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***Mycoplasma pneumoniae* and health outcomes in children with asthma**

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Introduction

Asthma, a heterogeneous disease that affects 8% of US children, imparts a significant economic burden on society and has a marked effect on the quality of life of affected patients.^{1,2} *Mycoplasma pneumoniae* (*Mp*) has been reported in children with acute exacerbations of asthma as well as in children with chronic asthma.^{3–5} *Mp* infection has been associated with both the initiation and persistence of asthma in children, although the pathogenic mechanisms are not well understood.^{6,7} Prior studies on the role of *Mp* in asthma have been limited by the difficulty in culturing this organism, the poor performance of *Mp* serology in defining active infection, and variable sensitivities of PCR assays in detecting *Mp*.

Our group has identified a 68-kDa protein toxin unique to *Mp* called the Community Acquired Respiratory Distress Syndrome Toxin (CARDS Tx) that possesses both vacuolating and adenosine diphosphate ribosyltransferase activity similar to pertussis toxin⁸. We developed assays to detect CARDS Tx by PCR and antigen capture (AC).^{9,10} CARDS Tx gene sequences are more sensitive for the detection of *Mp* by PCR than other gene targets such as *Mp* P1 adhesin (P1) and adenosine triphosphatase.^{11,12}

We previously reported that *Mp* detection was associated with increasing morbidity and airway inflammation in acute exacerbations of asthma and in refractory asthma in children.⁵ The purpose of this study was to determine the impact of *Mp* on asthma control and quality of life using assays to detect the presence of CARDS Tx protein and DNA in respiratory secretions in children 3–10 years old with persistent asthma.

Methods

Study Subjects

This single-center, prospective cohort study was conducted from March 2012 through December 2015 and was approved by the institutional review board. We obtained written informed consent from the parent or legal guardian of each subject. We enrolled 31 children 3–10 years of age with a physician diagnosis of asthma requiring asthma therapy. At the time of enrollment, subjects had stable asthma for at least one month. We enrolled subjects from pulmonary or general pediatric clinics during routine visits. The diagnosis of asthma was based on physician assessment according to national guidelines.¹³ Exclusion criteria included: bleeding disorders, pulmonary disease other than asthma or other active medical conditions (e.g. heart disease, immunodeficiency, chronic sinus infection, severe uncontrolled gastroesophageal reflux, malignancy, HIV infection). Subjects were monitored for 1–7 follow-up visits over a 24 month period of time.

Specimens were collected from the throat and nasopharynx using nylon-tipped swabs (Copan, Murrieta, CA) and placed into transport media containing vancomycin, amphotericin and colistin. We collected exhaled breath condensate (EBC) using the RTube (Respiratory Research, Austin, TX) and measured EBC pH with an Orion Ross® Micro pH electrode (Thermo Scientific, Beverly, MA) after argon degassing as described.¹⁴ All

samples were stored at 4 degrees C, transported within 24 hours for processing, and subsequently stored at -80 degrees C (throat, nasopharyngeal swabs) or -20°C (exhaled breath condensates) until analysis.

Surveys

We measured asthma symptoms and quality of life based on caregiver responses to validated questionnaires in English or Spanish. Symptom scores were measured using the Lara Asthma Symptom Scale [LASS], an 8-item questionnaire; scores range from 1 to 5, with higher scores representing more severe asthma symptoms over the past four weeks.¹⁵ The Quality of life (QOL) was measured using the Pediatric Asthma Caregiver's Quality of Life Questionnaire [PACQLQ], a 13-item questionnaire; scores range from 1 to 7, with higher scores representing better quality of life over the past 7 days. The minimal clinically important difference in scores is 0.5.¹⁶

We asked caregivers about their child's medication use, school days missed (for children enrolled in day care, preschool or school), health care utilization, level of allergic rhinitis symptoms (rating scale of 0–10), and exposure to environmental tobacco smoke over the past 12 months (enrollment) or since the last visit (follow up visits). We created several summary scores (See eMethods for more details). The environmental tobacco smoke score accounted for additive exposures inside and outside the home with more points for exposure to maternal smoking (potential range: 0–13). Health care utilization was the sum of emergency department visits, unscheduled doctor visits and hospitalizations. When applicable, outcome variables were calculated as the percentage of days in the past year or since the last visit, e.g. percentage of days with oral corticosteroid use, percentage of days absent from school and percentage of days with health care utilization. The controller medication score was based on parental report of use of inhaled corticosteroids (none=0, low=2, medium=4, high dose=6), long-acting beta agonists (none=0; any=2) and montelukast (none=0; any=0.5) (potential total score: 0–8.5); higher scores represent higher levels of controller medication use. The controller medication-symptom score (potential range: 1–13.5) was the sum of the controller medication score and the symptom score. The asthma severity index (determined at enrollment) was the sum of the controller medication-symptom score and total health care utilization for the year prior to enrollment, and was used to indicate chronic asthma severity. To calculate total health care utilization for this index, we counted the total number of episodes of health care utilization for the year prior to enrollment (i.e., the sum of emergency department visits, unscheduled doctor visits and hospitalizations.) We calculated BMI and BMI z-score using the SAS code provided by the CDC (ref: <http://www.cdc.gov/nccdphp/dnpao/growthcharts/resources/sas.htm>)

Detection of Mp DNA and protein

DNA from airway samples was purified using the Maxwell® 16 viral total nucleic acid purification kit (Promega) and the Maxwell MDx automated purification system (Promega) according to manufacturer's instructions. Real-time PCR for CARDS Tx (MPN372) was performed as described,^{9,10} with the limit of detection (LOD) set at 5 genomes. CARDS Tx protein was quantified using AC as previously described¹⁰ with LOD set at 0.078pg. *Mp* positive subjects were defined as those with any PCR or AC above the LOD, and *Mp*

negative subjects were those in whom testing at a given visit was negative. Analyses using either only PCR positive or only AC positive as an indication of *Mp* status were similar to using any PCR or AC as an indication of *Mp* status. We also determined the maximum level of CARDS Tx by AC and by PCR at each visit.

Statistical analysis

We assessed distributional characteristics of each variable. Descriptive statistics were expressed as means $\pm$ standard deviation or median and 25–75 percentile interquartile range, as appropriate. For continuous variables, the CARDS Tx PCR and AC levels below the LOD were set at one-half the LOD. EBC pH values ≤ 6.5 were excluded from analysis, since these values may reflect episodes of GER. Multi-level mixed effects models were used to assess associations between *Mp* status, study outcomes (symptom score, quality of life, health care utilization, school absenteeism, and oral corticosteroid use) over time. Generalized linear mixed models were used to analyze outcome data from non-Gaussian distributions. The likelihood of converting from *Mp* positive to negative, and vice-versa, on successive visits, was determined by examining *Mp* transition probabilities. Multi-level random effects models were also used to examine relationships between co-morbidities (atopy; ETS exposure) and the outcome variables, controlling for severity score at enrollment. All statistical tests were performed at the two-sided 0.05 level of statistical significance and all statistical analyses were conducted in SAS (Version 9.4, SAS Institute Inc. Cary, NC).

Results

Demographics

We enrolled 31 subjects (mean age 6.6 ± 2 years, range 3.2–10.8; 71% male) with asthma from a predominantly Hispanic population (Table 1). In the 12 months prior to enrollment, 19 (61.3%) subjects had at least one emergency department visit and 17 (54.8%) were hospitalized at least once; 17 (65%) had two or more courses of oral corticosteroids (range: 0–10 courses). Median percentage days with some health care utilization (emergency department visit, unscheduled doctor visit or hospitalization) was 1.1 [IQR 0.3–2.5] (range 0–4.7). Median percentage days on oral corticosteroids was 0.8 [IQR 0.3–1.1] (range 0–2.7). Median percentage school days absent was 1.4 [IQR, 0.3,3] (range 0–3).

The study spanned from 3/2012 to 12/2015. 28 subjects completed 2–8 visits for a total of 176 study visits for all subjects (mean no. visits per subject = 5.7 ± 2.8 SD; median 7 [IQR, 3–8]). Fifteen subjects completed all 8 visits, 9 completed 3–7 visits, 4 completed 2 visits, and three subjects completed only the enrollment visit.

At enrollment, 27 (90%) subjects were taking an inhaled corticosteroid, either alone (46.7%) or in combination with a long-acting beta agonist (43.3%). Median controller medication score was 6.5 [IQR, 2.5–8.5], (range 0–8.5). (As a point of reference, a child taking medium dose inhaled corticosteroids with a long-acting beta agonist would have a score of 6).

In terms of co-morbidities, 18 (58.1%) subjects reported a physician diagnosis of another atopic condition (allergic rhinitis, eczema or food allergy), 6 (19.4%) had a diagnosis of obstructive sleep apnea and 6 (19.4%) had a diagnosis of gastroesophageal reflux disease,

although none reported severe uncontrolled symptoms at enrollment. (See eFigure 1 for details). Thirteen (41.9%) subjects were exposed to environmental tobacco smoke (ETS) in their home (8; 25.8%) or regularly outside their home (5; 16.1%). Median ETS exposure score was 1 [IQR, 0–3] (range 0–7). (As a point of reference, occasional smoke exposure outside the home would result in a score of 1 and exposure to maternal smoking would result in a score of 5).

Outcomes

At enrollment, the mean asthma severity index for all subjects was 12.8 (± 7.5 SD, range 1.3–27.6), and subjects had a mean symptom score of 2.2 (± 0.8 SD, range 1–3.6). Overall, these scores reflect milder asthma symptoms.¹⁵ Subjects had a median QOL score of 5.6 (IQR, 3.7–6.5; range 2–7), indicating some impairment related to asthma, but better than scores reported previously for children with refractory asthma.^{5,17} Most subjects had variability in symptom scores and QOL scores while some maintained consistent scores throughout the 2 year study period (Figure 1). Within subjects coefficient of variation for symptom scores was 24% (95% CI 21, 29; range 0–51%); variation for QOL was 15% (95% CI 13, 29; range 0–31%). Overall, symptom scores improved significantly over the duration of the study ($P=0.03$), but with no significant difference in the rate of change by asthma severity index tertiles ($P=0.12$) (Figure 2). QOL scores also improved over time ($P=0.0015$). Other studies have noted improvement in asthma symptoms over time in children with asthma, even without intervention.^{18,19} (See eFigure 2 for histograms of outcome variables.)

Associations between outcome measures; association of co-morbidity measures with outcomes

After controlling for age, gender, BMI, ETS exposure and baseline asthma severity index, significant associations were observed between most of the outcome measures other than exhaled breath condensate pH (EBC pH) (Table 2). We also examined the relationship between co-morbidity measures and outcomes. Higher allergic rhinitis scores were associated with worse symptom scores ($P=0.004$) and QOL scores ($P=0.006$) as has been described.²⁰ Higher levels of ETS exposure were associated with lower levels of health care utilization ($P=0.012$) and fewer school days missed ($P=0.012$). These latter findings, while surprising, may reflect complex interactions with regard to health care utilization and/or confounders such as air pollution exposures as have been described.^{21–23} BMI z-score was not associated with any outcome variables.

Mp testing

Mp was detected in 20 (64.5%) of subjects at enrollment, based on detection of *Mp* by PCR and/or AC. In general, levels of *Mp* CARDS Tx detected by AC or PCR were considered low. For example, patients with pneumonia due to *Mp* typically have AC levels >10 pg/ml and PCR levels $>10,000$ genomes (unpublished data). In the current study, high levels of CARDS Tx were detected in only two samples (Figure 4). Subjects who were *Mp* negative had a 0.348 probability of becoming *Mp* positive on the following visit. Subjects who were *Mp* positive had a 0.579 probability of remaining *Mp* positive on the subsequent visit. The association between *Mp* status and prior *Mp* status on the previous visit was significant ($P=0.0058$).

We tracked the antibiotic use of subjects throughout the study. Thirty percent of subjects received antibiotics in the three months prior to a given study visit (18.3% penicillin class; 11.4% macrolide) for a total of 50 short courses of antibiotics (30 penicillin, 20 macrolide). Prior antibiotic treatment (any antibiotic or specific antibiotic group) was not associated significantly with *Mp* detection (or level of CARDS Tx by AC or PCR), or with outcomes at the subsequent study visit, although the study was underpowered to address this effect.

We sought to investigate the seasonal variation in *Mp* prevalence, since studies during a recent worldwide epidemic showed a higher winter/spring prevalence of *Mp* compared with other seasons.^{5,24,25} In the current study, which began at the end of this most recent worldwide epidemic, the rate of *Mp* positivity was initially high, but the overall percentages of *Mp* positive samples declined over the course of the study (Figure 3A), albeit not significantly. However, in contrast to our previous report and that of others, there was a significantly higher percentage of *Mp* positive samples in the three month period from May–July when compared to Nov–Jan ($p<0.001$), Feb–Apr ($p=0.008$) and Aug–Oct ($p=.02$) (Figure 3B).

Outcomes with *Mp*

We found no significant relationship between *Mp* detection (or level of CARDS Tx by AC or PCR) and outcomes except for controller medication score (Table 3; eTable1). Median controller medication scores were slightly higher in *Mp* negative compared with *Mp* positive subjects (6.5 [IQR, 2.5–8.5] versus 6.3 [IQR 2.5–8.5], $P=0.04$). Similarly, higher controller medication scores were associated with lower levels of AC, although this result was not statistically significant ($P=0.07$). (Other authors reported that in pediatric patients with acute exacerbation of asthma, *Mp* negative subjects had higher rates of inhaled corticosteroid use than *Mp* positive subjects.)⁴ Similarly, there was no significant relationship between *Mp* detection at a specific visit and outcomes at a subsequent visit.

Discussion

Mp infects the upper and lower respiratory tract causing pneumonia, bronchitis, and possibly asthma.^{6,26,27} Prior evidence has linked *Mp* to new-onset asthma⁶, exacerbations of asthma,^{4,6} chronic worsening of asthma and long-term decrements in pulmonary function.^{5,28} Biscardi et al. reported that *Mp* was associated with the initiation and recurrence of asthma exacerbations in children.⁶ In a previous study, we found high rates of *Mp* detection in children with acute asthma, refractory asthma and in healthy children without asthma. Moreover, we found some evidence that asthma control (measured by ACT scores), quality of life (measured by PAQLQ) and EBC pH were significantly lower in *Mp* positive pediatric subjects with refractory asthma compared with *Mp* negative subjects.⁵ The purpose of the current study was to determine the impact of *Mp* on asthma control and quality of life over time in a cohort of children with persistent asthma.

In the current study, we found a significant seasonal pattern for higher rates of *Mp* detection in the months of May through July. In a prior study, conducted from Dec 2009 through June 2011, we noted a significant increase in *Mp* detection during the winter/spring months, which appeared to correlate with a worldwide epidemic of *Mp*.^{5,24,27} At the beginning of

this study (Spring, 2012), we noted a higher rate of *Mp* positivity, which may have coincided with the end of that prior worldwide epidemic. Subsequently, the overall rates of *Mp* positivity declined. Thus, this study may have been conducted during a predominantly endemic phase of *Mp* infection.

We have shown a high rate of *Mp* detection in children with asthma and a high rate of persistence of *Mp* detection. However, we found no relationship between *Mp* detection (or level of CARDS Tx by AC or PCR) and asthma control, quality of life, or health care utilization, oral corticosteroid use and EBC pH. These findings are in contrast to our previous findings of an association between *Mp* detection and worse quality of life and asthma control and lower EBC pH in children with chronic refractory asthma and those with acute exacerbations of asthma.⁵ The potential impact of endemic *Mp* versus epidemic outbreaks of *Mp* on different subgroups of asthma patients remains unclear.

The salient findings of the current study are that low levels of detection of *Mp* do not correlate with significant morbidity in children with persistent asthma. We speculate that low levels of *Mp* may indicate chronic colonization or latent infection, as described by others^{29,30} or the presence of sufficient immunity to *Mp* that protects patients from significant morbidity due to *Mp*. Interestingly, mathematical modeling of *Mp* epidemic cycles suggests that duration of immunity influences the periodicity of *Mp* outbreaks and thus the timing of our study may have influenced our outcomes.³¹

This study had several limitations. While we collected substantial longitudinal data from 28 children, our overall number of subjects was low, which may have precluded the opportunity to reveal subtle changes in certain subpopulations of children with asthma. Although subjects had persistent asthma, most were receiving inhaled corticosteroids and subsequently had relatively mild symptoms and relatively good quality of life at enrollment. We cannot, therefore, offer conclusions about the impact of *Mp* on children with poorly controlled asthma. Overall, however, our data is reassuring that despite the high prevalence of *Mp* detection, morbidity related to *Mp* was low. This may serve to establish that low levels of *Mp* colonization or latent infection may not substantially impact overall asthma control. Thus, wide use of anti-*Mp* antibiotics in persistent asthma does not appear to be warranted. Although our study was not powered to identify an effect of antibiotics, this finding is consistent with a prior study that showed no substantial impact of clarithromycin in subjects with asthma and *Mp* infection, where lower airway sampling of *Mp* was employed.³²

In summary, children with persistent asthma had high rates of detection of *Mp*, as measured by the presence of CARDS Tx. Detection of *Mp* was not associated significantly with asthma symptoms, quality of life or health care utilization in this pediatric asthma cohort.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AC	antigen capture
BMI	Body Mass Index
CARDS Tx	Community Acquired Respiratory Distress Syndrome toxin
EBC	exhaled breath condensate
Mp	<i>Mycoplasma pneumoniae</i>
N	nasopharyngeal
PACQLQ; QOL	Pediatric Asthma Caregiver's Quality of Life Questionnaire
P1	P1 adhesin
PCR	polymerase chain reaction
T	throat

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Figure 1. Variability in Symptom Scores (LASS) [1A] and QOL [1B] by individual subjects. Boxes: 25th – 75th percentiles; whiskers: 95th percentile (or max/min values); circles: outliers beyond 95th %; asterisks: “extreme” outliers (> 3x the 25th–75th percentiles); middle line: median.

Figure 2.

Symptom Scores (LASS) over time by severity group.

$P=0.03$ for effect of time. $P=0.12$ for differences between severity groups. Analysis based on a multi-level repeated measures model.

Figure 3.

Mycoplasma pneumoniae positive status.

A. Prevalence of *Mycoplasma pneumoniae* (*Mp*) positive samples over time. Results shown as observed percentages with overlaid penalized B-spline curve. B. Percentage of subject who tested positive for *Mp* at each month during the study. Denominator=total visits in each month, regardless of year.

Figure 4.

Levels of CARDS toxin by PCR and AC. Levels of CARDS Tx detected in individual samples collected during the study demonstrate relatively low PCR (genomes) or AC (pg/ml) except for two samples. Individual data points represent the higher value for each assay collected from either nasal swab or throat swab.

Table 1**Study Population Baseline Characteristic**

Age (years) *	6.6 (2), (range:3.2–10.8)
Male Gender, No. (%)	22 (71)
Race, No. (%)	
White	25 (80.6)
African American	5 (16.1)
Multiple	1 (3.2)
Hispanic ethnicity, No. (%)	26 (83.9)
Parent education level, No. (%)	
Less than high school	6 (19.4)
High school completion	10 (32.3)
Some College or above	15 (48.4)
Weight (kg)	24 [IQR, 21–37] (range 15–61)
BMI z score	0.9 (1.44) (range –3.5 – 4.8)
Controller medication, No. (%)	
Inhaled corticosteroid alone	14 (46.7)
Low dose ICS	4 (13.3)
Medium dose ICS	4 (13.3)
High dose ICS	6 (20)
Inhaled corticosteroid + LABA	13 (43.3)
Low dose + LABA	1 (3.3)
Medium dose + LABA	7 (23.3)
High dose + LABA	5 (16.7)
Montelukast only	3 (10)
Controller medication score	6.5 [IQR, 2.5–8.5] (range 0–8.5)
Co-morbidity, No.(%)	
Atopy diagnosis	18 (58.1)
OSA diagnosis	6 (19.4)
GERD diagnosis	6 (19.4)
ETS exposure, No. (%)	
None	11 (35.5)
Occasional, outside home	7 (22.6)
Regular, outside home	5 (16.1)
Exposure inside home	8 (25.8)
Mycoplasma positive, No. (%)	20 (64.5)
Symptom scores (LASS)	2.2 (0.8) (range 1–3.6)
Quality of Life (PACQLQ)	5.6 [IQR,3.7–6.5] (range 2.2–7)
Severity score	12.8 (7.5) (range 1.3–27.6)

Abbreviations: BMI, body mass index (z score calculated using CDC SAS code); ETS, environmental tobacco smoke; GERD, gastroesophageal reflux disease; ICS, inhaled corticosteroid; LABA, long-acting beta agonist; LASS, Lara Asthma Symptom Scale; OSA, obstructive sleep apnea; PACQLQ, Pediatric Asthma Caregiver's Quality of Life Questionnaire

* Data are presented as mean (SD), median [25th–75th percentile interquartile range (IQR)] or number (%).

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Table 2Associations between outcome measures^a

		QOL	Med controller	Asthma Severity Index	HCU	OCS	School absences	EBC pH
Symptom score	Estimate	-0.36	0.07	0.05	0.08	0.05	0.06	0.13
	SE	0.04	0.02	0.01	0.03	0.04	0.02	0.13
	<i>P</i>	<0.001	0.005	<0.001	0.004	0.20	<0.001	0.33
QOL	Estimate		-0.04	-0.06	-0.15	-0.16	-0.08	-0.17
	SE		0.04	0.03	0.04	0.06	0.02	0.21
	<i>P</i>		0.32	0.03	<0.001	0.01	0.002	0.42
Med Controller	Estimate			0.22	0.14	0.15	0.07	0.06
	SE			0.04	0.09	0.13	0.05	0.39
	<i>P</i>			<0.001	0.11	0.22	0.18	0.88
Asthma Severity Index	Estimate				0.08	0.08	0.10	-0.002
	SE				0.02	0.04	0.03	0.01
	<i>P</i>				0.002	0.04	0.001	0.83
HCU	Estimate					0.31	0.14	-0.33
	SE					0.06	0.03	0.35
	<i>P</i>					<0.001	<0.001	0.33
OCS	Estimate						0.09	-0.51
	SE						0.04	0.42
	<i>P</i>						0.01	0.22
School absences	Estimate							-0.17
	SE							0.33
	<i>P</i>							0.61

^a *P* value based on multi-level repeated measures model. EBC pH, exhaled breath condensate pH; HCU, percentage of days with health care utilization; Med controller, medication controller score; OCS, percentage of days with oral corticosteroid usage; QOL, quality of life; School absence, percentage of days with school absences; SE, standard error.

Table 3Associations of outcome variables with *Mp* Status^a

Variable	<i>Mp</i> Status			P value
	Negative	Positive	Total	
Symptom Score				0.73
N	87	88	175	
Median [Q1,Q3]	1.6 [1.3,2.3]	1.8 [1.4,2.4]	1.8 [1.3,2.4]	
Min, Max	1, 4.3	1, 4.3	1, 4.3	
QOL				0.64
N	87	88	175	
Median [Q1,Q3]	6.5 [5.4,6.9]	6.1 [4.6,7]	6.3 [4.9,6.9]	
Min, Max	2.2, 7	2.2, 7	2.2, 7	
Med Controller Score				0.04
N	87	86	173	
Median [Q1,Q3]	6.5 [2.5,8.5]	6.3 [2.5,8.5]	6.5 [2.5,8.5]	
Min, Max	0, 8.5	0, 8.5	0, 8.5	
HCU				0.68
N	87	86	173	
Median [Q1,Q3]	0 [0.1,1]	0 [0.1,1]	0 [0.1,1]	
Min, Max	0, 19	0, 4.7	0, 19	
OCS				0.93
N	84	84	168	
Median [Q1,Q3]	0 [0,0.927]	0 [0,0.822]	0 [0,0.864]	
Min, Max	0, 10	0, 10.204	0, 10.204	
School absences				0.31
N	86	85	171	
Median [Q1,Q3]	0.92 [0,3.06]	0 [0,2.74]	0 [0,3.01]	
Min, Max	0, 23.81	0, 10	0, 23.81	
EBC pH				0.69
N	78	81	159	

Mp Status				
Variable	Negative	Positive	Total	P value
Median [Q1,Q3]	8.3 [8.11,8.5]	8.29 [8.11,8.46]	8.3 [8.11,8.48]	
Min, Max	7, 8.77	7.08, 8.76	7, 8.77	

^a P-value based on multi-level repeated measures model. Q1,Q3, 25–75 percentile interquartile range; EBC pH, exhaled breath condensate pH; HCU, percentage of days with health care utilization; Med controller, medication controller score; OCS, percentage of days with oral corticosteroid usage; QOL, quality of life; School absence, percentage of days with school absences.