Polymorphisms in the Mannan-Binding Lectin Gene Are Not Associated with Questionnaire-Reported Respiratory Tract Infections in Children

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Background. Low mannan-binding lectin (MBL) levels, caused by MBL2 polymorphisms, are suggested to contribute to susceptibility to respiratory tract infections (RTIs), particularly early in life. Large-scale replication of previous associations is needed, however. We investigated the association between MBL2 polymorphisms and the frequency of RTI in a large population-based birth cohort of white children.

Methods. The frequency of RTI was prospectively assessed by annual parental questionnaires until children were 4 years of age. Thirteen polymorphisms in MBL2 were determined in 987 Dutch children. Haplotypes, previously shown to be associated with functional levels of MBL, were constructed, and their associations with the frequency of RTI during year 1, year 2, and the first 4 years of life were assessed. High-producing, intermediate-producing, and deficient MBL2 genotypes were defined on the basis of exon 1 and Y/X promoter polymorphisms.

Results. No differences were found between investigated polymorphisms and haplotype frequencies in the population as a whole or between the groups with frequent, moderately frequent, or no RTIs reported. Deficient MBL2 genotypes were not associated with an increased risk of RTI (odds ratio, 0.71 [95% confidence interval, 0.25 to 2.05]) during years 1–4 of life. This was also true when year 1 and year 2 were studied separately.

Conclusion. These results suggest that, at the population level, MBL2 polymorphisms do not contribute to the risk of questionnaire-reported RTI in white children.
respiratory tract infections (RTIs) in children [10]. Although the findings of several studies, mostly hospital based, suggest that MBL2 genotypes associated with low levels of MBL lead to an increased risk of RTI [4, 11–15], other studies have failed to confirm this association [16–19]. The redundancy of the innate immune system, which may compensate sufficiently to prevent infection, could fail to limit the severity of infection when innate immunity is compromised by MBL deficiency. This may be why hospital-based studies tend to show an effect of MBL2 polymorphisms on susceptibility to infectious disease.

Two prospective population-based cohort studies have been published to date. The first is the study by Koch et al. [13], performed in 252 Eskimo children who were investigated weekly for the number of RTI episodes, from 6 weeks to 2 years of age. In this study, deficient MBL2 genotypes were found to be associated with a 2-fold increased risk for acute RTI, an observation that was restricted to children aged 6–17 months. That MBL deficiency might be relevant during that particular age period is thought to result from the fact that the adaptive immunity needed to protect against RTI is still immature in children <2 years of age but may compensate for MBL deficiency after that age. The second study is the recently published study by Müller et al. [19], performed in a large German birth cohort selected for the study of allergies. In this study, low-producing MBL2 genotypes were found not to be associated with an increased frequency of questionnaire-based RTI, which was calculated for the following age periods: 0–1, 0–2, 0–5, and 0–11 years. However, it should be mentioned that this study population did not have a homogenetic background, and haplotypes were not studied. Validation or replication of a study finding is essential to understand its significance and to sort true-positive from false-positive associations. Therefore, previous associations between MBL2 polymorphisms and RTI need to be replicated in a large independent population. To establish the actual role played by MBL2 polymorphisms in the susceptibility to RTI in white children, we studied the association between haplotypes of MBL2 and the occurrence of RTI in a population-based birth cohort of children of Dutch ancestry.

**METHODS**

**Study population.** Children were participants in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study, originally designed to investigate atopy and asthma. Details of the study design have been published elsewhere [20]. Recruitment took place in 1996 and 1997. A screening questionnaire on maternal allergy was completed by 10,232 pregnant women visiting 1 of 52 prenatal clinics in The Netherlands [21]. On the basis of 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. Questionnaires for parental completion were sent to the parents during pregnancy, when the children reached the ages of 3 and 12 months, and yearly thereafter. DNA was successfully collected from 1037 children. Of these children, 987 were of Dutch ancestry and were used for analysis; the 50 children of non-Dutch origin were excluded. The frequency of RTI among the children was included on the questionnaire, first at 1 year of age and then annually until 4 years of age. Data on genotypes and the frequency of RTI were complete in 896 Dutch children.

The general characteristics of the study population were comparable between participants and nonparticipants in the genetic study, apart from allergy in the mother (66% for participants vs. 20% for nonparticipants). Other variables were also investigated and found to be approximately equally distributed between participants and nonparticipants: maternal smoking during pregnancy, duration of pregnancy, birth weight, sex, breast-feeding for ≥12 weeks, day care, environmental tobacco smoke exposure, and existence of siblings (data not shown). The study was approved by the institutional review board, and parents gave informed consent.

**Polymorphisms in the MBL2 gene.** Genomic DNA was extracted from buccal swabs or blood by performing chloroform–2-propanol extraction. DNA was amplified using REPLI-g UltraFast technology (Qiagen). SNPs of MBL2 were selected on the basis of known functionality (n = 7) combined with haplotype-tagging SNPs that have a minor allele frequency of >0.1 (n = 6), selected from the publicly available database of the International HapMap Project [22]. The studied SNPs are listed in table 1. Genotyping was performed by competitive allele-specific polymerase chain reaction using KASPar genotyping chemistry, performed under contract by KBiosciences (http://www.kbiosciences.co.uk/). Previous functional analyses by Madsen et al. [5] and others, including Wiertsema et al. [4], demonstrated that the YA/O genotype has intermediate levels of functional MBL multimers. The YA/O genotype has been reported to be functionally different from YA/A and was recently reported to be beneficial for improved outcomes of severe infections [23]. Thus, exon 1 SNPs were combined with the promoter polymorphism Y/X to define high-producing (A/A), intermediate-producing (YA/O), and deficient (XA/O and O/O) MBL2 genotypes.

**Frequency of RTI.** Information on the frequency of RTI in children 1–4 years of age was collected from annual parental questionnaires using the following question: “How often did your child have serious respiratory tract and/or ear-nose-throat infections, such as flu, infection of the throat, middle ear, or sinuses, bronchitis or pneumonia, during the last 12 months?” Four answers were possible: never, 1–2 times, 3–5 times, and ≥6 times. On the basis of the answers given on the 4 consecutive annual questionnaires, 3 groups were defined according to the reported frequency of RTI. We defined frequent RTIs as ≥3 RTIs per year reported on 3 or 4 annual questionnaires (group 1).
Group 2 consisted of children without any reported RTIs during the 4 years. Group 3 consisted of the remaining children, who had a low to moderate frequency of RTI (1 or more RTIs reported on the 4 annual questionnaires but not frequent RTIs according to the study definition).

The periods of 0–12 months and 13–24 months of life were also analyzed separately, because an effect of MBL2 polymorphisms has been shown in these age groups particularly [4, 13]. Frequencies of MBL2 genotypes in children with 3 or more reported RTIs during the same year. The same analyses were done for the second year of life.

**Statistical analyses.** The genotype data were analyzed for deviations from Hardy-Weinberg equilibrium by means of the $\chi^2$ statistic. Haplotypes were estimated using PHASE software (version 2.1; available at http://stephenslab.uchicago.edu/software.html). Differences in haplotype and genotype frequencies (with 95% confidence intervals [CIs]) between children with frequent RTIs and those without reported RTIs were calculated. MBL2 haplotypes were finally divided into those associated with high (LYPA), intermediate (HYPA, LYQA, and LXPA-C), or low (LXPA-G, HYPD, LYPB, and LYQC) expression of MBL, to increase power. Crude odds ratios (ORs) with 95% CIs were estimated by means of logistic regression analyses per year and for years 1–4 cumulatively, to estimate the association between MBL2 haplotypes, deficient MBL2 genotypes, and categories of annual RTI frequency. Analyses were performed with SPSS software (version 12.0.2; SPSS). $P < .05$ was considered to indicate statistical significance.

### RESULTS

Of the 896 children in our final study population with complete information on genotypes and annual RTI rates, 55 (6%) fulfilled the criteria for frequent RTIs during the first 4 years of life, according to our definition (group 1). A total of 130 children (15%) did not have any reported RTIs in these years (group 2), and 711 children (79%) had a low to moderate frequency of reported RTIs (group 3). RTI rates were similar in the 50 children of non-Dutch origin who were excluded (data not shown). Minor allele frequencies of the 13 determined polymorphisms of MBL2 were similar to those previously reported in white Dutch subjects (table 1) [4, 24]. All SNPs adhered to Hardy-Weinberg expectations ($P > .05$). Data for both parents were available for 193 children. These data were analyzed for inheritance patterns, using family-based association tests, which showed inheritance errors (supposedly caused by genotyping errors) in <1% of the cases.

**MBL2 haplotype associations.** PHASE 2.1 software constructed 34 haplotypes from the genotype data. Strong linkage disequilibrium led to 7 well-known haplotypes based on MBL2 exon 1 (A or B/C/D) and H/L, Y/X, and P/Q promoter SNPs: HYPA, LYPA, LXPA, HYPD, LYPB, and LYQC (table 2). The LXPA haplotype can further be divided on the basis of the 3130G→C polymorphism in exon 4, resulting in 8 haplotypes that have been associated with functional MBL serum levels [4, 5]. Two haplotypes that have not been described previously were found at a low prevalence (LXPB, $n = 2$; HYQA, $n = 2$) and were excluded in further analyses. The observed haplotype frequencies with corresponding predicted MBL serum levels are listed in table 2; they are in agreement with previous findings in Dutch studies [4, 24].

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**Table 1.** MBL2 single-nucleotide polymorphisms (SNPs) studied in the Prevention and Incidence of Asthma and Mite Allergy study cohort.

| dbSNP identifier | SNP | Allele name | SNP type | MAF  |
|------------------|-----|-------------|----------|------|
| rs11003125       | −619G→C | H/L | Proximal promoter, Ht | 0.370 |
| rs7096206        | −290G→C | Y/X | Proximal promoter | 0.232 |
| rs7095891        | −66C→T | P/Q | 5’-UTR | 0.214 |
| rs5030737        | 154C→T, Arg52Cys | D | Exon 1, codon 52 | 0.066 |
| rs1800450        | 161G→A, Gly54Asp | B | Exon 1, codon 54, Ht | 0.135 |
| rs1800451        | 170G→A, Gly57Glu | C | Exon 1, codon 57 | 0.014 |
| rs12246310       | 297C→T | ... | Ht | 0.214 |
| rs10824793       | 1916A→G | ... | Ht | 0.449 |
| rs1838066        | 2071A→G | ... | Ht | 0.374 |
| rs1838065        | 2139A→G | ... | Ht | 0.376 |
| rs930507         | 3130G→C | ... | Exon 4, Ht | 0.185 |
| rs10824792       | 5190C→T | ... | Ht | 0.417 |
| rs2120132        | 5365C→T | ... | Ht | 0.250 |

**NOTE.** Ht, haplotype tagging; MAF, minor allele frequency (determined in a study population of Dutch white ethnicity); UTR, untranslated region.
No significant difference in haplotype frequencies was observed between the 2 extreme groups, that is, groups 1 and 2 (table 2). The differences in the percentages varied from 5 (95% CI, 14 to 4) for LYQA to 3 (95% CI, 4 to 10) for LYPB. Finally, analyses of 3 MBL2 haplotype groups (group 1 associated with high, group 2 with intermediate, and group 3 with low expression of MBL) showed similar results (data not shown).

### MBL2 genotype associations

Considering genotypes of exon 1, 547 children (61%) were homozygous for the wild-type A allele (genotype A/A), 37 (4%) were homozygous for variant alleles (genotype O/O), and 312 (35%) were heterozygous (genotype A/O) (table 3). Prevalences of exon 1 genotypes in children with frequent RTIs did not differ from those in children with no reported RTIs during years 1–4. The differences in the

### Table 2. Percentages of children with different haplotypes of MBL2, by frequency of questionnaire-reported respiratory tract infections (RTIs) during years 1–4.

| Haplotype | Predicted MBL serum level | All children (n = 896/1792) | No RTIs (n = 130/260) | Moderately frequent RTIs (n = 711/1422) | Frequent RTIs (n = 55/110) | Difference in percentage (95% CI) |
|-----------|---------------------------|-----------------------------|----------------------|----------------------------------------|--------------------------|--------------------------------|
| LYPA      | High                      | 73 (4)                      | 9 (3)                | 58 (4)                                 | 6 (6)                    | -2 (-7 to 3)                  |
| HYPB      | Intermediate              | 554 (31)                    | 90 (35)              | 428 (30)                               | 36 (33)                  | 2 (-9 to 13)                  |
| LYQA      | Intermediate              | 358 (20)                    | 47 (18)              | 286 (20)                               | 25 (23)                  | -5 (-14 to 4)                 |
| LXPAa     | C allele                  | 325 (18)                    | 48 (18)              | 256 (18)                               | 21 (19)                  | -1 (-10 to 8)                 |
| G allele  | Low                       | 96 (5)                      | 14 (5)               | 76 (5)                                 | 6 (6)                    | 0 (-5 to 5)                   |
| HYPD      | Low                       | 114 (6)                     | 18 (7)               | 91 (6)                                 | 5 (5)                    | 2 (-3 to 7)                   |
| LYPB      | Low                       | 249 (14)                    | 33 (13)              | 205 (14)                               | 11 (10)                  | 3 (-4 to 10)                  |
| LYQC      | Very low                  | 23 (1)                      | 1 (0)                | 22 (2)                                 | 0 (0)                    | 0 (0 to 1)                    |

NOTE. Indicated n values are no. of children/no. of haplotypes. CI, confidence interval; MBL, mannan-binding lectin.

a Predicted MBL level according to Madsen et al. [5] and Wiertsema et al. [4].

b Excluded were children with an ethnicity other than Dutch (n = 50), children with incorrect haplotypes (n = 2), and children without complete RTI information.

c Frequent RTIs were defined as 3 or more RTIs per year in 3 or 4 years during the first 4 years of life.

d Difference between the percentage for children with frequent RTIs and that for children with no RTIs.

e Haplotype LXPA is further divided on the basis of the 3130G→C polymorphism in exon 4 of MBL2 [4].

### Table 3. Association between MBL2 genotypes and frequency of reported respiratory tract infections (RTIs) during years 1–4.

| Genotype     | All childrena | No RTIs | Moderately frequent RTIs | Frequent RTIsb | Difference in percentage (95% CI) |
|--------------|---------------|---------|--------------------------|----------------|--------------------------------|
| Exon 1 polymorphisms |                |         |                          |                |                                |
| A/A          | 547 (61)      | 85 (65) | 421 (59)                 | 41 (75)        | -10 (-24 to 4)                 |
| A/O          | 312 (35)      | 38 (29) | 262 (37)                 | 12 (22)        | 7 (-6 to 20)                   |
| O/O          | 37 (4)        | 7 (5)   | 28 (4)                   | 2 (4)          | 1 (-5 to 7)                    |
| Promoter alleles includedc |                |         |                          |                |                                |
| High MBL     | 548 (61)      | 85 (65) | 422 (59)                 | 41 (75)        | -10 (-24 to 4)                 |
| Intermediate | 216 (24)      | 29 (22) | 178 (25)                 | 9 (16)         | 6 (-6 to 18)                   |
| Deficient MBL| 132 (15)      | 16 (12) | 111 (16)                 | 5 (9)          | 3 (-6 to 12)                   |
| All          | 896 (100)     | 130 (100)| 711 (100)               | 55 (100)       | -                               |

NOTE. The odds ratio for frequent RTIs comparing deficient MBL2 genotypes with intermediate-producing plus high-producing MBL2 genotypes is 0.71 (95% CI, 0.25 to 2.05), estimated on the basis of χ^2 statistics. CI, confidence interval; MBL, mannan-binding lectin.

a Excluded were children with an ethnicity other than Dutch (n = 50), children with presumably incorrect haplotypes (n = 2), and children without complete RTI information.

b Frequent RTIs were defined as 3 or more RTIs per year in 3 or 4 years during the first 4 years of life.

c Difference between the percentage for children with frequent RTIs and that for children with no RTIs.
Our study included only white children. RTIs are known to occur more frequently among Eskimo children [25]. The specific genetic background and an a priori high risk of RTI make the results of Koch et al. hard to generalize to white children. Second, RTIs were measured differently in both studies. We used questionnaire data to assess RTI, whereas Koch et al. used exact measures of numbers of RTI episodes assessed by clinical examination. Third, we analyzed haplotypes in relation to 3 MBL2 genotype groups (high producing, intermediate producing, and deficient) based on exon 1 and promoter SNPs, whereas Koch et al. used 2 groups (MBL sufficient and MBL insufficient) based on the same polymorphisms. Finally, we analyzed years 1 and 2 of life separately, but further determination of the vulnerable period, as done by Koch et al., was impossible with our annual data.

The second study showing an effect in a young age group was performed in The Netherlands by Wiertsema et al. [4]. This study included children (n = 383) seen in the hospital with a history of 2 or more previous episodes of acute otitis media (AOM). A positive association was found with recurrent AOM in children 12–24 months of age (n = 113) but not in older children (n = 131). That the latter study involved a cohort of children with a history of AOM, whereas we studied a birth cohort representing the general population, might explain the discrepancy between the results.

Several other studies have also shown an association between RTI and variant MBL2 genotypes [11, 12, 14, 15], but this association was not verified by others [16–19]. All of the studies that have shown a relationship have been hospital-based case-control studies, conducted in children with recurrent RTIs [14], children who had undergone adenoidectomy and/or tonsillectomy because of recurrent upper RTIs [15], or children with suspected immunodeficiency [11]. This phenomenon might be explained by a possible association between MBL deficiency and the severity of infectious diseases, although the supposition that MBL deficiency affects the severity of RTIs has not been accurately tested.

The results of our study are in line with the results of the recent study by Müller et al. [19] in a large German birth cohort, which was also used for studying allergy (Multicenter Atopy Study). This study (n = 749) did not find an association between MBL2 polymorphisms and risk of RTI. In addition to the exon 1 and promoter polymorphisms that were studied by Müller et al. in our study we included analyses with haplotypes of MBL2 and followed up a larger homogenous study population for a longer period of time (n = 896 at the 4-year vs. n = 749 at the 1-year follow-up time point).

Finally, unlike some previous investigations, we addressed RTI as a whole. The spectrum of infections in our study was wide: from otitis to pneumonia and bronchitis. The effect of MBL2 mutations may differ for different clinical manifestations or etiological agents. In a Dutch study, rhinovirus was the most common respiratory tract pathogen (24%), followed by influ-
enza virus type A (11%) and coronavirus (7%) [26]. Binding of MBL to influenza A virus involves recognition of oligosaccharides of the viral proteins hemagglutinin and neuraminidase [27]. MBL does not appear to recognize any specific rhinovirus structure; therefore, MBL deficiency may not have altered the incidence of RTI in our study if this pathogen was present in a large number of subjects.

The major strengths of our study are its large sample size, prospectively repeated questionnaires, and population-based sample. Ordinal regression analyses enabled us to study all children as well as to study only the extreme groups; both analyses showed similar results. MBL2 genotyping was performed according to recent standards. Exon 1 and promoter polymorphisms were included and were combined to create genotypes related to high, intermediate, and deficient MBL serum levels. Moreover, haplotypes were created on the basis of haplotype-tagging polymorphisms.

To appreciate our results, it is also necessary to consider some potential limitations. First, the PIAMA study was originally designed to study atopy and asthma. Exact numbers of RTI episodes, as analyzed by Koch et al. [13], were not recorded. Instead, we grouped the children according to frequency of RTI, as reported in annual questionnaires. Strictly speaking, RTI in our study indicates the presence of respiratory symptoms that the parents considered more serious than a common cold. This may lead to misclassification in the reported frequency of RTI, but the misclassification can be supposed to be nondifferential (i.e., to be independent of MBL2 genotype status). To study the validity of our definitions, we compared the questionnaire data on the reported frequency of RTI with data on the prescription of antibiotics and adenoidectomy and tonsillectomy. Antibiotic prescription was highly associated ($P < .01$) with frequent RTIs, as defined by the questionnaires, and a similar association was seen with adenoidectomy and tonsillectomy. Furthermore, as with the frequency of RTI, we observed no association of MBL2 genotype status with ear, nose, and throat surgery or antibiotic prescription rates.

Second, the overall response rate of the study was moderate (53%), and the study population may not be representative of the general population of newborns in the study areas. In studies of this kind, it is important that losses to follow-up be minimal. In the recruitment phase, it was made explicit to the prospective participants that a long-term commitment was necessary, which may have dampened enthusiasm to participate [20]. Fortunately, loss to follow-up has been relatively small, and we maintain that valid conclusions can still be reached despite the moderate magnitude of response.

Third, selection bias may have occurred if the association between MBL2 polymorphisms and frequent RTIs differed between the children included in this part of the study and those who were excluded from analysis because of missing information. Among the children who were not included, a higher percentage had nonallergic mothers. This difference is caused by the original design of the PIAMA study, not by selective dropout [20]. Nevertheless, we separated the children by maternal allergy and confirmed the lack of association between MBL2 polymorphisms and frequent RTIs in both groups (data not shown).

Moreover, selection bias is unlikely, because the MBL2 genotype distribution we found is similar to that in other studies involving white Dutch subjects and conforms to Hardy-Weinberg equilibrium.

Finally, we performed a power analysis to strengthen our negative findings. In this calculation, we assumed an allele frequency of 0.232 for the high-risk allele ($Y/X$ promoter SNP) and a prevalence of 6.1% for frequent RTIs, on the basis of our own data. The Genetic Power Calculator [28] showed that our number of cases ($n = 55$) was sufficient to exclude genotype-relative risks of 2 and 2.5, respectively, for heterozygotes and homozygotes for the high-risk allele (power of >80% to reject the null hypothesis at $\alpha = .05$). Genotypic risks of this size are in line with the findings of Koch et al. [13], and we consider relative risks of this size to be clinically relevant. Therefore, our study population seems sufficient to exclude clinically relevant effects of MBL2 polymorphisms on susceptibility to RTI.

We do not rule out the possibility that MBL2 polymorphisms may contribute to the severity of infections or, in specific circumstances of impaired immunity, to the complex genetic control of protective immunity to infection. Nevertheless, in most circumstances other components of the immune system may compensate for the lack of MBL.

In conclusion, this population-based birth cohort study in white children shows that MBL2 polymorphisms known to result in low serum levels of MBL do not seem to pose an increased risk of RTI during the first 4 years of life, as indicated by parental questionnaires. The effect of these polymorphisms was also absent in children <2 years of age. Thus, MBL deficiency seems to be of no clinical significance for the risk of RTI in white children at a population level.

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