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# Airflow limitation, asthma, and *Chlamydia pneumoniae*-specific heat shock protein 60

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**Background:** *Chlamydia pneumoniae* has been associated with asthma. It has also been suggested that *C pneumoniae* infection may lead to lung remodeling in a subset of asthmatic patients. Seroreactivity against *Chlamydia trachomatis* heat shock protein 60 (hsp60), a highly conserved, immunoreactive chaperone protein, is associated with immunopathologic abnormalities, leading to blinding trachoma and tubal infertility. This suggests that the host response to infection may affect chronic inflammatory damage to the eye and the fallopian tubes. The pathogenesis of *C trachomatis* disease associations is thought to include molecular mimicry (autoimmunity), direct activation of the innate immune response via the CD14/toll-like receptor 4 complex, or both.

**Objective:** To study whether airflow limitation in asthma in *C pneumoniae*-exposed individuals is associated with a specific antibody response to the *C pneumoniae* hsp60 molecule and not with a genus-specific response to the hsp60 molecule.

**Methods:** In a case-control study, we evaluated 138 *C pneumoniae*-exposed primary care patients (86 adult asthmatic cases and 52 nonasthmatic controls) for seroreactivity against a *C pneumoniae*-specific hsp60 fragment and against the *C trachomatis* hsp60 molecule. We analyzed associations with asthma and irreversible lung remodeling as measured by means of postbronchodilator forced expiratory volume in 1 second.

**Results:** Twenty-seven percent of asthmatic patients were *C pneumoniae* hsp60 seropositive vs 8% of controls ( $P < .01$ ). Controlling for age, sex, and smoking, *C pneumoniae* hsp60 seropositivity was associated with lower postbronchodilator forced expiratory volume in 1 second in asthmatic patients ( $P < .05$ ). No comparable associations were present for *C trachomatis* hsp60.

**Conclusions:** In individuals with evidence of previous exposure to *C pneumoniae* infection, a host antibody response against a *C pneumoniae* hsp60 fragment but not against *C trachomatis* hsp60 was associated with airflow limitation in adults with asthma.

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## INTRODUCTION

*Chlamydia pneumoniae* is a ubiquitous intracellular human respiratory pathogen that is associated with asthma and chronic obstructive pulmonary disease (COPD).<sup>1,2</sup> *C pneumoniae* infection has been associated with bronchial hyperreactivity,<sup>3,4</sup> new-onset asthma,<sup>5</sup> acute intermittent asthma,<sup>6,7</sup> chronic asthma,<sup>4,7</sup> and asthma severity.<sup>8–10</sup> Chlamydial infections are characterized by persistence and immunopathologic damage to host target tissues, including the lungs. Emerging evidence strongly suggests that a significant subgroup of patients diagnosed as having asthma will progress to chronic bronchitis, emphysema, and COPD.<sup>11</sup> It has been suggested that *C pneumoniae* is a causal factor in this process of asthma airway remodeling leading to COPD.<sup>12,13</sup> Asthma lung remodeling is defined as the development of changes in asth-

matic airways leading to irreversible airway obstruction (and hence, by definition, COPD). Remodeling histopathologic features include airway smooth muscle hypertrophy, myofibroblast proliferation, and excess extracellular matrix deposition or degradation, processes generally regarded as immunopathologic. There are in vitro and in vivo data demonstrating that *C pneumoniae* infection of these relevant human respiratory cells is capable of generating the cytokines and tissue factors implicated in lung remodeling.<sup>12</sup> There are also clinical and epidemiologic data showing that adult-onset asthma is associated with *C pneumoniae* infection<sup>12</sup> and with accelerated loss of lung function.<sup>14</sup> Retrospective<sup>15</sup> and prospective<sup>16</sup> studies have confirmed significant associations between *C pneumoniae* antibodies and an accelerated development of airflow limitation in adults with nonatopic asthma.

Inflammatory tissue damage leading to irreversible scarring has been studied extensively in established chlamydial diseases, including trachoma, pelvic inflammatory disease, and tubal infertility, which are caused by another chlamydial species, *Chlamydia trachomatis*. Scarring in these *C trachomatis* diseases is associated with seroreactivity against a 60-kDa *C trachomatis* heat shock protein (hsp60), suggesting that host responses to hsp60 may trigger autoimmune or inflammatory responses, resulting in immunopathologic abnormalities.<sup>17–19</sup> A study by Huittinen et al<sup>20</sup> showing that airflow limitation in adult asthma is associated with seroreactivity against the whole *C pneumoniae* hsp60 molecule

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raises the possibility that a similar pathologic mechanism exists for *C pneumoniae*-associated asthma. Because hsp60 sequences are highly conserved in various chlamydia species,<sup>21</sup> further investigations are warranted into whether the association between lung remodeling in asthma and the antibody response to *C pneumoniae* hsp60 is species specific or possibly due to nonspecific cross-reactivity with *C trachomatis* hsp60. Improved understanding of the pathophysiologic mechanisms underlying the association may lead to improved management of asthmatic patients to prevent the development of irreversible lung damage. We now report that asthma and airflow limitation are significantly associated with seroreactivity against a *C pneumoniae*-specific hsp60 fragment and are not associated with seroreactivity against the entire *C trachomatis* molecule.

## METHODS

### Patients

Asthmatic cases were sequentially enrolled during the course of usual care from the primary care family practice of 1 of the investigators (D.L.H.). Asthma was diagnosed according to American Thoracic Society criteria (typical symptoms triggered by a variety of stimuli plus objective evidence of reversible airway obstruction). All the asthmatic patients were ambulatory and nonhospitalized. Some presented with asthma exacerbations, and others were encountered with stable, persistent asthma symptoms. Nonasthmatic patients in the same clinical practice, all of whom had normal results of pulmonary function testing, provided control serum samples. Control patients were enrolled as part of practice-based case-control studies for asthma in the practice of the same investigator (D.L.H.). Nonasthmatic controls were either asymptomatic attendees for health maintenance visits or were seen for an acute cough illness without clinical or pulmonary function evidence of asthma. Detailed descriptions of the asthmatic case<sup>20,22</sup> and nonasthmatic control<sup>5</sup> clinical characteristics and *C pneumoniae* microimmunofluorescence (MIF) serologic findings have been published previously. The Dean Health System institutional human subjects committee approved the protocols, and the patients provided written informed consent.

### Serologic Analysis

We included only study subjects (asthmatic cases and nonasthmatic controls) with serologic evidence of previous infection by *C pneumoniae*, as defined by a serum IgG titer of 1:16 or greater in the MIF assay using purified elementary bodies of *C pneumoniae*.<sup>23</sup> Thus, only subjects with evidence of exposure to *C pneumoniae* were included to eliminate confounding effects of exposure status on interpretation of the results of hsp60 seroreactivity. The MIF assays were performed as described previously.<sup>22</sup> All the subjects provided a serum specimen that was tested by means of enzyme-linked immunosorbent assay (ELISA) for IgG antibodies against (1) the whole-molecule chlamydial hsp60 derived

from *C trachomatis* as a fusion protein with glutathione S-transferase (Ctr-hsp60), as described previously,<sup>19</sup> and (2) a cloned 22-kDA fragment of hsp60 from *C pneumoniae* (CpnFr-hsp60) encompassing amino acids 80 to 277. The fragment was cloned using degenerative primers previously shown to amplify a DNA fragment that can be used as a species-specific probe for identifying different hsp60 genes.<sup>24</sup> The amplified polymerase chain reaction products were digested with NdeI and SapI and were ligated into pCYB1. The recombinant plasmids were transformed into competent *Escherichia coli*, screened, and selected. *E coli* JM 109 containing the pCYB1 plasmids encoding the *C pneumoniae* fragment were expressed, and the fragment was purified using the IMPACT 1 (Intein Mediated Purification with an Affinity Chitin-binding Tag) kit (New England Biolabs, Ipswich, Massachusetts). This particular fragment was chosen because preliminary results indicated its superior ability to distinguish *C pneumoniae* from *C trachomatis* when tested against other cloned fragments. The purified recombinant protein/fragments were adsorbed onto 96-well microtiter plates at a concentration of 10 ng per well. The ELISA testing was performed without knowledge of patient clinical status or pulmonary function test results.

### Statistical Analysis

The ELISA results were analyzed as continuous and as binary variables. Log transformation of ELISA optical density (OD) results was performed before analyses of OD as a continuous variable. Pearson correlation and linear regression were used to compare OD relationships between the 2 tests. Analysis of variance was used to analyze OD as a dependent variable, with asthma status or pulmonary function as the independent variable. Logistic regression was used to analyze binary dependent variables (asthma/nonasthma or ELISA positive/negative). ELISA positive was defined as an OD of 0.2 or greater for Ctr-hsp60 (positive = Ctr+) and CpnFr-hsp60 (positive = Cpn+) tests. To control for clinical characteristics that are known to be associated with asthma, pulmonary function, or chlamydial hsp60 seroreactivity, all the models included age, sex, and (ever-) smoking as additional independent variables.

## RESULTS

Of 86 asthmatic cases with serologic evidence of previous exposure to *C pneumoniae*, 63 gave a history that their first asthma symptoms began in the context of an acute respiratory illness, such as bronchitis or pneumonia ("infectious asthma"). Of 52 nonasthmatic controls, 15 had uncomplicated acute bronchitis, and 37 were enrolled during asymptomatic health maintenance visits. There were no significant differences in hsp60 seroreactivities for asthmatic patients with and without infectious asthma or for controls with either uncomplicated acute bronchitis or health maintenance visits. Patient characteristics and serologic findings are presented in Table 1.

Table 1. Participant Characteristics and Serologic Findings

	Asthmatic cases (n = 86)	Nonasthmatic controls (n = 52)
Age, mean (SD), y	43.5 (15.4)	45.6 (9.4)
Sex, M/F, No.	37/49	35/17
Ever-smoking, yes/no, No.	30/48 <sup>a</sup>	5/47
Asthma began after acute respiratory illness, yes/no, No.	63/23	NA
Acute bronchitis/asymptomatic, healthy, No.	NA	15/37
FEV <sub>1</sub> , mean (SD), % of predicted		
Prebronchodilation	72.1 (20.6)	101.1 (9.1)
Postbronchodilation	87.0 (16.1)	ND
Cpn+, No. (%)	23 (26.7) <sup>b</sup>	4 (7.7)
Ctr+, No. (%)	13 (15.1)	5 (9.6)

Abbreviations: Cpn, *Chlamydia pneumoniae*-specific heat shock protein 60 fragment (CpnFr-hsp60); Ctr, *Chlamydia trachomatis* whole heat shock protein 60 molecule, as antigens in the enzyme-linked immunosorbent assay; FEV<sub>1</sub>, forced expiratory volume in 1 second; NA, not applicable; ND, not determined.

<sup>a</sup> Smoking data are missing for 8 asthmatic cases.

<sup>b</sup>  $P < .01$  for Cpn+ (asthma vs nonasthma).

There were no significant correlations between CpnFr-hsp60 OD and Ctr-hsp60 OD and no significant associations between being immunoreactive for CpnFr-hsp60 and Ctr-hsp60 for the entire study group or for cases or controls analyzed separately. In univariate analyses, CpnFr-hsp60 OD was significantly associated with asthma ( $P = .02$ ) and Ctr-hsp60 OD was not ( $P = .80$ ). Immunoreactivity of CpnFr-hsp60 was also significantly associated with asthma ( $P = .006$ ), whereas Ctr-hsp60 immunoreactivity was not ( $P = .23$ ) (Table 1). These results remained significant after controlling for age, sex, and smoking. In a logistic regression model controlling for age, sex, smoking, and Ctr-hsp60 immunoreactivity, the odds ratio for an association between CpnFr-hsp60 immunoreactivity and asthma was 2.5 (95% confidence interval, 1.3–4.6). Among the multivariate models, smoking was significantly and consistently associated with asthma, and female sex was significantly and consistently associated with asthma and Ctr-hsp60 immunoreactivity.

Table 2 presents airflow limitation results according to CpnFr-hsp60 serologic category for asthmatic cases and nonasthmatic controls. Regarding prebronchodilator forced expiratory volume in 1 second (FEV<sub>1</sub>), in univariate analyses, asthma ( $P < .001$ ) and CpnFr-hsp60 immunoreactivity ( $P = .003$ ) were associated with airflow limitation. In multivariate analyses including case/control status (asthma/nonasthma), age, sex, and smoking, asthma remained significant ( $P < .001$ ), but CpnFr-hsp60 did not achieve significance ( $P = .09$ ). Age ( $P = .01$ ) and smoking ( $P = .002$ ) were also significantly associated with prebronchodilator FEV<sub>1</sub> in the multivariate model.

Regarding postbronchodilator FEV<sub>1</sub>, in univariate analyses (assuming no change in postbronchodilator FEV<sub>1</sub> for controls), asthma ( $P < .001$ ) and CpnFr-hsp60 immunoreactivity ( $P = .003$ ) were significantly associated with airflow limitation. In multivariate analyses including case/control status (asthma/nonasthma), age, sex, and smoking, asthma ( $P < .001$ ) and CpnFr-hsp60 ( $P = .04$ ) remained significantly associated with airflow limitation. Age and smoking ( $P < .001$  for both) were also significantly associated with postbronchodilator FEV<sub>1</sub> in the multivariate model.

## DISCUSSION

Current classification schemes for adult obstructive airway disease syndromes generally regard asthma and COPD as separate entities. A cardinal characteristic of asthma is reversible airway obstruction, whereas airway obstruction in COPD is defined as irreversible despite treatment. Smoking is the major, but not likely the only, risk factor for COPD. Evidence from the Tucson Epidemiologic Study of Airway Obstructive Disease (TESAOD) indicates that COPD consists of 2 distinct entities, termed *smoking-associated COPD* and *asthma with chronic airway obstruction*.<sup>25</sup> The TESAOD investigators recently reported that asthma is a strong risk factor (in relative and absolute terms) for the subsequent development of chronic bronchitis, emphysema, and COPD.<sup>11</sup> In this TESAOD report, the adjusted relative risk for asthma was 12.5 (95% confidence interval, 6.8–22.8), and 21.6% of asthmatic patients developed COPD. By comparison, the relative risk for smoking was 2.9. Results of long-term,

Table 2. *Chlamydia pneumoniae* hsp60 Serologic Category and Airflow Limitation

	Cpn+	Cpn–	P value <sup>a</sup>
Pre-BD FEV <sub>1</sub> , mean (SD), % of predicted			
Nonasthmatic controls	99.5 (13.6) n = 4	101.3 (8.8) n = 48	NS
Asthmatic cases	66.8 (23.2) n = 23	74.1 (19.4) n = 63	NS
Post-BD FEV <sub>1</sub> , mean (SD), % of predicted <sup>b</sup>			
Nonasthmatic controls	99.5 (13.6) n = 4	101.3 (8.8) n = 48	NS
Asthmatic cases	80.6 (18.9) <sup>a</sup> n = 22	89.5 (14.3) <sup>a</sup> n = 57	.04

Abbreviations: BD, bronchodilation; Cpn+, CpnFr-hsp60 ELISA OD of 0.2 or greater; Cpn–, CpnFr-hsp60 ELISA OD less than 0.2; CpnFr-hsp60, *Chlamydia pneumoniae*-specific hsp60 fragment as antigen in the ELISA test; ELISA, enzyme-linked immunosorbent assay; FEV<sub>1</sub>, forced expiratory volume in 1 second; hsp60, heat shock protein 60; NS, not significant; OD, optical density.

<sup>a</sup> Cpn+ vs Cpn– (controlled for case-control status, age, sex, and smoking).

<sup>b</sup> The post-BD FEV<sub>1</sub> values were obtained only for asthmatic subjects; post-BD FEV<sub>1</sub> values for nonasthmatic controls are assumed to be equal to pre-BD values.



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prospective, population-based epidemiologic studies of randomly selected individuals (such as the TESAOD) are more generalizable than are results derived from clinical observations, usually cross-sectional, of populations referred to academic specialty settings, on which current classification schemes for obstructive airway syndromes seem to be based.

We included only adult asthmatic cases and nonasthmatic controls with serologic evidence of previous *C pneumoniae* infection by MIF to ensure that all the participants had a history of *C pneumoniae* exposure. We further evaluated the species specificity of the association of *C pneumoniae* hsp60 with lung remodeling by examining the immunoreactivity of these patients against a *C pneumoniae*-specific fragment of hsp60 (CpnFr-hsp60) and against the *C trachomatis* hsp60 whole molecule (Ctr-hsp60). The *C pneumoniae* hsp60 and *C trachomatis* hsp60 homologues of the *E coli* GroEL hsp contain 544 amino acids that are 91.4% identical; the *C pneumoniae* 80- to 277-amino acid sequence fragment, which was used as antigen in this study, shares 94.4% identity with its *C trachomatis* counterpart.<sup>26,27</sup> We found that antibodies against CpnFr-hsp60 were significantly associated with asthma and with airflow limitation. We found no comparable associations for Ctr-hsp60, which strongly suggests that the associations are species specific. Furthermore, the CpnFr-hsp60 prevalence in asthma was greater than 25% (Table 1), suggesting a potential pathologic role in many adults with asthma.

Antibodies against CpnFr-hsp60 were associated with prebronchodilator and postbronchodilator airflow limitation, although the association with prebronchodilator FEV<sub>1</sub> was no longer significant after multivariate analysis. Prebronchodilator FEV<sub>1</sub> reflects the combination of asthma clinical severity and irreversible airflow limitation. It has been suggested that postbronchodilator FEV<sub>1</sub> is a better marker for irreversible airflow limitation than is prebronchodilator FEV<sub>1</sub>.<sup>28</sup> An even better measure of irreversible airflow limitation (not performed in this study) is postbronchodilator FEV<sub>1</sub> obtained after 2 weeks of maximal bronchodilator therapy with oral corticosteroids, which alleviates obstruction due to inflammatory cellular infiltrates that do not respond to inhaled bronchodilator alone. Additional limitations of this study include the lack of a systematic evaluation of allergy skin test positivity in this primary care population; also, data on active smoking, secondhand cigarette smoke exposure, and duration of asthma were incomplete. Future studies should include these variables. The prevalence of ever-smoking in this community-based sample of asthmatic patients is consistent with the high prevalence reported previously in a large population-based cohort<sup>11</sup>; the lower smoking prevalence in the nonasthmatic controls may reflect the proportion of more health-conscious patients attending for health maintenance visits. The smoking imbalance was accounted for in the multivariate analyses. With these limitations in mind, the present results are consistent with an association of CpnFr-hsp60 seroreactivity with asthma lung remodeling or possibly with remodeling and asthma symptom severity. This interpretation re-

quires confirmation because we do not know to what extent this airflow limitation may be further reversible with more aggressive management, as discussed earlier in this paragraph.

The mechanisms underlying chlamydial hsp60 and disease associations are unknown but are believed to include chlamydial hsp60-mediated immunopathologic abnormalities via infectious triggering of autoimmunity due to molecular mimicry<sup>29</sup> and direct activation of innate inflammatory immune responses via the CD14/toll-like receptor 4 complex.<sup>30</sup> It is generally acknowledged that recent worldwide increases in asthma must be attributable to a very strong environmental factor(s), which may include the ubiquitous prevalence of *C pneumoniae* infection worldwide.<sup>31</sup> On the other hand, it is also acknowledged that susceptibility to asthma has a strong genetic component. *C pneumoniae* IgA seroreactivity, a putative marker for recurrent or persistent infection, is associated with asthma and other lower respiratory tract illnesses, including acute uncomplicated bronchitis,<sup>22,32</sup> whereas *C pneumoniae* hsp60 seroreactivity seems to be associated only with asthma.<sup>20</sup> Whether CpnFr-hsp60 seroreactivity reflects genetically determined innate immune responses that can promote an immunopathologic host response to *C pneumoniae* infection is unknown but deserves further investigation.

The case for persistent infection may be inferred from antibody responses as a marker of ongoing infection, analogy to *C trachomatis* studies,<sup>33</sup> and pilot treatment trial results.<sup>34</sup> In a monkey model of chronic pelvic inflammatory disease,<sup>33</sup> tubal damage was associated with evidence of *C trachomatis* infection in the fallopian tubes. Recently, a pilot randomized clinical trial<sup>34</sup> reported that 6 weekly doses of the azalide macrolide azithromycin significantly improved asthma symptoms and that the improvement persisted for 3 months after treatment (study end point). In that study, *C pneumoniae* antibodies also predicted clinical symptoms at follow-up. A larger trial, with 1-year follow-up, is currently ongoing (<http://clinicaltrials.gov/show/NCT00266851>). If *C pneumoniae* infection is indeed causal in asthma severity and lung remodeling, then treatment might alleviate asthma symptom severity and prevent the development of airway remodeling and COPD as an added benefit. Much longer trial follow-up than 1 year will be necessary to investigate this possibility.

In summary, epidemiologic evidence suggests that approximately 20% of asthmatic patients will develop irreversible airflow limitation (COPD).<sup>11</sup> The present results confirm and extend those of a previous study<sup>20</sup> associating *C pneumoniae* hsp60, asthma, and airflow limitation by showing that the association is specific for a *C pneumoniae* hsp60 fragment and is not present for *C trachomatis* hsp60. These data suggest that the anti-*C pneumoniae* hsp60 fragment should be studied prospectively as a potential biomarker for COPD. Long-term clinical trials are also justified to investigate the effects of antichlamydial treatment on asthma and on the development of COPD.

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