

The Suppressive Role of *Streptococcus pneumoniae* Colonization in Acute Exacerbations of Childhood Bronchial Asthma

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Keywords

Asthma · Bacterial colonization · Childhood · Cytokine · *Streptococcus pneumoniae*

Abstract

Introduction: Little is known about the association between bacterial infections and exacerbations of bronchial asthma. **Objective:** To elucidate the effect of bacterial infections on bronchial asthma, we examined pharyngeal bacterial colonization, duration of wheezing, and serum levels of cytokines and chemokines during acute exacerbations of asthma in children. **Methods:** Potential bacterial pathogens were investigated in pharyngeal samples and viruses obtained from nasal secretions of 111 children who were outpatients and/or in patients with acute exacerbations of asthma (mean/median age: 2.8/2.6, respectively). We also measured serum levels of 27 different cytokines/chemokines. **Results:** Pharyngeal bacterial cultures were positive in 110 of 111 children. The 3 major bacterial pathogens were *Streptococcus pneumoniae* (29.7%), *Moraxella catarrhalis* (11.7%), and *Haemophilus influenzae* (10.8%). *M. catarrhalis* was detected more frequently in patients with pneumonia. Furthermore, patients with *S. pneumoniae* colonization had significantly shorter wheezing episodes than those without it. In contrast, the duration of wheezing did not differ significantly among cases with other bacteria such as *M. catarrhalis* and *H. influenzae*. Furthermore, the length of wheezing episode in patients with *S. pneumoniae* colonization showed significant inverse correlation with peripheral white blood cell count, neutrophil count, and C-reactive protein, while there was no significant correlation between duration of wheezing and these 3 parameters among patients with *M. catarrhalis* or *H. influenzae*. Among the 27 cytokines/chemokines, only serum tumor necrosis factor (TNF)- α was significantly lower in patients with *S. pneumoniae* colonization than in those without it. **Conclusions:** These results suggested that pharyngeal *S. pneumoniae* colonization plays a suppressive role on the pathophysiology during acute exacerbations of asthma.

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mophilus influenzae (10.8%). *M. catarrhalis* was detected more frequently in patients with pneumonia. Furthermore, patients with *S. pneumoniae* colonization had significantly shorter wheezing episodes than those without it. In contrast, the duration of wheezing did not differ significantly among cases with other bacteria such as *M. catarrhalis* and *H. influenzae*. Furthermore, the length of wheezing episode in patients with *S. pneumoniae* colonization showed significant inverse correlation with peripheral white blood cell count, neutrophil count, and C-reactive protein, while there was no significant correlation between duration of wheezing and these 3 parameters among patients with *M. catarrhalis* or *H. influenzae*. Among the 27 cytokines/chemokines, only serum tumor necrosis factor (TNF)- α was significantly lower in patients with *S. pneumoniae* colonization than in those without it. **Conclusions:** These results suggested that pharyngeal *S. pneumoniae* colonization plays a suppressive role on the pathophysiology during acute exacerbations of asthma.

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Introduction

Various exacerbating factors such as viruses, bacteria, mycoplasma, and chlamydia are associated with bronchial asthma. In particular, viral infections are major causes of both the development of bronchial asthma and subsequent exacerbations [1, 2]. However, information on the association between bacterial infection and exacerbations of asthma is lacking.

Previous studies have shown that bacterial colonization is linked to a greater risk of wheezing recurrences and the seriousness of respiratory tract illnesses, including exacerbations of asthma [3, 4]. Kloepper et al. [4] reported that *Streptococcus pneumoniae* and *Moraxella catarrhalis* are associated with increased cold symptoms and moderate exacerbations of asthma. Another study showed that children with recurrent wheezing and coexistent bacterial colonization and viral infection had greater rates of cyanosis and longer hospital stays than those with viral infection only [5].

Conversely, animal models have demonstrated that killed *S. pneumoniae* administered to the airways suppresses eosinophilic inflammation and hyperresponsiveness [6]. It has also been reported that *S. pneumoniae* infection or treatment with killed *S. pneumoniae* suppresses the characteristic features of allergic airway disease, including responses by Th2 cytokines and antibodies, the accumulation of peripheral and pulmonary eosinophils, and goblet cell hyperplasia in mouse models of ovalbumin-induced allergic airway disease [7]. These 2 reports demonstrate that bacterial infections, particularly with *S. pneumoniae*, have a suppressive effect on bronchial asthma. However, the detailed associations between bacterial infection and asthma remain controversial.

We investigated whether bacterial colonization is related to the duration of wheezing, peripheral white blood cell count, C-reactive protein, and levels of several cytokines and chemokines in serum among cases of acute exacerbations of childhood asthma.

Materials and Methods

Patients and Study Characteristics

This retrospective study involved children ($n = 111$, 74 boys, 37 girls; mean/median age 2.8/2.6 years; range 10 months to 6 years) with acute exacerbations of asthma who were treated as outpatients and/or inpatients at Tokai University Hospital, Hadano Red Cross Hospital, or Gunma Children's Medical Center between January 1, 2008 and December 31, 2016. All patients were diagnosed with bronchial asthma according to Japanese guidelines; asthma was confirmed on the basis of recurrent wheezing and dys-

Table 1. Patient characteristics

	Asthma
<i>n</i>	111
Age, year (mean/median)	2.8/2.6
Gender (male), %	66.7
Family history of asthma, %	57.4
Eczema, %	36.1
Food allergy, %	25.9
≥1 positive aeroallergen, %	58.9

pnea on 3 or more independent occasions, and reversible bronchoconstriction was identified [8]. We collected clinical data on the severity of the exacerbations, duration of wheezing, and complication of pneumonia. Acute asthma was diagnosed by physician in the emergency department based on evidence of wheezing and dyspnea. Mild exacerbation was defined as mild wheezing, stable disease, no dyspnea, and $S_pO_2 \geq 96\%$. Moderate exacerbation was defined as wheezing and dyspnea, apparent retraction, and S_pO_2 from 92 to 95%. Severe exacerbation was defined as more severe wheezing and dyspnea with $S_pO_2 \leq 91\%$. Exclusion criteria were congenital heart disease, chronic lung disease, or evidence of a foreign body. These criteria were set because these conditions can affect asthma-related outcome measures.

Bacterial Cultures and Virus Detection

Pharyngeal samples were collected via swabs from the throats of 111 patients during acute exacerbations of asthma. The samples were cultured immediately at the hospitals and the microorganisms were identified by examining the morphology of the colonies using Gram stain techniques. Bacterial isolates (*S. pneumoniae*, *M. catarrhalis*, and *Haemophilus influenzae*) were identified by standard microbiological protocols [9]. Nasal aspirates were also obtained from the patients and analyzed via antigen detection kits for respiratory syncytial (RS) virus (Becton Dickinson, Fukushima, Japan), influenza virus types A and B (Denka-Seiken, Gosen, Japan), adenovirus (Tauns, Izunokuni, Japan), and human metapneumovirus (Meiji Seika Pharma, Uji, Japan). Remaining secretions were frozen at -80°C until reverse transcription-polymerase chain reaction examinations and direct DNA sequencing analysis, as described previously [10]. Polymerase chain reaction products were analyzed by automatic electrophoresis (MCE-202 MultiNA; Shimadzu, Kyoto, Japan) [11].

Serum Total IgE and Allergen-Specific IgE

Automated fluoroenzyme immunoassay (FEIAUnicap® 100, Phadia AB, Uppsala, Sweden) was used to measure serum levels of total IgE and allergen-specific IgE against inhalant allergens, including *Dermatophagoides farinae/pteronyssinus*, house dust, Japanese cedar, orchard grass, ragweed, cat and dog dander, *Alternaria*, and *Cladosporium*, as described previously [12]. Allergen-specific IgE values $\geq 0.35 \text{ U}_A/\text{mL}$ (Class I) were considered positive, and the sensitivity for the detection of total IgE was 2.00 IU/mL . Allergic sensitization was defined as having one or more positive values for allergen-specific IgE.

Fig. 1. Bacteria detected in childhood asthma during acute exacerbations (**a**). Among 111 samples from patients with asthma exacerbations, *S. pneumoniae* was detected in 33 cases (29.7%), *M. catarrhalis* in 13 (11.7%), *H. influenzae* in 12 (10.8%), other bacteria in 52 (46.8%), and no bacteria in 1 (0.9%). Comparison of bacteria detected between cases of complicated pneumonia (**b**) and those without (**c**). *M. catarrhalis* was detected significantly more frequently in patients with complicated pneumonia. Data were analyzed using chi-square test statistics and Fisher's exact test for categorical variables. * $p < 0.05$ versus no pneumonia.

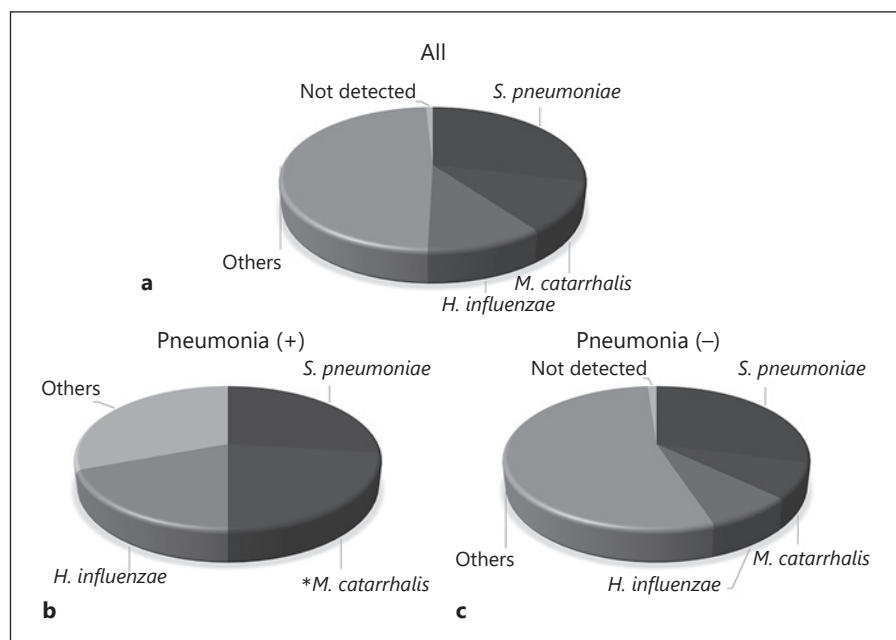
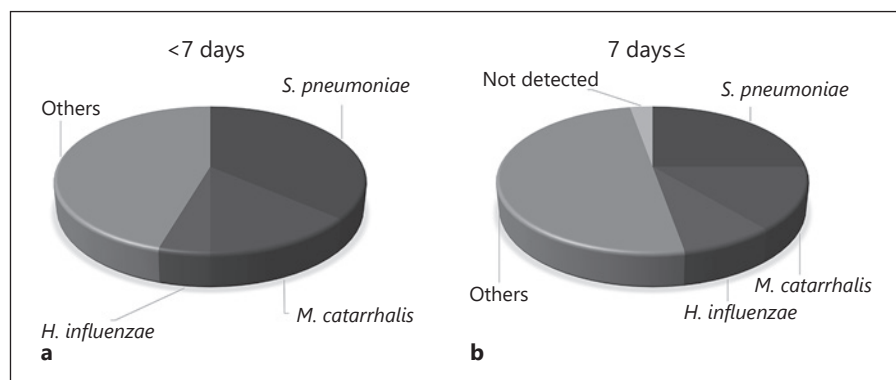


Fig. 2. Comparison of bacteria detected to duration of wheezing (**a**, **b**). The results showed that *S. pneumoniae* tended to be more frequently detected in patients whose wheezing episodes were <7 days although there was no significant difference between them. Data were analyzed using chi-square test statistics and Fisher's exact test for categorical variables.



Serum Cytokines and Chemokines

Peripheral white blood cell counts including neutrophils and eosinophils, C-reactive protein, and 27 serum cytokines/chemokines including interleukin (IL)-1 β , IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, and IL-17, interferon (IFN)- γ , IFN- γ -induced protein-10, tumor necrosis factor (TNF)- α , granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1, macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , eotaxin, regulated on activation normal T expressed and secreted chemokine (RANTES), platelet-derived growth factor bb, fibroblast growth factor basic, and vascular endothelial growth factor were measured in samples from patients with asthma not under systemic corticosteroid treatment at the time of examination. A multi-cytokine detection system, Bio Plex (Bio-Rad, Hercules, CA, USA), was used to quantify serum cytokines and chemokines, according to the manufacturer's instructions, with a Luminex System (Luminex Corporation, Austin, TX, USA), and calculated via Bio-Plex software (Bio-Rad), as described previously [10].

Statistical Analyses

The characteristics of the patients were evaluated by the Pearson χ^2 test and Fisher's exact test for categorical variables. Unpaired data were analyzed by the Mann-Whitney U test. Correlation coefficients were calculated using Spearman rank correlation coefficient analysis. Values for $p < 0.05$ (two-sided) were considered statistically significant. All analyses were performed using SPSS for Windows (version 25.0, SPSS Japan Inc., Tokyo, Japan).

Results

Bacterial Cultures and Virus Detection

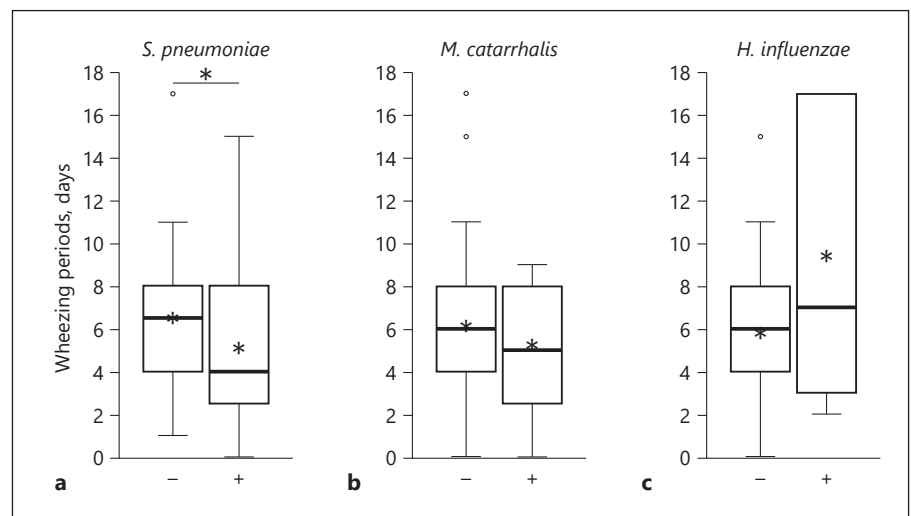
Patient characteristics are shown in Table 1. Among the 111 pharyngeal samples from patients with acute asthma exacerbations, *S. pneumoniae* was detected in 33 cases (29.7%), *M. catarrhalis* in 13 (11.7%), *H. influenzae* in 12

Table 2. Association of rhinovirus and RS virus detection, wheezing periods, and cytokine with bacterial quantity

	Bacterial quantity detected (CFUe)	Rhinovirus detection, %	RS virus detection, %	Wheezing periods, days	Cytokine
<i>S. pneumoniae</i>	0	34	23	6.5	
	1–100	17	44	5.3	*TNF- α ↓
	≥100	63	13	4.6	*IL-6↑, *MIP1- β ↑
					*IL-1ra↑
	All	31	35	*5.0	*TNF- α ↓
<i>M. catarrhalis</i>	0	34	29	6.1	
	1–100	33	17	5.9	
	≥100	0	0	4.6	
					*IL-6↑, *IL-8↓
	All	25	13	5.3	*IL-10↓, *IL-7↓
<i>H. influenzae</i>	0	33	27	5.8	
	1–100	50	50	4.0	*IL-7↑
	≥100	0	0	*17.0	
	All	33	33	8.3	*IL-7↑

The significant differences of each parameter in bacterial quantity versus 0 CFUe are shown (Mann-Whitney U test, * $p < 0.05$). CFUe, colony-forming unit equivalents; IL, interleukin; MIP, macrophage inflammatory protein; IL-1ra, IL-1 receptor antagonist; TNF, tumor necrosis factor; RS, respiratory syncytial.

Fig. 3. Bacteria detected in relation to duration of wheezing episode (a–c). Wheezing episodes were significantly shorter in patients with *S. pneumoniae* than those without (a). No significant differences in the lengths of wheezing episodes were found whether *M. catarrhalis* or *H. influenzae* were detected or not (b, c). Medians are represented by horizontal bars. Data were analyzed using the Mann-Whitney U test. * $p < 0.05$.



(10.8%), other bacteria in 52 (46.8%), and no bacteria in 1 (0.9%; Fig. 1a). *M. catarrhalis* was detected significantly more often in patients with complicated pneumonia than in the other patients (Fig. 1b, c). Detection of these 3 bacterial pathogens was not altered regardless of allergic status and the level of severity of exacerbation (data not shown). In addition, there were no significant differences of duration of wheezing between allergic and not allergic patients. The median duration of wheezing among the 111 patients in this

study was 7 days; therefore, we compared the bacteria detection rates to duration of wheezing. The results showed that *S. pneumoniae* tended to be more frequently detected in patients whose wheezing episodes were <7 days although there was no significant difference between them (Fig. 2).

Among patients with acute exacerbations, 2 major viruses were detected, that is, rhinovirus in 24 (21.6%) and RS virus in 27 (24.3%). There were no significant differences between the bacterial and virus detection rates.

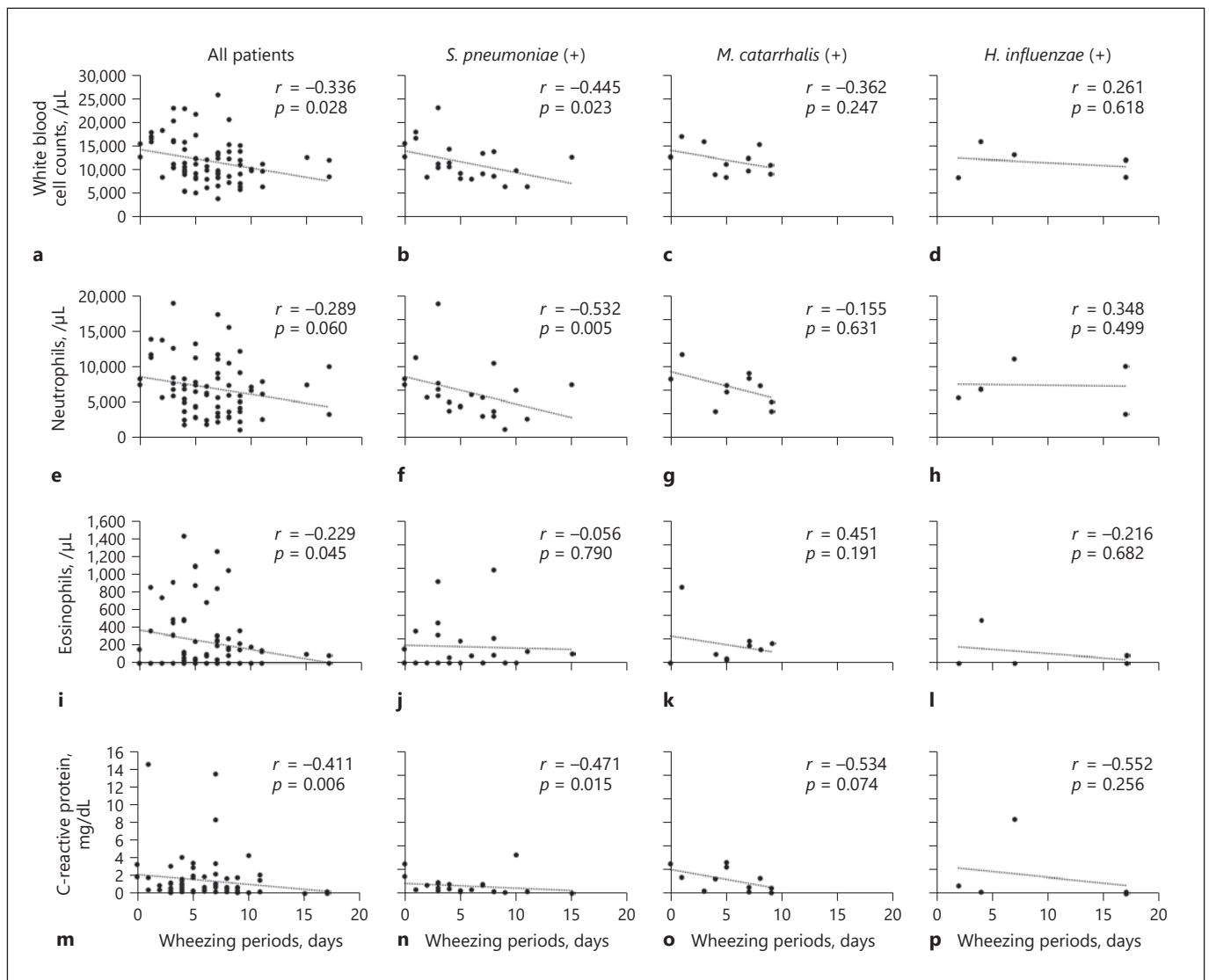


Fig. 4. Correlation between wheezing episodes and other parameters during acute exacerbations of asthma (a–p). In all patients, the duration of wheezing episode showed significant inverse correlation with peripheral white blood cell counts, eosinophils, and C-reactive protein (a, i, m). In patients with *S. pneumoniae*, wheezing duration showed significant inverse correlation with peripheral

white blood cell counts, neutrophils, and C-reactive protein (b, f, n). In patients with *M. catarrhalis* and *H. influenzae*, wheezing duration showed no significant correlation with those parameters. Data were analyzed via the Spearman rank correlation coefficient method.

We next examined the association between the virus detected and quantity of bacteria. We divided bacteria detected into greater and <100 colony-forming unit equivalents. No significant association was observed with the virus detection rate (Table 2).

We analyzed the bacteria detected in relation to duration of wheezing. Episodes of wheezing were significantly shorter in patients with *S. pneumoniae* than in the other patients (Table 2, Fig. 3a). In contrast, no significant

differences in wheezing episodes were observed between cases with *M. catarrhalis* or *H. influenzae* (Table 2, Fig. 3b, c). Furthermore, in patients with *S. pneumoniae*, the duration of wheezing correlated inversely and significantly with peripheral white blood cell count, neutrophils, and C-reactive protein (Fig. 4b, f, n). In contrast, in those with *M. catarrhalis* and *H. influenzae*, the duration of wheezing did not correlate with these parameters (Fig. 4).

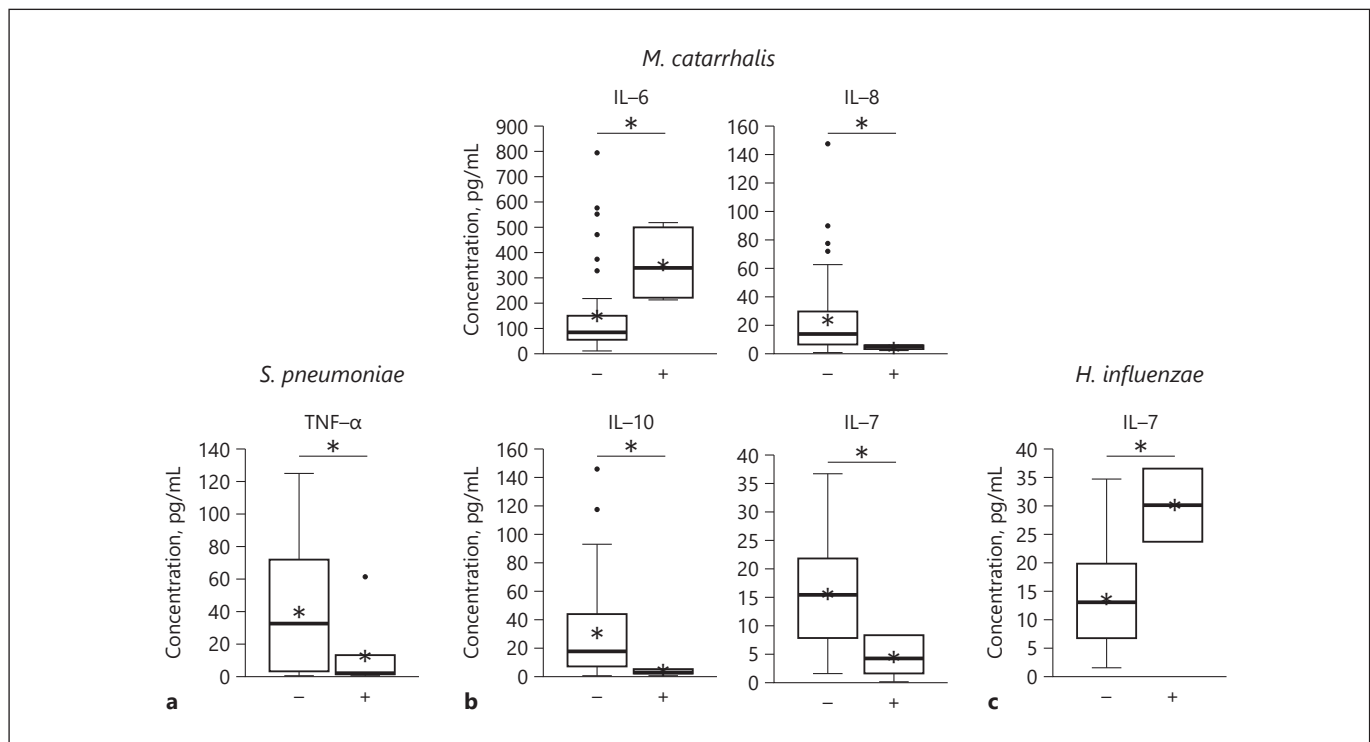


Fig. 5. Comparison of cytokines and chemokines in asthma during acute exacerbations between patients with or without *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* (a–c). Of the 27 cytokines/chemokines, TNF-α was lower in patients with *S. pneumoniae* than in those without (a). IL-6 was higher in patients with *M. catarrhalis* than in patients without while IL-7, IL-8, and IL-10 levels were

lower (b). Only IL-7 was higher in cases of *H. influenzae* (c). No significant differences among the other cytokine/chemokines were observed. Medians are represented by horizontal bars. Mann-Whitney U test was used to analyze the data. * $p < 0.05$. IL, interleukin; TNF, tumor necrosis factor.

Serum Cytokines/Chemokines

We compared the difference in peripheral white blood cell counts including neutrophil and eosinophil, C-reactive protein, and 27 serum cytokines/chemokines between allergic and not allergic patients. There was no significant difference in white blood cell count including neutrophil and eosinophil, and C-reactive protein between them. On the other hand, of 27 serum cytokines/chemokines, only IFN-γ was significantly lower in allergic patients than not allergic patients (6.2 ± 2.7 vs. 16.6 ± 8.7 , $p = 0.03$). However, no significant differences were found in subgroups of patients such as *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* positive patients between allergic and not allergic patients (data not shown). Next, we evaluated the differences in bacteria-specific values for 27 cytokine/chemokines. Only TNF-α was lower in cases of *S. pneumoniae*, than in those without (Fig. 5a). IL-6 was higher in cases where *M. catarrhalis* was detected, while IL-7, IL-8, and IL-10 were lower (Fig. 5b). In cases of *H. influenzae*, only IL-7 was at a higher concentra-

tion (Fig. 5c). No significant differences were observed among the other cytokine/chemokines (data not shown). We also analyzed the association between cytokines/chemokines and quantity of bacteria. Cytokines/chemokines in each quantity of bacteria that are significantly higher or lower than no bacteria are shown in Table 2.

Discussion

We found that pharyngeal *S. pneumoniae* colonization was associated with shorter episodes of wheezing in acute exacerbations of childhood asthma. Previous studies have shown that bacterial colonization, specifically by *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae*, has an impact on the intensity of respiratory symptoms such as stuffy or runny nose, cough, and wheezing, in addition to acute exacerbations of asthma [3, 4, 13]. In contrast, the length of wheezing episode appears independent of the bacterial or viral species found in pharyngeal aspi-

rates [14]. However, we found that the episodes of wheezing were shorter in patients with *S. pneumoniae* colonization during acute exacerbations of asthma. Studies in animal models have demonstrated that the intratracheal administration of whole killed *S. pneumoniae* during or after sensitization to ovalbumin significantly reduces the development of eosinophilia in the blood and the subsequent recruitment of eosinophils into bronchoalveolar fluid, while it improves dynamic lung compliance and peripheral airflow; in other words, it suppresses resistance in the small airways of the lungs [6, 15]. Another study showed that vaccination with *S. pneumoniae* resulted in an annual mean reduction of 56% in the frequency of acute exacerbations among children with asthma [16].

S. pneumoniae components are recognized by toll-like receptor (TLR) 2 and TLR4, both of which induce inflammatory cell responses via adaptor protein MyD88. Accordingly, Thorburn et al. [15] demonstrated in mouse models that TLR2, TLR4, and MyD88 signaling pathways contribute to the suppression of eosinophilic and splenocyte Th2 responses to various degrees in the reduction of airway hyperresponsiveness mediated by *S. pneumoniae*. They also showed that intratracheal administration of *S. pneumoniae* suppresses serum IL-5 and IL-13 released from T cells in mediastinal lymph nodes and splenocytes [15]. Agonists of TLR4 show anti-asthmatic effects in murine models, such as eosinophilia, inhibition of airway hyperresponsiveness, and Th2 cytokine production upon the activation of TLR-dependent MyD88 [17]. These findings collectively suggest that *S. pneumoniae* is involved in the suppression of acute asthma exacerbations.

To clarify the mechanism underlying the suppressive effect of *S. pneumoniae* on asthma, we investigated serum cytokines and chemokines in children with asthma. We found that the production of TNF- α in serum was significantly lower in patients with *S. pneumoniae* than in those without. We believe that our report is the first to show an association between pharyngeal colonization by *S. pneumoniae* in patients with childhood asthma and serum TNF- α . TNF- α , a proinflammatory cytokine, is largely produced by activated macrophages, but can also be produced to a lesser extent by B cells, T cells, NK cells, Kupffer cells, glial cells, and adipocytes [18]. It regulates cellular processes such as proliferation, differentiation, apoptosis, inflammation, and the immune response [19]. Jeong et al. [20] reported that in the lungs and blood of TNF- α knockout mice, *S. pneumoniae* counts were higher by approximately hundred-

fold compared with the wild-type, which resulted in early death and low survival rates. In this context, TNF- α plays a critical role in the resolution of *S. pneumoniae* and protects the host from systemic infection by *S. pneumoniae*.

M. catarrhalis is a dominant pathogenic species found within the airway microbial communities in asthma patients and is known to initiate pulmonary inflammation or exacerbate established cases [21, 22]. In this study, no significant differences in duration of wheezing episodes were found between patients either with or without *M. catarrhalis*. A previous report revealed that recognition of *M. catarrhalis* by multiple TLRs, such as TLR4 and TLR9, triggers the release of the proinflammatory cytokines IL-6 and TNF- α in murine macrophages [23]. Al-nahas et al. [24] reported that airway infection by *M. catarrhalis* in mice provokes a strong inflammatory response consisting of massive neutrophilic infiltration, high levels of IL-6 and TNF- α , and moderate CD4⁺ T-cell-derived IFN- γ and IL-17 levels. Although our results showed that the production of IL-6 in patients with *M. catarrhalis* was significantly higher than that in patients without, there were no significant differences in the levels of TNF- α and IL-17 between the 2 groups.

IL-6 mediates proinflammatory and anti-inflammatory immune responses through trans- and classical signaling, respectively [25]. In contrast to TNF- α , neutralization of IL-6 increases local and systemic TNF- α in addition to the levels of early IL-1 β and IL-17 in bronchoalveolar fluid [24]. The same study also reported that anti-IL-6 treatment in wild-type mice infected with *M. catarrhalis* intranasally resulted in a slightly greater number of mice with moribund symptoms, which suggests that IL-6 exerts anti-inflammatory effects during pulmonary infections with *M. catarrhalis* [24].

H. influenzae, a common bacterium of the respiratory tract, is one of the species most frequently isolated from the airways of patients with neutrophilic asthma [26]. Previous reports have shown that the symptoms of respiratory tract infection do not differ significantly whether nasopharyngeal *H. influenzae* is detected or not [27]. We demonstrated that there was no difference in the length of wheezing episode and cytokines/chemokines, except for IL-7, between patients either with or without *H. influenzae*; however, Essilfie et al. [28] demonstrated that infection with *H. influenzae* in allergic airways disease in mouse models provokes neutrophilic asthma, which is dependent on IL-17. Interestingly, it has been reported that both live *H. influenzae* infections and the administration of killed *H. influenzae* in allergic airways disease sup-

press the features of eosinophilic asthma, including eosinophilic inflammation and allergen-induced Th2 cytokines [29]. This finding may support the hypothesis that exposure to bacteria plays a suppressive role in eosinophilic bronchial asthma.

In conclusion, our data raises the possibility that pharyngeal colonization by *S. pneumoniae* may play a suppressive role during acute exacerbations of childhood asthma. This study was limited in that it was observational and retrospective. Prospective studies are required to identify the exact mechanism by which *S. pneumoniae* colonization suppresses acute exacerbations of bronchial asthma. These studies might serve to help doctors and researchers better understand the pathogenesis of the disease and may lead to the development of other immunoregulatory therapies to prevent and treat asthma.

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Statement of Ethics

This study was approved by the Ethics Committee of Tokai University Hospital, Hadano Red Cross Hospital, and Gunma Children's Medical Center. Parents of the patients provided informed consent and additional consent was obtained from children considered old enough (usually 9 years of age and older).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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Author Contributions

Y.K. and M.K. conducted the study design, collected samples, performed data analysis, wrote the first draft, and finalized the manuscript. Y.Y., T.K., K.S., M.N., M.E., H.T., and K.H. collected samples and performed data analysis. H.M. interpreted the results. All authors read and approved the final manuscript.

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