RESEARCH PAPER

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Streptococcus pneumoniae pharyngeal colonization in school-age children and adolescents with cancer

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Patients with cancer, particularly those with hematologic malignancies, are at an increased risk of invasive pneumococcal disease (IPD) and they are included in the list of subjects for whom pneumococcal vaccination is recommended. The main aim of this study was to evaluate *Streptococcus pneumoniae* colonization in school-aged children and adolescents with cancer to determine the potential protective efficacy of 13-valent pneumococcal conjugate vaccine (PCV13). An oropharyngeal swab was obtained from 277 patients (age range 6-17 years) with cancer during routine clinical visits and analyzed for *S. pneumoniae* using real-time polymerase chain reaction. *S. pneumoniae* was identified in 52 patients (18.8%), including 47/235 (20.0%) with hematologic malignancies and 5/42 (11.9%) with solid tumors. Colonization declined significantly with an increase in age (odds ratio [OR] 0.34, 95% confidence interval [CI] 0.16-0.71, and OR 0.30, 95% CI 0.11-0.82 in children aged 10-14 and $\geq$15 years, respectively, as compared to those $<$10 years). Carriage was more common among patients with leukemia or lymphoma than in children with solid tumors. Co-trimoxazole prophylaxis was significantly associated with reduced pneumococcal carriage (OR 0.41, 95% CI 0.19–0.89). A total of 15/58 (25.9%) and 26/216 (12.0%) children were colonized by PCV13 serotypes among cancer patients previously vaccinated and not vaccinated with 7-valent pneumococcal conjugate vaccine (PCV7), respectively. In conclusion, this study indicates that children and adolescents with cancer are frequently colonized by *S. pneumoniae*. Because most of the carried serotypes are included in PCV13, this vaccine is presently the best solution to reduce the risk of IPD in these patients.

Introduction

Pediatric patients with cancer, particularly those with hematologic malignancies, are at an increased risk of invasive pneumococcal disease (IPD). This has been evidenced since the 1980s and was recently confirmed in a number of epidemiological studies.[1-4] Combining data from a nationwide surveillance for IPD and the German childhood cancer registry, Meisel et al. showed that children with acute lymphoblastic leukemia (ALL) had a more than 10-fold higher risk for IPD compared with the general pediatric population.[5] Hjuler et al. studied the risk for IPD in children 0–17 y old with underlying chronic disease and found that children with cancer had an adjusted relative risk of 19.0 compared with children who did not have cancer.[6] These findings explain why children with cancer worldwide are included in the list of subjects for whom the prevention of pneumococcal infections via vaccine administration is strongly recommended,[7] although their immune response can be suboptimal at least in some patients.[8] Currently, 3 different vaccines are available: the 23-valent polysaccharide vaccine (PPV23), the 10-valent pneumococcal conjugate vaccine (PCV10) and 13-valent pneumococcal conjugate vaccine (PCV13). For children with cancer 6–18 y old, it is suggested that if they have never received PCV13, then they should be given a dose of this vaccine before or after the recommended PPV23 dose, regardless of whether they were previously vaccinated with the 7-valent pneumococcal conjugate vaccine (PCV7).[7] However, the true effect of PCV13 in children with cancer has not been established. A reliable information at this regard could be obtained by monitoring pharyngeal pneumococcal colonization and by evaluating the coverage offered by

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this vaccine. Even if several factors determine the risk for developing pneumococcal disease, pneumococcal colonization is a pre-requisite for IPD development.[9] Moreover, it has been evidenced that pneumococcal conjugate vaccine administration reduces the risk of IPD just by reducing the pharyngeal carriage of pneumococcal serotypes included in the administered vaccine.[10] On the other hand, the measurement of pneumococcal vaccine efficacy on the basis of pneumococcal carriage has been suggested as a useful method able to overcome the problems deriving by the evaluation of immune response.[9] To the best of our knowledge, there aren't data on pneumococcal colonization in children with cancer and due to their risk of IPD this information in relation to pneumococcal conjugate vaccine administration seems extremely important.

The main aim of this study was to evaluate *Streptococcus pneumoniae* colonization in a group of school-aged children and adolescents with cancer to determine the theoretical risk of IPD in these patients and the potential protective efficacy of PCV13. Moreover, because the first pneumococcal conjugate vaccine, PCV7, was administered to less than 50% of the pediatric population, including children with a chronic severe underlying disease, in Italy until 2009,[11] a carriage evaluation of children who were born before that year will permit carriage comparisons in vaccinated and unvaccinated cancer patients and enable the measurement of the long-term effect of PCV7.

Results

Characteristics of the 277 enrolled children with cancer (183 males, 66.1%; mean age ± standard deviation, 11.1 ± 3.5 years) are shown in **Table 1**. One hundred 2four (44.8%) were children <10 years old, 102 (36.8%) were children 10–14 y old, and 51 (18.4%) were children ≥15 years old. Two hundred thirty-five (84.8%) children had hematologic malignancy, and 42 (15.2%) had a solid tumor. A total of 159 (57.4%) patients were receiving maintenance therapy, and 118 (42.6%) had discontinued treatment since less than 6 months. Prophylaxis with co-trimoxazole was administered to 220 (79.4%) children, whereas 57 (20.6%) did not receive this drug. *S. pneumoniae* was identified in the swabs of 52 subjects (18.8%). Carriers were significantly younger than non-carriers (67.3% vs 39.6%, respectively, were <10 years old; $p < 0.01$). There were no differences between carriers and non-carriers with regard to gender, ethnicity, number of siblings, parental smoking habit, gestational age, birth weight, exclusive breast-feeding, allergy history, and meningococcal vaccination. Only influenza vaccination was significantly more common among carriers than non-carriers (13.5% vs 3.1%; $p = 0.007$).

Associations between demographic and clinical characteristics and pneumococcal carriage are shown in **Table 2**. Colonization was strictly age-related, and it declined significantly with an increase in age of the enrolled children (OR 0.34, 95% CI 0.16-0.71, and OR 0.30, 95% CI 0.11-0.82 in children 10-14 old and ≥15 years old, respectively, as compared to those <10 years old). By contrast, colonization was not affected by gender, the presence of siblings, parental smoking habit, the presence or absence of chemotherapy, hospitalization within the last 3 months, administration of anti-infective agents within the last 3 months, and the occurrence of infections within the last 3 months. Carriage was more common among patients with leukemia or lymphoma, but the difference compared with children

Table 1. Demographic and clinical characteristics of 277 children and adolescents with cancer according to pneumococcal carriage

	All children (n = 277)	Carriers (n = 52)	Non-carriers (n = 225)	p-value
Age at enrolment				
<10	124 (44.8)	35 (67.3)	89 (39.6)	
10-14	102 (36.8)	12 (23.1)	90 (40.0)	
≥15	51 (18.4)	5 (9.6)	46 (20.4)	<0.01
Sex				
Male	183 (66.1)	34 (65.4)	149 (66.2)	
Female	94 (33.9)	18 (34.6)	76 (33.8)	0.91
Ethnicity				
Caucasian	257 (92.8)	50 (96.1)	207 (92.0)	
Non-Caucasian	20 (7.2)	2 (3.9)	18 (8.0)	0.39
Diagnosis				
Leukemia/lymphoma	235 (84.8)	47 (90.4)	188 (83.6)	
Other cancers	42 (15.2)	5 (9.6)	37 (16.4)	0.22
Phase				
Maintenance/In therapy	159 (57.4)	24 (46.1)	135 (60.0)	
Therapy stopped within the last 6 months	118 (42.6)	28 (53.9)	90 (40.0)	0.07
Co-trimoxazole prophylaxis				
No	57 (20.6)	18 (34.6)	39 (17.3)	
Yes	220 (79.4)	34 (65.4)	186 (82.7)	**<0.01**
No. of siblings				
0	61 (22.0)	16 (30.8)	45 (20.0)	
1	133 (48.0)	22 (42.3)	111 (49.3)	
2	56 (20.2)	9 (17.3)	47 (20.9)	
≥3	27 (9.8)	5 (9.6)	22 (9.8)	0.28
Parental smoking habit				
Both non-smokers	168 (60.6)	31 (59.6)	137 (60.9)	
At least one smoker	109 (39.4)	21 (40.4)	88 (39.1)	0.87
Gestational age (weeks)[a]				
<37	18 (6.5)	4 (7.8)	14 (6.2)	
≥37	258 (93.5)	47 (92.2)	211 (93.8)	0.75
Birth weight (g)[a]				
<2,500	10 (3.6)	1 (2.0)	9 (4.0)	
≥2,500	265 (96.4)	50 (98.0)	215 (96.0)	0.69
Exclusive breastfeeding				
No	61 (22.0)	12 (23.1)	49 (21.8)	
Yes	216 (78.0)	40 (76.9)	176 (78.2)	0.84
Infections (last 3 months)				
No	222 (80.1)	46 (88.5)	176 (78.2)	
Yes	55 (19.9)	6 (11.5)	49 (21.8)	0.10
Allergy				
No	230 (83.0)	46 (88.5)	184 (81.8)	
Yes	47 (17.0)	6 (11.5)	41 (18.2)	0.25
Meningococcal vaccination[a]				
No	201 (73.6)	36 (69.2)	165 (74.7)	
Yes	72 (26.4)	16 (30.8)	56 (25.3)	0.42
Influenza vaccination during current season				
No	263 (94.9)	45 (86.5)	218 (96.9)	
Yes	14 (5.1)	7 (13.5)	7 (3.1)	**0.007**

[a]Some missing values.

Table 2. Association between selected demographic and clinical characteristics and pneumococcal carriage in children with cancer

	OR (95% CI)[a]
Age at enrollment	
<10	1 (reference)
10-14	0.34 (0.16-0.71)
≥15	0.30 (0.11-0.82)
Sex	
Male	1 (reference)
Female	1.06 (0.52-2.15)
Diagnosis	
Leukemia/lymphoma	1 (reference)
Other cancers	0.53 (0.18-1.52)
Phase	
Maintenance/In therapy	1 (reference)
Therapy stopped within the last 6 months	1.35 (0.67-2.75)
Co-trimoxazole prophylaxis	
No	1 (reference)
Yes	0.41 (0.19-0.89)
Siblings	
No	1 (reference)
Yes	0.52 (0.24-1.11)
Parental smoking habit	
Both non-smokers	1 (reference)
At least one smoker	0.90 (0.46-1.76)
Infections (last 3 months)	
No	1 (reference)
Yes	0.48 (0.19-1.24)
Anti-infective therapy (last 3 months)	
No	1 (reference)
Yes	0.52 (0.26-1.03)

[a]Multivariate models included terms for age at enrollment, gender, siblings, parental smoking, disease phase, co-trimoxazole prophylaxis and diagnosis as well as other clinical characteristics.
CI: confidence interval; OR: odds ratio.

who had solid tumors did not reach statistical significance (OR 0.53; 95% CI 0.18–1.52). Co-trimoxazole prophylaxis was significantly associated with reduced pneumococcal carriage (OR 0.41, 95% CI 0.19–0.89).

The relationship between pneumococcal vaccination status and pneumococcal carriage in the population as a whole and the 2 younger age groups (there were too few vaccinated children in the group aged ≥15 years) is shown in **Table 3**. The proportion of carriers in the entire population of any pneumococcal serotype, any serotype included in PCV7 and the 6 additional serotypes included in PCV13 were higher in PCV7-vaccinated than in unvaccinated children, but these differences were not statistically significant (OR 1.70, 95% CI 0.79–3.65; OR 1.51, 95% CI 0.65–3.49; and OR 2.69, 95% CI 0.62–11.7 for carriers of any pneumococcal serotype, carriers of any serotype in PCV7, and carriers of any of the 6 additional serotypes in PCV13, respectively). Sub-analysis of the group <10 years old revealed similar results, with higher carriage rates in vaccinated children but without statistically significant differences between groups.

Individual serotypes identified by vaccination status are shown in **Table 4**. A total of 15 out of 58 children vaccinated with

Table 3. Relationship between pneumococcal vaccination status and pneumococcal carriage in children with cancer.[ab]

	Vaccinated with PCV7 (n = 58)	Not vaccinated against pneumococcus (n = 216)	OR (95% CI)
Pneumococcal carrier status			
Any serotype			
Non-carriers	39 (67.2)	183 (84.7)	1 (reference)
Carriers	19 (32.8)	33 (15.3)	1.70 (0.79-3.65)
Serotypes in PCV7			
Non-carriers	43 (74.1)	190 (88.0)	1 (reference)
Carriers	15 (25.9)	26 (12.0)	1.51 (0.65-3.49)
Six additional serotypes in PCV13			
Non-carriers	53 (91.4)	211 (97.7)	1 (reference)
Carriers	5 (8.6)	5 (2.3)	2.69 (0.62-11.7)
Subgroup aged	(n = 39)	(n = 85)	
Any serotype			
Non-carriers	22 (56.4)	67 (78.8)	1 (reference)
Carriers	17 (43.6)	18 (21.2)	2.05 (0.81-5.20)
Serotypes in PCV7			
Non-carriers	25 (64.1)	71 (83.5)	1 (reference)
Carriers	14 (35.9)	14 (16.5)	1.79 (0.66-4.91)
Six additional serotypes in PCV13			
Non-carriers	34 (87.2)	83 (97.6)	1 (reference)
Carriers	5 (12.8)	2 (2.4)	4.33 (0.63-29.5)
Subgroup aged 10-14 years	(n = 14)	(n = 86)	
Any serotype			
Non-carriers	13 (92.9)	75 (87.2)	1 (reference)
Carriers	1 (7.1)	11 (12.8)	0.76 (0.08-7.47)
Serotypes in PCV7			
Non-carriers	14 (100.0)	78 (90.7)	1 (reference)
Carriers	0 (0.0)	8 (9.3)	NE
Six additional serotypes in PCV13			
Non-carriers	14 (100.0)	84 (97.7)	1 (reference)
Carriers	0 (0.0)	2 (2.3)	NE

[a]ORs adjusted for age at enrollment, gender, siblings, parental smoking, disease phase, co-trimoxazole prophylaxis and diagnosis.
[b]No information concerning the pneumococcal vaccination status of three children.
CI: confidence interval; NE: not estimable; OR: odds ratio; PCV7: 7-valent pneumococcal conjugate vaccine; PCV13: 13-valent pneumococcal conjugate vaccine.

PCV7 (25.9%) and 26 out of 216 (12.0%) of those not vaccinated with PCV7 were colonized by PCV13 serotypes. Prevalence of colonization with more than one PCV13 serotype was higher - though not statistically significant - among children who have received PCV7 (OR 2.30, 95% CI 0.80–6.61). Serotypes 19F and 4 were the most frequently identified serotypes in vaccinated and unvaccinated patients. A very low number of patients were colonized exclusively by non-PCV13 serotypes. Moreover, in this case, the number was higher in the vaccinated group, although this difference did not reach statistical significance.

Table 4. Carriage of specific pneumococcal subtypes in children with cancer according to pneumococcal vaccination status[a]

	Vaccinated with PCV7 (n = 58)	Not vaccinated against pneumococcus (n = 216)	OR (95% CI)[b]
Carriers of PCV13 serotypes	15 (25.9)	26 (12.0)	1.60 (0.68-3.73)
Number of PCV13 serotypes carried			
1	6 (10.3)	13 (6.0)	0.91 (0.28-2.97)
≥2	9 (15.5)	13 (6.0)	2.30 (0.80-6.61)
Serotype carried			
Positive for serotype 1	0 (0.0)	1 (0.5)	NE
Positive for serotype 3	1 (1.7)	1 (0.5)	4.15 (0.09-183.0)
Positive for serotype 4	6 (10.3)	6 (2.8)	3.11 (0.77-12.49)
Positive for serotype 5	2 (3.4)	2 (0.9)	2.82 (0.30-26.92)
Positive for serotype 6A	1 (1.7)	0 (0.0)	NE
Positive for serotype 6B	0 (0.0)	1 (0.5)	NE
Positive for serotype 7F	0 (0.0)	1 (0.5)	NE
Positive for serotype 9V	1 (1.7)	4 (1.8)	1.09 (0.09–13.68)
Positive for serotype 14	0 (0.0)	0 (0.0)	NE
Positive for serotype 18C	0 (0.0)	0 (0.0)	NE
Positive for serotype 19A	2 (3.4)	0 (0.0)	NE
Positive for serotype 19F	14 (24.1)	25 (11.6)	1.43 (0.60–3.40)
Positive for serotype 23F	0 (0.0)	1 (0.5)	NE
Carriers of non-PCV13 serotypes	4 (6.9)	7 (3.2)	2.06 (0.52–8.09)

[a]Three subjects had missing information on pneumococcal vaccination.
[b]ORs adjusted for age at enrollment, gender, siblings, parental smoking, disease phase, co-trimoxazole prophylaxis and diagnosis. For the analyses on single serotypes, the reference category is non-carrier of each corresponding serotype.
CI: confidence interval; NE: not estimable; OR: odds ratio; PCV7: 7-valent pneumococcal conjugate vaccine.

Discussion

This study shows that approximately 20% of school-age children and adolescents with cancer who received maintenance therapy or who have discontinued chemotherapy within the last 6 months were colonized by *S. pneumoniae*. Unfortunately, in this study, evaluation of pneumococcal colonization in a comparable group of healthy children was not included and this does not permit to evaluate whether children with cancer were differently colonized in comparison to normal subjects living in the same geographic area with limited pneumococcal vaccination coverage. Moreover, no data are available in Italy on pneumococcal colonization rates in patients with cancer prior to PCV7 licensure. Furthermore, the study was undertaken over a 6 month period, although the colonization may vary by season. In addition, the lack of antibody levels in vaccinated children and of clinical data in this specific population represents further limits. However, pneumococcal colonization is a pre-requisite for IPD and its evaluation represents an alternative method to analyze pneumococcal vaccine efficacy,[12] that seems particularly useful in a population like that of patients with cancer who show significant reduction in systemic antibodies. The detection of this rate of pneumococcal colonization was obtained by the use of the most accurate available methods to identify *S. pneumoniae*. Respiratory secretions were collected from the oropharynx which has been demonstrated to be the most effective site for detecting *S. pneumoniae* in older children and adults.[13] A flocked nylon fiber tip was used because previous studies have shown that this ensures the highest rate of detection of *S. pneumoniae*, particularly in comparison with the more widely used Dacron and rayon swabs).[14] *S. pneumoniae* was identified by means of molecular methods that, albeit with some exceptions,[15] have been found to be significantly more reliable than traditional non-enriched cultures in routine practice.[16] Furthermore, to improve the detection of *S. pneumoniae* without increasing the risk of false-positive results, both the *lytA* and *cpsA* genes were amplified.[17] However, a relevant role in conditioning high colonization rates of children with cancer could derive from the state of immunodeficiency associated with cancer and its treatment that results in impaired antibody protection against pneumococci. Lehrnbecher et al. assessed the spontaneous reconstitution of humoral immunity against pneumococcal antigens in a total of 53 children treated for acute lymphoblastic leukemia who had previously never received any pneumococcal vaccine.[18] These authors found that at 3 and 9 months after the completion of chemotherapy, most patients had levels of specific antibodies to pneumococcal antigens that were below the putative correlate of protection. Unfortunately, we did not measure pneumococcal serotype-specific antibodies in our patients, but the evaluation of oropharyngeal pneumococcal carriage represents an alternative useful method to evaluate IPD potential risk and pneumococcal conjugate vaccine efficacy.

The colonization rate was higher in younger patients and declined with age. Moreover, it was significantly reduced in patients receiving co-trimoxazole prophylaxis. Both of these findings were expected. A progressive reduction in pneumococcal colonization with increasing age is a common finding in otherwise healthy children.[19,20] This observation has been attributed to the continuous exposure to circulating pneumococcal serotypes that evokes a protective immunity and the reduced role of risk factors, which can significantly enhance the horizontal spread of pneumococcal strains. Moreover, the reduction of pneumococcal carriage in cancer patients who receive co-trimoxazole prophylaxis is consistent with previous findings that in Italy this antibiotic remains effective against more than 70% of pneumococcal

strains, although its prolonged use is accompanied by a selection of resistant strains.[21] Unfortunately, an evaluation of co-trimoxazole susceptibility of colonizing *S. pneumoniae* was not performed, and it was not possible to determine whether this problem also occurred in the children enrolled in this study.

Pneumococcal carriage was higher in children with hematologic malignancies than in patients with other types of cancer. Although this difference was not statistically significant due to the low number of children with solid tumors enrolled in the study, it was consistent with the findings obtained in previous studies, which demonstrated a greater risk of IPD in children with leukemia or lymphoma compared with those who had other types of cancer. Recently, Meisel et al. reported that the adjusted risk ratio (RR) of IPD was approximately 6 times higher (52.1) in patients with hematologic cancer than in those with non-hematologic malignancies, although this second group was at an increased risk of this disease (8.9) compared with healthy subjects.[5]

Influenza vaccination in the current season was received significantly more often by pneumococcal carriers than by non-carriers. We do not have a clear explanation for this finding. It is well-known that influenza vaccination has a role in prevention of pneumococcal infection[22,23] and in our study performed in patients with cystic fibrosis,[24] the majority of whom were vaccinated against influenza, influenza vaccination coverage was similar between carriers and non-carriers.

Considering the data collected in this study, it appears that at least for the 7 serotypes included in PCV7, the duration of protection against carriage is relatively short. In this study, the colonization rates were slightly higher in children who had received PCV7 in the first year of life than in unvaccinated patients. Furthermore, colonizing serotypes were found in both groups, particularly the serotypes that were included in this vaccine. Although sampling was limited to a single time point and pneumococcal colonization overtime as well as specific antibodies were not evaluated, these data indicate that vaccine efficacy against colonization does not persist among school-aged children who were previously immunized with PCV7 when the vaccine uptake is low as occurred in Italy until 2009. A similar finding was recently reported in a study involving healthy school-aged children and adolescents[25] and was considered to be the consequence of a progressive decrease of the antibody concentrations that are evoked within the first year of life but, when the children reach school age, are no longer adequate to avoid colonization. Because each serotype has different putative correlates of protection,[26] it is likely that those serotypes that need the highest concentrations to be eliminated are those that are more frequently carried. This finding is confirmed both by the results obtained in a study performed in healthy subjects and by this study because the most commonly detected serotype was 19F, which has been reported to have a very high correlate of protection against IPD.[27] However, the potential reduction of pneumococcal conjugate vaccines effectiveness in preventing pharyngeal colonization does not mean that the vaccine can lose its ability to prevent IPD in a relatively short time. The antibody concentrations required to avoid the risk of IPD are significantly lower than those that are required to prevent carriage, and a progressive reduction of antibody levels can only have a marginal impact on vaccine effectiveness against severe disease. However, further studies regarding these issues are urgently needed.

In conclusion, this study indicates that children and adolescents with cancer are frequently colonized by *S. pneumoniae*. This finding highlights a possible risk of IPD. Because most of the carried serotypes are included in PCV13, this vaccine is presently the best solution to reduce the risk of IPD in these patients.

Methods

Patient enrollment and swab collection

The Ethics Committees of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, and other participating hospitals (located in Monza, Pavia and Rome) approved the protocol of this multi-center study. Children and adolescents were enrolled after written parental informed consent, and written assent was obtained from subjects aged $\geq$8 years.

Patients 6–17 y old with a diagnosis of cancer who regularly attended the pediatric cancer center of each hospital between January 1, 2014, and June 30, 2014, and who were considered compliant with the protocols used in each center for the different types of cancer were considered eligible for this study. Among these children, those who had received maintenance therapy for either a hematologic malignancy or a solid tumor and patients who had discontinued any anti-cancer treatment since less than 6 months were selected. Subjects with an underlying disease different from cancer, those with a clinically evident respiratory infection at the time of selection and those receiving antibiotics for any reason were excluded.

Clinical and laboratory data of each enrolled child for the previous 3 months were retrieved from clinical records and recorded in an electronic database that was specifically prepared for the study.

The patients' pneumococcal vaccination status was established by consulting the official vaccination chart, which was issued by the Vaccination Service of the Region in which the patients lived. The pneumococcal immunization schedule recommended by the Italian Ministry of Health prior to 2011, which is the period in which the enrolled subjects could have received the pneumococcal vaccine, involved 3 doses of PCV7 in the first year of life, or 2 in the second year, or a single dose between the child's second and fifth years.[28] Patients were considered fully vaccinated with PCV7 if one of these recommendations was fulfilled by the time of enrollment; they were not considered fully vaccinated if they had started but not completed the vaccination schedule. The latter group comprised only 1% of the enrolled subjects, and it was not compared with the groups of fully vaccinated or unvaccinated children.

From the enrolled children, oropharyngeal samples were obtained using an ESwab kit that contained a polypropylene screw-capped tube filled with 1 mL of liquid Amies medium

(Brescia, Copan, Italy). Sampling was performed by pressing the tongue downward to the floor of the mouth with a spatula, and the tonsillar arches and the posterior wall of the oropharynx were swabbed without touching the sides of the mouth. All swabs were immediately refrigerated at −20°C and transported within one week to the central laboratory, where they were processed within 2 hours of arrival.

Identification of *S. pneumoniae*

Bacterial genomic DNA was extracted from the samples (250 μL) using a NucliSENS easyMAG automated extraction system (BioMérieux, Bagno a Ripoli, Florence, Italy) and a generic protocol. Samples were tested for the autolysin-A-encoding (*lytA*) and *wzg* (*cpsA*) genes of *S. pneumoniae* using real-time polymerase chain reaction (PCR) as previously described.[29] The detection level of this test was 16 genome copies, and each sample was tested in triplicate. Samples were considered positive if at least 2 of the 3 tests revealed the presence of both genes. No internal amplification control was used in the reaction to maximize sensitivity, but an external control was performed. Real-time PCR-negative specimens were also tested for the presence of an RNase P-encoding gene to exclude PCR inhibition and DNA extraction failure.

All positive cases were serotyped using primers and probes that were designed using GenBank database sequences (www.ncbi.nlm.nih.gov) of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F (i.e., serotypes in PCV13), and were synthesized by TIB Molbiol (Genoa, Italy) as previously described.[30] Analytical specificity was pre-evaluated using computer-aided analyses in the Primer-blast (www.ncbi.nlm.nih.gov/tools/primer-blast) and BLAST software (www.blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the collected sequences with all of the sequences listed under 'bacteria' and 'Homo sapiens'.

Statistical analysis

Groups were compared using the X^2 or Fisher's exact test as appropriate. Ordered categorical data were compared using a Cochran-Armitage test for trends. Multivariate odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using unconditional multiple logistic regression models to measure associations between i) pneumococcal vaccination and pneumococcal carrier status and ii) selected demographic and clinical characteristics and pneumococcal carrier status. Adjustments were made for the following covariates, which were defined *a priori*: age, gender, ethnicity, presence of siblings, and parental smoking habits, disease phase, co-trimoxazole prophylaxis and type of cancer diagnosis, as well as other clinical characteristics. Stratified analyses were also conducted for age subgroups (<10, 10-14, and ≥15 years). All analyses were 2-tailed, and *p*-values of 0.05 or less were considered statistically significant. All analyses were performed using SAS version 9.2 (Cary, NC, USA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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