

Infectious *Chlamydia pneumoniae* is Associated With Elevated Interleukin-8 and Airway Neutrophilia in Children With Refractory Asthma

Katir K. Patel, MS,* Alfin G. Vicencio, MD,† Zhongfang Du, MD,† Kalliope Tsirilakis, MD,† Paul S. Salva, MD, PhD,‡ and Wilmore C. Webley, PhD*

Background: Neutrophilic asthma is thought to be less responsive than eosinophilic asthma to anti-inflammatory therapies including corticosteroids. *Chlamydia pneumoniae* has been implicated in asthma, possibly by induction of interleukin (IL)-8. We hypothesized that IL-8 is increased in the bronchoalveolar lavage (BAL) fluid from children with asthma and *C. pneumoniae*.

Methods: BAL fluid was analyzed for *C. pneumoniae* and IL-8 using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay from 2 asthma patient populations in the Bronx, NY and Massachusetts with an average age of 8 and 8.7 years old, respectively. For comparison, samples were also analyzed for *C. trachomatis* and *Mycoplasma* 16s DNA.

Results: Of 18 Bronx samples analyzed, 6 (33%) were PCR-positive for *C. pneumoniae*, 10 (56%) for *C. trachomatis*, and 8 (44%) for *Mycoplasma* 16s DNA. IL-8 from *C. pneumoniae*-positive samples was 3.3-fold higher compared with negative samples ($P = 0.003$). There was no difference between patients tested for *C. trachomatis* or *Mycoplasma*. Of 84 Massachusetts samples analyzed, 42 (50%) were PCR-positive for *C. pneumoniae*, 42 (50%) for *C. trachomatis*, and 13 (16%) for *Mycoplasma*. IL-8 concentration from *C. pneumoniae*-positive samples was 10.49-fold higher compared with negative samples ($P = 0.0001$). As in the Bronx cohort, there were no differences between patients tested for *C. trachomatis* or *Mycoplasma*. Lastly, BAL neutrophilia predicted the presence of *C. pneumoniae* but not *Mycoplasma* or *C. trachomatis*.

Conclusions: Children with asthma who were PCR-positive for *C. pneumoniae* demonstrated elevated concentrations of IL-8 and neutrophils in BAL fluid compared with similar patients who were positive for *C. trachomatis* or *Mycoplasma* organisms, but PCR-negative for *C. pneumoniae*. Undiagnosed *C. pneumoniae* infection in children may therefore contribute to poorly controlled asthma via induction of IL-8.

Key Words: *Chlamydia pneumoniae*, IL-8, refractory asthma, children

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There is increasing evidence that inflammatory phenotype in asthma partly dictates both severity of disease and response to therapy.^{1,2} For example, patients with neutrophilic asthma, as

measured by induced sputum or bronchoalveolar lavage (BAL) fluid, tend to be less responsive to traditional asthma medications including inhaled and oral corticosteroids.^{3–5} Although the precise mechanism for these observations is currently unclear, some postulate that undiagnosed chronic bacterial infection may contribute to this phenomenon by induction of specific proinflammatory cytokines including interleukin (IL)-8, a potent neutrophil chemoattractant and activating factor.⁶ Interleukin-8 is known to be released by monocytes,⁷ macrophages,⁸ fibroblasts,⁸ and airway epithelial cells.⁹ Increased levels of IL-8 have been described in chronic neutrophilic airway inflammation in patients with refractory asthma, cystic fibrosis, and other chronic airway diseases.^{4,10–12} Although acute exposures to airway irritants, including particulate matter, viruses, and endotoxin have been shown to induce production of IL-8, it is unclear whether chronic, undiagnosed infections can lead to a sustained increase in this inflammatory cytokine, and thus contribute to ongoing disease.¹³

Chlamydia pneumoniae, an obligate intracellular bacterium, has also been implicated in poorly controlled asthma.^{14,15} In addition, earlier investigations have demonstrated that *C. pneumoniae* can enhance IL-8 secretion,¹⁶ and that this secretion may be initiated by adherence of the organism to human airway epithelial cells.¹⁷ Confirmation of infection can be difficult, and thus true prevalence of infection is unknown. As such, the specific role for *C. pneumoniae* in chronic respiratory diseases including asthma is also unclear.¹⁸

In previous investigations, we used modified culture techniques and molecular methods to demonstrate a high prevalence of *C. pneumoniae* in BAL fluid from children with asthma.^{14,15} It is unclear from these earlier studies whether the presence of *C. pneumoniae* alters the cytokine profile in the airway of such patients. Thus, we sought to determine whether *C. pneumoniae* was associated with IL-8 concentrations in BAL fluid from children with poorly controlled asthma. Portions of this manuscript were presented as a poster presentation at the 2009 American Thoracic Society International Meeting.¹⁹

METHODS

Patient Population

All patients came from a referral-based pulmonary practice because of poorly controlled asthma at the Pediatric Respiratory Medicine Department, Children's Hospital at Montefiore Albert Einstein College of Medicine, Bronx, NY as well as Pediatric Pulmonology of Western New England and Baystate Medical Center, Springfield, MA. Children were referred for bronchoscopy after thorough evaluation did not yield a definitive diagnosis or after medical management did not produce an adequate improvement in their clinical condition. The asthmatic children—as defined by the NHLBI guidelines²⁰—were taking high doses of corticosteroid, long-acting bronchodilators, and a leukotriene inhibitor.²¹ Patients ranged in age from 0 to 16 years old with an

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From the *Department of Microbiology, University of Massachusetts Amherst Massachusetts, Amherst, MA; †Section of Pediatric Respiratory Medicine, Children's Hospital at Montefiore, Albert Einstein College of Medicine, Bronx, NY; and ‡Departments of Pediatric Pulmonology, Baystate Medical Center, Springfield, MA.

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Address for correspondence: Wilmore C. Webley, PhD, Department of Microbiology, University of Massachusetts, 639 North Pleasant St, 203 Morrill IV N, Amherst MA 01003. E-mail: wilmore@microbio.umass.edu.

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average age of 8.7 years old for the Bronx patients and 8.0 years old for the Massachusetts patient cohort.

Sample Collection

We obtained banked BAL samples that were collected from patients undergoing flexible bronchoscopy with lavage under a separate Institutional Review Board-approved protocol. We specifically sought to identify samples collected from patients with poorly controlled asthma and no other identifiable pathology contributing to their persistent symptoms. BAL was performed according to the normal protocol for our institution and as previously reported.^{14,15} Briefly, the right middle lobe or lingula was lavaged with a total of 3 mL/kg of normal saline divided into 3 equal aliquots (maximum aliquot of 20 mL). If a patient required lavage in a separate lobe for clinical purposes (ie, recurrent pneumonia in a different lobe), the sample obtained at that time was used. Additional lavage was not performed for investigational purposes. Up to 5 mL of recovered fluid was immediately frozen and stored in -80°C until analysis.

Polymerase Chain Reaction

Genomic DNA was isolated from BAL using the QIAMP DNA Blood mini kit (Qiagen). Polymerase chain reaction (PCR) was used to detect DNA of both *Chlamydia* and *Mycoplasma*. We used the following primer sets to determine the presence and prevalence of *C. pneumoniae* and *C. trachomatis*, respectively: Cpn201 and 202 primers (product 207 bp) and *Ct* using primers *Ct* sense and *Ct* antisense (504 bp) as previously reported.¹⁴ The PCR products were then separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. *Mycoplasma* 16S was performed using commercial kits (Maxim Biotech, Inc. *Mycoplasma* 16S rRNA kit-SP-10521 and PCR Primer Amplification Kit), according to the manufacturers guidelines.

Performing IL-8 Enzyme-Linked Immunosorbent Assay

Immediately after bronchoscopy, BAL samples were processed and stored at -80°C until analysis. Concentration of IL-8 was determined in BAL fluid using BD OptEIA Human-IL-8 enzyme-linked immunosorbent assay Kit II (BD Biosciences, San Jose, CA), according to the manufacturer's instructions.

Cell Counts

BAL cell counts and differentials were performed according to standard techniques in the hospital's Hematology Clinical Laboratory at Baystate Medical Center (Springfield, MA) and Children's Hospital at Montefiore, Albert Einstein College of Medicine (Bronx, NY) as previously reported.¹⁴ Briefly, cell enumeration was performed manually using a cell-counting chamber under phase microscopy with results expressed as "number of cells per cubic millimeter." BAL differential counts were performed using Wright-stained cytospin preparations of BAL which were then examined under oil-immersion microscopy (50 $\times$ or 100 $\times$). Results were expressed on the basis of a 100 cell count survey.

Statistical Analysis

All statistical analyses for IL-8 concentration among the different groups of the patient population were performed using Microsoft Excel. Statistical associations were determined with Fisher exact test and χ^2 with Yates correction (SPSS 15.0 software). A *P* value of 0.05 was considered significant and all tests were 2-tailed. Sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPVs) were performed using the Clinical Calculator 1 online program at: <http://faculty.vassar.edu/lowry/VassarStats.html>.

TABLE 1. Bronx, NY Patient Demographics

Bronx	<i>C. pneumoniae</i> Positive (%) (n = 6)	<i>C. trachomatis</i> Positive (%) (n = 10)	<i>Mycoplasma</i> Positive (%) (n = 8)
Age (yr)	9.0	10.0	10.3
Gender			
Male	4 (66.7)	6 (60.0)	5 (62.5)
Female	2 (33.3)	4 (40.0)	3 (37.5)
Race			
Black	1 (17)	6 (60.0)	2 (25.0)
Hispanic	5 (83)	4 (40.0)	6 (75.0)
Asthma	6 (100.0)	10 (100.0)	8 (100.0)
Medication			
Inhaled steroids	6 (100.0)	6 (60.0)	4 (50.0)
Oral steroids	2 (33.3)	1 (10.0)	1 (12.5)
Other*	4 (66.7)	3 (30.0)	3 (37.5)

*Other medicines at the time of sample collection included leukotriene receptor antagonist and long-acting beta agonists (LABA).

RESULTS

Bronx Patients

Of 31 BAL samples collected and banked between July 2007 and July 2008, 20 were obtained from patients with poorly controlled asthma and no identifiable airway pathology contributing to persistent respiratory symptoms. Of these 20 patients, 2 were excluded from analysis because their asthma was complicated by allergic bronchopulmonary aspergillosis. Thus, a total of 18 samples were analyzed (12 males and 6 females). All patients included in the study were diagnosed with moderate to severe persistent asthma that was poorly controlled, as defined by the current NIH guidelines.²² All were chronically treated with high-dose inhaled corticosteroids, although 2 patients self-discontinued these medications before bronchoscopy. With the exception of 1 child, all patients in this cohort were also receiving or had previously been managed with additional therapies including leukotriene receptor antagonists (LTRA) and/or long-acting beta agonists (LABA). Two patients were receiving omalizumab at the time of sample collection. Age range was between 2 and 16 years old (Table 1).

Massachusetts Patients

We previously analyzed *C. pneumoniae*, *C. trachomatis*, and *Mycoplasma* status in a large cohort of patients undergoing clinically indicated bronchoscopy in Massachusetts.^{14,15} Because of the initial results obtained from our smaller Bronx cohort, we reviewed data from a select subcohort of these samples to identify similar patients who underwent bronchoscopy because of poorly controlled asthma and no alternate diagnosis for persistent symptoms. This cohort consists of patients mainly from western Massachusetts. For comparison we also tested 7 samples from patients with culture-documented bacterial bronchitis and asthma. We identified 84 patients who underwent flexible bronchoscopy with lavage for poorly controlled asthma and no other diagnosis to explain persistent respiratory symptoms. Patients aged between 8 months and 18 years. At the time of bronchoscopy, all were being prescribed high doses of inhaled corticosteroids, and all were either receiving or had not responded to adjunct therapies including LABA, LTRA, omalizumab, or antireflux medications. Of these patients, 74 were asthmatics and IL-8 positivity had a significant correlation with basement membrane thickening based on biopsy taken during bronchoscopy. There were no significant correlations between IL-8 positivity and age, gender, or race (Table 2).

TABLE 2. Characteristics of Study Population in Massachusetts Cohort

Massachusetts	<i>C. pneumoniae</i> Positive (%) (n = 42)	<i>C. trachomatis</i> Positive (%) (n = 42)	<i>Mycoplasma</i> Positive (%) (n = 13)
Age (yr)	8.1	7.2	6.4
Gender			
Male	25 (59.5)	24 (57.1)	6 (46.1)
Female	17 (40.5)	18 (42.9)	7 (53.9)
Race			
Caucasian	25 (59.5)	31 (73.8)	6 (46.1)
Black	6 (14.3)	6 (14.3)	4 (30.8)
Hispanic	11 (26.2)	5 (11.9)	3 (23.1)
BMTH			
Positive	32 (76.2)	25 (59.5)	6 (46.1)
Negative	4 (9.5)	9 (21.4)	3 (23.1)
Not done	6 (14.3)	8 (19.1)	4 (30.8)
Asthma	42 (100.0)	42 (100.0)	13 (100.0)
Medication			
Inhaled steroids	23 (54.8)	17 (40.5)	6 (46.1)
Oral steroids	10 (23.8)	3 (7.1)	2 (15.4)
Other*	19 (45.2)	22 (52.4)	5 (38.5)

*This includes acid reflux and other asthma medications.
BMTH indicates basement membrane thickening.

IL-8 Concentration and *C. pneumoniae* in Bronx Patients

Of the 18 Bronx samples analyzed, 6 (33%) were PCR-positive for *C. pneumoniae*. IL-8 concentrations from *C. pneumoniae*-positive samples were 3.3-fold higher on average compared with *C. pneumoniae*-negative samples (Fig. 1A; 167.0 ± 56 pg/mL vs. 51.0 ± 60 pg/mL, respectively; $P = 0.0003$). In comparison, 10 samples (56%) were PCR-positive for *C. trachomatis* and 8 (44%) were positive for *Mycoplasma*. When IL-8 was analyzed in relation to *C. trachomatis* and *Mycoplasma* positivity, no differences were observed between DNA positive and negative samples (79.5 ± 81 pg/mL vs. 102.4 ± 82 pg/mL, and 101.0 ± 74 pg/mL vs. 114.2 ± 88 pg/mL, respectively; Fig. 1B, C).

IL-8 Concentration and *C. pneumoniae* in Massachusetts Patients

Similar, but more striking results were seen in the Massachusetts patients. Of the 84 samples analyzed, 42 (50%) were PCR-positive for *C. pneumoniae* (note that 21 of 42 samples were positive for both *C. pneumoniae* and *C. trachomatis*). IL-8 concentration from *C. pneumoniae*-positive samples was 10.49-fold higher on average compared with *C. pneumoniae*-negative patient samples (Fig. 2A; 129.0 ± 65 pg/mL vs. 12.3 ± 28 pg/mL, respectively, $P = 0.0001$). There was no difference in gender distribution, age or medications being prescribed at the time of sample collection between *C. pneumoniae* PCR-positive and negative patients (Table 2). In comparison, 42 samples (50%) were positive for *C. trachomatis* and 13 (16%) were positive for *Mycoplasma*. As in the Bronx cohort, there were no significant differences in IL-8 levels between DNA positive and negative samples patients tested for *C. trachomatis* or *Mycoplasma* (76.0 ± 79 pg/mL vs. 73.6 ± 79 pg/mL and 60.7 ± 51 pg/mL vs. 66.5 ± 73 pg/mL, respectively; Fig. 2B, C).

Because PCR positivity may not equate with viability of organisms, we reviewed previous culture data for the Massachusetts patients.^{14,15} Consistent with the PCR data from both the Bronx and Massachusetts cohorts, we found that culture-positive samples demonstrated a 9.85-fold increase in IL-8 values compared with culture-negative samples (Fig. 3; 113.3 ± 69 pg/mL vs. 11.5 ± 30 pg/mL, respectively; $P = 0.0001$). It should be noted

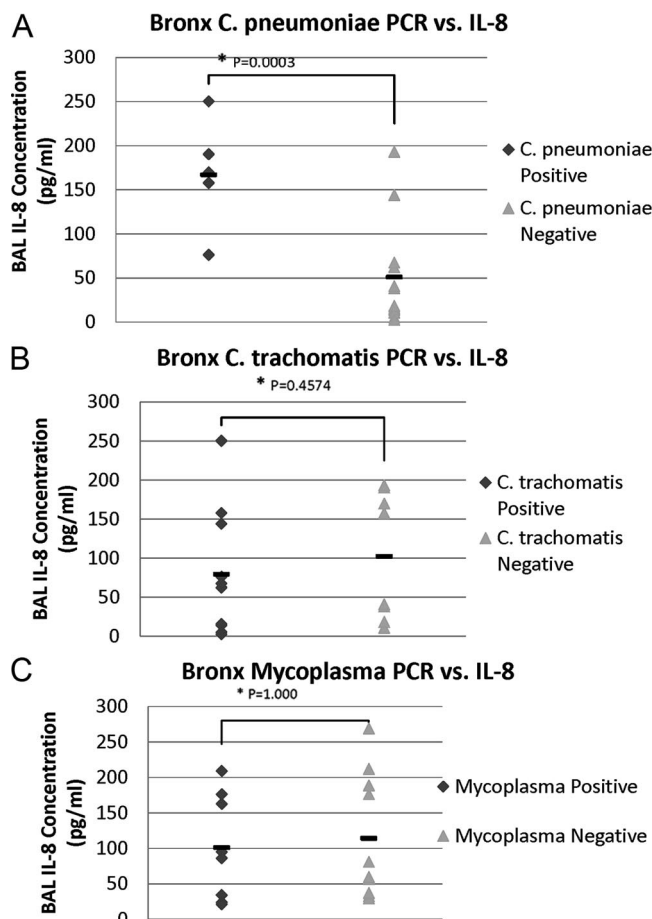


FIGURE 1. Elevated IL-8 in the BAL of *C. pneumoniae* positive Bronx patients. *C. pneumoniae*-positive patient samples had an IL-8 concentration that was 3.3 fold higher on average compared with *C. pneumoniae*-negative samples, $P = 0.0003$ (graph A). *C. trachomatis* and *Mycoplasma*-positive samples did not demonstrate significant differences, compared with negative samples (graphs B and C, respectively). Horizontal lines represent the mean concentration for each group and symbols represent individual patient samples in the positive or negative column.

that because of the limitations of the culture techniques, these cultures do not differentiate between *C. trachomatis* and *C. pneumoniae* organisms.

Lastly, to determine whether there were differences in IL-8 concentrations between *C. pneumoniae* and other common bacterial pathogens, we tested 7 asthmatic patients from our Massachusetts cohort, who were also diagnosed with bacterial bronchitis by culture methods in the clinical laboratory. These patients had one or more of the following microbes in their BAL fluid: *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Neisseria species*, and *Streptococcus pneumoniae*. IL-8 concentrations in the asthmatic patients who did not harbor these organisms was 2.0 fold higher when compared with those of the bacterial bronchitis patient subcohort described above (86.1 ± 74 vs. 42.9 ± 50 , $P = 0.0001$; Fig. 4). When *C. pneumoniae*-positive asthmatic patients were compared with patients displaying bacterial bronchitis, we found a 2.87-fold higher IL-8 level for the *C.*

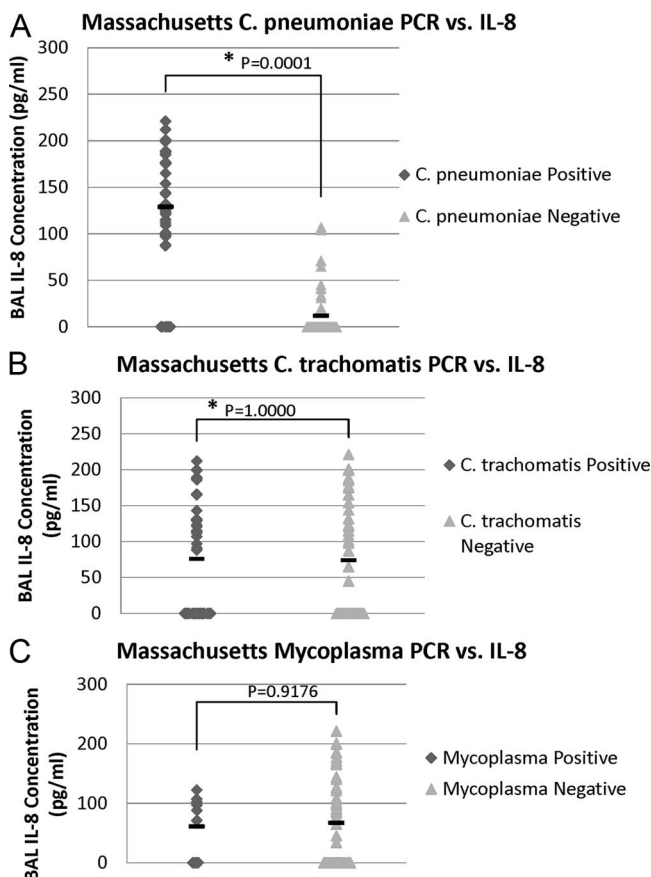


FIGURE 2. Elevated IL-8 in the BAL of *C. pneumoniae* positive Massachusetts patients. *C. pneumoniae*-positive patients had a 10.48 fold higher IL-8 concentration compared with *C. pneumoniae*-negative ones (graph A). There was no significant difference in IL-8 concentrations for *C. trachomatis* and *Mycoplasma* DNA positive patients (graphs B and C, respectively). Means are represented by horizontal lines.

pneumoniae-positive asthmatic patients, suggesting that IL-8 elevation at the levels seen in this cohort might be more closely linked to *C. pneumoniae* presence than general bacterial infection of the airways (129.3 ± 62 vs. 45.0 ± 56 , Fig. 4) $P = 0.0001$.

BAL Neutrophilia Predict *C. pneumoniae* Infection

Because we found an association between *C. pneumoniae* status and IL-8 levels in both patient populations, and since the molecular and modified culture techniques used in our study may not be readily available in the clinical setting, we then sought to determine whether BAL neutrophilia could predict the presence of *C. pneumoniae*. Because of the limited number of samples collected from Bronx patients, we were unable to draw any reliable conclusions in this patient population. Bronchial neutrophilia ($>10\%$ of neutrophils) in the Massachusetts patient cohort, however, was significantly associated with the presence of *C. pneumoniae* DNA (Fisher exact test, 2-tailed $P = 0.0001$). The overall sensitivity, specificity, PPV, and NPV of $>10\%$ BAL neutrophils/mL to predict *C. pneumoniae* infection were 93%, 76%, 80%, and 91%, respectively. Neither *C. trachomatis* nor *Mycoplasma* DNA was a strong predictor of airway neutrophilia with

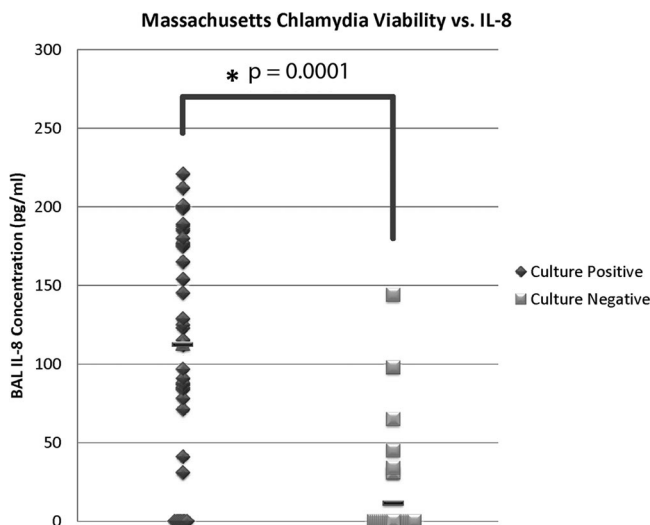


FIGURE 3. Elevated IL-8 is strongly associated with culturable *Chlamydia*. Culture positive patients had average IL-8 levels of 113.3 ± 69 pg/mL versus 11.5 ± 30 pg/mL for culture negative patient samples ($P = 0.0001$, Fisher exact test, T-test).

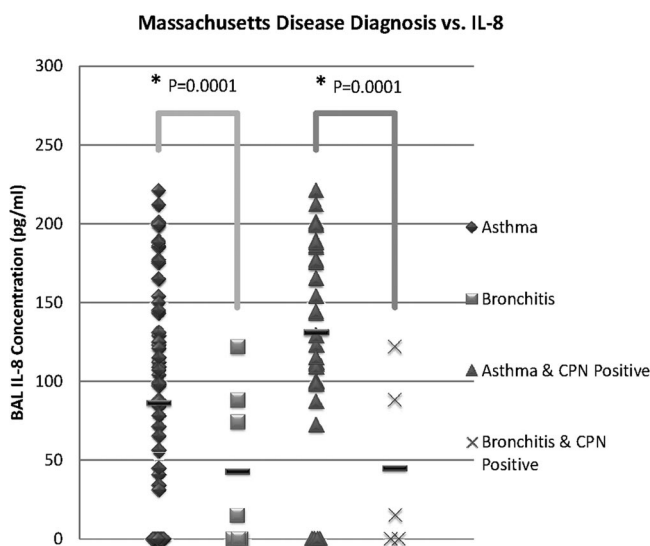


FIGURE 4. Asthmatic patients with *C. pneumoniae* have higher IL-8 levels than bacterial bronchitis patients. The data also show that asthma patients who were *C. pneumoniae* positive had a 2.87 fold higher IL-8 level compared with bronchitis patients.

sensitivity of 55% and 53%, specificity 38% and 40%, PPV 46% and 14%, NPV 45% and 82%, respectively.

DISCUSSION

Patients with poorly controlled asthma have persistent symptoms despite treatment with inhaled corticosteroids and other adjunct therapies including LABA and LTRA.^{22,23} While the majority of patients with asthma demonstrate enhanced allergic inflammatory responses, some patients with poorly controlled

disease exhibit a nonallergic, T_H1 -dominant inflammatory phenotype that may be less responsive to traditional anti-inflammatory therapy.^{3–5} Currently, the mechanisms underlying different inflammatory phenotypes in asthma are unknown. In the present study, we used a convenience sample of patients with poorly controlled asthma who underwent flexible bronchoscopy and found that BAL fluid from patients who were DNA positive for *C. pneumoniae* demonstrated elevated IL-8 concentrations compared with patients who showed negative results for *C. pneumoniae*. None of these patients was suspected of having *C. pneumoniae* in their lower respiratory secretions by clinical measures (ie, active pneumonia). Because this pattern was seen in 2 distinct patient populations but did not hold true for *C. trachomatis* or *Mycoplasma*, it seems less likely that this association simply reflects a generalized response to airway colonization with atypical bacteria, but rather a response to *C. pneumoniae*. As such, it seems plausible that *C. pneumoniae* may represent a subphenotype of poorly controlled asthma that is associated with a nonallergic (ie, IL-8-associated) inflammatory response. We also demonstrated that airway neutrophilia may be an indicator for *C. pneumoniae* infection, particularly when conventional cultures fail to identify other bacterial organisms in the lower airway. Because IL-8 is a known neutrophil chemotactic factor, and since *C. pneumoniae* is known to induce IL-8,^{24,25} the association with neutrophilia is not surprising. However, because the molecular and modified culture techniques used to identify *C. pneumoniae* in our investigation may not be readily available in the clinical setting, BAL fluid cell counts may become a useful screening tool during evaluation of poorly controlled asthma.

There are important limitations to our investigation. Because we obtained samples from a convenient population of poorly controlled asthmatics who underwent flexible bronchoscopy, our results may not be applicable to the majority of patients whose disease is easily controlled with intermittent use of beta agonists or low-dose inhaled corticosteroids. Nevertheless, our findings may be important for the minority of patients whose symptoms remain poorly controlled despite standard treatment. Current recommendations for such patients focus on escalation of existing therapy and identification of comorbid conditions including gastroesophageal reflux, allergic disorders, sinusitis, or obstructive sleep apnea.²³ *C. pneumoniae* may represent an additional comorbid condition that is under diagnosed, and importantly, one for which additional adjunct therapies could potentially be designed. An additional limitation to our study is that we were unable to analyze true control samples (for example, children with no respiratory disease who are undergoing elective surgery), which has been a common limitation in similar pediatric BAL studies.^{26–28} Nevertheless, an important underlying objective of our study was to characterize a potential subphenotype of poorly controlled asthma, namely that associated with *C. pneumoniae*. As such, our control group consists of patients with poorly controlled asthma who do not demonstrate evidence of *C. pneumoniae* in the lower airway. Macrolide therapy is widely employed in the clinical setting for community acquired pneumonia and other respiratory illnesses including sinusitis. Since prolonged courses of macrolide antibiotics are increasingly used in chronic inflammatory diseases such as cystic fibrosis, its efficacy, and safety profiles are well documented.^{29,30} From the current investigation, it is unclear whether patients with poorly controlled asthma who are also harboring *C. pneumoniae* would benefit from the addition of macrolide antibiotics. However, a previous investigation in adults used clarithromycin as an adjunct treatment for refractory asthma, documenting a reduction in IL-8 levels during treatment as well as clinical benefits that were most dramatic for patients with neutrophil-

predominant asthma (as opposed to allergic/eosinophilic disease).³¹ In this study, the mechanism of action was presumed to involve the anti-inflammatory properties of macrolides. However, given our current work, there is a possibility that some of these patients, particularly those with neutrophil-predominant inflammation, were unknowingly infected with *C. pneumoniae*. A similar large-scale investigation in pediatric patients demonstrated no benefit of azithromycin for children with moderate-to-severe persistent asthma.³² Unlike the previous study in adults, however, these investigators did not perform analysis based on inflammatory phenotype, and only upper airway secretions were tested for the presence of *C. pneumoniae*. Further, it has been well documented that *C. pneumoniae* preferentially colonizes the lower airways, has a propensity to cause chronic or latent infections that may not be adequately treated with standard courses of therapy used for community-acquired pneumonia or bacterial sinusitis and requires prolonged courses of therapy.^{33–36} On the basis of our current work and the previous work of others, we propose additional investigations focusing on the evaluation and management of children with poorly controlled asthma which include analysis of inflammatory phenotype and, based on the observed phenotype, further analysis for the presence of *C. pneumoniae* in the lower respiratory tract.

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