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Association of CD14 Promoter Polymorphism with Otitis Media and Pneumococcal Vaccine Responses

S. P. Wiertsema,[1,2] S.-K. Khoo,[2] G. Baynam,[2] R. H. Veenhoven,[3] I. A. Laing,[2] G. A. Zielhuis,[4] G. T. Rijkers,[1,5] J. Goldblatt,[2,6] P. N. LeSouëf,[2] and E. A. M. Sanders[1]*

Department of Pediatric Immunology, University Medical Center Utrecht, Utrecht, The Netherlands[1]; School of Paediatrics and Child Health, University of Western Australia, Perth, Australia[2]; Department of Pediatrics, Spaarne Hospital, Hoofddorp, The Netherlands[3]; Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands[4]; Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, The Netherlands[5]; and Genetic Services of Western Australia, Perth, Australia[6]

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Innate immunity is of particular importance for protection against infection during early life, when adaptive immune responses are immature. CD14 plays key roles in innate immunity, including in defense against pathogens associated with otitis media, a major pediatric health care issue. The T allele of the CD14 C-159T polymorphism has been associated with increased serum CD14 levels. Our objective was to investigate the hypothesis that the CD14 C-159T allele is protective against recurrent acute otitis media in children. The association between the CD14 promoter genotype and the number of acute otitis media episodes was evaluated both retrospectively and prospectively in a cohort of 300 children. Serotype-specific immunoglobulin G (IgG) antibody responses after pneumococcal vaccinations were examined according to CD14 genotype to compare immune responsiveness across genotypes. An age-dependent association was found: compared with that for CC homozygotes aged between 12 to 24 months, TT homozygotes had fewer episodes of acute otitis media (79 versus 41%, respectively; $P = 0.004$); this relationship was absent in older children. Additionally, TT homozygotes showed higher serotype-specific anti-pneumococcal IgG antibody levels. Our data suggest that genetic variation in CD14, a molecule at the interface of innate and adaptive immune responses, plays a key role in the defense against middle ear disease in childhood and in pneumococcal vaccine responsiveness. These findings are likely to be important to these and other immune-mediated outcomes in early life.

Otitis media (OM) is the most common reason for children under 3 years of age to visit general practitioners and represents a major pediatric health care issue (16, 44). Bacteria, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and viruses, including respiratory syncytial virus, play roles in the onset of inflammation of the middle ear cavity. Innate defense systems that are capable of quickly distinguishing potential pathogens from self-structures are important for protection from infection, particularly at early age when adaptive immune responses to many microorganisms, including encapsulated bacteria, are immature (50).

CD14 plays a central role in the innate immune defense against many microorganisms causing otitis media. First characterized as the endotoxin lipopolysaccharide receptor (53), CD14 also appears to be involved in responses to lipoteichoic acid from gram-positive bacteria (7), peptidoglycan of both gram-positive and gram-negative bacteria (18), mycobacteria, and viruses (12). The CD14 crystal structure provides a basis for this ligand diversity (24). The involvement of CD14 in macrophage responses against purified capsular polysaccharide of *S. pneumoniae*, the most important bacterial pathogen associated with OM, makes CD14 a biologically plausible candidate to investigate for its function in the immune defense against middle ear infection (46). CD14 is expressed in membrane-bound form (mCD14) on the cell surfaces of monocytes and neutrophils, but it is also present in serum as a soluble protein. Soluble CD14 (sCD14) has the ability to confer pathogen responsiveness to cells that do not constitutively express CD14 on their membranes (36, 49) and to enhance mCD14-mediated responses to both lipopolysaccharide and peptidoglycan (13). CD14 is also an important molecule for the optimal functioning of Toll-like receptor 4 (29), a principal membrane-signaling molecule through which mammals sense infection (3).

A functional polymorphism in the promoter of the CD14 gene (C-159T) has been described previously (2). The T allele is associated with enhanced transcriptional activity in a monocytic reporter assay and increased sCD14 levels in epidemiological studies (2, 22, 28).

Since increased sCD14 levels would enhance immune responsiveness, we hypothesized that the T allele would be protective against middle ear infections. To this end, a cohort of children with histories of recurrent acute otitis media (AOM) was used to analyze the number of children suffering from an otitis-prone condition, which was defined as having had four or more AOM episodes per year, according to CD14 C-159T genotype (20). Furthermore, the presence or absence of AOM recurrence according to CD14 genotype was studied prospectively. Immunoglobulin G (IgG) serotype-specific antibody responses to pneumococcal vaccinations were investigated in relation to the CD14 promoter genotype to compare immune responsiveness across genotypes.

* Corresponding author. Mailing address: Department of Pediatric Immunology, UMCU, Lundlaan 6, KC03.063.0, 3584 EA, Utrecht, The Netherlands. Phone: 31 30 2504000. Fax: 31 30 2505350. E-mail: l.sanders@umcutrecht.nl.

TABLE 1. General and immunologic characteristics of the children under study for occurrence of AOM

Parameter	Value of parameter for children with genotype			*P* value
	CD14 CC (n = 83)	CD14 CT (n = 149)	CD14 TT (n = 68)	
Mean age (95% CI)	3.2 (2.8–3.5)	3.1 (2.8–3.4)	2.8 (2.4–3.1)	0.3
No. (%) of boys	50 (60.2)	87 (58.4)	48 (70.6)	0.2
No. (%) breastfed for ≥3 mo	36 (43.4)	63 (42.3)	29 (42.6)	0.9
Mean no. of siblings (95% CI)	1.1 (0.9–1.3)	1.2 (1.0–1.3)	1.0 (0.8–1.2)	0.6
No. (%) of children attending day care				
12–24 mo old	10 (35.7)	24 (46.2)	13 (44.8)	0.7
24–48 mo old	22 (81.5)	49 (85.9)	21 (84.0)	0.9
No. (%) in homes where smoking occurs	23 (27.7)	47 (31.5)	29 (42.6)	0.1
Geometric mean				
IgM level (g/liter)	1.4	1.4	1.3	0.5
IgA level (g/liter)	0.7	0.8	0.7	0.4
IgG level (g/liter)	9.3	9.2	8.8	0.6
IgG1 level (g/liter)	7.2	7.3	7.2	0.9
IgG2 level (g/liter)	0.9	0.9	0.9	0.6
IgG3 level (g/liter)	0.4	0.4	0.4	0.9
IgG4 level (g/liter)	0.3	0.2	0.1	0.06
IgE (kU/liter)	16.9	17.7	8.8	0.01

MATERIALS AND METHODS

Study population to investigate occurrence of AOM. The number of AOM episodes experienced in the last year was analyzed according to CD14 genotype in 300 children who participated in a randomized controlled trial on the clinical efficacies of pneumococcal vaccines in the prevention of AOM (47). All children were between 1 and 7 years of age and had histories of two or more physician-documented AOM episodes in the year before study entry.

In the original study, half of the subjects received pneumococcal vaccines, whereas the other half was randomized to receive hepatitis A or B vaccinations. As it is assumed that hepatitis vaccination has no significant impact on AOM recurrence, we prospectively analyzed the presence or absence of AOM recurrence according to CD14 genotype in the 161 subjects who received hepatitis vaccines during the 18-month follow-up period.

Study population to investigate pneumococcal vaccine responses. The association between the CD14 promoter polymorphism and the serotype-specific IgG response to pneumococcal conjugate, followed by pneumococcal polysaccharide vaccinations, was investigated for 71 children who were participating in two large vaccination trials investigating the prevention of recurrence of otitis media by pneumococcal vaccinations in The Netherlands (41, 47).

The first group consisted of 48 children, aged 2 to 7 years, with histories of two or more physician-diagnosed episodes of acute otitis media in the previous 12 months, who were randomly selected from the study population participating in the randomized controlled vaccination study described above. These children received the 7-valent pneumococcal conjugate vaccine (Prevnar; Wyeth Pharmaceuticals, Philadelphia, PA), followed by the 23-valent pneumococcal polysaccharide vaccine 6 months later (Pneumune; Wyeth Pharmaceuticals, Philadelphia, PA).

The second group consisted of 23 children, aged 2 to 7 years, who had suffered from at least two prolonged (lasting 3 months or longer) episodes of bilateral otitis media with effusion (OME) documented by an ear, nose, and throat specialist and who were therefore referred for ventilation tube placement. These children were also vaccinated with the 7-valent pneumococcal conjugate vaccine, followed by the 23-valent pneumococcal polysaccharide vaccine, but with an interval of 4 months.

The seven-valent pneumococcal conjugate vaccine (PCV7) consisted of 2 μg each of capsular polysaccharides from pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to mutant nontoxic diphtheria toxin (CRM_{197}). The 23-valent pneumococcal polysaccharide vaccine (PPV23) consisted of 25 μg of capsular polysaccharides from each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

These studies were approved by the participating hospitals' and institutions' ethics committees. Parental written informed consent was obtained for all subjects.

DNA extraction and genotyping. Genomic DNA was extracted from whole blood collected at study entry by using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany). Patients were genotyped for the CD14 C-159T polymorphism by using PCR and restriction enzyme digestion with AvaII (Promega Corporation, Madison, WI) as previously described by Baldini and coworkers (2).

Measurement of pneumococcal serotype-specific antibodies and immunoglobulins. Blood samples for the determination of pneumococcal antibodies were obtained 4 weeks after the pneumococcal polysaccharide booster vaccination. Serum was separated and stored at −20°C until analysis. Postvaccination IgG antibody levels to all PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) were measured by enzyme-linked immunosorbent assay as described previously (39). Minimal detection levels for these pneumococcal serotypes were 0.03, 0.09, 0.12, 0.61, 0.05, 0.11, and 0.08 μg/ml, respectively. All sera were preincubated overnight at 4°C with pneumococcal cell wall polysaccharide in diluting buffer to block nonspecific anti-cell wall polysaccharide antibodies (50 μg/ml; Statens Serum Institute, Copenhagen, Denmark) (33). The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardization (37).

Serum immunoglobulin (IgM, IgA, and IgG) levels and IgG subclasses were measured by rate nephelometry in sera obtained at the time of study entry and stored at −20°C until analysis. Total serum IgE levels were determined by ImmunoCAP (Pharmacia, Uppsala, Sweden).

Statistical methods. To assess differences in general and immunological characteristics, analysis of variance (for age, immunoglobulin levels, and IgG subclass levels), chi-square (for gender, breastfeeding, day care attendance, and smoking), and Kruskal-Wallis tests (for siblings) were used.

For retrospective analyses, children were categorized based on the number of AOM episodes in the year before study entry. Since having had four or more AOM episodes per year was defined as the otitis-prone condition (20), children were classified as those with two to three AOM episodes per year or those with four or more AOM episodes per year. For the prospective analyses, the outcome measured was the presence or absence of AOM recurrence. Chi-square analyses were employed to investigate differences between groups according to CD14 genotype in both retrospective and prospective analyses.

To test differences between antibody levels after pneumococcal vaccinations according to CD14 genotype, a general linear model with adjustments for age, gender, type of ear disease, and log total IgE level was used. Additionally, a model recessive for the C allele was employed, with adjustments as described above. *P* values of <0.05 were considered statistically significant. SPSS 12.0.1 for Windows (SPSS, Inc., Chicago, IL) was used for all analyses.

RESULTS

General characteristics and immunological data on the cohort with recurrent AOM are described in Table 1. There were no statistically significant differences across the CD14 genotype for age or gender, serum immunoglobulin M (IgM), IgA, IgG, and IgG subclass levels, or environmental risk factors

TABLE 2. Association between CD14 C-159T genotype and categorized number of AOM episodes according to age

Genotype	No. (%) of subjects with the indicated no. of AOM episodes[a]					
	Total cohort		Participants aged			
			12 to 24 mo		>24 mo	
	2–3 Episodes	≥4 Episodes	2–3 Episodes	≥4 Episodes	2–3 Episodes	≥4 Episodes
CD14 CC	26 (31)	57 (69)	6 (21)	22 (79)	20 (36)	35 (64)
CD14 CT	50 (34)	99 (66)	12 (23)	40 (77)	38 (39)	59 (61)
CD14 TT	32 (47)	36 (53)	17 (59)	12 (41)	15 (38)	24 (62)

[a] Retrospectively obtained data from the year before study entry. *P* values for the comparison of numbers of individuals with no recurrence of AOM with numbers of individuals with ≥1 recurring AOM episode according to the three genotype groups by using chi-square analyses are 0.5 for the total cohort, 0.04 for participants 12 to 24 months of age, and 0.5 for participants >24 months of age. *P* values for the comparison of numbers of individuals with no recurrence of AOM with numbers of individuals with ≥1 recurring AOM episode according to the homozygous CC and TT genotypes by using chi-square analyses are 0.3 for the total cohort, 0.01 for participants 12 to 24 months of age, and 0.5 for participants >24 months of age.

known to predispose people to a recurrence of AOM. Notably, total serum IgE levels were significantly higher in CC and CT carriers than in TT homozygotes ($P = 0.01$) (Table 1).

There were significantly fewer otitis-prone TT homozygotes between 12 to 24 months of age than CD14 CC homozygotes (79 versus 41%; $P = 0.004$) (Table 2). These TT homozygotes had a significantly lower mean number of AOM episodes of 4.3 (95% confidence interval [CI], 3.3 to 5.3) compared to 5.7 (95% CI, 4.5 to 6.9) in CC homozygotes. This association was not found in children older than 24 months of age ($P = 0.8$). In the prospective analyses, CD14 C-159TT homozygotes younger than 24 months also had significantly less recurrence of AOM than CC homozygotes (36 versus 81%; $P = 0.01$) (Table 3).

Since innate immunity influences adaptive immune responses (19), we investigated pneumococcal serotype-specific IgG antibody responsiveness after combined pneumococcal polysaccharide and conjugate vaccinations according to CD14 genotype in a group of children with recurrent or prolonged OM. General characteristics and serum immunoglobulin levels

TABLE 3. Association between CD14 C-159T genotype and categorized number of AOM episodes according to age[b]

Genotype	No. (%) of subjects with the indicated no. of AOM episodes[a]					
	Total cohort		Participants aged			
			12 to 24 mo		>24 mo	
	No AOM episodes	≥1 AOM episode	No AOM episodes	≥1 AOM episode	No AOM episodes	≥1 AOM episode
CD14 CC	17 (37)	29 (63)	3 (19)	13 (81)	14 (47)	16 (53)
CD14 CT	37 (48)	40 (52)	11 (41)	16 (59)	26 (52)	24 (48)
CD14 TT	18 (47)	20 (53)	9 (64)	5 (36)	9 (38)	15 (62)

[a] Prospectively obtained data during 18 months of follow-up in children who did not receive pneumococcal vaccinations. *P* values for the comparison of numbers of individuals with 2 or 3 AOM episodes with numbers of individuals with ≥4 AOM episodes according to the three genotype groups by using chi-square analyses are 0.09 for the total cohort, 0.002 for participants 12 to 24 months of age, and 0.9 for participants >24 months of age. *P* values for the comparison of numbers of individuals with 2 or 3 AOM episodes with numbers of individuals with ≥4 AOM episodes according to the homozygous CC and TT genotypes by using chi-square analyses are 0.05 for the total cohort, 0.004 for participants 12 to 24 months of age, and 0.8 for participants >24 months of age.
[b] Age of control subjects at the start of the pneumococcal vaccine study.

TABLE 4. General characteristics and serum immunoglobulin levels according to CD14 genotype of the children under study for antibody responses

Parameter	Value of parameter for children with genotype			*P* value
	CD14 CC ($n = 19$)	CD14 CT ($n = 39$)	CD14 TT ($n = 13$)	
Mean age (95% CI)	4.2 (3.5–4.9)	4.5 (3.9–5.1)	4.1 (2.9–5.2)	0.7
No. of boys (%)	13 (68.4)	28 (71.8)	5 (38.5)	0.1
No. of AOM (%)	14 (73.7)	26 (66.7)	8 (61.5)	0.8
Geometric mean (g/liter)				
IgM level	1.4	1.3	1.4	0.7
IgA level	1.0	1.1	0.8	0.6
IgG level	10.2	10.0	9.4	0.8
IgG1 level	8.3	8.1	8.0	0.9
IgG2 level	1.2	1.3	1.2	0.9
IgG3 level	0.5	0.5	0.5	0.9
IgG4 level	0.4	0.3	0.2	0.4

of this study cohort according to genotype are shown in Table 4. There were no significant differences in the distribution of genotypes by age or gender, and also, total IgM, IgA, IgG, and IgG subclass levels did not differ significantly according to genotype. As we have previously shown that both groups of children with either AOM or OME display similar antibody responses after vaccination (52), these groups were pooled for the analysis of antibody responses.

CC homozygotes, i.e., homozygotes of the genotype associated with a higher number of AOM episodes, had lower IgG responses against all serotypes of the seven-valent pneumococcal conjugate vaccine than did those of either the CT or the TT genotype (Fig. 1). Antibody levels were significantly lower in CC homozygotes than in CT/TT individuals for pneumococcal serotypes 9V ($P = 0.02$), 18C ($P = 0.04$), and 23F ($P = 0.02$), and there was a nonsignificant trend for serotype 4 ($P = 0.09$).

DISCUSSION

An age-dependent association was found between the CD14 C-159T polymorphism and the number of AOM episodes in

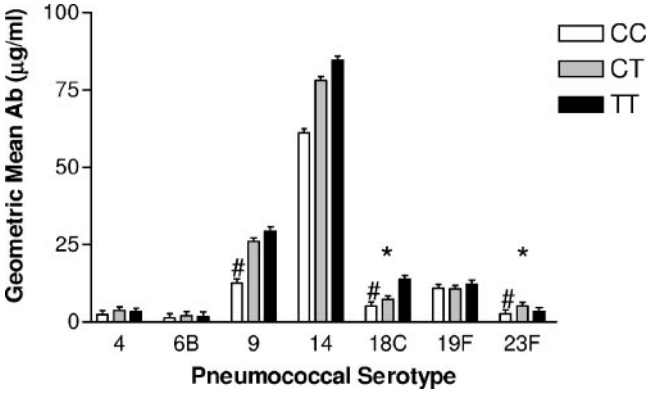

FIG. 1. Geometric mean-adjusted IgG antibody (Ab) titers against seven pneumococcal serotypes after pneumococcal conjugate vaccination, followed by pneumococcal polysaccharide booster vaccination in CD14 CC (open bars), CT (gray bars), and TT (closed bars) carriers (error bars indicate standard errors). *, P was <0.05, comparing CC versus CT versus TT using a general linear model; #, P was <0.05, comparing CC versus CT/TT using a general linear model.

a cohort of 300 children with histories of recurrent AOM. In children up to 24 months of age, TT homozygotes had fewer AOM episodes in prospective and retrospective analyses than did CC homozygotes. Importantly, TT homozygotes had higher specific IgG responses to pneumococcal conjugate vaccine serotypes in a group of children with AOM and OME.

The higher antibody levels in TT carriers suggest a more vigorous adaptive immune response against *S. pneumoniae*, one of the main pathogens in AOM. This enhanced immune response may contribute to the reduced number of episodes of middle ear infections. That increased IgG antibody production may be related to the reduced middle ear infection frequency is supported by the findings that, after pneumococcal vaccinations, IgG anticapsular antibodies protect against acute otitis media in infants (4, 15) and that otitis-prone patients have lower total IgG levels (48) as well as lower pneumococcal polysaccharide vaccine responses (11, 14, 38, 39).

That CD14, as a pivotal molecule in innate immunity, plays a more prominent role in early life is in accordance with a previous report of age-dependent effects of CD14 C-159T polymorphism (34). Polymorphisms in other innate immunity genes, such as mannan-binding lectin, also have the highest impacts early in life (45, 51). The association of mannan-binding lectin polymorphisms with the risk of upper respiratory tract infections in early childhood was greatest in children aged 6 to 17 months (25). The finding of an age-dependent effect of the genetic variation in innate immune molecules being most evident in early childhood is consistent with the prime importance of the innate immune system between 6 and 24 months of age. During this period of development, maternally derived antibodies have waned and the adaptive immune system is immature, particularly with respect to polysaccharide antigen responses (50). Conversely, with the subsequent maturation of adaptive immune responses, genetic variation in innate immune responses may be of lesser importance, and hence, associations with these variants may become less evident.

Determining the mechanism by which CD14 influences the magnitude of pneumococcal-specific antibody responses and the development of otitis media is of considerable importance but at present remains unclear. In preliminary experiments, however, we observed higher interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-10 cytokine levels in supernatants of unstimulated peripheral blood mononuclear cell cultures from CD14 C-159TT homozygote donors than those from cultures from CC homozygotes. Furthermore, the addition of recombinant sCD14 to peripheral blood mononuclear cell cultures from CC donors resulted in levels of these cytokines comparable with those observed in TT homozygotes, suggesting that sCD14 is positively associated with proinflammatory cytokine secretion. These findings are consistent with the proinflammatory role of sCD14 in bacterial meningitis in mice and with the finding that the administration of recombinant sCD14, concomitant with *S. pneumoniae*, results in an enhanced release of IL-6 and TNF-α (5). The protective role of these proinflammatory cytokines in the innate immune defense against bacteria is well established (54), and it is known that innate immune responses have a profound influence on the quality and intensity of the subsequent adaptive immune response (19, 31, 32). Furthermore, an important role for these proinflammatory cytokines, i.e., IL-6, TNF-α, and IL-10, in enhancing humoral immune responses to polysaccharide antigens has been described previously (6, 8, 23, 30). Therefore, enhanced innate and adaptive immune responses in TT homozygotes upon natural exposure to AOM pathogens, secondary to increased sCD14 levels, might explain the lower otitis media frequency observed in these TT homozygote subjects.

An additional finding, which is in accord with previous reports (2, 17, 27), was lower total IgE levels in TT homozygotes, i.e., in the group of subjects with higher pneumococcal-specific IgG levels. Our data suggest an inverse relationship between IgG and IgE levels according to CD14 genotype. The associations of genetic variation in CD14 with atopy (27) and that atopy may predispose people to increased susceptibility to pneumococcal or respiratory tract infections (21, 26, 43) and impaired pneumococcal vaccine responses (1) further supports the importance of atopy to these outcomes. Environmental factors, such as breastfeeding, number of siblings, day care attendance, and tobacco smoke exposure, which are known to contribute to the development of recurrent otitis media (10), were equally distributed across the CD14 genotype in our current study, so they are not likely to explain our findings. Our data therefore suggest that the effect of CD14 polymorphism is separate from and in addition to these known environmental factors.

A limitation of our study is that we investigated the number of otitis episodes and antibody responses in overlapping but not identical cohorts. Future research in other cohorts, specifically in a large prospective birth cohort, is required to further investigate the impact of innate immunity pathways associated with CD14 in relation to infection susceptibility and pneumococcal vaccine responses at various ages. Our findings could conceivably be attributable to another polymorphism in linkage disequilibrium with the studied polymorphism, but there are now several lines of evidence to suggest that the polymorphism we studied is the most likely cause of our findings (49). Since the CD14 promoter polymorphism may be associated both with the clinical course of recurrent AOM in young children and with antibody responses upon pneumococcal vaccination, further studies of additional components of this innate immune pathway, such as Myd88 (42), IRAK4 (35), NEMO (40), and IκBα (9), are warranted.

In conclusion, our data suggest that genetic variation in CD14, a molecule at the interface of innate and adaptive immune responses, plays a key role in the defense against middle ear disease in childhood and in determining pneumococcal vaccine responsiveness. These findings are likely to be important to these and other immune-mediated outcomes in early life.

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ERRATUM

Association of CD14 Promoter Polymorphism with Otitis Media and Pneumococcal Vaccine Responses

S. P. Wiertsema, S.-K. Khoo, G. Baynam, R. H. Veenhoven, I. A. Laing, G. A. Zielhuis, G. T. Rijkers, J. Goldblatt, P. N. LeSouëf, and E. A. M. Sanders

Department of Pediatric Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; School of Paediatrics and Child Health, University of Western Australia, Perth, Australia; Department of Pediatrics, Spaarne Hospital, Hoofddorp, The Netherlands; Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegan, The Netherlands; Department of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, The Netherlands; and Genetic Services of Western Australia, Perth, Australia

Volume 13, no. 8, p. 892–897, 2006. Page 894: The second and third sentences in footnote *a* of Table 2 should be replaced by the second and third sentences in footnote *a* of Table 3, and the second and third sentences in footnote *a* of Table 3 should be replaced by the second and third sentences in footnote *a* of Table 2.