

Functional polymorphisms in the mannan-binding lectin 2 gene: Effect on MBL levels and otitis media

Selma P. Wiertsema, PhD,^a Bjorn L. Herpers, MD,^b Reinier H. Veenhoven, MD, PhD,^d Marcel M. M. Salimans, MD, PhD,^e Henk J. T. Ruven, PhD,^c Elisabeth A. M. Sanders, MD, PhD,^a and Ger T. Rijkers, PhD^{a,b} Utrecht, Nieuwegein, Hoofddorp, and Amersfoort, The Netherlands

Background: Mannan-binding lectin (MBL) can bind to microorganisms, initiating the lectin pathway of complement activation. Aberrant MBL serum levels, caused by *MBL2* gene polymorphisms, are a possible risk factor for recurrent infections. Within the 7 common *MBL* haplotypes, still considerable variation in MBL serum levels exists.

Objective: To investigate functional MBL levels and *MBL2* polymorphisms in a large cohort of children with recurrent acute otitis media.

Methods: Twelve genetic variants in the *MBL2* gene and functional MBL serum levels were determined in a cohort of children with recurrent acute otitis media. Haplotypes were constructed and associated with functional MBL serum levels and the number of otitis episodes in the previous year. **Results:** The 7 common *MBL2* haplotypes mainly determine the level of functional MBL in serum. In addition, the 3130G>C single nucleotide polymorphism, located in exon 4, further significantly influenced functional MBL levels within the LXPA haplotype. LXPA carriers with 3130G showed a significantly lower geometric mean functional MBL serum level of 0.19 $\mu\text{g/mL}$ compared with 0.70 $\mu\text{g/mL}$ in 3130C carriers ($P = .026$). Nonwild-type *MBL2* carriers between 12 and 24 months had a significantly increased number of otitis episodes (5.1/y) compared with wild-type *MBL2* carriers (4.1/y; $P = .027$). In older children, this association was not found anymore. **Conclusion:** Additional single nucleotide polymorphisms within the 7 common haplotypes can further explain the observed variation in functional MBL serum levels. MBL seems to be of particular clinical importance during early childhood, when maternally derived antibodies have waned, and protective adaptive immunity is not well developed yet.

Clinical implications: Single nucleotide polymorphisms in the promoter region, in exon 1, and in exon 4 of *MBL2* contribute to increased risk for otitis media in children younger than 2 years. (J Allergy Clin Immunol 2006;117:1344-50.)

Key words: Mannan-binding lectin, complement, otitis media, functional mannan-binding lectin serum levels, polymorphisms, haplotypes

Mannan-binding lectin (MBL) is a C-type lectin with a collagen-like domain¹ that plays an important role in innate immune defense against infections.² MBL functions as a pattern-recognition receptor that can bind highly conserved structures on a wide range of micro-organisms, known as pathogen-associated molecular patterns.^{3,4} MBL subunits fold into oligomers, which, on binding of mannan or alternative carbohydrate ligands, recruit MBL-associated serine proteases. This complex initiates the lectin pathway of complement activation.⁵⁻⁸ Compared with other components of the complement system, there is an enormous range in serum MBL concentrations in human beings, from 0.1 to 10,000 ng/mL. MBL deficiency, defined as an MBL serum concentration below 100 ng/mL, has been estimated to occur in 5% to 10% of the general white population.⁹ This condition seems to be associated with an increased risk for infections, especially when a coexisting primary or secondary immune deficiency is present and during the immunologic frail period of early childhood, when the adaptive immune system has not yet matured and protective maternally derived antibodies have waned.¹⁰⁻¹² Furthermore, MBL insufficiency is thought to influence autoimmune conditions, like SLE and rheumatoid arthritis, and the severity and course of several other diseases, like AIDS, hepatitis, and cystic fibrosis.^{10,13-15}

The differences in circulating serum MBL levels can to a large extent be explained by genetic variation in the *MBL2* gene, which encodes MBL.¹⁶ Three single nucleotide polymorphisms (SNPs) in exon 1, known as the D, B, and C alleles, all interfere with the formation of higher MBL oligomers, leading to alterations in circulating MBL levels (Arg52Cys, D-variant; Gly54Asp, B-variant; Gly57Glu, C-variant).¹⁷⁻¹⁹ An *MBL* coding region carrying any of the 3 polymorphisms is collectively termed "0," whereas the wild-type allele is referred to as "A."²⁰ In addition, 3 pairs of allelic dimorphisms in the downstream promoter and the 5'-untranslated region of

From ^athe Department of Pediatric Immunology, University Medical Center Utrecht; ^bthe Department of Medical Microbiology & Immunology, and ^cthe Department of Clinical Chemistry, St Antonius Hospital, Nieuwegein; ^dthe Department of Pediatrics, Spaarne Hospital, Hoofddorp; and ^ethe Department of Medical Microbiology, Meander Medical Center, Amersfoort. Supported by The Netherlands Organization for Health Research and Development ZonMW (grant numbers 002828480 and 90461092) and the Netherlands Health Insurance Co Zilveren Kruis-Achmea.

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Reprint requests: Ger T. Rijkers, PhD, Department of Paediatric Immunology, Wilhelmina Children's Hospital/University Medical Center Utrecht, Lundlaan 6, KC03.063.0, 3584 EA Utrecht, The Netherlands. E-mail: g.rijkers@antonius.net.

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Abbreviations used

AOM: Acute otitis media
DGGE: Denaturing gradient gel electrophoresis
MBL: Mannan-binding lectin
OM: Otitis media
SNP: Single nucleotide polymorphism
SSP-PCR: PCR with sequence-specific primers

the *MBL* gene have been described to further affect MBL serum levels: –619G>C (H/L), –290G>C (Y/X), and –66C>T (P/Q).^{20–22} Many other polymorphisms within the *MBL2* gene exist; whether these SNPs also influence functional MBL serum levels is unknown.²³ Because strong linkage disequilibrium exists between the known genetic *MBL* variants, currently only 7 common haplotypes have been described.^{20,24}

The most frequently diagnosed infection in early childhood is otitis media (OM), in which viral pathogens, especially respiratory syncytial virus, as well as bacterial pathogens like *Streptococcus pneumoniae* and *Haemophilus influenzae* are involved.^{25,26} MBL activity could contribute to protection against bacterial pathogens involved in the development of OM, and likewise, MBL deficiency could be one of the factors predisposing to recurrent OM. We studied haplotypes of *MBL2* exon 1 and Y/X promoter polymorphisms in a well defined group of 383 children with a history of recurrent acute OM (AOM). Haplotypes were associated with functional MBL serum levels and with the number of AOM episodes the child had in the previous year. In addition, haplotypes composed of 12 polymorphisms across the *MBL2* gene were investigated for their influence on functional serum MBL levels in these children with recurrent AOM.

METHODS

Study population

Serum and DNA were available from children with a history of AOM participating in a randomized controlled pneumococcal vaccine efficacy trial as described in detail elsewhere.²⁷ Inclusion criteria were age between 1 and 7 years and a history of 2 or more physician-diagnosed AOM episodes in the previous year. Previous pneumococcal vaccination was an exclusion criterion. The ethical committees of the participating hospitals previously approved the study. Written informed parental consent was obtained from all subjects. Serum and whole blood for DNA isolation were collected at study entry, before any vaccination was given. Serum and blood were stored at –20°C until analysis.

MBL2 exon 1 and Y/X promoter haplotyping

Genomic DNA was extracted from whole blood by using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and stored at –20°C until analysis. Haplotypes of *MBL2* exon 1 and Y/X promoter polymorphisms were determined by using a previously described denaturing gradient gel electrophoresis (DGGE) assay with slight modifications in a nested PCR protocol.²⁸ Two PCR assays specific for the promoter X haplotype (forward primer ATT TGT TCT CAC

TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; 25 cycles, annealing temperature 63°C) or Y haplotype (forward primer TTT GTT CTC ACT GCC ACG) were run. The PCR products were diluted 1:100 in distilled water. *MBL2* exon 1 was amplified from these dilutions with an extra GC-clamp attached (forward primer with clamp ccg ccc gcc gcg ccc cgc gcc cgg ccc gcc gcc ccc gcc ccG TGT TCA TTA ACT GAG ATT AAC CTT C; reverse primer CAG AAC AGC CCA ACA CG). The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. On electrophoresis, all exon 1 haplotypes have a unique pattern of migration. The corresponding Y/X promoter haplotypes could be inferred from the presence or absence of a product in the nested PCR.

SNPs in the *MBL2* gene

Eleven SNPs in the *MBL2* gene were determined by using PCR with sequence-specific primers (SSP-PCR). SNPs and primer sequences are described in Table I.

Because of the reported difficulties in SSP-PCR for the –619G>C (H/L) SNP (rs11003125),²⁰ this SNP was determined by using a TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, Calif). Two locus-specific PCR primers (forward primer GGA GTT TGC TTC CCC TTG GT; reverse primer GGG CCA ACG TAG TAA GAA ATT TCC A) and 2 allele-specific oligonucleotide TaqMan probes (CAA GCC TGT CTA AAA C-Vic; AAG CCT GTG TAA AA C-Fam) were used.

Functional MBL levels

Functional MBL levels in serum were determined by using the assay described by Kuipers et al.²⁹ This assay is based on the principle of yeast-induced bystander lysis of chicken erythrocytes. In short, serum samples were diluted and incubated with a standardized amount of freshly cultured baker's yeast (*Saccharomyces cerevisiae*) to activate the MBL pathway. To standardize for complement components of the classic, amplification, and terminal route, MBL-deficient serum was added. Chicken erythrocytes were used as the target for hemolysis because of MBL pathway activation. Hemoglobin release was measured in an ELISA reader at 405 nm. Percentages of hemolysis were calculated by using controls for 100% (water lysed) and 0% (buffer control) hemolysis. MBL activity is expressed as μg equivalent MBL per mL, relative to a reference human serum pool with an established antigenic MBL level of 1.67 $\mu\text{g}/\text{mL}$.

Statistical analyses

Differences in functional MBL concentrations according to *MBL2* exon1 and Y/X haplotypes were tested for significance by using a general linear model. Because our study population consists of 60% boys and because MBL activity increases with age, age and sex were corrected for in the model. The difference in the mean number of documented AOM episodes in the previous year according to haplotype was tested for significance by using Mann-Whitney *U* test. Haplotypes for the 12 SNP positions were estimated from unphased genotype data using the Bayesian statistical method in PHASE 2.1 (<http://www.stat.washington.edu/stephens/software.html>).^{30,31} Two not previously described haplotypes (HYPB, 3 haplotypes; LYQB, 1 haplotype) were found. These 4 additional haplotypes might be imperfectly constructed using PHASE, or these individuals might have been incorrectly genotyped. Therefore, these subjects were not included in statistical analyses.

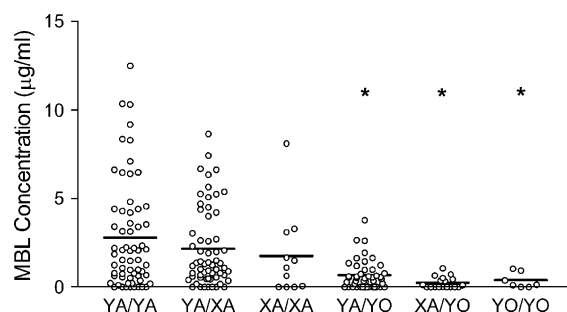
Differences in functional MBL levels according to the phased haplotypes were tested for significance by using a general linear model correcting for age and sex.

TABLE I. SNPs determined using SSP-PCR

dbSNP identifier	SNP*	Amino acid change	Name	Primer
rs930506	−5156A>G	Promoter		R-AAAGGCAGCTCAGTAGTTAGGAT R- AAGGCAGCTCAGTAGTTAGGAC F-CAATGATGGTCACCCACTAGG
rs920725	−1640C>T	Promoter		R-CCTTACGGGCATCGCCAA R- CTTACGGGCATCGCCAG F-GTGATGATAGCACAACCGTGT
rs7084554	−418A>G	Promoter		R-CTCCATGTCCTCTTCGGGT R- TCCATGTCCTCTTCGGGC F-GCTAGGCTGCTGAGGTTTCTT
rs7096206	−290G>C	Promoter	Y/X	F-CATTGTGTTCTACTGCCACG/C R-GATGCCAGAGAATGAGAGCTG
rs7095891	−66C>T	Promoter	P/Q	F-GTAGGACAGAGGGCATGCTT F- TAGGACAGAGGGCATGCTC R-GATGCCAGAGAATGAGAGCTG
rs5030737	154C>T	D52C	D-variant	R- T (C) CCTTGGTG (C) CATCACG† R- CT (T) CCTTGGTG (C) CATCACG† R- CT (C) CCTTGGTG (T) CATCACG† R-TTCT (T) CCTTGGTG (T) CATCACG† R- CT (C/T) CCTTGGTG (C/T) CATCACA† F-ACGGTCCCATTGTTCTCACT
rs1800450	161G>A	G54D	B-variant	R-CCCTTTTCT (C/T) CCTTGGTG† R- CCCTTTTCT (C/T) CCTTGGTG† F-ACGGTCCCATTGTTCTCACT
rs1800451	170G>A	G57E	C-variant	R-ACCTGGTTCCTTTTCTT R- CCTGGTTCCTTTTCTC F-ACGGTCCCATTGTTCTCACT
rs1982266	760C>T	Intron		F-TCCTGGGTCAAGGTCTCCT F- CCTGGGTCAAGGTCTCCC R-TTTTCCAGGGTCTCCTTTTG
rs930507	3130G>C	L126L		F-CCACTTTTTCACATTTAGGGCTG/C R-ATAGGGAACCTCACAGACGGC
rs2506	5887T>G			R-TGTCTTTCAGGAGTCTATACCTGA R- GTCTTTCAGGAGTCTATACCTGC F-ATGAGGTTTCTACTGGGACCAC

*Base pair substitutions are defined based on the coding strand.

†Different primers were used in 1 run for overlapping sequences in exon 1.

**FIG 1.** Functional MBL concentrations ($\mu\text{g/mL}$) according to MBL haplotype. * $P < .01$ compared with wild-type haplotype YA/YA.SPSS 12.0.1 for windows (SPSS Inc, Chicago, Ill) was used for all statistical analyses. $P < .05$ was considered significant.

RESULTS

Functional and genetic MBL analyses were performed in material from a pneumococcal vaccine efficacy trial,

in which 383 children were initially included. Of 244 of these children, DNA was available to determine haplotypes of *MBL2* exon 1 and Y/X promoter polymorphisms using DGGE. More than 98% of the patients were of white ethnicity. Functional MBL concentrations were determined in available serum from 204 of these 244 subjects. We correlated haplotypes of these individuals with their serum MBL level. Highest geometric mean functional MBL levels were observed in serum of wild-type YA/YA haplotype carriers (Fig 1). Mean serum MBL levels were lower when *MBL2* exon 1 and/or Y/X promoter polymorphisms were present. Differences in MBL serum levels between YA/YO, XA/YO, or YO/YO and wild-type YA/YA haplotype carriers were statistically significant ($P < .01$).

In addition, functional serum MBL concentrations were investigated according to haplotypes constructed of 12 polymorphisms in the *MBL2* gene in 195 individuals with full genotype data on these 12 SNP positions (Fig 2). PHASE 2.1 software constructed 33 different haplotypes from the genotype data. On the basis of the *MBL2* exon

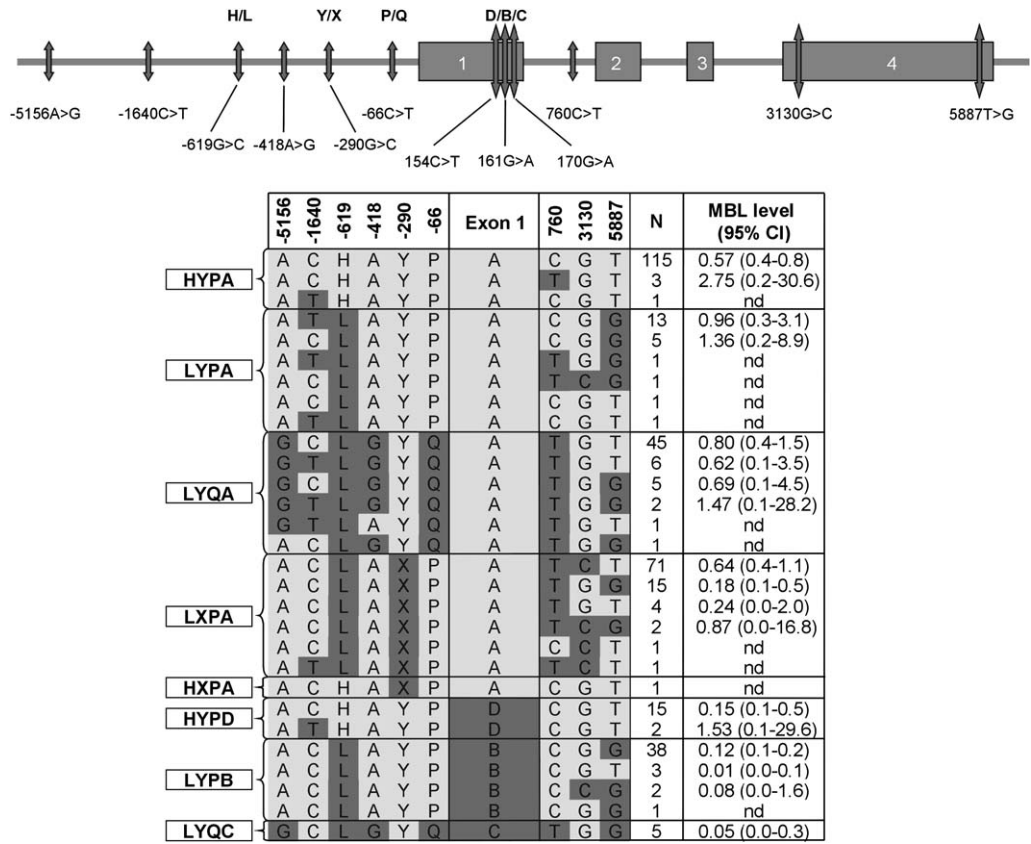


FIG 2. Schematic representation of SNPs studied across the *MBL2* gene and overview of haplotypes with functional MBL levels ($\mu\text{g/mL}$) constructed from genotype data on the 12 SNP positions. *N* indicates frequency of haplotypes. *nd*, Mean MBL level is not determined when haplotype frequency (*N*) is 1. Wild-type positions are indicated in light grey, polymorphic positions in dark grey.

1 and the H/L, Y/X, and P/Q promoter SNPs, the 7 common (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, and LYQC) and 1 of the rare (HXPA) haplotypes were obtained.^{20,32} Of 181 children with haplotype data, functional MBL levels were available. We now investigated geometric mean functional MBL concentrations according to the 8 recognized haplotypes. MBL levels were lower when an *MBL2* exon 1 polymorphism was present (Table II). Of the additional 6 SNPs that were studied, the exon 4 SNP 3130G>C, common within the LXPA haplotype, was found to affect functional MBL levels. Haplotypes with 3130G showed a significantly lower geometric mean functional MBL serum level of 0.19 $\mu\text{g/mL}$ (95% CI, 0.07-0.5) compared with 0.70 $\mu\text{g/mL}$ (95% CI, 0.4-1.2) in haplotypes including 3130C carriers ($P = .026$; Fig 2). The 12 SNP haplotype analysis revealed linkage between several SNPs. The P/Q SNP at position -66 was linked with the -418A>G promoter SNP and with the -5156A>G promoter SNP (Fig 2). Clinical relevance of the *MBL2* haplotypes was investigated by comparing the mean number of AOM episodes in the previous year between carriers of the wild-type YA/YA haplotype with nonwild-type carriers (Table III). For the group as a whole, there was no difference in the

number of previous AOM episodes according to haplotype. Because MBL is thought to be of particular importance in young children, we split the cohort on the basis of age. Indeed, in the group of youngest children between 12 and 24 months of age, wild-type carriers had significantly fewer AOM episodes compared with nonwild-type carriers ($P = .027$). For the older children, the *MBL* haplotype had no effect on the number of AOM episodes ($P = .89$).

DISCUSSION

Mannan-binding lectin levels and function vary greatly within the general population. Part of this variation can be explained by polymorphisms within the structural gene as well as by promoter polymorphisms. Yet, because considerable variation in MBL activity exists even within the 7 common *MBL* haplotypes, it has been speculated that additional variants may contribute to functional MBL levels. To address this issue, we have correlated the 7 recognized *MBL2* gene haplotypes as well as several new polymorphisms within existing haplotypes with functional serum MBL levels. Indeed we found that a common SNP in exon 4 (3130G>C) regulates functional MBL

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TABLE II. Geometric mean MBL levels ($\mu\text{g/mL}$) and 95% CIs according to recognized haplotypes

	N	95% CI	Mean functional MBL level ($\mu\text{g/mL}$)	P values*					
				LYPA	LYQA	LXPA	HYPD	LYPB	LYQC
HYPA	119	0.62	0.42-0.91	.2	.4	.4	.05	<.001	.009
LYPA	22	1.18	0.48-2.89		.5	.07	.01	<.001	.003
LYQA	60	0.84	0.49-1.44			.1	.02	<.001	.004
LXPA	94	0.47	0.30-0.73				.1	<.001	.02
HYPD	17	0.20	0.07-0.57					.3	.2
LYPB	44	0.10	0.05-0.20						.5
LYQC	5	0.05	0.01-0.32						

*P values comparing these levels are calculated by using a general linear model correcting for age and sex.

TABLE III. Association between MBL haplotype and number of AOM episodes from retrospectively obtained data

	Total cohort	12-24 mo	>24 mo
YA/YA			
Number of children	76	32	44
Median AOM episodes	3	3	3
Mean AOM episodes (95% CI)	4.3 (3.7-4.9)	4.1 (3.2-5.0)	4.4 (3.6-5.2)
Non-YA/YA			
Number of children	168	81	87
Median AOM episodes	4	4	3
Mean AOM episodes (95% CI)	4.7 (4.3-5.1)	5.1 (4.5-5.6)	4.4 (3.8-5.0)
P value*	.12	.027	.89

*P values are determined comparing the number of AOM episodes between wild-type and nonwild-type carriers using Mann-Whitney U test.

serum levels within the LXPA haplotype. Furthermore, we showed that functional *MBL2* gene polymorphisms are of clinical importance with respect to susceptibility to AOM in children younger than 2 years old when adaptive immune responses are still immature and the child is without maternally derived protective antibodies. These data extend previous data on the relation between *MBL* genotype and function in children with recurrent respiratory infections.³³

Bernig et al²³ published 87 polymorphic sites across the *MBL2* gene. The high frequency of *MBL2* polymorphic haplotypes in certain ethnic populations suggests a possible selective advantage for heterozygosity.^{20,21} Carriers of *MBL2* genetic variants may be protected against intracellular replicating organisms like *Mycobacteria* spp and *Leishmania* spp, or the lower MBL levels serve to reduce the harmful effects of extreme complement activation via the lectin pathway.³⁴⁻³⁷

In the study by Bernig et al,²³ a high degree of linkage between 51 SNPs with a minor allele frequency greater than 0.05, occurring in a 2-block structure, was observed.²³ We also found a high degree of linkage in our current study, especially within the 5' end of the *MBL2* gene. Recently, Bernig et al³⁸ redefined the 5' block boundary from position -2703 to position -4687. This is

in agreement with our observed linkage of the more 5' SNP at position -5156 with the SNPs at positions -418 and -66 of the *MBL2* promoter. The boundaries of the 5' block may even lie further downstream in the promoter region, and future analysis beyond the -5156 boundary, possibly including neighboring genes, may be required for optimal correlation of *MBL2* haplotypes with MBL serum levels.

The density of common SNPs is comparable for the 2-block structures identified, but the region that separates the 2 blocks shows an increased recombination rate and thus probably includes a recombination hot spot.^{23,38} Within this recombination hot spot, we now identified an SNP (3130G>C) that apparently influences functional MBL levels within the LXPA haplotype. Compared with homozygous carriers of the other common haplotypes (HYPA, LYPA, or LYQA), LXPA homozygotes display a much larger variation in MBL levels.²⁴ Our finding that the 3130G>C SNP further splits up the LXPA haplotype in 2 haplotypes with different MBL activities could be the basis for that large variation. Subtyping of LXPA homozygotes for the 3130G>C SNP would further establish the relation between this particular genotype and MBL function.

The 3130G>C SNP has previously been shown to be mainly observed within the LXPA haplotype in white subjects.²³ In other ethnicity groups, 3130C can also be found in the HYPD, LYPA, and LYQC haplotypes.²³ Our study population consists for approximately 95% of white children, and we thus found the 3130C primarily within the LXPA haplotype.

The 3130G>C SNP in exon 4, which encodes the carbohydrate recognition domain, does not lead to an amino acid substitution. However, it is known that non-coding SNPs might influence exon splicing or stability of the mRNA.^{23,39} Alternatively, Bernig et al²³ showed the 3130G>C SNP to be in linkage disequilibrium with the intronic IVS3+709G>C SNP. Possibly this SNP or another SNP in linkage with 3130G>C causes the difference in functional MBL serum levels we observed within the LXPA haplotype.

Previously variant *MBL2* genotypes as well as low circulating levels of MBL have been associated with susceptibility to invasive pneumococcal and meningococcal disease, immunodeficiency, and severe infections.⁴⁰⁻⁴⁵

In addition, several studies showed an association of upper respiratory tract infections and/or AOM with aberrant MBL levels and/or genotypes,^{11,46-48} but this was not confirmed by others.^{49,50} These conflicting results might be caused by variation in age of subjects under study, because in one prospective study in children from Greenland, the maximal effect of MBL deficiency was observed in children between 6 and 17 months of age, whereas the effect could not be demonstrated in younger or older children.¹¹ The fact that we find MBL relevance only below the age of 24 months makes sense, because adaptive immunity against polysaccharide encapsulated bacteria, a main group of causative pathogens for AOM, matures around this age.⁵¹ Similar to Koch et al,¹¹ we included the Y/X promoter polymorphism in our clinical association study, whereas other studies only investigated the structural *MBL2* exon 1 variants. Because others and we showed that the Y/X promoter polymorphism further downregulates functional MBL levels, this also could explain the conflicting data in literature.²⁰ Finally, the studies that did not find an association between OM and *MBL2* genotype were rather small ($n = 73$, $n = 89$), especially when looking at OM subgroups.^{49,50} Probably large and well characterized cohorts of defined ethnicity are necessary to establish the effect of a single gene in a multifactorial disease like OM.

Overall, our data show that both recognized and new *MBL2* polymorphisms influence functional MBL serum levels and are of clinical relevance in young children with recurrent AOM. Possibly additional SNPs within common *MBL2* haplotypes are of clinical importance. Therefore, future association studies will probably require a more locus-wide approach, as already suggested by Bernig et al.²³ Because data on *MBL2* haplotype distribution in a childhood population without recurrent OM are not available, and healthy adult populations will include persons with a history of recurrent OM, a large prospective birth cohort is probably required to determine the relative contribution of *MBL2* haplotypes to the development of recurrent OM.

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