

## ORIGINAL ARTICLE

Polymorphisms in the *TLR6* gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease

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Little is known about the etiology of childhood acute lymphoblastic leukemia (ALL). The presence of atopic disease has been shown to protect against developing childhood ALL. The aim of this study was to examine whether single nucleotide polymorphisms (SNPs) in innate immunity genes previously associated with atopic disease, can elucidate the inverse association between childhood ALL and atopic disease. We studied 525 children, including 192 with childhood ALL, 149 with atopic disease and 184 healthy control subjects. We compared genotype distributions of 29 SNPs in genes of *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10* and *CD14* between the three groups and corrected for multiple testing. The genotype distributions of two SNPs in the *TLR6* gene, rs5743798 and rs6531666, differed significantly between children with ALL, children with atopic disease and control subjects. Particularly in children with atopic eczema, risk alleles for atopic disease were observed more often than in control subjects, and less often in children with ALL than in control subjects. These findings support the immune surveillance hypothesis as an explanation for the protective association of atopic disease on childhood ALL. Further investigation is warranted to examine in more detail the role of innate immunity in the development of childhood ALL.

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**Keywords:** childhood acute lymphoblastic leukemia; atopic disease; *TLR6*; polymorphisms; innate immunity

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common form of cancer among children, which accounts for about 30% of all cancer cases in children under the age of 15 years. There is a specific age peak at 2–5 years, mainly due to an excess of pre-B ALL cases in this age range. Little is known about the etiology of childhood ALL. Interestingly, there is growing evidence of an inverse association between childhood ALL and atopic disease.<sup>1–3</sup> Many epidemiological studies have shown a protective association of atopic disease on the risk of childhood ALL. The presence of an atopic condition is thought to increase the vigilance of the immune system in monitoring for, identifying and eliminating malignant cells.<sup>4</sup> Therefore, atopy may lead to early elimination of malignant cells and thus prevent the development of malignancies like childhood ALL.

A multifactorial background has been suggested for atopic disease with genetic as well as environmental factors contributing to disease susceptibility. Various studies examined the role of innate immunity genes on the risk of atopic disease. Particularly single nucleotide polymorphisms (SNPs) in *toll-like receptor 2* (*TLR2*), *TLR4*, *TLR6*, *TLR9*, *TLR10* and *cluster of differentiation 14*

(*CD14*)<sup>5–9</sup> have been associated with atopic disease. TLRs are membrane receptors that act as the gatekeepers of the innate immunity by recognizing microbial components and initiating activation of an adequate immune response. *CD14* acts as a co-receptor, along with *TLR4*, for the detection of bacterial lipopolysaccharide. Variations in genes encoding *TLRs* and *CD14* are suggested to alter the capability to recognize microbes or alter the amount of gene product, leading to inadequate immune responses and increasing the susceptibility for atopic disease.<sup>5,9</sup> In the case of childhood ALL, much less is known about the role of genes involved in innate immunity.<sup>10</sup> Examining the genetic basis of the inverse association between childhood ALL and atopic disease might help towards elucidating the etiology of childhood ALL.

The aim of this study was to examine whether genetic variations in the genes of *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10* and *CD14* are associated with the inverse association between childhood ALL and atopic disease. We hypothesized that risk alleles for atopic disease are observed more often in atopic children than in healthy controls, and are observed less often in children with ALL than in healthy controls.

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## MATERIALS AND METHODS

### Study population

This study was conducted in three cohorts. The first cohort included 210 children, aged 0–15 years, with ALL who were diagnosed between 1984 and 2001 in Beatrix Children's Hospital of University Medical Center Groningen in Groningen, the Netherlands, or between 1997 and 2001 in Wilhelmina Children's Hospital of University Medical Center Utrecht in Utrecht, The Netherlands. Eighteen subjects were excluded because of their non-Caucasian background.

The second cohort included 149 atopic children, aged 0–8 years, derived from the PIAMA study,<sup>11</sup> see Supplementary Table S1. The PIAMA study is a prospective birth cohort study on Prevention and Incidence of Asthma and Mite Allergy. The participating children were born between May 1996 and December 1997 in three regions of the Netherlands. They were selected using a validated screening questionnaire with questions on asthma and respiratory allergies administered to the mother at her first visit to the midwife. The midwives were requested to ask every new pregnant woman to fill in the screening questionnaire. A total of 10 232 pregnant women completed the questionnaire and 2949 (29%) of them reported asthma or respiratory allergy and were defined as 'allergic'. Based on this screening, 7862 women were invited to participate, and 4146 agreed and gave informed consent. After birth, the baseline study population consisted of 3963 children. All children from allergic mothers ( $n = 1327$ ) and a random sample of children from non-allergic mothers ( $n = 663$ ) were selected for more extensive investigation, including DNA collection at 4 years of age. DNA was obtained from 1046 of these children and genotypes were obtained from 1037 children. Children with a non-Caucasian ethnicity were excluded from the analyses ( $n = 51$ ). Atopic disease was determined by elevated specific IgE levels ( $\geq 0.70$  IU/ml) and/or positive allergy skin test, in combination with asthma and/or eczema, as these more stringent definitions of atopy were used in epidemiological studies observing a reverse association between childhood ALL and atopy. Specific IgE to mite (*Dermatophagoides pteronyssinus*), cat (Fel d1), dog (Can f1), grass (*Dactylis glomerata*), birch (*Betula verrucosa*) and mould (*Alternaria alternata*) was measured by radioallergosorbent test. In PIAMA, allergy skin tests were performed with: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria alternata*, mixed grass pollen, mixed tree pollen, cat and dog. A positive skin test was defined as a mean wheal diameter of  $\geq 3$  mm in response to one or more allergens provided the diameter was negative ( $< 3$  mm) and the positive control was positive ( $\geq 3$  mm). Asthma was defined as an episode of wheezing or dyspnea, or use of inhalation steroids in the past 12 months at the age of 8 years. Eczema was defined as having reported an itching rash at least twice in the past 12 months, intermittently present at the usual eczema locations (for example, elbows, back of the knees, front of the ankles, around the eyes or ears, in the neck), for at least 2 years in a row in the age 0–8 years. In all, 82 children (55%) were diagnosed with atopic asthma, and 115 (77.2%) with atopic eczema. Forty-eight of the children (32%) were diagnosed both with asthma and eczema.

The third cohort consisted of 184 healthy control children, aged 0–8 years, also derived from the PIAMA cohort. These children did not have positive skin tests, their specific serum IgE levels were not increased ( $< 0.70$  IU/ml), and they were not diagnosed with either asthma or eczema.

The local medical ethics committees of participating institutes approved all studies. Written informed consent was obtained from all participants and/or their parents.

### Genotyping

Genomic DNA was extracted from buccal swabs, blood or bone-marrow samples. DNA extraction from blood or bone-marrow samples was performed by using the NucleoSpin<sup>®</sup> Blood L kit (Macherey-Nagel, Düren, Germany). For frozen samples the NucleoSpin Tissue kit (Macherey-Nagel) was used. Both kits were used according to the manufacturer's protocol. For DNA isolation from buccal swabs, the protocol described in Meulenbelt et al.<sup>12</sup> was used. DNA was amplified using REPLI-g UltraFast technology (Qiagen, Venlo, The Netherlands). We selected (potential) functional SNPs complemented with known haplotype-tagging SNPs in the genes for *TLR2*

(rs3804099, rs3804100, rs4969480), *TLR4* (rs4986790, rs6478317, rs10759932, rs11536878, rs11536889, rs1927911, rs10759931, rs2770150), *TLR6* (rs1039559, rs5743788, rs5743810, rs6531666, rs5743798), *TLR9* (rs352140, rs187084, rs5743836), *TLR10* (rs4274855, rs10856839, rs11096956, rs11096957, rs11466652, rs4129009) and *CD14* genes (rs2563298, rs2569190, rs256191, rs5744455). Genotyping was performed by Competitive Allele-Specific PCR using KASPar genotyping chemistry, performed under contract by K-Biosciences (Herts, UK). Quality of genotype data was guaranteed by standards of K-Biosciences with extensive quality control as described before.<sup>13</sup>

The genotype data of the healthy control subjects were analyzed for deviations from Hardy-Weinberg equilibrium using 1 degree of freedom chi-square tests. None of the SNPs deviated from HWE ( $P < 0.01$ ). On an average, the call rate was 97%, but one SNP (rs6531666) had a call rate of 86%. We therefore performed an extensive quality control on this SNP: the allele frequencies were similar between the cohorts and compared with near-lying SNPs ( $P > 0.05$ ), and genotypes were in Hardy-Weinberg equilibrium ( $P \geq 0.01$ ). Thus, as the quality of this SNP was good, we included it in the analyses. The 29 SNPs selected for *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10* and *CD14*, data source and allele frequencies are shown in Table 1. Pair-wise linkage disequilibrium (LD) was assessed between single SNPs by using the  $r^2$  statistic as implemented in Haploview version 4.1 (Broad Institute, Cambridge, UK).

### Data analyses

Differences in distribution of genotype and allele frequencies were tested using 4 degree of freedom chi-square test. To account for multiple testing, we calculated the false discovery rate as described by Benjamini and Hochberg,<sup>14</sup> using a  $P$ -value of 0.05 as a cutoff value. Further, we performed 10 000 permutation tests to correct for false-positives, where we compared the genotype distributions between ALL versus atopic patients for studied SNPs using permute module, implemented in PLINK software (free software, available at <http://pngu.mgh.harvard.edu/purcell/plink/>). The two subgroups in the atopic cohort, that is, asthmatic children and eczematous children, were analyzed separately in an additive model by using chi-square statistics. Subsequently, we compared children with childhood ALL and children with atopic disease separately with healthy controls by using logistic regression, to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) for the best-fitting model (that is, dominant, recessive or additive).  $P$ -values  $< 0.05$  were considered statistically significant. The statistical software package used was SPSS 16.0 (SPSS, Chicago, IL, USA).

## RESULTS

### Study population

Table 2 shows the patient characteristics of the three cohorts. A total of 525 children were included in the study: 192 children with childhood ALL (61% male), 149 children with atopic disease (55% male) and 184 healthy control children (48% male). There was a significant difference in sex distribution among the three cohorts ( $P = 0.041$ ).

### Association analysis

Table 3 shows the genotype and allele frequencies of the 29 SNPs among the three cohorts. The genotype distribution of two SNPs in the *TLR6* gene, rs6531666 ( $P = 0.0025$ ) and rs5743798 ( $P = 0.00047$ ), was significantly different among children with ALL, control subjects and children with atopic disease. Both SNPs sustained correction for multiple testing. When correcting for sex, both rs6531666 and rs5743798 remained significantly different among the three cohorts ( $P = 0.0035$  and  $P = 0.0006$ , respectively). The largest differences in genotype distributions were observed between children with ALL and children with atopic disease. The minor alleles of both rs6531666 and rs5743798 were associated with an increased risk of atopic disease when comparing with the controls, and at the same time associated with a decreased risk of

**Table 1.** SNPs genotyped in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10* and *CD14*, and their location, minor allele frequency, call rate and deviation from Hardy-Weinberg equilibrium in controls ( $n = 184$ )

| Gene         | Location | SNP        | Allele | SNP type                            | MAF  | Call rate | HWE P-value |
|--------------|----------|------------|--------|-------------------------------------|------|-----------|-------------|
| <i>TLR2</i>  | 4q32     | rs3804099  | T/C    | Ht, exon, synonymous, 199Asn        | 0.47 | 96        | 0.03        |
|              |          | rs3804100  | T/C    | Ht, exon, synonymous, 450Ser        | 0.06 | 98        | 0.48        |
|              |          | rs4696480  | T/A    | Ht, promoter                        | 0.46 | 98        | 0.35        |
| <i>TLR4</i>  | 9q32-q33 | rs4986790  | A/G    | Exon, nonsynonymous, Asp299Gly      | 0.07 | 97        | 0.26        |
|              |          | rs6478317  | A/G    | Ht, promoter                        | 0.32 | 98        | 0.35        |
|              |          | rs10759932 | T/C    | Ht, promoter                        | 0.11 | 97        | 0.94        |
|              |          | rs11536878 | C/A    | Ht, intron                          | 0.12 | 98        | 0.32        |
|              |          | rs11536889 | G/C    | Ht, 3'UTR                           | 0.15 | 98        | 0.11        |
|              |          | rs1927911  | C/T    | Ht, intron                          | 0.24 | 99        | 0.10        |
|              |          | rs10759931 | G/A    | Ht, promoter                        | 0.40 | 95        | 0.11        |
|              |          | rs2770150  | T/C    | Ht, promoter                        | 0.28 | 96        | 0.36        |
| <i>TLR6</i>  | 4p14     | rs1039559  | T/C    | Ht, intron                          | 0.47 | 95        | 0.72        |
|              |          | rs5743788  | C/G    | Ht, intron                          | 0.48 | 97        | 0.37        |
|              |          | rs5743810  | C/T    | Ht, exon, nonsynonymous, Ser249Pro  | 0.41 | 96        | 0.19        |
|              |          | rs6531666  | T/C    | Ht, intron                          | 0.28 | 86        | 0.23        |
|              |          | rs5743798  | C/T    | Ht, intron                          | 0.25 | 96        | 0.72        |
| <i>TLR9</i>  | 3p21.3   | rs352140   | T/C    | Ht, exon, synonymous, G2848A        | 0.43 | 97        | 0.85        |
|              |          | rs187084   | T/C    | Ht, intron                          | 0.45 | 96        | 0.15        |
|              |          | rs5743836  | T/C    | Ht, intron                          | 0.14 | 97        | 0.59        |
| <i>TLR10</i> | 4p14     | rs4274855  | G/A    | Ht, 5'UTR                           | 0.20 | 98        | 0.18        |
|              |          | rs10856839 | A/C    | Ht, 5'UTR                           | 0.17 | 97        | 0.21        |
|              |          | rs11096956 | G/T    | Ht, exon, synonymous                | 0.24 | 95        | 0.79        |
|              |          | rs11096957 | A/C    | Ht, exon, non-synonymous, Asn241His | 0.39 | 95        | 0.71        |
|              |          | rs11466652 | A/G    | Ht, synonymous                      | 0.14 | 97        | 0.76        |
|              |          | rs4129009  | A/G    | Ht, exon, Ile2322Val                | 0.20 | 98        | 0.31        |
| <i>CD14</i>  | 5q31.1   | rs2563298  | C/A    | Ht, 3'UTR                           | 0.26 | 98        | 0.09        |
|              |          | rs2569190  | G/A    | Ht, 5'UTR                           | 0.45 | 97        | 0.05        |
|              |          | rs2569191  | T/C    | Ht, promoter                        | 0.46 | 98        | 0.08        |
|              |          | rs5744455  | C/T    | Ht, promoter                        | 0.26 | 98        | 0.12        |

Abbreviations: Ht, haplotype-tagging; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism; UTR, untranslated region.

**Table 2.** Patient characteristics of the three cohorts

|                                 | ALL<br>N (%) | Controls<br>N (%) | Atopic<br>disease<br>N (%) | P-<br>value |
|---------------------------------|--------------|-------------------|----------------------------|-------------|
| Total number of patients        | 192          | 184               | 149                        |             |
| Male (%)                        | 115 (61%)    | 88 (48%)          | 82 (55%)                   | 0.041       |
| Age, years-median (range)       | 4.4 (0-15)   | 8 <sup>a</sup>    | 8 <sup>a</sup>             |             |
| <i>ALL subtype</i>              |              |                   |                            |             |
| B-lineage ALL                   | 163 (84.9%)  |                   |                            |             |
| T-lineage ALL                   | 20 (10.4%)   |                   |                            |             |
| Infant ALL                      | 2 (1.0%)     |                   |                            |             |
| Undifferentiated                | 2 (1.0%)     |                   |                            |             |
| Unknown                         | 5 (2.6%)     |                   |                            |             |
| <i>Cytogenetic aberrations</i>  |              |                   |                            |             |
| Hyperdiploid (> 50 chromosomes) | 50 (26.0%)   |                   |                            |             |
| T(12;21)                        | 20 (10.4%)   |                   |                            |             |
| <i>Atopic symptom</i>           |              |                   |                            |             |
| Asthma                          |              |                   | 82 (55%)                   |             |
| Eczema                          |              |                   | 115 (77%)                  |             |
| Asthma and eczema               |              |                   | 48 (32%)                   |             |
| Elevated IgE levels             |              |                   | 145 (97%)                  |             |
| Positive Skin Tests             |              |                   | 115 (77%)                  |             |

Abbreviation: ALL, acute lymphoblastic leukemia. <sup>a</sup>The control and atopic children were examined annually until the age of 8 years.

ALL when comparing with the controls. After 10 000 permutation tests, both rs6531666 ( $P = 0.013$ ) and rs5743798 ( $P = 0.0004$ ) sustained their significant differences in their genotype frequencies between ALL and atopic patients (see Supplementary Table S2). SNPs in the other *TLR* and/or *CD14* genes were not associated with childhood ALL or atopy.

The frequencies of the minor alleles in rs6531666 and rs5743798 were significantly different among the three groups. For rs6531666, the frequency of the minor allele was 23% in children with ALL, 29% in the control subjects and 33% in the atopic children ( $P = 0.028$ ). For rs5743798, the frequency of the minor allele was 19% in children with ALL, 25% in the healthy controls and 34% in the atopic children ( $P = 0.0001$ , which sustained correction for multiple testing).

Figure 1 presents the genotype effects (ORs and 95% CIs) of the heterozygous and homozygous minor allele genotype of *TLR6* SNPs rs6531666 and rs5743798 compared with the homozygous major allele genotype on the risk of ALL and atopic disease, using the healthy control subjects as reference group. Subjects homozygous for the minor allele (C) of rs6531666 showed a borderline significant decreased risk of childhood ALL (OR 0.39, 95% CI, 0.12-1.20,  $P = 0.099$ ), whereas they had significantly increased risk of atopic disease (OR 2.61, 95% CI, 1.07-6.39,  $P = 0.035$ ). The same trend was observed for subjects homozygous for the minor allele (T) of rs5743798, with a borderline significant decreased risk of childhood ALL (OR 0.42, 95% CI, 0.14-1.28,  $P = 0.13$ ) and a significantly increased risk of atopic disease (OR 2.88, 95% CI, 1.26-6.58,  $P = 0.012$ ).

As the atopic children and the control subjects were examined annually until the age of 8, we also performed the analysis

**Table 3.** Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10* and *CD14* among children with ALL, healthy control subjects and children with atopic disease

| Gene        | SNP        | Genotype | ALL N (%) | Control N (%) | Atopic disease N (%) | P-value        | FDR                |
|-------------|------------|----------|-----------|---------------|----------------------|----------------|--------------------|
| <i>TLR2</i> | rs3804099  | TT       | 51 (28)   | 48 (27)       | 35 (24)              |                |                    |
|             |            | TC       | 94 (52)   | 102 (57)      | 77 (53)              |                |                    |
|             |            | CC       | 37 (20)   | 28 (16)       | 34 (23)              | 0.472          | NS                 |
|             |            | C allele | 46%       | 44%           | 50%                  | 0.401          | NS                 |
|             | rs3804100  | TT       | 170 (90)  | 165 (90)      | 120 (84)             |                |                    |
|             |            | TC       | 18 (10)   | 18 (10)       | 22 (15)              |                |                    |
|             |            | CC       | 1 (1)     | 0 (0)         | 1 (1)                | 0.331          | NS                 |
|             |            | C allele | 5%        | 5%            | 8%                   | 0.136          | NS                 |
|             | rs4696480  | TT       | 42 (23)   | 60 (33)       | 52 (36)              |                |                    |
|             |            | TA       | 99 (54)   | 83 (46)       | 65 (45)              |                |                    |
|             |            | AA       | 44 (24)   | 38 (21)       | 29 (20)              | 0.102          | NS                 |
|             |            | A allele | 51%       | 44%           | 42%                  | 0.065          | NS                 |
| <i>TLR4</i> | rs4986790  | AA       | 168 (89)  | 151 (85)      | 126 (88)             |                |                    |
|             |            | AG       | 20 (11)   | 28 (16)       | 17 (12)              |                |                    |
|             |            | GG       | 0 (0)     | 0 (0)         | 1 (1)                | 0.314          | NS                 |
|             |            | G allele | 5%        | 8%            | 7%                   | 0.392          | NS                 |
|             | rs6478317  | AA       | 83 (44)   | 85 (47)       | 73 (50)              |                |                    |
|             |            | AG       | 82 (44)   | 74 (41)       | 59 (41)              |                |                    |
|             |            | GG       | 22 (12)   | 22 (12)       | 13 (9)               | 0.785          | NS                 |
|             |            | G allele | 34%       | 33%           | 29%                  | 0.470          | NS                 |
|             | rs10759932 | TT       | 140 (75)  | 144 (80)      | 120 (83)             |                |                    |
|             |            | TC       | 45 (24)   | 33 (18)       | 25 (17)              |                |                    |
|             |            | CC       | 1 (1)     | 3 (2)         | 0 (0)                | 0.192          | NS                 |
|             |            | C allele | 13%       | 11%           | 9%                   | 0.398          | NS                 |
|             | rs11536878 | CC       | 150 (79)  | 139 (78)      | 114 (77)             |                |                    |
|             |            | CA       | 35 (18)   | 39 (22)       | 29 (20)              |                |                    |
|             |            | AA       | 5 (3)     | 1 (1)         | 5 (3)                | 0.411          | NS                 |
|             |            | A allele | 12%       | 11%           | 13%                  | 0.784          | NS                 |
|             | rs11536889 | GG       | 134 (72)  | 129 (72)      | 104 (70)             |                |                    |
|             |            | GC       | 44 (24)   | 49 (27)       | 42 (28)              |                |                    |
|             |            | CC       | 7 (4)     | 1 (1)         | 2 (1)                | 0.195          | NS                 |
|             |            | C allele | 16%       | 14%           | 16%                  | 0.843          | NS                 |
|             | rs1927911  | CC       | 111 (58)  | 109 (61)      | 92 (63)              |                |                    |
|             |            | CT       | 65 (34)   | 57 (32)       | 46 (31)              |                |                    |
|             |            | TT       | 15 (8)    | 14 (8)        | 9 (6)                | 0.919          | NS                 |
|             |            | T allele | 25%       | 24%           | 22%                  | 0.641          | NS                 |
|             | rs10759931 | GG       | 70 (38)   | 62 (35)       | 48 (34)              |                |                    |
|             |            | GA       | 87 (48)   | 76 (43)       | 71 (51)              |                |                    |
|             |            | AA       | 26 (14)   | 38 (22)       | 21 (15)              | 0.318          | NS                 |
|             |            | A allele | 38%       | 43%           | 40%                  | 0.364          | NS                 |
|             | rs2770150  | TT       | 98 (54)   | 92 (53)       | 67 (46)              |                |                    |
|             |            | TC       | 69 (38)   | 73 (42)       | 63 (43)              |                |                    |
|             |            | CC       | 15 (8)    | 10 (6)        | 15 (10)              | 0.429          | NS                 |
|             |            | C allele | 27%       | 27%           | 32%                  | 0.252          | NS                 |
| <i>TLR6</i> | rs1039559  | TT       | 47 (26)   | 53 (30)       | 44 (31)              |                |                    |
|             |            | TC       | 87 (48)   | 85 (48)       | 69 (49)              |                |                    |
|             |            | CC       | 47 (26)   | 38 (22)       | 29 (20)              | 0.716          | NS                 |
|             |            | C allele | 50%       | 46%           | 45%                  | 0.345          | NS                 |
|             | rs5743788  | CC       | 45 (25)   | 49 (27)       | 43 (30)              |                |                    |
|             |            | CG       | 92 (50)   | 96 (53)       | 69 (47)              |                |                    |
|             |            | GG       | 47 (26)   | 36 (20)       | 34 (23)              | 0.628          | NS                 |
|             |            | G allele | 51%       | 46%           | 47%                  | 0.483          | NS                 |
|             | rs5743810  | CC       | 63 (35)   | 61 (34)       | 54 (38)              |                |                    |
|             |            | CT       | 82 (45)   | 94 (53)       | 66 (46)              |                |                    |
|             |            | TT       | 36 (20)   | 24 (13)       | 23 (16)              | 0.437          | NS                 |
|             |            | T allele | 43%       | 40%           | 39%                  | 0.625          | NS                 |
|             | rs6531666  | TT       | 104 (56)  | 72 (49)       | 49 (41)              |                |                    |
|             |            | TC       | 76 (41)   | 66 (45)       | 54 (45)              |                |                    |
|             |            | CC       | 5 (3)     | 9 (6)         | 15 (13)              | <b>0.0025</b>  | <b>Significant</b> |
|             |            | C allele | 23%       | 29%           | 33%                  | <b>0.0283</b>  |                    |
|             | rs5743798  | CC       | 118 (65)  | 100 (56)      | 66 (46)              |                |                    |
|             |            | CT       | 60 (33)   | 68 (38)       | 59 (41)              |                |                    |
|             |            | TT       | 5 (3)     | 10 (6)        | 19 (13)              | <b>0.00047</b> | <b>Significant</b> |
|             |            | T allele | 19%       | 25%           | 34%                  | <b>0.00011</b> |                    |
| <i>TLR9</i> | rs352140   | TT       | 67 (37)   | 57 (32)       | 45 (31)              |                |                    |
|             |            | TC       | 85 (46)   | 88 (49)       | 74 (51)              |                |                    |
|             |            | CC       | 31 (17)   | 36 (20)       | 26 (18)              | 0.772          | NS                 |
|             |            | C allele | 40%       | 44%           | 43%                  | 0.509          | NS                 |

**Table 3** (Continued)

| Gene  | SNP        | Genotype | ALL N (%) | Control N (%) | Atopic disease N (%) | P-value | FDR |
|-------|------------|----------|-----------|---------------|----------------------|---------|-----|
| TLR10 | rs187084   | TT       | 57 (31)   | 50 (28)       | 44 (30)              |         |     |
|       |            | TC       | 88 (48)   | 98 (55)       | 71 (49)              |         |     |
|       |            | CC       | 37 (20)   | 31 (17)       | 30 (21)              | 0.767   | NS  |
|       |            | C allele | 45%       | 45%           | 45%                  | 0.985   | NS  |
|       | rs5743836  | TT       | 133 (72)  | 138 (76)      | 107 (75)             |         |     |
|       |            | TC       | 45 (24)   | 40 (22)       | 34 (24)              |         |     |
|       |            | CC       | 7 (4)     | 4 (2)         | 2 (1)                | 0.667   | NS  |
|       |            | C allele | 16%       | 13%           | 13%                  | 0.490   | NS  |
|       | rs4274855  | GG       | 123 (64)  | 112 (62)      | 100 (69)             |         |     |
|       |            | GA       | 54 (28)   | 56 (31)       | 42 (29)              |         |     |
|       |            | AA       | 14 (7)    | 12 (7)        | 3 (2)                | 0.245   | NS  |
|       |            | A allele | 21%       | 22%           | 17%                  | 0.161   | NS  |
|       | rs10856839 | AA       | 131 (69)  | 124 (69)      | 97 (69)              |         |     |
|       |            | AC       | 55 (29)   | 47 (26)       | 39 (28)              |         |     |
|       |            | CC       | 3 (2)     | 8 (5)         | 5 (4)                | 0.592   | NS  |
|       |            | C allele | 17%       | 18%           | 17%                  | 0.930   | NS  |
|       | rs11096956 | GG       | 110 (59)  | 98 (56)       | 86 (63)              |         |     |
|       |            | GT       | 65 (35)   | 65 (37)       | 47 (34)              |         |     |
|       |            | TT       | 13 (7)    | 12 (7)        | 4 (3)                | 0.472   | NS  |
|       |            | T allele | 24%       | 25%           | 20%                  | 0.270   | NS  |
|       | rs11096957 | AA       | 66 (36)   | 57 (33)       | 55 (39)              |         |     |
|       |            | AC       | 91 (50)   | 88 (51)       | 72 (51)              |         |     |
|       |            | CC       | 27 (15)   | 29 (17)       | 15 (11)              | 0.571   | NS  |
|       |            | C allele | 39%       | 42%           | 36%                  | 0.302   | NS  |
|       | rs11466652 | AA       | 136 (74)  | 130 (73)      | 111 (76)             |         |     |
|       |            | AG       | 46 (25)   | 45 (25)       | 31 (21)              |         |     |
|       |            | GG       | 2 (1)     | 4 (2)         | 5 (3)                | 0.588   | NS  |
|       |            | G allele | 14%       | 15%           | 14%                  | 0.890   | NS  |
|       | rs4129009  | AA       | 125 (66)  | 112 (62)      | 103 (71)             |         |     |
|       |            | AG       | 52 (27)   | 57 (32)       | 39 (27)              |         |     |
|       |            | GG       | 13 (7)    | 11 (6)        | 3 (2)                | 0.219   | NS  |
|       |            | G allele | 21%       | 22%           | 16%                  | 0.104   | NS  |
| CD14  | rs2563298  | CC       | 95 (51)   | 85 (47)       | 86 (59)              |         |     |
|       |            | CA       | 86 (46)   | 85 (47)       | 54 (37)              |         |     |
|       |            | AA       | 5 (3)     | 11 (6)        | 6 (4)                | 0.139   | NS  |
|       |            | A allele | 26%       | 30%           | 23%                  | 0.129   | NS  |
|       | rs2569190  | GG       | 58 (32)   | 53 (29)       | 36 (25)              |         |     |
|       |            | GA       | 80 (44)   | 101 (56)      | 82 (56)              |         |     |
|       |            | AA       | 42 (23)   | 26 (14)       | 29 (20)              | 0.078   | NS  |
|       |            | A allele | 46%       | 43%           | 48%                  | 0.413   | NS  |
|       | rs2569191  | TT       | 59 (32)   | 53 (29)       | 36 (25)              |         |     |
|       |            | TC       | 81 (44)   | 101 (56)      | 82 (56)              |         |     |
|       |            | CC       | 46 (25)   | 28 (15)       | 28 (15)              | 0.061   | NS  |
|       |            | C allele | 47%       | 43%           | 47%                  | 0.512   | NS  |
|       | rs5744455  | CC       | 108 (57)  | 96 (53)       | 72 (49)              |         |     |
|       |            | CT       | 66 (35)   | 77 (43)       | 66 (45)              |         |     |
|       |            | TT       | 14 (7)    | 8 (4)         | 9 (6)                | 0.312   | NS  |
|       |            | T allele | 25%       | 26%           | 29%                  | 0.554   | NS  |

Abbreviations: ALL, acute lymphoblastic leukemia; FDR, false discovery rate; NS, not significant; SNP, single nucleotide polymorphism. Bold values emphasize significance.

including only the children with ALL who were 0–8 years ( $n=157$ ). Although numbers were lower, this analysis still showed that the genotype distributions of the same two *TLR6* SNPs, that is, rs6531666 ( $P=0.008$ ) and rs5743798 ( $P=0.0009$ ), differed significantly among the three groups. Supplementary Table S3 shows the genotype distributions of the children with ALL who were 0–8 years, compared with the controls and the atopic children.

It has been suggested that different subtypes of ALL, in particular, B-lineage ALL, may have different etiologies. Therefore, we performed separate analyses for the groups of children with B-lineage ALL and the children with T-lineage ALL. The results for the group with B-lineage ALL ( $n=163$ ) were similar to the entire ALL group: the *TLR6* SNPs rs6531666 and rs5743798 were significantly different among the three groups ( $P=0.005$  and  $P=0.001$ , respectively). Only SNP rs5743798 sustained correction for multiple

testing. ORs did not reach significant levels. When comparing the group with T-lineage ALL ( $n=20$ ) to the controls and the atopic children, none of the SNPs sustained correction for multiple testing, which may be due to the small number of T-ALL patients.

Among the 149 children with atopic disease, 82 (55%) were diagnosed with asthma. When comparing the genotype frequencies of asthmatic children and children with ALL with the controls, the genotype distribution of both *TLR6* SNP rs6531666 and rs5743798 were significantly different among the three cohorts ( $P=0.01$  and  $P=0.02$ , respectively). Both SNPs did not sustain correction for multiple testing. Of the 149 atopic children, 115 (77%) were diagnosed with eczema. When comparing the children with eczema and the children with ALL with the control subjects, again both *TLR6* SNP rs6531666 and rs5743798 were significantly different among the three groups ( $P=0.001$  and  $P<0.001$ , respectively). Both SNPs sustained correction for multiple testing.

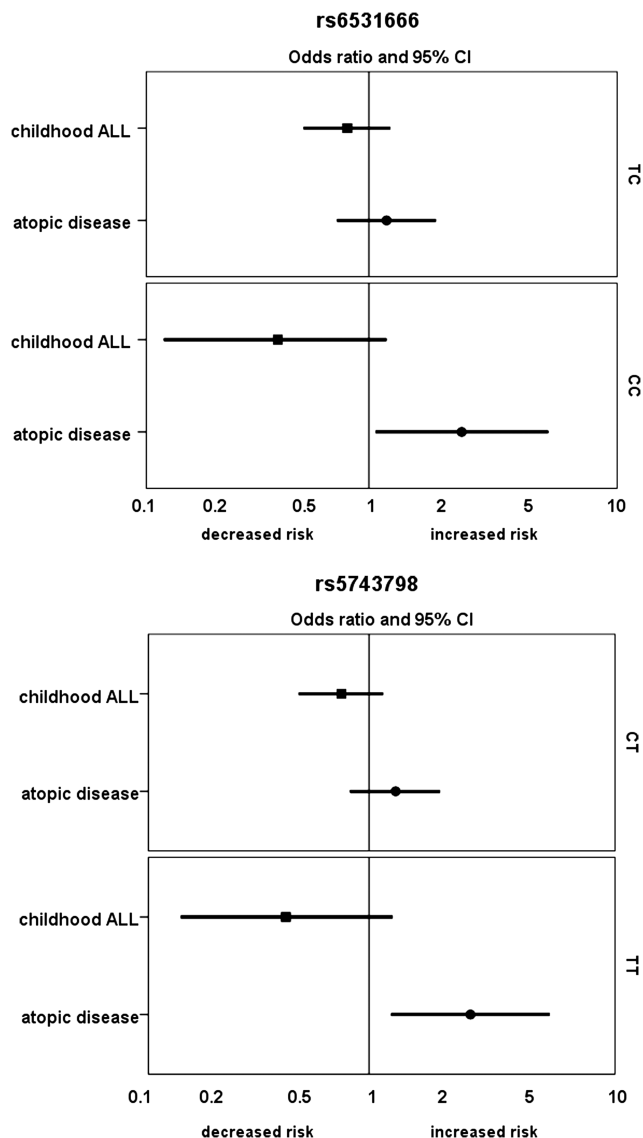


**Figure 1.** Genotype effects (OR and 95% CI) of the heterozygous and homozygous minor allele genotype of *TLR6* SNPs rs6531666 and rs5743798 compared with the homozygous major allele genotype on the risk of ALL and atopic disease, using the healthy control subjects as a reference group.

Finally, we constructed an LD plot of the *TLR6* gene, including the SNPs that were associated with the inverse association between childhood ALL and atopic disease. As can be seen in the LD plot of *TLR6*, rs5743798 and rs6531666 show moderately high LD with an  $r^2$  of 0.77 (Figure 2). Minor alleles of these SNPs were both associated with an increased risk of atopy and a decreased risk of childhood ALL, as would be expected with this LD.

## DISCUSSION

In the present study, we show that two polymorphisms in the *TLR6* gene are associated with the inverse association between childhood ALL and atopic disease. At the same time, compared with healthy controls, the minor alleles of these two *TLR6* SNPs are associated with an increased risk of atopic disease, as they are also associated with a decreased risk of childhood ALL. Ours was the first study to provide a possible genetic explanation for the

protective association of atopic disease on the occurrence of childhood ALL.

Different hypotheses have been proposed to explain the inverse relationship between childhood ALL and atopic disease. The principal factor linking childhood ALL and atopic disease seems to be the rate at which the immune system matures. Hypotheses proposed by Greaves and Kinlen suggest an etiological role for the immune system in the development of childhood leukemia via delayed exposure and abnormal response to childhood infections.<sup>15,16</sup> A hypothesis that has been proposed concerning the inverse association between atopic disease and childhood ALL is the so-called immune surveillance hypothesis. This hypothesis implies that the innate immune system recognizes antigens of malignant cells as foreign and mounts a response to them by triggering adaptive immune responses, which prevent a majority of potential cancers from developing.<sup>17</sup> As the vigilance of the immune system may be increased in the presence of atopic disease,<sup>4</sup> this could eliminate malignant cells, and prevent the development of malignancies, such as childhood ALL.

Atopic disease is associated with a predominance of T-helper 2 (Th2) cells, essential for the production of IgE, versus T helper 1 (Th1) cells. All infants are born with a Th2-dominated immune profile, characterized by interleukin 4 (IL-4), IL-5, IL-9, IL-10 and IL-13 production. By the age of 2 years, nonatopic infants have gradually migrated to a Th1-dominant profile, which is characterized by: IL-12, IL-18, interferon-gamma and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), whereas infants who develop atopy fail to make this Th2-to-Th1 transition. It has been suggested that one of the driving forces for this immune shift is microbial exposure, which induces innate immunity cells, such as dendritic cells, to produce cytokines important for the development of Th1 responses.<sup>18</sup> Dendritic cells express TLRs and are susceptible to TLR ligands, including microbial stimuli, as well as endogenous ligands. Genetic variation in *TLRs* may influence the activation of T-regulatory cells (responsible for suppressing Th2 responses), and/or skewing of the Th1–Th2 balance. We propose that the two SNPs in the *TLR6* gene as found in our study are related to an altered shift in the Th1–Th2 balance, causing an increased risk of developing atopic disease, which by means of increased vigilance protects against childhood ALL.

Ligands for TLR6 include diacyl lipopeptides from *Mycoplasma*, yeast zymosan from *Saccharomyces cerevisiae*, and lipoteichoic acid from group B *Streptococci* and *Staphylococci*, which are often found in the upper respiratory tract.<sup>19</sup> Earlier studies found that the production of tumour necrosis factor  $\alpha$  elicited by zymosan and gram-positive bacteria<sup>20</sup> is inhibited in *TLR6* knockout mice, as is its production in response to mycoplasmal macrophage-activating lipopeptide-2 from *Mycoplasma fermentans*.<sup>21</sup> These data suggest that the *TLR6* gene controls Th1 differentiation, whereas the absence of TLR6-mediated signals generates Th2 responses. Moreover, Kormann *et al.*<sup>5</sup> showed another SNP located in the *TLR6* gene, that is, rs5743789, to be associated with increased mRNA expression. Carriers of the minor allele of this SNP showed increased Th1 cytokine expression, and reduced Th2 cytokine production after stimulation with its ligand. Unfortunately, at the beginning of our study, there was no information available on the possible importance of *TLR6* SNP rs5743789, and it was therefore not included in our study. Alternatively, the *TLR6* SNPs may be associated with a decreased risk of childhood ALL more directly via expression of TLR6 on leukemic cells, as has previously been shown for B-chronic lymphoblastic leukemia cells.<sup>22</sup> Future research is warranted to define the association between TLR6 and childhood ALL cells.

Recently, various genome-wide association studies have been performed in order to find risk genes for atopic disease.<sup>23</sup> Although several candidate gene studies did find *TLR6* polymorphisms to be associated with atopy,<sup>5,8,24</sup> this has to date not yet been confirmed in genome-wide association studies. However,

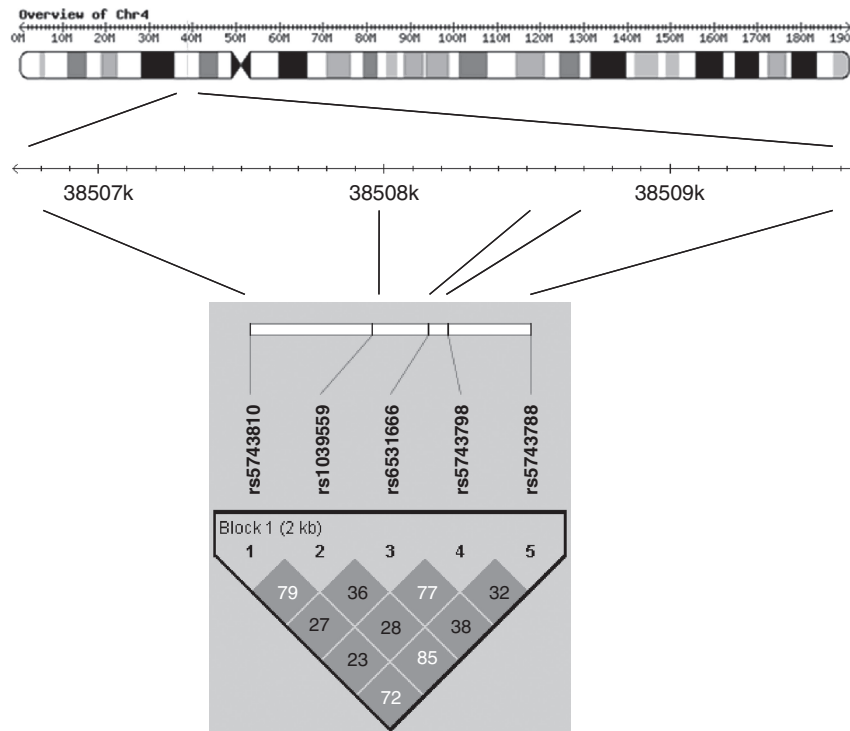


**Figure 2.** Genetic layout of *TLR6* with LD plot ( $r^2$ ).

a genome-wide association study of atopic disease in combination with positive skin tests has not been performed to date. Based on previous epidemiological studies, we specifically defined atopic disease relatively strictly by elevated IgE levels and/or positive skin tests in combination with asthma and/or eczema.<sup>5,6,24–26</sup> This strengthens our results. Unfortunately, for the children with ALL, no information was available on atopy, as IgE levels and skin tests are not performed regularly in children with leukemia. However, since measuring IgE levels may be influenced by immunosuppressive treatment regimens, measuring IgE levels for the purpose of this study did not seem useful.

As a wide range of atopic conditions exist, it is conceivable that the analysis of allergic subtypes is more accurate than the overall allergy estimate in describing the association, even though numbers are smaller. When comparing the genotype frequencies of the subgroups of children with asthma and eczema to childhood ALL, the strongest inverse association was found between eczematous children and children with childhood ALL. This finding is supported by the literature.<sup>1–3,27</sup>

This study has some limitations. Although the minor allele frequencies of the two *TLR6* SNPs (0.25 and 0.28) are considered common, observations were made in a limited number of subjects and therefore need to be interpreted with caution. Even though we showed a robust association between two SNPs in the *TLR6* gene and the inverse association between childhood ALL and atopic disease, one could argue that these results were chance findings. This is, however, not very likely as we corrected for multiple testing and performed permutation analysis. Nevertheless, the common problem in candidate gene studies of false positive findings could not be ruled out altogether. Therefore, our study needs to be replicated in study populations of the same or, preferably, larger sample sizes. Furthermore, the control population was derived from the study on Prevention and Incidence of Asthma and Mite Allergy (PIAMA). However, the control population was selected after careful screening for absence of atopic disease, by specific IgE levels, skin-prick tests, asthma and eczema, to make sure our control cohort was not biased.

Moreover, the question arises whether the SNPs in the *TLR6* gene that we found are relevant with respect to their function. This clearly requires further study. They are both haplotype-tagging SNPs, implying the effect can be direct or indirect. A direct effect may come from a direct biological influence of (one of) the SNPs, even though they are located in introns. It is now well established that introns may be related to the regulation of gene expression. An indirect effect can be achieved by tagging of a region of the gene that is functional.

## CONCLUSION

We examined the etiology of childhood ALL from a new genetic perspective, and found two *TLR6* polymorphisms to be associated with the inverse association between childhood ALL and atopic disease. Our findings support the immune surveillance hypothesis as an explanation for the protective effect of atopic disease on childhood ALL and provide new insight into the mechanism of the inverse association between childhood ALL and atopic disease. Further investigation is warranted to replicate our findings and to examine in more detail the role of innate immunity in the development of childhood ALL.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

Karin GE Miedema designed and performed the research, analyzed and interpreted data, performed statistical analysis and wrote the manuscript. Wim JE Tissing designed research, performed research, analyzed and interpreted data and wrote the manuscript. Esther M te Poele designed and performed the research, collected data and revised the manuscript. Willem A Kamps interpreted data and revised the manuscript. Behrooz Z Alizadeh and Marjan Kerkhof analyzed and interpreted data, performed statistical analysis and revised the manuscript. Johan HC de Jongste, Henriëtte A Smit, Anne P de Pagter and Marc Bierings performed research, collected data and revised the manuscript. H Marike Boezen designed research and revised the manuscript. Dirkje S Postma and Eveline SJM de Bont designed the research, interpreted data and revised the manuscript. Gerard H Koppelman designed the research, interpreted data and wrote the manuscript.

## REFERENCES

- Schuz J, Morgan G, Bohler E, Kaatsch P, Michaelis J. Atopic disease and childhood acute lymphoblastic leukemia. *Int J Cancer* 2003; **105**: 255–260.
- Hughes AM, Lightfoot T, Simpson J, Ansell P, McKinney PA, Kinsey SE et al. Allergy and risk of childhood leukaemia: results from the UKCCS. *Int J Cancer* 2007; **121**: 819–824.
- Dahl S, Schmidt LS, Vestergaard T, Schuz J, Schmiegelow K. Allergy and the risk of childhood leukemia: a meta-analysis. *Leukemia* 2009; **23**: 2300–2304.
- Turner MC, Chen Y, Krewski D, Ghadirian P. An overview of the association between allergy and cancer. *Int J Cancer* 2006; **118**: 3124–3132.
- Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J et al. Toll-like receptor heterodimer variants protect from childhood asthma. *J Allergy Clin Immunol* 2008; **122**: 86–92.
- Smit LA, Siroux V, Bouzigon E, Oryszczyn MP, Lathrop M, Demenais F et al. CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults. *Am J Respir Crit Care Med* 2009; **179**: 363–368.
- Reijmerink NE, Bottema RW, Kerkhof M, Gerritsen J, Stelma FF, Thijs C et al. TLR-related pathway analysis: novel gene-gene interactions in the development of asthma and atopy. *Allergy* 2010; **65**: 199–207.
- Hoffjan S, Stemmler S, Parwez Q, Petrasch-Parwez E, Arinir U, Rohde G et al. Evaluation of the toll-like receptor 6 Ser249Pro polymorphism in patients with asthma, atopic dermatitis and chronic obstructive pulmonary disease. *BMC Med Genet* 2005; **6**: 34.
- Daley D, Lemire M, Akhabir L, Chan-Yeung M, He JQ, McDonald T et al. Analyses of associations with asthma in four asthma population samples from Canada and Australia. *Hum Genet* 2009; **125**: 445–459.
- Han S, Lan Q, Park AK, Lee KM, Park SK, Ahn HS et al. Polymorphisms in innate immunity genes and risk of childhood leukemia. *Hum Immunol* 2010; **71**: 727–730.
- Brunekreef B, Smit J, de Jongste J, Neijens H, Gerritsen J, Postma D et al. The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results. *Pediatr Allergy Immunol* 2002; **13** (Suppl 15): 55–60.
- Meulenbelt I, Droog S, Trommelen GJ, Boomsma DI, Slagboom PE. High-yield non-invasive human genomic DNA isolation method for genetic studies in geographically dispersed families and populations. *Am J Hum Genet* 1995; **57**: 1252–1254.
- Bottema RW, Reijmerink NE, Kerkhof M, Koppelman GH, Stelma FF, Gerritsen J et al. Interleukin 13, CD14, pet and tobacco smoke influence atopy in three Dutch cohorts: the allergenic study. *Eur Respir J* 2008; **32**: 593–602.
- Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med* 1990; **9**: 811–818.
- Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. *Nat Rev Cancer* 2006; **6**: 193–203.
- Kinlen LJ. Epidemiological evidence for an infective basis in childhood leukaemia. *Br J Cancer* 1995; **71**: 1–5.
- Burnet M. Cancer; a biological approach. I. The processes of control. *Br Med J* 1957; **1**: 779–786.
- Kramer U, Heinrich J, Wjst M, Wichmann HE. Age of entry to day nursery and allergy in later childhood. *Lancet* 1999; **353**: 450–454.
- Ishii KJ, Coban C, Akira S. Manifold mechanisms of Toll-like receptor-ligand recognition. *J Clin Immunol* 2005; **25**: 511–521.
- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci USA* 2000; **97**: 13766–13771.
- Takeuchi O, Kawai T, Muhlrath PF, Morr M, Radolf JD, Zychlinsky A et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* 2001; **13**: 933–940.
- Rozkova D, Novotna L, Pytlík R, Hochova I, Kozak T, Bartunkova J et al. Toll-like receptors on B-CLL cells: expression and functional consequences of their stimulation. *Int J Cancer* 2010; **126**: 1132–1143.
- Weidinger S, Baurecht H, Naumann A, Novak N. Genome-wide association studies on IgE regulation: are genetics of IgE also genetics of atopic disease? *Curr Opin Allergy Clin Immunol* 2010; **10**: 408–417.
- Reijmerink NE, Kerkhof M, Bottema RW, Gerritsen J, Stelma FF, Thijs C et al. Toll-like receptors and microbial exposure: gene-gene and gene-environment interaction in the development of atopy. *Eur Respir J* 2011; **38**: 833–840.
- 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.
- Novak N, Yu CF, Bussmann C, Maintz L, Peng WM, Hart J et al. Putative association of a TLR9 promoter polymorphism with atopic eczema. *Allergy* 2007; **62**: 766–772.
- Wen W, Shu XO, Linet MS, Neglia JP, Potter JD, Trigg ME et al. Allergic disorders and the risk of childhood acute lymphoblastic leukemia (United States). *Cancer Causes Control* 2000; **11**: 303–307.

Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)

**Table S1.** The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) recruitment scheme

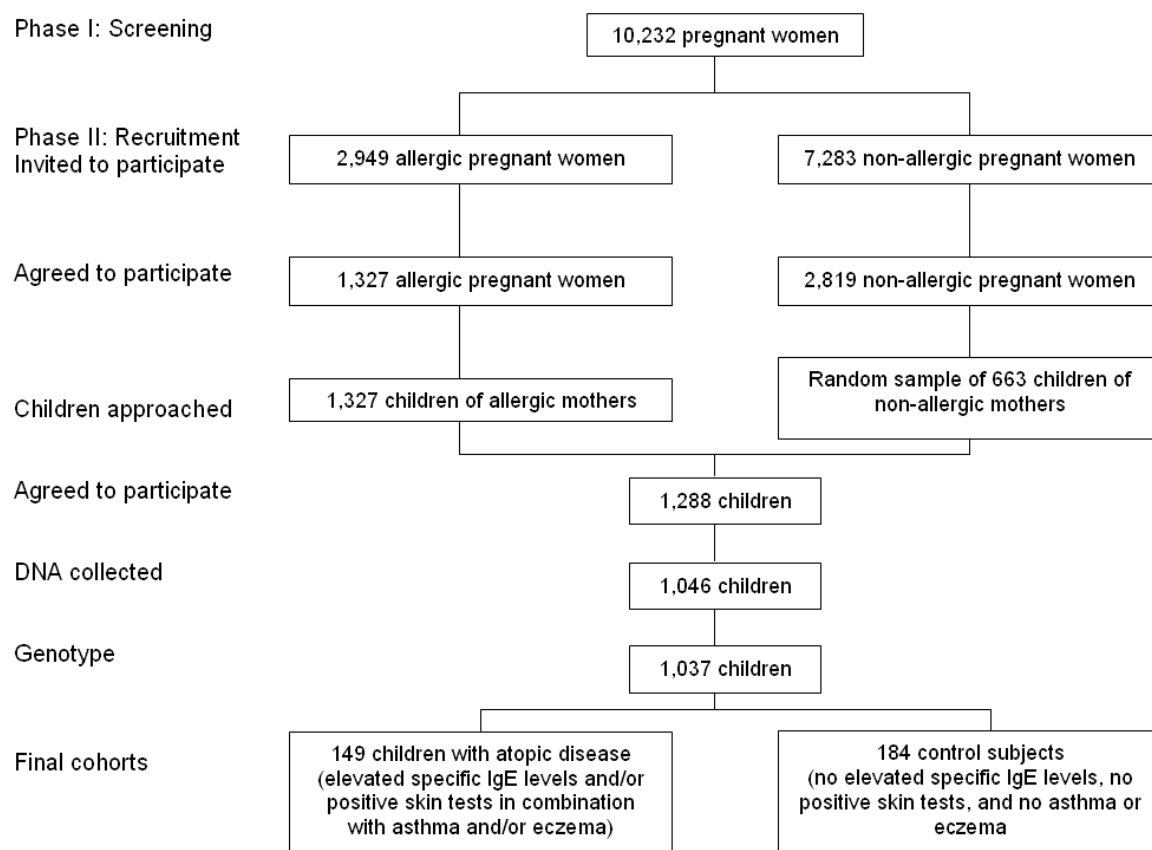


**Table S2.** 10,000 permutation analysis of the 29 SNPs

| <b>SNP</b> | <b>P value</b> | <b>Permutation<br/>p value</b> | <b>Corrected<br/>permutation<br/>p value</b> |
|------------|----------------|--------------------------------|----------------------------------------------|
| rs3804099  | 0.65           | 0.34                           | 0.99                                         |
| rs3804100  | 0.26           | 0.12                           | 0.91                                         |
| rs4696480  | 0.04           | 0.031                          | 0.48                                         |
| rs4986790  | 0.49           | 0.48                           | 1                                            |
| rs6478317  | 0.49           | 0.24                           | 0.99                                         |
| rs10759932 | 0.20           | 0.083                          | 0.87                                         |
| rs11536878 | 0.88           | 0.65                           | 1                                            |
| rs11536889 | 0.28           | 0.97                           | 1                                            |
| rs1927911  | 0.67           | 0.37                           | 0.99                                         |
| rs10759931 | 0.76           | 0.54                           | 1                                            |
| rs2770150  | 0.38           | 0.19                           | 0.97                                         |
| rs1039559  | 0.42           | 0.20                           | 0.98                                         |
| rs5743788  | 0.59           | 0.39                           | 0.99                                         |
| rs5743810  | 0.66           | 0.41                           | 0.99                                         |
| rs6531666  | 0.00040        | 0.00020                        | 0.013                                        |
| rs5743798  | 0.000097       | 0.0001                         | 0.00040                                      |
| rs352140   | 0.57           | 0.40                           | 1                                            |
| rs187084   | 0.98           | 0.86                           | 1                                            |
| rs5743836  | 0.41           | 0.37                           | 0.99                                         |
| rs4274855  | 0.09           | 0.13                           | 0.90                                         |
| rs10856839 | 0.51           | 0.64                           | 1                                            |
| rs11096956 | 0.27           | 0.23                           | 0.99                                         |
| rs11096957 | 0.53           | 0.36                           | 0.99                                         |
| rs11466652 | 0.27           | 0.91                           | 1                                            |
| rs4129009  | 0.12           | 0.11                           | 0.87                                         |
| rs2563298  | 0.22           | 0.32                           | 0.99                                         |
| rs2569190  | 0.12           | 0.59                           | 1                                            |
| rs2569191  | 0.07           | 0.83                           | 1                                            |
| rs5744455  | 0.19           | 0.31                           | 0.99                                         |

The observed p value for a differential genotype frequencies in patients with atopy compared to ALL patients and the corresponding permutation snp-wise p values and p values that are corrected for multiple testing.

**Table S3.** Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14* among children with ALL in the age 0-8 years, healthy control subjects, and children with atopic disease

| Gene | SNP        | Genotype | ALL<br>N (%) | Control<br>N (%) | Atopic<br>disease<br>N (%) | P value | FDR |
|------|------------|----------|--------------|------------------|----------------------------|---------|-----|
| TLR2 | rs3804099  | TT       | 41 (27)      | 48 (27)          | 35 (24)                    |         |     |
|      |            | TC       | 75 (50)      | 102 (57)         | 77 (53)                    |         |     |
|      |            | CC       | 34 (23)      | 28 (16)          | 34 (23)                    | 0.390   | NS  |
|      | rs3804100  | C allele | 48%          | 44%              | 50%                        | 0.395   | NS  |
|      |            | TT       | 143 (92)     | 165 (90)         | 120 (84)                   |         |     |
|      |            | TC       | 12 (8)       | 18 (10)          | 22 (15)                    |         |     |
|      | rs4696480  | CC       | 1 (1)        | 0 (0)            | 1 (1)                      | 0.189   | NS  |
|      |            | C allele | 5%           | 5%               | 8%                         | 0.082   | NS  |
|      |            | TT       | 36 (24)      | 60 (33)          | 52 (36)                    |         |     |
|      |            | TA       | 80 (52)      | 83 (46)          | 65 (45)                    |         |     |
| TLR4 | rs4986790  | AA       | 36 (24)      | 38 (21)          | 29 (20)                    | 0.226   | NS  |
|      |            | A allele | 50%          | 44%              | 42%                        | 0.124   | NS  |
|      |            | AA       | 137 (88)     | 151 (85)         | 126 (88)                   |         |     |
|      | rs6478317  | AG       | 18 (12)      | 28 (16)          | 17 (12)                    |         |     |
|      |            | GG       | 0 (0)        | 0 (0)            | 1 (1)                      | 0.432   | NS  |
|      |            | G allele | 6%           | 8%               | 7%                         | 0.580   | NS  |
|      | rs10759932 | AA       | 72 (47)      | 85 (47)          | 73 (50)                    |         |     |
|      |            | AG       | 64 (41)      | 74 (41)          | 59 (41)                    |         |     |
|      |            | GG       | 19 (12)      | 22 (12)          | 13 (9)                     | 0.872   | NS  |
|      | rs11536878 | G allele | 33%          | 33%              | 29%                        | 0.575   | NS  |
|      |            | TT       | 117 (76)     | 144 (80)         | 120 (83)                   |         |     |
|      |            | TC       | 38 (25)      | 33 (18)          | 25 (17)                    |         |     |
|      | rs11536889 | CC       | 0 (0)        | 3 (2)            | 0 (0)                      | 0.095   | NS  |
|      |            | C allele | 12%          | 11%              | 9%                         | 0.348   | NS  |
|      |            | CC       | 124 (80)     | 139 (78)         | 114 (77)                   |         |     |
|      | rs1927911  | CA       | 27 (17)      | 39 (22)          | 29 (20)                    |         |     |
|      |            | AA       | 5 (3)        | 1 (1)            | 5 (3)                      | 0.329   | NS  |
|      |            | A allele | 12%          | 11%              | 13%                        | 0.787   | NS  |
|      |            | GG       | 112 (74)     | 129 (72)         | 104 (70)                   |         |     |
|      |            | GC       | 35 (23)      | 49 (27)          | 42 (28)                    |         |     |
|      |            | CC       | 5 (3)        | 1 (1)            | 2 (1)                      | 0.302   | NS  |
|      |            | C allele | 15%          | 14%              | 16%                        | 0.841   | NS  |
|      |            | CC       | 92 (59)      | 109 (61)         | 93 (63)                    |         |     |
|      |            | CT       | 52 (33)      | 57 (32)          | 46 (31)                    |         |     |
|      |            | TT       | 12 (8)       | 14 (8)           | 9 (6)                      | 0.950   | NS  |

|       |            |          |          |          |          |        |             |
|-------|------------|----------|----------|----------|----------|--------|-------------|
| TLR6  | rs10759931 | T allele | 24%      | 24%      | 22%      | 0.711  | NS          |
|       |            | GG       | 58 (38)  | 62 (35)  | 48 (34)  |        |             |
|       |            | GA       | 70 (46)  | 76 (43)  | 71 (51)  |        |             |
|       |            | AA       | 23 (15)  | 38 (22)  | 21 (15)  | 0.412  | NS          |
|       |            | A allele | 38%      | 43%      | 40%      |        |             |
|       |            | TT       | 81 (54)  | 92 (53)  | 67 (46)  |        |             |
|       | rs2770150  | TC       | 56 (37)  | 73 (42)  | 63 (43)  | 0.417  | NS          |
|       |            | CC       | 13 (9)   | 10 (6)   | 15 (10)  |        |             |
|       |            | C allele | 27%      | 27%      | 32%      | 0.266  | NS          |
|       | rs1039559  | TT       | 36 (24)  | 53 (30)  | 44 (31)  |        |             |
|       |            | TC       | 77 (52)  | 85 (48)  | 69 (49)  |        |             |
|       |            | CC       | 36 (24)  | 38 (22)  | 29 (20)  | 0.706  | NS          |
|       |            | C allele | 50%      | 46%      | 45%      |        |             |
|       |            | CC       | 34 (23)  | 49 (27)  | 43 (30)  | 0.391  | NS          |
|       | rs5743788  | CG       | 80 (53)  | 96 (53)  | 69 (47)  |        |             |
|       |            | GG       | 36 (24)  | 36 (20)  | 34 (23)  | 0.599  | NS          |
|       |            | G allele | 51%      | 46%      | 47%      |        |             |
|       | rs5743810  | CC       | 51 (35)  | 61 (34)  | 54 (38)  | 0.508  | NS          |
|       |            | CT       | 71 (48)  | 94 (53)  | 66 (46)  |        |             |
|       |            | TT       | 26 (18)  | 24 (13)  | 23 (16)  | 0.734  | NS          |
|       | rs6531666  | T allele | 42%      | 40%      | 39%      |        |             |
|       |            | TT       | 82 (54)  | 72 (49)  | 49 (41)  | 0.821  | NS          |
|       |            | TC       | 67 (44)  | 66 (45)  | 54 (45)  |        |             |
| TLR9  |            | CC       | 4 (3)    | 9 (6)    | 15 (13)  | 0.008  | NS          |
|       |            | C allele | 25%      | 29%      | 33%      |        |             |
|       |            | CC       | 95 (63)  | 100 (56) | 66 (46)  | 0.018  | NS          |
|       | rs5743798  | CT       | 54 (36)  | 68 (38)  | 59 (41)  |        |             |
|       |            | TT       | 3 (2)    | 10 (6)   | 19 (13)  | 0.0009 | Significant |
|       |            | T allele | 20%      | 25%      | 34%      |        |             |
|       | rs352140   | TT       | 57 (38)  | 57 (32)  | 45 (31)  | 0.0005 | Significant |
|       |            | TC       | 69 (46)  | 88 (49)  | 74 (51)  |        |             |
|       |            | CC       | 25 (17)  | 36 (20)  | 26 (18)  | 0.690  | NS          |
|       | rs187084   | C allele | 39%      | 44%      | 43%      |        |             |
|       |            | TT       | 46 (31)  | 50 (28)  | 44 (30)  | 0.423  | NS          |
|       |            | TC       | 73 (49)  | 98 (55)  | 71 (49)  |        |             |
|       |            | CC       | 31 (21)  | 31 (17)  | 30 (21)  | 0.796  | NS          |
|       |            | C allele | 45%      | 45%      | 45%      |        |             |
|       |            | TT       | 109 (71) | 138 (76) | 107 (75) | 0.992  | NS          |
|       | rs5743836  | TC       | 39 (26)  | 40 (22)  | 34 (24)  |        |             |
|       |            | CC       | 5 (3)    | 4 (2)    | 2 (1)    | 0.768  | NS          |
|       |            | C allele | 16%      | 13%      | 13%      |        |             |
| TLR10 | rs4274855  | GG       | 100 (64) | 112 (62) | 101 (69) | 0.439  | NS          |
|       |            | GA       | 46 (30)  | 56 (31)  | 42 (29)  |        |             |
|       |            | AA       | 10 (6)   | 12 (7)   | 3 (2)    |        |             |
|       |            |          |          |          |          |        |             |

|      |            |          |          |          |          |       |    |
|------|------------|----------|----------|----------|----------|-------|----|
| CD14 | rs10856839 | A allele | 21%      | 22%      | 16%      | 0.160 | NS |
|      |            | AA       | 111 (72) | 124 (69) | 97 (69)  |       |    |
|      |            | AC       | 41 (27)  | 47 (26)  | 39 (28)  |       |    |
|      |            | CC       | 3 (2)    | 8 (5)    | 5 (4)    | 0.779 | NS |
|      |            | C allele | 15%      | 18%      | 17%      |       |    |
|      |            | GG       | 89 (58)  | 98 (56)  | 86 (63)  |       |    |
|      | rs11096956 | GT       | 55 (36)  | 65 (37)  | 47 (34)  | 0.512 | NS |
|      |            | TT       | 10 (7)   | 12 (7)   | 4 (3)    |       |    |
|      |            | T allele | 24%      | 25%      | 20%      |       |    |
|      | rs11096957 | AA       | 54 (36)  | 57 (33)  | 55 (39)  | 0.267 | NS |
|      |            | AC       | 76 (50)  | 88 (51)  | 72 (51)  |       |    |
|      |            | CC       | 21 (14)  | 29 (17)  | 15 (11)  |       |    |
|      |            | C allele | 39%      | 42%      | 36%      | 0.578 | NS |
|      |            | AA       | 115 (75) | 130 (73) | 111 (76) |       |    |
|      |            | AG       | 36 (24)  | 45 (25)  | 31 (21)  |       |    |
|      |            | GG       | 2 (1)    | 4 (2)    | 5 (3)    | 0.714 | NS |
|      |            | G allele | 13%      | 15%      | 14%      |       |    |
|      |            | AA       | 101 (65) | 112 (62) | 104 (71) |       |    |
|      | rs4129009  | AG       | 44 (28)  | 57 (32)  | 39 (27)  | 0.814 | NS |
|      |            | GG       | 10 (7)   | 11 (6)   | 3 (2)    |       |    |
|      |            | G allele | 21%      | 22%      | 15%      |       |    |
|      | rs2563298  | CC       | 80 (53)  | 85 (47)  | 86 (59)  | 0.094 | NS |
|      |            | CA       | 69 (45)  | 85 (47)  | 55 (37)  |       |    |
|      |            | AA       | 3 (2)    | 11 (6)   | 6 (4)    |       |    |
|      |            | A allele | 25%      | 30%      | 23%      | 0.121 | NS |
|      |            | GG       | 46 (31)  | 53 (29)  | 36 (25)  |       |    |
|      |            | GA       | 67 (45)  | 101 (56) | 82 (56)  |       |    |
|      | rs2569190  | AA       | 36 (24)  | 26 (14)  | 29 (20)  | 0.120 | NS |
|      |            | A allele | 47%      | 43%      | 48%      |       |    |
|      |            | TT       | 46 (30)  | 53 (29)  | 36 (25)  |       |    |
|      | rs2569191  | TC       | 68 (44)  | 101 (56) | 82 (56)  | 0.369 | NS |
|      |            | CC       | 40 (26)  | 28 (15)  | 28 (15)  |       |    |
|      |            | C allele | 48%      | 43%      | 47%      |       |    |
|      | rs5744455  | CC       | 87 (57)  | 96 (53)  | 72 (49)  | 0.080 | NS |
|      |            | CT       | 57 (37)  | 77 (43)  | 66 (45)  |       |    |
|      |            | TT       | 10 (7)   | 8 (4)    | 9 (6)    |       |    |
|      |            | T allele | 25%      | 26%      | 29%      | 0.107 | NS |
|      |            |          |          |          |          |       |    |

NB: SNP – single nucleotide polymorphism; ALL – acute lymphoblastic leukemia; FDR – false discovery rate; NS – not significant