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The 4G/4G Plasminogen Activator Inhibitor-1 Genotype Is Associated With Frequent Recurrence of Acute Otitis Media

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ABSTRACT

OBJECTIVES. Plasminogen activator inhibitor-1 counterregulates cell migration, adhesion, and tissue repair. The *PAII* 4G/5G promoter polymorphism has an effect on expression levels of *PAII*. After a first acute otitis media episode, children are at increased risk for a next episode. Because the *PAII* 4G allele is associated with higher plasminogen activator inhibitor-1 production and, hence, decreased tissue repair, we hypothesize that this allele may contribute to increased recurrence of acute otitis media.

PATIENTS AND METHODS. The *PAII* 4G/5G polymorphism was genotyped in 348 Dutch children aged 1 to 7 years who were suffering from recurrent acute otitis media and participating in a randomized, controlled trial and 463 healthy control subjects, representative of the general population.

RESULTS. No significant difference in *PAII* genotype distribution between the whole acute otitis media group and control subjects was observed. However, children with the *PAII* 4G/4G genotype had an increased risk of more frequent acute otitis media episodes compared with those who were homozygous for the 5G variant, also after correction for cofactors. This finding was attributable to children <4 years of age.

CONCLUSIONS. Our findings suggest that the *PAII* 4G/4G genotype is associated with an increased risk for the otitis-prone condition, potentially because of impaired healing after a previous otitis media episode.

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Key Words

plasminogen activator inhibitor-1, polymorphism, otitis media

Abbreviations

AOM—acute otitis media
PAI-1—plasminogen activator inhibitor-1
OR—odds ratio
UPA—urokinase plasminogen activator
TM—tympanic membrane

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ACUTE OTITIS MEDIA (AOM) is the most common infection in children, but the number of AOM episodes varies markedly between patients. Approximately 10% to 15% of all children suffer from ≥ 4 episodes per year and are considered otitis prone.¹⁻³ One of the most prominent risk factors seems to be a first episode of AOM early in life predisposing to a next event.^{4,5} Other issues affecting the risk of developing recurrent middle ear infections include host factors like gender, age, atopy, and ethnic background.⁶ Environmental factors associated with recurrent AOM episodes are microbial exposure, like in day care or from older siblings, or triggers, like lack of breastfeeding or exposure to tobacco smoke. Often there is a family history of otitis media.^{4,6,7} Twin studies have shown a heritability of 57% for acute ear infections and 72% for chronic ear infections.⁸⁻¹² Furthermore, the correlation for recurrent otitis media is twofold higher in monozygotic twins (65%–71%) compared with dizygotic twins (25%–34%).¹³ Genetic factors involved in inflammation and tissue repair may play a role in the development of recurrent AOM episodes and the otitis-prone condition. Delayed downregulation of the inflammatory response to infections in the middle ear cavity may play a role in priming the individual for additional AOM episodes. Plasminogen activator inhibitor-1 (PAI-1) is known to have an important role in fibrinolysis.^{14,15} In addition, PAI-1 is involved in the inflammatory reaction, inhibits cell adhesion and migration, and delays tissue repair.^{16,17} The 4G/5G insertion-deletion polymorphism at position -675 of the *PAII* gene is known to influence the expression of PAI-1.^{18,19} Individuals homozygous for the 4G allele have higher PAI-1 concentrations in peripheral blood than those with the 4G/5G or 5G/5G genotypes.^{20,21} The *PAII* 4G allele has been reported previously to be associated with asthma, as well as with increased morbidity and mortality in meningococcal disease.^{20,22-25}

We hypothesized that the *PAII* 4G/4G genotype, known to result in high PAI-1 concentrations, which is associated with decreased tissue integrity, is associated with recurrence of otitis media. In this study, we investigated the association between this *PAII* polymorphism and recurrent AOM. Our results show that 4G homozygous individuals have an increased risk for an otitis-prone condition, especially under the age of 4 years.

METHODS

Participants

Patients who initially participated in a randomized, controlled study on prevention of recurrent AOM by pneumococcal vaccinations were included in this study.²⁶ Parents were informed about the study by primary care physicians, pediatricians, and otolaryngologists from across the Netherlands. Children were enrolled in the study after obtaining approval of the medical ethical

committee of the Utrecht University Medical Centre and informed consent from the parents or guardians. DNA was available from 348 Dutch white children, 1 to 7 years of age, who were suffering from recurrent AOM defined as ≥ 2 AOM episodes in the preceding year. The number of AOM episodes before vaccination was based both on parental report (AOM defined as having ≥ 1 of the symptoms: acute earache, new-onset otorrhea, irritability, and fever) and on clinical information of the diagnosis by a physician. In the present study cohort, 122 children suffered from 2 to 3 otitis media episodes in the year preceding inclusion, whereas 226 suffered from ≥ 4 episodes (otitis-prone condition).³ No children with < 2 AOM episodes were included in the vaccination trial. In addition, children with primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, chromosomal abnormalities such as Down syndrome, and craniofacial abnormalities such as cleft palate were excluded from the study. The history of atopy, defined as eczema, hay fever, recurrent wheezing, or asthma, and the number of AOM episodes in the year before inclusion were assessed by the questionnaire filled out by all of the patients and their parents at inclusion in the study. Otorrhea duration was assessed in 214 children for whom *PAII* genotypes were available. Patient characteristics are described in Table 1. Healthy adult Dutch white control subjects ($n = 463$) were derived from the Dutch Blood Bank Sanquin. No records of previous history regarding AOM were available for these control subjects; however, in the general population, a total number of ≤ 3.2 AOM episodes is expected in childhood.³ This implies that control subjects will have had, on average, much fewer AOM episodes per year than the patients. Blood was sampled for DNA isolation. DNA isolation from donor buffy coats was performed according to standard protocols using a QIAamp DNA mini kit (Qiagen, Valencia, CA).

Experimental Procedures

PAII -675 genotype (rs1799889) was determined using single base extension analysis, a technique commonly used in our laboratory (ABI Prism, SNaPshot Multiplex kit; Applied Biosystems, Foster City, CA). The genomic region of interest was amplified using polymerase chain reaction (37 cycles; annealing temperature of 60°C; total volume of 10 μ L) with the following primers: forward, 5'-CAGCCAGACAAGGTTGTT-3', and reverse 5'-CCACCCGGTGCTCT-3'. Amplification resulted in a 265-nucleotide polymerase chain reaction product. After purification with Shrimp Alkaline Phosphatase and Exonuclease I (both from Amersham Biosciences, Uppsala, Sweden), a single base extension with fluorescently labeled dideoxynucleotide triphosphates was performed using the reverse primer 5'-TTTTGATACACGGCT-GACTCCCC-3'. The poly-T tail allows us to distinguish primers in a multiplex analysis for which this assay was

TABLE 1 Characteristics of Patients With Recurrent AOM

Characteristics	2–3 AOM Episodes (N = 122)	≥4 AOM Episodes (N = 226)	P ^a
Male gender, n (%)	84 (69)	128 (57)	.03
Age, median (range), y	2.42 (1.0–6.3)	2.10 (1.0–7.0)	.40
Age group (12–48 mo), n (%)	84 (69)	180 (80)	.03
Age at first AOM, geometric mean (SD), mo	10.1 (2.0)	7.7 (2.1)	.001
Breastfeeding ≥3 mo, n (%)	57 (47)	96 (43)	.50
Tobacco smoke exposure indoors, n (%)	41 (34)	70 (31)	.63
Day care			
At age 12–24 mo, n/N (%)	22/44 (50)	42/99 (42)	.47
At age 25–48 mo, n/N (%)	70/78 (90)	113/127 (89)	1.00
No. of siblings, median (range); mean	1.0 (0–3); 0.93	1.0 (0–7); 1.17	.02
Family history positive for AOM			
Parents (121/225), n (%)	79 (65)	131 (58)	.21
Siblings, n/N (%)	48/86 (56)	98/182 (54)	.79
Atopy, n (%) ^b	54 (44)	117 (52)	.22

^a Fisher's exact test, Mann-Whitney *U* test, and *t* test were performed when appropriate.

^b Atopy was defined as having eczema, hay fever, or recurrent wheezing or asthma.

originally designed. The products generated were analyzed on an ABI Prism 3100 genetic analyzer using GeneScan Analysis Software (Applied Biosystems). When no clear genotype was obtained or no consensus was reached between 2 independent investigators, genotypes were excluded from the analyses.

Statistical Analyses

Statistical analyses were performed by using SPSS 11.0 (SPSS Inc, Chicago, IL). A comparison of genotype frequencies was made between patients and blood donor control subjects assuming a multiplicative model.²⁷ Binomial variables were analyzed using Pearson's χ^2 test or Fisher's exact test when appropriate. Continuous variables were compared for the different genotypes using the Mann-Whitney *U* test (nonparametric) or *t* test (normal distribution). Within the patient sample, only genotype distributions of children suffering from 2 to 3 episodes per year before vaccination were compared with those of individuals who had ≥4 episodes after correction for age at study entry, gender, number of siblings, and the log-transformed age at the time of the first episode of AOM, using binary logistic regression. The log-transformed age at the time of the first AOM episode was included in the analysis, because age at the first AOM episode itself did not show a normal distribution and an early first infection predisposes to a second AOM episode. A child who has had a first AOM episode at a younger age has had a longer period of time to develop multiple AOM episodes than a child of the same age who suffered from the first infection at a later age. Because the interaction between the age at the first AOM episode and the age of inclusion was significant, this was accounted for in the analyses. Because the pathogenesis of AOM in young children may differ from older children because of an immature immune system and different anatomic features such as the Eustachian tube, we also

analyzed separately the frequencies of the number of AOM episodes in the year before inclusion in patients 1 to 4 years of age and patients >4 years. Presence of atopy did not differ between the otitis-prone children and those with fewer episodes. Because asthma cannot, by definition, be diagnosed in children <6 years of age, which applies to the main fraction of our patient cohort, recurrent wheezing and asthma as reported by the parents were considered the same in the statistical analyses. In addition to the multiplicative model, the effect of *PAII* genotype on the number of AOM episodes was also assessed comparing 4G/4G homozygotes with those homozygous for the 5G allele.

RESULTS

The *PAI1* 4G/4G Genotype Is Not Associated With Initial Susceptibility to AOM

The *PAI1* –675 polymorphism was successfully genotyped in 463 control subjects and 317 children with recurrent AOM. *PAI1* genotype frequencies in the control population were in Hardy-Weinberg equilibrium, and the allele frequencies were similar to results published previously.²⁴ The distribution of genotypes in control subjects was as follows: 5G/5G, 99 (21.4%); 4G/5G, 241 (52.1%); and 4G/4G, 123 (26.6%). A significant difference in genotype distribution was only found between control subjects and those patients suffering from ≥4 episodes of AOM ($P = .02$). No difference was observed between patients having 2 to 3 AOM episodes a year and control subjects ($P = .34$; Table 2).

Related Epidemiologic Findings

In our patient population, log-transformed age at first AOM, gender, and the number of siblings were related to AOM recurrence rates (Table 1). Children who suffered from ≥4 episodes in the year before inclusion had their

TABLE 2 Comparison of *PAI1* 4G/5G Genotype Distribution in Patients With Recurrent Otitis Media and Control Subjects

<i>PAI1</i> 4G/5G	Control Subjects (N = 463), n (%)	2–3 Episodes of AOM (N = 115), n (%)	≥4 Episodes of AOM (N = 202), n (%)	4G/4G vs 5G/5G		2–3 vs ≥4, Crude OR (P) ^d	2–3 vs ≥4, Adjusted OR (P) ^{c,d}
				Control vs 2–3, OR (P) ^a	Control vs ≥4, OR (P) ^b		
All patients						1.59 (.01)	1.52 (.03)
5G/5G	99 (21.4)	26 (22.6)	28 (13.9)	1.00 ^e	1.00 ^e	1.00 ^e	1.00 ^e
4G/5G	241 (52.1)	65 (55.6)	109 (54.0)	1.03 (.92)	1.60 (.05)	1.56 (.16)	1.57 (.18)
4G/4G	123 (26.6)	24 (20.9)	65 (32.2)	0.74 (.34)	1.87 (.02)	2.52 (.01) ^f	2.31 (.03) ^f
Patients <4 y						1.75 (.01) ^d	1.72 (.02) ^d
5G/5G		18 (23.4)	25 (15.8)		1.00 ^e	1.00 ^e	1.00 ^e
4G/5G		48 (62.3)	85 (53.8)			1.28 (.50)	1.32 (.47)
4G/4G		11 (14.3)	48 (30.4)			3.14 (.01) ^f	2.97 (.02) ^f
Patients ≥4 y						1.53 (.21) ^d	1.17 (.69) ^d
5G/5G		8 (21.1)	3 (6.8)			1.00 ^e	1.00 ^e
4G/5G		17 (44.7)	24 (54.5)			3.77 (.08)	5.25 (.06)
4G/4G		13 (34.2)	17 (38.6)			3.49 (.11) ^f	2.50 (.30) ^f

^a Data are for patients with 2 to 3 episodes of AOM versus control subjects: $P = .45$ (χ^2).

^b Data are for patients with ≥4 episodes of AOM versus control subjects: $P = .05$ (χ^2).

^c Adjusted ORs depicted were corrected for age at study entry, gender, number of siblings, and the log-transformed age at the time of the first episode of AOM.

^d ORs represent the effect of the *PAI1* 4G/5G polymorphism (binary logistic regression analysis, multiplicative model).

^e The reference OR is 1.00.

^f Data are for 4G homozygous versus 5G homozygous patients, crude or adjusted, binary logistic regression analysis. In this analysis, the *PAI1* –675 polymorphism was included in the model as a categorical variable.

first AOM episode at a significantly younger age compared with the children suffering from 2 to 3 episodes. Although the majority in the total group of children with AOM were boys, girls were overrepresented in the group of children suffering from ≥4 AOM episodes when compared with the group of children with 2 to 3 AOM episodes. The number of siblings was higher in the otitis-prone group compared with the children with 2 to 3 episodes. Age at inclusion was not significantly different when comparing the group of children suffering from 2 to 3 AOM episodes with the group with ≥4 episodes. However, when children were divided into groups under and over the age of 4 years, the distribution of AOM frequency differed significantly (Fig 1). The occurrence of atopy and the presence of a family history positive for AOM did not differ between children with 2 to 3 vs ≥4 episodes of AOM.

The *PAI1* 4G/4G Genotype Is Associated With the Otitis-Prone Condition in Children <4 Years of Age

Comparing patients having 2 to 3 AOM episodes to those with ≥4, the log-odds ratio (OR) of the 4G/4G versus 5G/5G genotype was ~2 times the logOR of the 4G/5G versus 5G/5G genotype (OR: 2.52; logOR: 0.4 and OR: 1.52; logOR: 0.19, respectively; Table 2). Hence, the 4G allele probably has a multiplicative effect on AOM recurrence rate.²⁷ After correction for confounding factors as described in “Statistical Analyses,” we still found that the *PAI1* 4G/4G genotype was associated with recurrence of disease in patients ($P = .01$, crude OR: 1.59; $P = .03$, adjusted OR: 1.52). The *PAI1* 4G/4G individuals had an increased risk for ≥4 AOM episodes compared with patients homozygous for the 5G variant ($P = .01$, crude OR: 2.52; $P = .03$, adjusted OR: 2.31). Because the

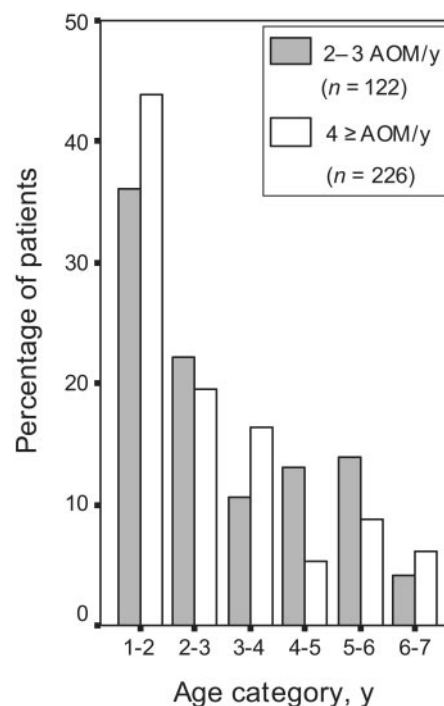


FIGURE 1

Distribution of the number of AOM episodes for different age categories at the time of inclusion. The number of patients (age category in years) for the group of children with 2 to 3 AOM episodes in the year before inclusion is 44 (1–2), 27 (2–3), 13 (3–4), 16 (4–5), 17 (5–6), and 5 (6–7). The number of patients (age category) for the group of children with ≥4 AOM episodes in the year before inclusion is 99 (1–2), 44 (2–3), 37 (3–4), 12 (4–5), 20 (5–6), and 14 (6–7). The distribution of 2 to 3 vs ≥4 AOM episodes differs between patients <4 and >4 years of age ($P = .03$).

distribution of the number of AOM episodes differed between the groups of children 1 to 4 years of age and >4 years of age, these groups were also analyzed separately (Fig 1). The association of the *PAI1* 4G/4G geno-

type with the otitis-prone condition was only found in the group of children 1 to 4 years of age ($P = .01$, crude OR: 1.75; $P = .02$, adjusted OR: 1.72; Table 2). Comparing the children with *PAII* 4G/4G genotype with those homozygous for the 5G allele yielded a crude OR of 3.14 ($P = .01$; adjusted OR: 2.97, $P = .02$). The *PAII* 4G/4G genotype was not significantly associated with the otitis-prone condition in children >4 years of age. The number of days with otorrhea showed a trend toward longer duration in the 4G homozygous children (geometric mean days [SD] for genotypes: 5G/5G, 5.3 [3.9]; 4G/5G, 6.5 [4.0]; and 4G/4G, 7.2 [4.2]). This was, however, not significant ($P = .66$, analysis of variance).

DISCUSSION

In this study we show that, in the patient group with a history of ≥ 2 AOM episodes, children 1 to 4 years of age, with the *PAII* 4G/4G genotype, which is related to high PAI-1 levels, have increased risk for more frequent recurrent AOM.^{18,19} The association is significant even after correction for possible confounding factors, with only a minimal change in ORs. PAI-1 inhibits cellular migration and invasion, processes essential in tissue repair in inflammatory disease.^{16,17} Hence, higher PAI-1 levels might result in longer persistence of tissue inflammation or abnormally repaired tissue prone to bacterial colonization and new infections.

Two possible mechanisms for PAI-1 interaction and inhibition of tissue repair have been proposed. First, PAI-1 may inhibit a proteolytic cascade at the cell surface that results in matrix destruction, allowing cellular migration and invasion for tissue repair. The proteolytic cascade mechanism involves the activation of surface-bound plasminogen by surface-bound urokinase plasminogen activator (uPA). PAI-1 can bind to uPA on the cell surface and inhibit its proteolytic activity and, hence, tissue repair.^{15,28,29} A second proposed pathway involves competitive binding of PAI-1 and integrin to vitronectin. Both share the same binding site region on vitronectin. Vitronectin/integrin interaction is needed for cell migration and adhesion. The inhibiting effect of PAI-1 is negated when excess nonsurface-bound uPA forms a complex with PAI-1 resulting in a release of PAI-1 from vitronectin, which is then available for interaction with integrin, favoring cell adhesion.³⁰

A possible role for high PAI-1 concentrations in the stronger inhibition of healing of inflamed otolaryngeal tissue that may, thus, favor an otitis-prone condition is supported by the results of Hansson et al³¹ that imply a further role for PAI-1 and plasminogen in tissue repair. They have shown that perforated tympanic membranes (TMs) of plasminogen-deficient mice exhibit reduced healing 15 days after myringectomy compared with TM from wild-type mice. Intravenous administration of plasminogen, in plasminogen-deficient mice, resulted in restored TM healing even if plasminogen was administered

30 days after perforation. The distribution of inflammatory cells in the perforated TM differed significantly between plasminogen-deficient and wild-type mice in vivo. Plasminogen-deficient mice with initially normal TMs showed spontaneous development of middle ear effusion. Interestingly, in an otherwise healthy 10-year-old boy with a history of chronic otitis media, low levels of serum plasminogen were detected.³² Interaction of PAI-1 with uPA prohibits catalytic activation of plasminogen to plasmin. High PAI-1 levels might, therefore, result in similar clinical findings as seen in plasminogen deficiency.

Furthermore, in skin wound healing, PAI-1, uPA, and uPA receptor are expressed at the site of regenerative epithelial outgrowths at the edge of the wound.^{33,34} Plasminogen-deficient mice showed impaired wound healing.³⁵ In addition, Chen et al³⁶ reported that uPA expression is increased on infection with *Streptococcus pneumoniae* in an otitis media model in the rat. *S pneumoniae* is one of the major causative microbes of otitis media in humans.³⁷⁻³⁹ We recognize that these findings do not prove a role for PAI-1 in AOM, but we do feel that they deserve further attention. Additional functional assays in an otitis model are needed to further investigate the precise role of PAI-1 in AOM. These studies would preferably also be directed toward analyzing the therapeutic role of PAI-1 inhibitors and plasminogen in overcoming the effect of high PAI-1 expression and their effects on recurrence of otitis media.

We found that *PAII* 4G/4G had no effect on the initial susceptibility to AOM as reflected by the lack of significant differences between patients with AOM and control subjects. This fits in our concept of tissue repair where only once an episode of AOM has occurred may impaired tissue healing in 4G homozygous individuals facilitate microbial reinfection and the recurrence of otitis media. We realize that the control group was not an age-matched group without any AOM episodes, which may have biased our results. However, occurrence of AOM in the control subjects was <2 episodes per year based on the prevalence of AOM in childhood. The association between the *PAII* 4G/4G genotype and the otitis-prone condition remained significant even after correction of the effect of the polymorphism on AOM recurrence rates for gender, number of siblings, and both age and age at first AOM for the children <4 years of age. After 4 years of age we found a difference in the distribution of the number of AOM episodes compared with the younger children. AOM (and recurrence) has its peak in the first years of life. Ongoing recurrence in older children most likely selects a specific group that differs from the larger group of 10% to 15% of all children with recurrent AOM at early age. We possibly selected for the older patients as a result of the nature of the vaccination trial in which the patients included were

more often otitis-prone children and particularly those at older age who had ongoing recurrent disease.

We only studied 1 genetic polymorphism in a complex multifactorial disease. As for many risk factors, only a small increase in risk can be found, which may be related to other risk factors as well. These may be both genetic and environmental. The balance of the different risk factors will determine the phenotype observed. Studying multiple factors simultaneously does, however, require large numbers of individuals. It must be mentioned that the association found in this study may reflect the result of linkage disequilibrium, meaning that not the *PAII* polymorphism itself but a genetic variation linked to this polymorphism is the actual disease-associated genetic variant. Additional studies are, therefore, required to confirm our findings. However, our data show that studies of genes involved in the inflammatory responses and tissue repair will be of significance in understanding recurrent AOM.

CONCLUSIONS

Individuals homozygous for the 4G allele show an increased risk for an otitis-prone condition, especially under the age of 4 years. Impaired tissue integrity, because of high PAI-1 levels in *PAII* 4G homozygous individuals once an episode of AOM has occurred, may possibly facilitate bacterial colonization and, thereby, the recurrence of otitis media.

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