1</td>
<td>7.1 (4.6) / 369</td>
<td>NA</td>
<td>8.6 (3.9) / 226</td>
</tr>
<tr>
<td>age 2</td>
<td>36.1 (5.1) / 714</td>
<td>NA</td>
<td>18.6 (3.9) / 207</td>
</tr>
<tr>
<td>age 4</td>
<td>65.1 (5.2) / 750</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>age 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Specific IgE to indoor allergens (%) / n</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age 6</td>
<td>NA</td>
<td>25.5 / 137</td>
<td>NA</td>
</tr>
<tr>
<td>age 8</td>
<td>26.9 / 748</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

SD=standard deviation. n=numbers available for analysis. NA=+ not available.
Table 2
Results of MDR analysis evaluating TLRs, TLR related genes and proxy measurements of high microbial exposure.

<table>
<thead>
<tr>
<th>Number of Variables in Model&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Best model</th>
<th>Mean CV&lt;sup&gt;b&lt;/sup&gt; consistency</th>
<th>Mean prediction error (%)</th>
<th>Pperm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>OR&lt;sup&gt;d&lt;/sup&gt; p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Gene(s) and/or environmental factors</strong></td>
<td><strong>SNP(s)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total IgE 1-2 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Siblings</td>
<td>-</td>
<td>8.2</td>
<td>0.44</td>
<td>0.004</td>
</tr>
<tr>
<td>2</td>
<td>Sibling, TLR6</td>
<td>rs1039559</td>
<td>2.6</td>
<td>0.45</td>
<td>0.04</td>
</tr>
<tr>
<td>3</td>
<td>Day-care, Sibling, LBP</td>
<td>rs2232596</td>
<td>3.0</td>
<td>0.43</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Total IgE 6-8 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>LBP</td>
<td>rs745144</td>
<td>4.6</td>
<td>0.46</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>TLR10, LY96(MD2)</td>
<td>rs11096957, rs7838114</td>
<td>2.0</td>
<td>0.42</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>Siblings, CD14, LBP</td>
<td>rs2915863, rs745144</td>
<td>2.4</td>
<td>0.45</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Specific IgE to indoor allergens 6-8 years</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>TLR1</td>
<td>rs5743604</td>
<td>4.2</td>
<td>0.45</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>TLR6, TLR6</td>
<td>rs5743798, rs5743810</td>
<td>4.4</td>
<td>0.45</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>TLR1, LBP, TLR5</td>
<td>rs5743594, rs6025085, rs851186</td>
<td>4.2</td>
<td>0.40</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of variables in model; <sup>b</sup> CV=cross-validation; <sup>c</sup> Significance of prediction error (empirical p-value based on 1000 permutations); <sup>d</sup> Odds Ratios and (95% confidence intervals) after logistic
regression, with p-value. No or 1 sibling and/or wild type genotype in a recessive model was used as reference category. Logistic regression not performed on three factor interactions.

**Figure 1**
Figure of the TLRs and TLR associated proteins analyzed in this study. TLR1 and 6 form heterodimers with TLR2.
Figure 2
Mean IgE at age 1, 2, 4 and 8 years in the three birth cohorts, children with no or 1 sibling compared to children with two or more siblings.

● / ○ PIAMA; ■ / □ PREVASC; ▲ / △ KOALA; (■ ▲ ●) no or 1 sibling versus (□ △ ○) two or more siblings. Error bars represent mean and 95% CI.
Figure 3
Gene-environment interaction of TLR6 and sibling exposure. Best 2 variables in model, total IgE at age 1-2 years OR= odds ratio and 95% confidence interval are shown.
Figure 4
Graphical display of interaction observed in MDR analysis between day-care, sibling and rs2232596 (LBP).

Cells with a light background show combinations with decreased risk to develop high total IgE, cells with a dark background show combinations with an increased risk to develop high total IgE. Horizontally: AA, AG and GG represent the alleles of rs2232596, underneath 0 and 1 represent no vs yes day-care. Vertically: 0,1, and 2 represent 0,1 and 2 or more older siblings.
Reference List


