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Nasopharyngeal polymicrobial colonization during health, viral upper respiratory infection and upper respiratory bacterial infection

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Abstract

Objectives—We sought to understand how polymicrobial colonization varies during health, viral upper respiratory infection (URI) and acute upper respiratory bacterial infection to understand differences in infection-prone vs. non-prone patients.

Methods—Nasopharyngeal (NP) samples were collected from 74 acute otitis media (AOM) infection-prone and 754 non-prone children during 2094 healthy visits, 673 viral URI visits and 631 AOM visits. Three otopathogens *Streptococcus pneumoniae* (*Spn*), Nontypeable *Haemophilus influenzae* (*NTHi*), and *Moraxella catarrhalis* (*Mcat*) were identified by culture.

Results—NP colonization rates of multiple otopathogens during health were significantly lower than during viral URI, and during URI they were lower than at onset of upper respiratory bacterial infection in both AOM infection-prone and non-prone children. AOM infection-prone children had higher polymicrobial colonization rates than non-prone children during health, viral URI and AOM. Polymicrobial colonization rates of AOM infection-prone children during health were equivalent to that of non-prone children during viral URI, and during viral URI were equivalent to that of non-prone during AOM infection. *Spn* colonization was positively associated with *NTHi* and *Mcat* colonization during health, but negatively during AOM infection.

Conclusion—The infection-prone patients more frequently have multiple potential bacterial pathogens in the NP than the non-prone patients. Polymicrobial interaction in the NP differs during health and at onset of infection.

Keywords

polymicrobial colonization; acute otitis media; otitis prone children; viral upper respiratory infection

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Introduction

The pathogenesis of upper and lower respiratory bacterial infections involves complex interactions among bacteria, respiratory viruses, and the host innate immune responses (1–4). Bacterial-viral interactions play a critical role in bacterial infection pathogenesis such as acute otitis media (AOM) (2, 5, 6). Viral upper respiratory tract infection (URI) is considered as the most important predictor for an upper respiratory bacterial infection such as AOM, unquestionably outweighing all other risk factors (1, 2, 5, 6). Approximately >90 % of upper respiratory bacterial infections have a symptomatic viral URI at the time of clinical presentation with an upper respiratory bacterial infection (4, 5, 7).

The most common pathogens that cause upper and lower respiratory bacterial infections are *Streptococcus pneumoniae* (*Spn*), Nontypable *Haemophilus influenzae* (*NTHi*), and *Moraxella catarrhalis* (*Mcat*) (4, 8–12). During health, *Spn*, *NTHi* and *Mcat* nasopharyngeal (NP) colonization is asymptomatic and elicits mild NP epithelial inflammation that allows controlled immune surveillance in the NP, leading to the clearance of colonization without developing immunopathology (13–15). When a viral URI occurs, an inflammatory response induced by viral co-infection promotes increased growth of *Spn*, *NTHi* and/or *Mcat* if the organism is already colonized in the NP, or facilitates colonization and escalation of growth of new otopathogens (14–16).

Spn, *NTHi* and *Mcat* colonization in the NP is a necessary pre-requisite for infection due to these organisms. In a previous study we have shown that approximately 17% of healthy young children are colonized with two or more of the three major respiratory bacterial pathogens (*Spn*, *NTHi*, *Mcat*) (17). The major difference in NP colonization was seen in polymicrobial colonization, not single species colonization when comparing healthy children to children with AOM (17). Bacterial pathogens have different interaction patterns at onset of an upper respiratory bacterial infection and during health (17). Co-colonization influences antibody responses (18) and outcomes of infection (19). The objectives of this study were to characterize polymicrobial NP colonization during health, at onset of a viral URI and at onset of AOM as well as to determine if differences occurred in infection-prone vs. non-prone patients.

Material and Methods

Experimental Design

This study derives from a 10-year (2006–2015) prospective longitudinal evaluation of immunity to *Spn* and *NTHi* NP colonization and AOM in young children. Healthy children without any previous episodes of AOM were enrolled at 6 months of age from Rochester, NY. NP samples were collected at 6 healthy visits at 6, 9, 12, 15, 18 and 24 month of age, and classified into healthy and URI visits according to whether or not the children presented with viral URI symptoms such as sore throat, nasal congestion, runny nose, sneezing, cough, and low-grade fever. Whenever the children were diagnosed as having AOM, tympanocentesis was performed and MEF samples were collected to confirm the clinical diagnosis. When a child experienced 3 episodes of AOM infections within a 6 month time span or 4 episodes of AOM infections in a 12 month time span they were designated as

AOM infection-prone and because the diagnosis was confirmed by tympanocentesis-derived middle ear fluid culture we have termed these children stringently-defined otitis prone in our prior publication (20). Three bacterial pathogens (*Spn*, *NTHi* and *Mcat*) were identified by culture. AOM diagnosis, sample collection and bacterial pathogen identifications were performed as described previously (17, 21). All children received the standard vaccination regimen in the United States, including the PCV-7 (before 2010) or PCV-13 (since 2010) vaccine (Prevnar, Pfizer Pharmaceuticals, Collegeville, PA) at the recommended ages. The study was approved by the Institutional Review Board of Rochester General Hospital. Written informed consent was obtained from parents and guardians of all children in the study.

Statistics

The rates of NP colonization among children of the same age at healthy, URI and AOM visits were compared using the Fisher exact test and GraphPad Prism software. Bacterial interactions were analyzed using repeated measures logistic regression models. Predicted outcomes of colonization with *Spn*, *NTHi* and *Mcat* were examined using multivariate logistic regression. Generalized estimating equations were used to model exchangeable correlation among subjects. Four logistic regression models (healthy, URI, AOM visits and overall) were calculated by using R version 2.13.2 (www.r-project.org/). To examine the effects of covariates on each of the 3 pathogens, we modeled NP colonization of each pathogen separately by using the remaining 2 pathogens as predictors and including an interaction term. For each model, we estimated odds ratios (ORs) for the response pathogen given the presence of each predictor pathogen alone, then jointly; synergistic associations between bacteria are indicated by $OR > 1$; competitive association, by $OR < 1$. The absence of both predictor pathogens was used as the reference condition. A $p < 0.05$ was considered significant.

Results

Study cohort

This analysis involved a total of 2094 healthy visits, 673 URI visits and 631 AOM visits. Samples were taken from 74 infection-prone children and 680 non-prone children at 6 to 24 month of age. The characteristics of the patients are summarized in Table 1.

Comparison of NP colonization Among Health, URI and AOM visits

We compared NP colonization rates with single species, multiple species or any combination of the three potential bacterial pathogens (*Spn*, *NTHi* and *Mcat*) among three states (healthy, viral URI and AOM) in infection-prone and non-prone patients. We found that colonization at healthy visits was less frequent than at URI visits, and colonization at URI visits was less frequent than at a visit when AOM was diagnosed in both infection-prone and non-prone patients (Figure 1). In infection-prone children, overall NP colonization rates with any species of the three otopathogens at healthy visits (69.0% (116/168)) was significantly lower than URI visits (87.1% (81/93)) which was significantly lower than AOM visits (97% (229/236)) (all $p < 0.01$, Fig 1A, 1D). The differences in NP colonization among the three states were in polymicrobial colonization two or more of the three otopathogens) (Fig. 1C,

1E), but not in single species colonization (Fig. 1B). The overall rates of polymicrobial colonization in infection-prone children were 31.0% (52/168) at healthy visits, 49.5% (46/93) at URI visits and 62.1% (110/177) at AOM visits (all $P < 0.01$). Overall single species colonization rates were 38.1% (64/168) at healthy visits, 37.6% (35/93) at viral URI visits and 41.8% (74/177) at AOM visits (all $p > 0.5$) (Fig. 1B). Similar results were obtained in non-prone children (Fig. 1F–1J). Overall colonization rates by any of the three otopathogens, as well as polymicrobial colonization rates at AOM visits were greater than at URI visits and rates at URI visits were greater than at healthy visits (all $p < 0.001$) (Fig. 1I, 1J).

The overall colonization rates of *Spn* during health (28.5% (569/2094)) were less than at URI visits (45.6% (307/673)) and rates at URI visits were less than at AOM visits (53.8% (340/631)) (all $p < 0.01$). Overall colonization rates of *NTHi* during health (8.8% (185/2094)) were less than at URI visits (19.8% (133/673)) rates at URI visits were less than at AOM visits (45.1% (285/631)) (all $p < 0.0001$). Overall colonization rates of *Mcat* during health (30.1% (643/2094)) were less than at viral URI (52% visits (350/673)) ($p < 0.0001$), but there was no difference between viral URI and at AOM visits ($p = 0.8$) (Figure 2).

Multivalent regression analysis showed that both viral URI and AOM were positively associated with NP colonization by each individual otopathogen (all $p < 0.05$) except AOM states with *Mcat* colonization ($p > 0.05$). The strongest association was identified between AOM and *NTHi* colonization (OR=3.45, z score =9.10, $p < 0.00001$). Both viral URI and AOM were strongly and positively associated with polymicrobial colonization (all $p < 0.0001$) (Table 2).

Comparison of NP colonization between AOM infection-prone and non-prone patients

Infection-prone children had significantly higher rates of NP colonization with bacterial respiratory pathogens than non-prone children. The differences between infection-prone and non-prone were children in colonization with multiple species (Fig. 3E–3H) but not in single species colonization (Fig 3A–3D). The percentage of multiple species colonization in infection-prone children during health, URI and AOM visits were all higher than non-prone children across the ages (Fig. 3D–3G). The overall polymicrobial colonization rates in infection-prone children were significant higher than non-prone children during health (31.0% (52/168) vs. 14.4% (253/1754), $p < 0.0001$), at URI visits (49.5% (46/93) vs. 33.6% (180/535), $p = 0.005$) and at AOM visits (62.1% (110/177) vs. 45.5% (148/325), $p = 0.0004$) (Fig. 2H). Polymicrobial colonization rates in both infection-prone and non-prone during health were less than rates at URI visits and rates at URI visits were less than AOM visits. However, the polymicrobial colonization rates of infection-prone children during healthy visits were as high as the polymicrobial colonization rates in non-prone children at URI visits (31.0% (52/168) vs. 33.6% (180/535), $p = 0.57$); and the polymicrobial colonization rates of infection-prone children at URI visits were as high as the polymicrobial colonization rates of non-prone children at the onset of AOM (49.5% (46/93) vs. 45.5% (148/325), $p = 0.56$) (Fig 3G). Similar results were obtained when we observed average number of colonization events with bacterial respiratory pathogens (Fig. 3I–3L). There were no significant differences between infection-prone and non-prone children in single species

colonization at healthy, at URI visits or AOM visits across ages (Fig.3A–3C) nor overall (Fig. 3D) (all p values >0.5).

We previously reported that colonization rates of *Spn*, *NTHi* and *Mcat* in infection-prone children during healthy visits were significantly higher than non-prone children (22). Here we further compared the colonization rates at URI visits and AOM. We found that at URI visits, NP colonization rates of infection prone children were significantly higher than non-prone children for *Spn* (29.6% (67/95) vs. 43.6% (252/578), $p=0.01$) and *NTHi* (57.9% (55/95) vs. 18.2% (105/578), $p=0.02$), but not for *Mcat* (49.5% (151/249) vs. 52.4% (303/578), $p=0.68$). During AOM, infection-prone children had higher colonization rates with *Mcat* (60.6% (151/249) vs. 45.0% (172/382), $p=0.0001$), and trended higher for *Spn*, (58.6% (146/249) vs. 50.8% (194/382), $p=0.06$) and *NTHi* (48.6% (121/249) vs. 42.9% (164/28), $p=0.17$). Overall (all three states) infection-prone children had significantly higher colonization rates in *Spn*, *NTHi*, and *Mcat* (all $p<0.0001$) (Figure 4).

Multivariate regression analysis showed that the infection-prone condition was strongly and positively associated with NP colonization of each individual respiratory bacterial pathogen as well as polymicrobial colonization (all $OR>1$, $p<0.001$) (Table 2).

Interactions of bacterial otopathogens in the NP during health, URI and AOM

Since we previously found that bacterial interaction in the NP differed during health and at onset of AOM, we generated four different models with different datasets: during health, at URI visits, AOM and with all states together (Table 3). At healthy visits, *Spn* and *NTHi* colonization and *Spn* and *Mcat* colonization were positively associated with each other. When examining *Spn* colonization as the predicted outcome, *Spn* colonization was positively associated with *Mcat* ($OR=1.77$, $p<0.0001$) colonization, and trended toward a positive association with *NTHi* ($OR=1.46$, $P=0.06$). When examining *NTHi* colonization as the predicted outcome, *NTHi* colonization was positively associated with *Spn* ($OR=1.52$, $P=0.04$). When examining *Mcat* colonization as the predicted outcome, *Mcat* colonization was positively associated with *Spn* ($OR=1.84$, $P<0.0001$). The infection-prone condition was positively associated with all three respiratory bacterial pathogens colonizing the NP (all $p<0.05$). No significant association was identified between *NTHi* and *Mcat* colonization ($P>0.05$) (Table 3 Model I).

At URI visits, *Spn* and *Mcat* colonization was positively associated each other when *Mcat* colonization was examined as the predicted outcome ($OR=1.41$, $P=0.04$) or trended toward a positive association when *Spn* colonization was examined as the predicted outcome ($OR=1.33$, $P=0.09$). *NTHi* and *Mcat* colonization trended toward a negative association with each other when *Mcat* ($OR=0.58$, $p=0.06$) and *NTHi* colonization ($OR=0.63$, $p=0.09$) was examined as the predicted outcomes. No significant association was identified between *Spn* and *NTHi* colonization when *Spn* ($OR=1.36$, $p=0.25$) and *NTHi* colonization ($OR=1.48$, $p=0.15$) was examined as the predicted outcomes (Table 3 Model II).

At AOM visits, *Spn* and *NTHi*, as well as *NTHi* and *Mcat* colonization were negatively associated with each other (all $OR<1$, $p<0.00001$). However, *Spn* and *NTHi* co-colonization was positively associated with *Mcat* colonization ($OR=2.45$, $p=0.008$), and *Spn* and *Mcat*

colonization positively associated with *NTHi* (OR=2.17, $p=0.02$), and *NTHi* and *Mcat* co-colonization positively associated with *Spn* (OR=2.27, $p=0.02$). Overall (all data together), *Spn* and *Mcat* colonization was positively associated each other (OR>1, $P<0.00001$), while *NTHi* and *Mcat* colonization were negatively associated each other (OR<1, $p<0.0001$) (Table 3 Model III).

Discussion

Microbial pathogens often coexist or compete with multiple other microbial species in their natural environments (23). The NP is a polymicrobial reservoir in which a variety of commensal bacteria and potential bacterial pathogens colonize (10, 24–27). Polymicrobial colonization is common in children with AOM (28, 29), and also other upper respiratory infections such as sinusitis (4, 30), and lower respiratory infections such as pneumonia (31–33). Polymicrobial infections are associated with increased infection severity and poorer patient outcomes (34). However, microbial pathogens are generally studied as individual manifestations (23, 34). To the best of our knowledge, this study is the first report to characterize polymicrobial colonization in the NP of infection-prone children. We found that differences in NP colonization between infection-prone and non-prone children most frequently involved increased multiple otopathogen colonization, not single species colonization. The infection-prone condition was very strongly associated with higher polymicrobial colonization rates during health, at URI and AOM visits.

The pathogenesis of respiratory bacterial infections begins in the NP and is triggered by a viral co-infection that facilitates the transition from asymptomatic bacterial carriage to an infection (3–5, 10, 27). During health, *Spn*, *NTHi* and *Mcat* are commensal bacteria (35, 36) that do not cause sufficient inflammation or breach epithelial barriers which would lead to an infection (23, 36). It is only during times of epithelial disruption or immune dysfunction that *Spn*, *NTHi* and *Mcat* cause infection (23, 36). Viral URI can enhance bacterial colonization by causing NP inflammation, changing physiological properties of NP epithelial cells, and modulating host innate and adaptive immune responses (5, 23, 36, 37). In this study, we found that the major differences between health, URI and AOM visits involved the rates of polymicrobial colonization, not single species colonization in the NP. URI and AOM were strongly associated with higher polymicrobial colonization. These results demonstrate that polymicrobial colonization plays a critical role in pathogenesis of AOM, especially in infection-prone children. Due to the increased frequency of polymicrobial colonization, infection-prone children are more susceptible than non-prone children to developing AOM from URI. Also due to immunological deficiency (20, 22), AOM infection-prone children have insufficient innate and adaptive immune responses after natural colonization with potential otopathogens and AOM to prevent recurrent colonization and infection. The etiological bacteria therefore persist in the NP of infection-prone children more so than non-prone children (38).

Polymicrobial interaction is critical for pathogenesis of respiratory bacterial infection (2, 5, 17, 23, 31, 34). Polymicrobes can interact synergistically or compete with each other to impact progress of infection by up- or down-regulating of virulence traits, altering the infected niche, or modulating the host immune response (5, 34). In this study we

investigated the interaction of three bacterial pathogens (*Spn*, *NTHi* and *Mcat*) at three different health states. We found that *Spn* and *NTHi*, as well as *Spn* and *Mcat* interacted positively during health, but negatively during AOM. The results were consistent with our previous studies (17). We also found that at URI visits, *Mcat* colonization were positively associated with *Spn* colonization, but negatively with *NTHi*. Interestingly, during AOM, although the three respiratory bacterial pathogens had a strong negative association with each other, when two respiratory bacterial pathogens colonized in the NP, they were positively associated with colonization of the third respiratory bacterial pathogen. These results highlight the complexity of NP polymicrobial interactions in the NP.

AOM infection-prone children are a subset of the population (approximately 10%) of young children who have three or more episodes of AOM in six months, or four or more episodes in a 12 month period, all confirmed by tympanocentesis (20). Our previous studies have shown that AOM infection-prone children have immune deficiencies in systemic and mucosal antibody responses to NP colonization, as well as AOM, poor memory B-cell and T-helper cell generation, poor responses to routine pediatric vaccinations (diphtheria toxoid, tetanus toxoid, pertussis toxoid, filamentous hemagglutinin, pertactin and hepatitis B vaccines), and lower innate inflammatory responses to respiratory viruses than non-prone children (20, 39). Similarly, patients who experience recurrent acute sinusitis, recurrent acute exacerbations of chronic bronchitis and multiple episodes of community-acquired pneumonia may have immune deficiencies as we have discovered in otitis prone children. This would be an important area for future research.

This study has some limitations. NP microbial ecology includes a broad variety of microbiota (10, 24–27). We investigated only the three major potential bacterial respiratory pathogens that cause AOM. We did not prove that the symptoms during URI visits were caused by viruses and we did not evaluate the full microbiota or bacterial-viral interactions or host immunity-otopathogen interactions that have proved critical for clinical outcomes (6). Further studies are needed in those areas of research and role of respiratory microbiome in maintaining health and pathogenesis of infection.

In conclusion, in this study we have shown that polymicrobial NP colonization and bacterial-bacterial interaction in pathogenesis of upper respiratory bacterial infection differs during times of health, viral URI and at onset of AOM as well as between infection-prone and non-prone patients. The infection-prone patient more frequently has multiple potential bacterial pathogens in the NP than the non-prone patient.

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Highlights

1. NP polymicrobial colonization rates during health are < during URI < during AOM.
2. Otitis-prone child have higher polymicrobial colonization rates than non-prone child.
3. Polymicrobial interactions in the NP differ during health, URI and AOM.
4. *Spn* is associated with *NTHi* or *Mcat* positively during health, negatively during AOM.

Figure 1. Comparison of NP colonization of single and multiple species of otopathogens between healthy, URI and AOM visits

NP samples were collected from children at prospective healthy visits, URI and AOM at 6–24 months of age. The three bacterial potential pathogens *Spn*, *NTHi* and *Mcat* were identified by standard culture. The percentiles of NP colonization with any species of the three otopathogens, single species (B,G) and polymicrobial colonization (> two species) were compared by Fisher exact test between health, URI and AOM visits in AOM infection-prone and non-prone children. A–E, AOM infection-prone children; F–J, Non-prone children: A and F, any species colonization rates over time; D and G, single species colonization rates over time; C and H polymicrobial colonization rates over time; D and I, overall colonization rates with any species; E and J, overall polymicrobial colonization rates.

Figure 2. Comparison of NP colonization rates of each otopathogens between healthy, URI and AOM visits

NP samples were collected from children at prospective healthy visits, onset of URI and AOM at 6–24 months of age. The three bacterial potential pathogens *Spn*, *NTHi* and *Mcat* were identified by standard culture. The percentiles of NP colonization of *Spn*, *NTHi*, and *Mcat* by Fisher exact test between health, URI and AOM visits in all children.

Figure 3. Comparison of NP colonization between AOM infection-prone and non-prone children
 NP samples were collected from children at prospective healthy visits, URI and AOM at 6–24 months of age. The three potential bacterial otopathogens *Spn*, *NTHi* and *Mcat* were identified by standard culture. The percentiles of single species colonization (any of the three species), polymicrobial colonization (> two species) and average number of species colonizing in the NP were compared by Fisher exact test between AOM infection-prone and non-prone at health, URI and AOM visits. A-D, % single colonization; G-H, % polymicrobial colonization; I-L, average numbers of species colonized in the NP. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Figure 4. Comparison of NP colonization rates of each otopathogens between AOM infection-prone and non-prone children

NP samples were collected from children at prospective healthy visits, URI and AOM at 6–24 months of age. The three bacterial potential pathogens *Spn*, *NTHi* and *Mcat* were identified by standard culture. The percentiles of NP colonization of *Spn*, *NTHi*, and *Mcat* by Fisher exact test between AOM infection-prone children and non-prone children during health, URI and AOM visits

Table 1

Characteristics of study cohorts

	Overall	Infection-prone	Non-prone	P values (infection-prone vs. non-prone)
Total children	754 (100%)	74 (9.8%)	680 (90.2%)	<0.0001
Gender				
Female	357 (47.3%)	23(31.1%)	334 (49.2%)	0.003
Male	397 (52.7%)	51(68.9%)	346 (50.8%)	
Race				
White	520 (68.9%)	61(82.4%)	459 (67.5%)	0.008
Non-white	234 (32%)	13 (17.6%)	221 (32.5%)	
Brest feeding [*]				
> 6M	104 (15.5%)	3 (4.4%)	101 (16.7%)	0.52
< 6M	181 (26.9%)	23 (33.8%)	158 (26.1%)	
Formula	387 (57.6%)	42 (61.8%)	345 (57.2%)	
Daycare attendance [*]				
Yes	238 (34.2%)	36 (62.1%)	202 (31.7%)	<0.0001
No	457 (65.8%)	22 (37.9%)	435 (68.3%)	
Sibling				
Yes	472(62.6%)	45 (61%)	428 (62.9%)	0.71
No	282(37.4%)	29 (39%)	252 (37.1%)	
Home smoker				
Yes	98(13.0%)	5 (6.8%)	93 (13.7%)	0.10
No	656 (90.2%)	69 (93.2%)	587 (86.3%)	
Total visits (samples) ^{**}	3398 (100%)	542 (16.0%)	2856 (84.0%)	<0.0001
Visits of each healthy state group ^{**}				
Healthy total visits	2094 (61.6%)	198 (36.5%)	1896 (66.4%)	<0.0001

	Overall	Infection-prone	Non-prone	P values (infection-prone vs. non-prone)
visits per child	2.78	2.67	2.79	/
total visits	673 (19.8%)	95 (17.5%)	578 (20.2%)	0.15
visits per child	0.89	1.28	0.85	/
total visits	631 (18.5%)	249 (15.9%)	382 (13.4%)	0.12
visits per child	0.84	3.36	0.56	/

* daycare and breastfeeding date of some children were missing.

** NP samples were collected at all visits

Association of AOM infection-prone, URI and AOM with NP colonization of potential bacterial pathogens

Table 2

	Outcomes							
	<i>Spn</i>		<i>NTHi</i>		<i>Mcat</i>		Polymicrobial colonization	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
Infection-prone	1.71 (1.30–2.24)	0.0001	1.95 (1.42–2.67)	0.00004	1.58 (1.23–2.04)	0.00035	2.18 (1.67–2.84)	<0.00001
URI	1.67 (1.41–1.98)	<0.00001	2.09 (1.61–2.72)	<0.00001	2.20 (1.83–2.64)	<0.00001	2.46 (2.02–3.00)	<0.00001
AOM	1.32 (1.03–1.68)	0.02	3.45 (2.64–4.51)	<0.00001	1.08 (0.87–1.35)	0.49	1.58 (1.25–1.99)	0.00011

Bold: statically significant difference. OR>1, positive association; OR<1, negative association.

Table 3

Interactions between potential pathogens of AOM in the NP

	Outcomes					
	<i>Spn</i>		<i>NTHi</i>		<i>Mcat</i>	
	OR (95%CI)	P value	OR (95%CI)	P value	OR (95%CI)	P value
Model I (Health)	<i>Spn</i>	/				
	<i>NTHi</i>	1.46 (0.98–2.18)	0.06	1.52 (1.01–2.27)	0.04	1.84 (1.48–2.3)
	<i>Mcat</i>	1.77 (1.42–2.2)	<0.00001	0.87 (0.55–1.37)	0.55	0.86 (0.54–1.368)
	<i>Spn X NTHi</i>	/	/	/	/	/
	<i>Spn X Mcat</i>	/		1.39 (0.72–2.7)	0.33	1.45 (0.76–2.78)
	<i>NTHi X Mcat</i>	1.41 (0.76–2.61)	0.27			/
Model II (URI)	Infection-prone	1.58 (1.08–2.31)	0.020	3.22 (2.11–4.91)	<0.00001	1.46 (1.02–2.07)
	<i>Spn</i>	/		1.48 (0.87–2.53)	0.15	1.41 (1.01–1.97)
	<i>NTHi</i>	1.36 (0.8–2.31)	0.25	/		0.58 (0.33–1.02)
	<i>Mcat</i>	1.33 (0.95–1.85)	0.09	0.63 (0.36–1.09)	0.09	/
	<i>Spn X NTHi</i>	/		/		1.5 (0.7–3.19)
	<i>Spn X Mcat</i>	/		1.32 (0.62–2.77)	0.48	/
Model III (AOM)	<i>NTHi X Mcat</i>	1.61 (0.98–2.66)	0.06	/		/
	Infection-prone	1.44 (0.69–3.01)	0.34	2.03 (1.14–3.61)	0.02	0.80 (0.5–1.28)
	<i>Spn</i>	/		0.35 (0.22–0.56)	0.00001	0.74 (0.48–1.16)
	<i>NTHi</i>	0.34 (0.21–0.55)	0.00001	/		0.22 (0.13–0.37)
	<i>Mcat</i>	0.78 (0.5–1.21)	0.28	0.24 (0.15–0.4)	<0.00001	/
	<i>Spn X NTHi</i>	/		/		2.45 (1.26–4.74)
Model IV (Overall)	<i>Spn X Mcat</i>	/		2.17 (1.13–4.14)	0.02	/
	<i>NTHi X Mcat</i>	2.27 (1.17–4.39)	0.02	/		/
	Infection-prone	1.37 (0.96–1.95)	0.09	1.6 (1.09–2.35)	0.02	2.1 (1.53–2.89)
	<i>Spn</i>	/		1.01 (0.78–1.31)	0.95	1.57 (1.31–1.87)
	<i>NTHi</i>	0.93 (0.71–1.22)	0.58820	/		0.52 (0.38–0.72)
	<i>Mcat</i>	1.49 (1.25–1.76)	<0.00001	0.55 (0.41–0.74)	0.00006	/
	<i>Spn X NTHi</i>	/		/		1.32 (0.88–1.98)
						0.18

Outcomes						
<i>Spn</i>			<i>NTHi</i>			<i>Mcat</i>
OR (95%CI)	P value		OR (95%CI)	P value	OR (95%CI)	P value
<i>Spn X Mcat</i> <i>NTHi X Mcat</i> Infection-prone	/		1.12 (0.74–1.69)	0.58	/	
	1.34 (0.92–1.97)	0.13	/		/	
	1.71 (1.3–2.24)	0.0001	1.95 (1.42–2.67)	0.00004	1.58 (1.23–2.04)	0.0004

Bold: statically significant difference. OR>1, positive association; OR<1, negative association.