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

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ABSTRACT: 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.

To assess whether interactions between microbial exposures and genes encoding TLRs (and related genes) result in atopy, genes, environmental factors and gene–environment interactions of 66 single-nucleotide polymorphisms (SNPs) of 12 genes (*TLR 1–6*, *9* and *10*, *CD14*, *MD2*, lipopolysaccharide-binding protein (*LBP*) and *Dectin-1*), and six proxy parameters of microbial exposure (sibship size, pets (three different parameters), day-care and intrauterine and childhood tobacco smoke exposure) were analysed for association with atopic phenotypes in 3,062 Dutch children (the Allergenic study).

The presence of two or more older siblings increased the risk of developing high total immunoglobulin (Ig)E levels at different ages. This risk increased further in children aged 1–2 yrs 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).

Larger sibship size is associated with increased total IgE levels. Furthermore, complex two- and three-factor interactions exist between genes and the environment. The *TLRs* and related genes interact with proxy parameters of high microbial exposure in atopy development.

KEYWORDS: Atopy, endotoxin, gene–environment interaction, genetic polymorphism, genetic risk factors

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, and farm and raw milk exposure) have shown consistent protective effects against the development of atopic diseases [1–3].

Microbial products are recognised and bound by Toll-like receptors (TLRs), germline-encoded receptors that are widely expressed, *e.g.* by antigen-presenting cells such as macrophages and dendritic cells, as well as regulatory T-cells and epithelial cells [4]. These receptors form either homo- or heterodimers in order to bind diverse microbial products [5]. Each TLR 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 respiratory syncytial virus [6]. TLR activation stimulates T-regulatory cells and/or skews the T-helper (Th)1/Th2 balance towards Th1 [6–8].

Evidence for (single)-gene and gene–environment interaction between these *TLRs*, related genes (such as *CD14*) and proxy measurements of high microbial exposure in the development of atopy has been shown [9–12]. However, aside

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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, the Child, Parent, Health, Focus on Lifestyle and Predisposition (KOALA) cohort, the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort and the Prevention of Asthma in Children (PREVASC) cohort. In these three cohorts, consistent genetic associations as well as consistent gene–environment interactions have previously been identified [12]. We used the following atopy phenotypes: 1) total immunoglobulin (Ig)E at age 1–2 yrs; 2) total IgE at 6–8 yrs; and 3) specific IgE to indoor allergens at 6–8 yrs. For this purpose, we used the unbiased data mining approach known as multifactor dimensionality reduction (MDR), which 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. The local medical ethics committees of participating institutes approved the study. All parents provided written informed consent.

IgE measurements

Total IgE levels were determined in capillary or venous blood collected at ages 1, 4 and 8 yrs in PIAMA, ages 1, 2, 4 and 6 yrs in PREVASC, and ages 1 and 2 yrs 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 yrs, and 6 and 8 yrs. In the subgroup of children with IgE measurements available at both 1 and 2 yrs, 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 yrs. Positive specific IgE was defined as specific IgE >0.35 IU·mL^{-1} for house dust mite, dog or cat (indoor allergens) at age 6 yrs (PREVASC) or 8 yrs (PIAMA).

Single-nucleotide polymorphism selection and genotyping

We selected haplotype tagging (ht) single-nucleotide polymorphisms (SNPs) and SNPs of special interest due to previous association or functionality (online supplementary table 2). SNPs of *TLR* genes (*TLR 1–6, 9* and *10*) and accessory genes (*CD14, MD2* (Human Unidentified Gene-Encoded large proteins database name *LY96*), LPS binding protein (*LBP*) and *Dectin-1*) (fig. 1 and online supplementary table 1). These *TLR* genes were selected based on their function to bind diverse microbial products, and/or their 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 online supplementary table 1.) htSNPs (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, Hilden, Germany). Genotyping was performed by competitive allele-specific PCR using KASPar^{TM} genotyping chemistry, performed under contract by K-Biosciences (Hoddesdon, UK). 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; $\geq$4 h 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 3 months of pregnancy; and 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-squared 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; Computational Genetics

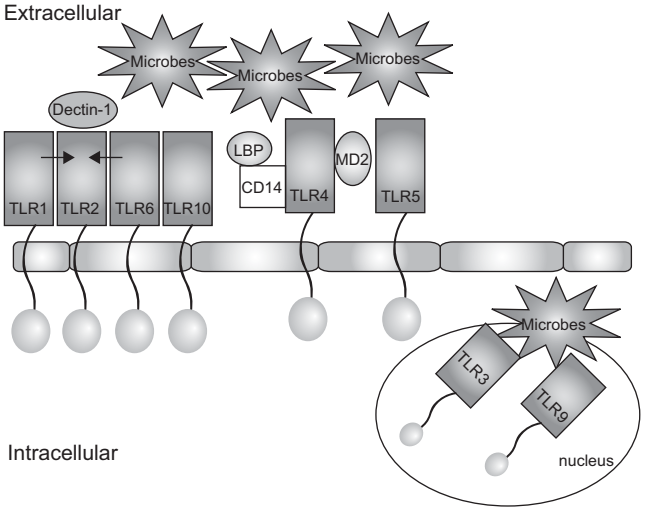

FIGURE 1. The Toll-like receptors (TLRs) and TLR-associated proteins analysed in this study. TLR1 and 6 form heterodimers with TLR2. LBP: lipopolysaccharide-binding protein.

TABLE 1 Characteristics of children participating in the Allergenic study

Characteristics	Cohort		
	PIAMA	PREVASC	KOALA
Participants in Allergenic study	1037 (25.0)	374 (49.8)	1651 (58.1)
Ethnicity Dutch origin	95.1	95.7	95.2
Male	51.2	49.2	50.6
Family history			
Atopy mother	66.5	51.4	33.3
Asthma mother	16.7	31.4	8.8
Atopy father	31.6	47.7	36.4
Asthma father	7.4	21.9	10.0
Environmental exposures			
Mother smoking last trimester of pregnancy %	10.8	9.1	4.5
ETS at home first yr	23.2	21.5	11.5
Pet (dog and/or cat) first yr	39.1	33.3	39.8
Dog first yr	14.2	22.9	19.4
Cat first yr	28.7	12.9	24.5
Number of older siblings at birth			
0	51.5	40.1	41.9
1	34.8	42.7	40.9
⩾2	13.7	17.2	15.2
Total IgE IU·mL^{-1}			
Age 1 yr	7.1±4.6/369	8.6±3.9/226	6.0±3.6/687
Age 2 yrs	NA	11.7±3.9/358	11.9±5.7/694
Age 4 yrs	36.1±5.1/714	18.6±3.9/207	NA
Age 6 yrs	NA	22.5±5.5/218	NA
Age 8 yrs	65.1±5.2/750	NA	NA
Specific IgE to indoor allergens			
Age 6 yrs	NA	137 (25.5)	NA
Age 8 yrs	748 (26.9)	NA	NA

Data are presented as n (%), % or geometric mean±SD (n). PIAMA: Prevention and Incidence of Asthma and Mite Allergy cohort; PREVASC: Prevention of Asthma in Children cohort; KOALA: Child, Parent, Health, Focus on Lifestyle and Predisposition cohort; ETS: environmental tobacco smoke; IgE: immunoglobulin E; NA: not available.

Laboratory, Dartmouth Medical School, Hanover, NH, USA) as described in the online repository and used previously [18–20].

htSNPs, environmental exposures and sex were included in the MDR analysis. Best models were computed for one, two and three factors. Since we were specifically interested in gene–environment interactions, we also used the landscape option in case an environmental factor were not 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 two-factor interaction results from MDR analysis. In the logistic regression analyses, an interaction term of the two variables was included. Graphical displays were used to interpret the three-factor interactions. The single SNP associations and association between total IgE and sibship size were analysed using ANOVA and linear regression. The linear regression was corrected for atopic status of the parents, smoke exposure, pet exposure, sex, breastfeeding and day-care attendance. The ANOVA and linear regression analyses were performed using SPSS 14.0 (SPSS, Chicago, IL, USA). The Graphs displayed were created by GraphPad Prism (GraphPad, La Jolla, CA, USA).

RESULTS

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

Single SNP analysis

Online supplementary table 3 shows the association between single SNPs and total and specific IgE.

MDR analysis

Total IgE at age 1–2 yrs

Of all genetic and environmental factors, the environmental factor ''siblings'' was selected by MDR as best predictor in the single-factor model (p-value after 1,000 permutations of the data (p_{perm})=0.004; table 2). Children having two or more older siblings had an increased risk for high IgE at age 1–2 yrs compared with one or no older siblings after birth.

TABLE 2 Results of multifactor dimensionality reduction analysis evaluating Toll-like receptors (TLRs), TLR-related genes and proxy measurements of high microbial exposure

Number of variables in model	Best model		Mean CV consistency	Mean prediction error %	p_{perm}[#]	OR (95% CI)[¶]	p-value
	Gene(s) and/or environmental factors	SNP(s)					
Total IgE age 1-2 yrs							
1	Siblings		8.2	0.44	0.004	2.3 (1.6–3.3)	0.000008
2	Siblings, *TLR6*	rs1039559	2.6	0.45	0.04	4.6 (2.1–9.7)	0.00008
3	Day-care, Siblings, *LBP*	rs2232596	3.0	0.43	0.007	NA[+]	
Total IgE 6–8 yrs							
1	*LBP*	rs745144	4.6	0.46	0.13	1.3 (1.0–1.7)	0.07
2	*TLR10*, *LY96* (*MD2*)	rs11096957, rs7838114	2.0	0.42	0.03	0.4 (0.2–0.6)	0.0006
3	Siblings, *CD14*, *LBP*	rs2915863, rs745144	2.4	0.45	0.03	NA[+]	
Specific IgE to indoor allergens 6–8 yrs							
1	*TLR1*	rs5743604	4.2	0.45	0.04	0.6 (0.5–0.9)	0.005
2	*TLR6*, *TLR6*	rs5743798, rs5743810	4.4	0.45	0.08	0.7 (0.3–1.5)	0.5
3	*TLR1*, *LBP*, *TLR5*	rs5743594, rs6025085, rs851186	4.2	0.40	0.02	NA[+]	

SNP: single-nucleotide polymorphism; CV: cross validation; Ig: immunoglobulin; LBP: lipopolysaccharide-binding protein; NA: not available. [#]: significance of prediction error (empirical p-value based on 1,000 permutations); [¶]: after logistic regression with no or one older sibling and/or wild-type genotype in a recessive model used as the reference category; [+]: logistic regression was not performed on three-factor interactions.

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 different ages (for KOALA at age 1 and 2 yrs, for PIAMA and PREVASC separately at age 4 yrs and for PIAMA at age 8 yrs (fig. 2). For PIAMA at age 1 yr and PREVASC at age 1, 2 and 6 yrs there was a similar trend, but this was not significant (fig. 2).

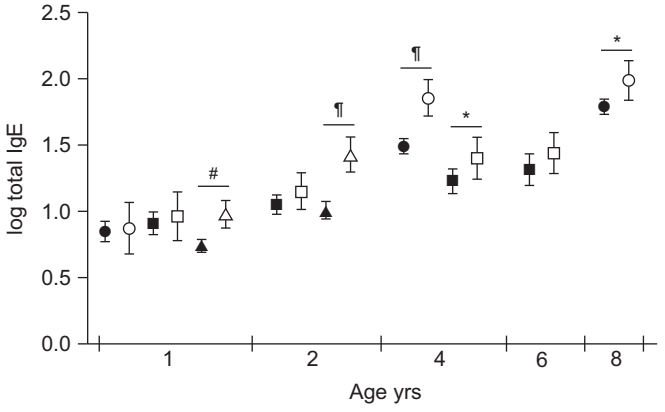

FIGURE 2. Mean immunoglobulin (Ig)E levels at age 1, 2, 4 and 8 yrs in the three birth cohorts: children with no or one older sibling (●, ■ and ▲) compared with children with two or more older siblings (○, □ and △). Circles: PIAMA; squares: PREVASC; triangles: KOALA. Error bars represent 95% confidence intervals. *: $p \leqslant 0.05$; [#]: $p \leqslant 5 \times 10^{-4}$; [¶]: $p \leqslant 5 \times 10^{-7}$.

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

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

Total IgE at age 6–8 yrs

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

TLR10 (rs11096957) and *MD2* (rs7838114) were selected as the best two-factor model ($p_{perm}=0.03$) (table 2). In particular, children carrying the minor allele of rs7838114 and the wild-type allele of rs11096957 were protected from the development of high IgE at age 6–8 yrs. Siblings and *TLR10* (rs11096957) emerged as the best predictive gene–environment interaction. However, this was not statistically significant.

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

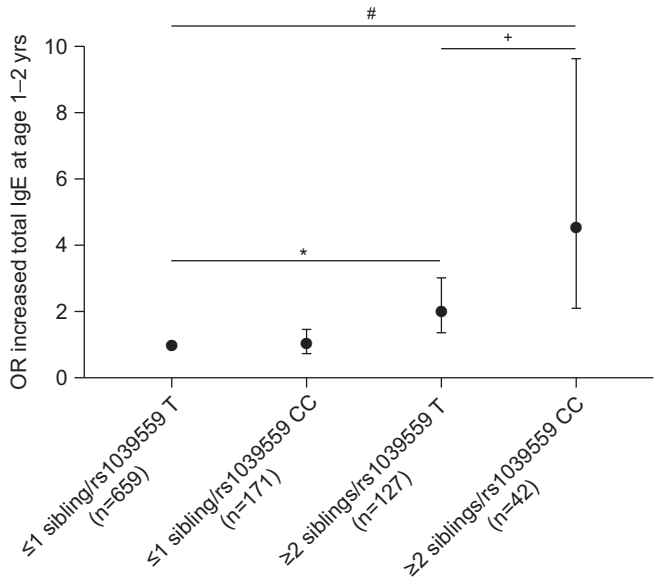

FIGURE 3. Gene–environment interaction of Toll-like receptor (*TLR*)*6* (rs1039559) and older sibling exposure. *: $p \leq 0.05$; #: $p \leq 5 \times 10^{-4}$; +: $p = 0.06$.

Specific IgE to indoor allergens at 6–8 yrs

TLR1 (rs5743604) was selected by MDR as the best predictor in the single-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 two-factor model, but this was not significant ($p_{perm} = 0.08$) (table 2). Siblings and *TLR10* (rs11096957) emerged as the best gene–environment interaction, but this was not significant.

TLR1 (rs5743594), *LBP* (rs6025083) and *TLR5* (rs851186) were selected by MDR as the best three-factor model ($p_{perm} = 0.02$), which indicated a complex three-way gene–gene interaction (table 2).

DISCUSSION

To our knowledge, this is the first study that identifies genes, environmental factors and gene–environment interactions of TLRs 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 two or more older siblings and increased total serum IgE levels. This was observed at four different ages in all three birth cohorts (fig. 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 [21], who showed a protective effect of larger sibship size on the development of hay fever. Thereafter, 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, only a few studies have reported 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

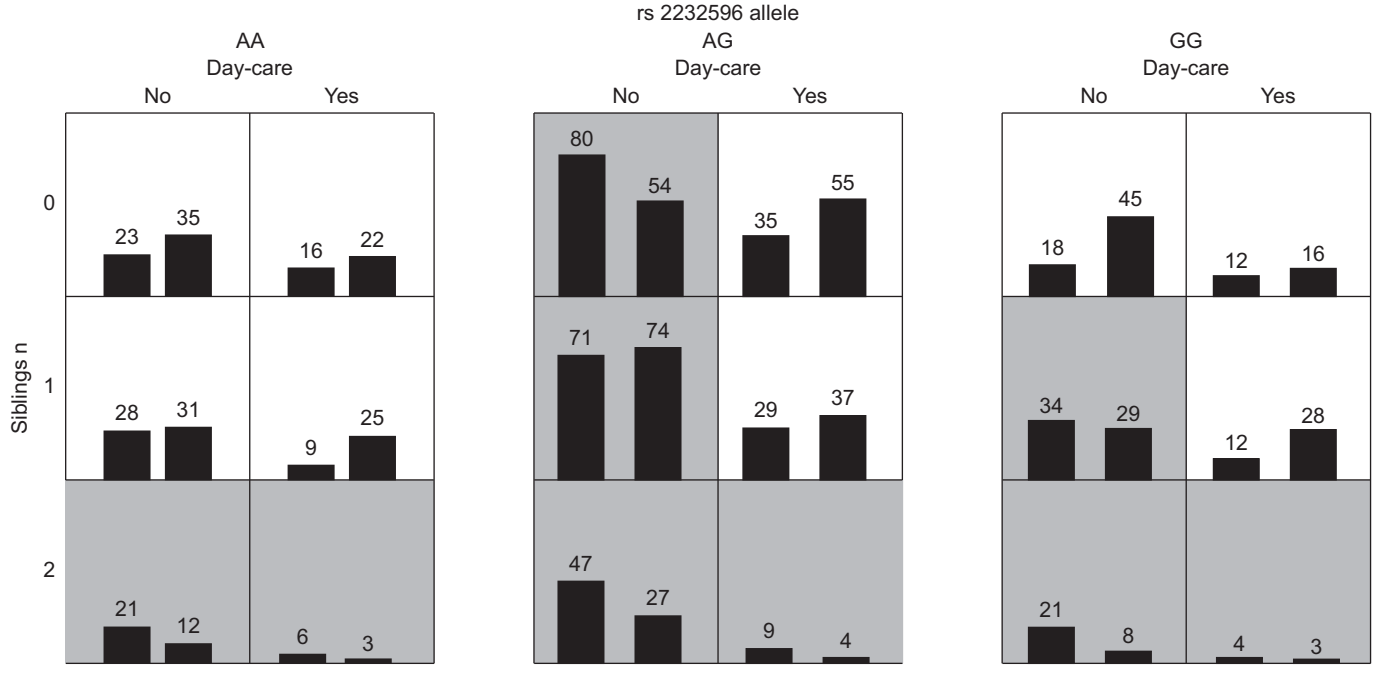

FIGURE 4. Interaction observed in multifactor dimensionality reduction analysis between day-care, older sibling exposure and lipopolysaccharide binding protein (*LBP*) (rs2232596). Cells with a light background show combinations with decreased risk of developing high total immunoglobulin (Ig)E, cells with a dark background show combinations with an increased risk of developing high total IgE. AA, AG and GG represent the alleles of *LBP* (rs2232596). Numbers in graphs represent number of subjects with specific genotype and exposures.

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 has been reported [22, 28]. 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 these should include a comparison of total serum IgE.

We additionally showed that gene–environment interaction exists between an SNP located in *TLR6* and sibship size with respect to the development of total IgE at age 1–2 yrs. TLR6 forms a heterodimer with TLR2 and TLR2/6 heterodimers recognise lipopeptides, often found in the upper respiratory tract. A recent study by KORMANN *et al.* [29] 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 Th1 cytokine expression and reduced Th2 cytokine production after stimulation with the TLR6 ligand [29]. In our data, a gene–environment interaction became apparent with an SNP (rs1039559) not genotyped by KORMANN *et al.* [29]. We showed an increased risk for high total IgE in children carrying the minor allele of rs1039559 and having two or more older siblings. Since this SNP was not investigated by KORMANN *et al.* [29], it is difficult to compare our results. However, our data lend further support to the role of *TLR6* in atopic diseases and indicate 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 against the development of high total IgE levels at age 1–2 yrs, predominantly in children heterozygous for rs2232596, but not in children with more than one older sibling. The protective effect of day-care on total IgE development has been shown before [30, 31]. We confirm and extend this observation by showing that the ''day-care effect'' also depends on genetics 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 three-factor gene–environment interaction for total IgE at age 1–2 yrs is located in the *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 [32]. To our knowledge, no associations with SNPs located in LBP and atopic phenotypes have been published before. MICHEL *et al.* [33] 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) [33]. We hypothesise 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 has 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 Th1 instead of Th2 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 yrs than at age 6–8 yrs.

We and others have previously shown 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 [9, 12]. This interaction did not emerge 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 realise that MDR identifies the models that describe the data best, but that it does not exclude other models.

To date, it is not known exactly 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–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 (combinations 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 has made the analysis of complex gene–gene and gene–environment interaction feasible, since it is designed to compress multidimensional genetic data into a single dimension [13]. This is a promising method to analyse complex interactions in large datasets [34]. 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 that 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. Nevertheless, bias resulting from the presence of three populations cannot 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 TLR7 and TLR8 in future analysis. It would also have been of considerable interest to analyse the same SNPs studied 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 acknowledge that replication of these results is crucial, particularly as we were

unable to perform an independent replication study. We chose instead to analyse the entire cohort to optimise our study's 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 analysing the protective effect of farm life in central Europe [1]. Our study did not include enough children living on farms 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 two- and three-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 carried out to replicate these findings, and functional studies could be useful to investigate the exact biological mechanism of these gene–environment interactions.

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STATEMENT OF INTEREST

A statement of interest for D.S. Postma can be found at www.erj.ers journals.com/site/misc/statements.xhtml

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