

# *Chlamydia pneumoniae* Infection and Inflammation in Adults with Asthma

T. Sävykoski<sup>a</sup> T. Harju<sup>b</sup> M. Paldanius<sup>a</sup> H. Kuitunen<sup>b</sup> A. Bloigu<sup>a</sup>  
E. Wahlström<sup>d</sup> P. Ryttilä<sup>e</sup> V. Kinnula<sup>b,f</sup> P. Saikku<sup>a,c</sup> M. Leinonen<sup>a</sup>

<sup>a</sup>Department of Microbiology, National Public Health Institute, and Departments of <sup>b</sup>Internal Medicine and <sup>c</sup>Medical Microbiology, University of Oulu, Oulu; <sup>d</sup>Department of Vaccines, National Public Health Institute, <sup>e</sup>Department of Allergy, Skin and Allergy Hospital, Helsinki University Central Hospital, and <sup>f</sup>Department of Medicine, Pulmonary Division, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

## Key Words

Asthma · *Chlamydia pneumoniae* · Heat shock protein · C-reactive protein

## Abstract

**Background:** *Chlamydia pneumoniae* infection and immune response to the *C. pneumoniae* heat shock protein 60 (CpHsp60) have been suggested to be associated with asthma. **Objectives:** To study whether a slightly elevated C-reactive protein (CRP) level as a marker of low-grade systemic inflammation has a role in this association, we collected serum and sputum samples from 103 asthma patients with disease severity ranging from mild to moderate and from 30 healthy volunteers. **Methods:** IgA and IgG antibodies to *C. pneumoniae* elementary bodies (CpEB) and CpHsp60 were measured by enzyme immunoassay. Serum CRP levels were measured with a rapid two-site ultra-sensitive assay based on time-resolved immunofluorometry. **Results:** The asthma patients, especially those with moderate asthma, had higher serum IgA antibody levels to CpHsp60 than the healthy controls (test for trend,  $p = 0.05$ ), whereas antibody levels to CpEB antigen did not differ between the study groups. CRP levels were higher in both asthma groups compared to the control group and moreover, the patients with moderate

asthma had higher CRP levels than those with mild asthma (test for trend,  $p < 0.01$ ). The subjects with a slightly elevated CRP level, defined as  $\geq 1.8$  mg/l, had higher CpEB IgA ( $p = 0.001$ ), CpEB IgG ( $p = 0.008$ ) and CpHsp60 IgA ( $p = 0.023$ ) antibody levels in serum compared to the subjects with lower CRP levels. **Conclusions:** Slightly elevated CRP levels as a marker of low-grade systemic inflammation may be associated with *C. pneumoniae* infection in asthma patients.

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## Introduction

Although viral respiratory infections have been implicated as triggers of asthma [1], less is known about the role of bacterial infections in the initiation or promotion of asthmatic inflammation. Respiratory illnesses, such as bronchitis and pneumonia, often precede the onset of asthma, suggesting that respiratory tract infections may, in addition to asthma exacerbations, also contribute to the initiation of asthma [2]. *Chlamydia pneumoniae* is an obligate intracellular bacterium causing upper and lower respiratory infections throughout the world [3]. All chlamydial infections frequently remain persistent and several chronic inflammatory diseases have been presumptive-

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Dr. Maija Leinonen  
National Public Health Institute  
PO box 310, FI-90101 Oulu (Finland)  
Tel. +358 8 537 6235, Fax +358 8 537 6222  
E-Mail maija.leinonen@ktl.fi

ly associated with *C. pneumoniae* infection. There is growing evidence to link *C. pneumoniae* infection with asthma: a chronic infection caused by *C. pneumoniae* has been suggested to be linked to adult-onset asthma [4], to influence the severity of asthma [5], and to increase asthma exacerbations [6, 7].

Heat shock proteins are evolutionarily highly conserved proteins that absorb stressful stimuli and are produced by both host and microbial cells in stressful conditions, e.g. during infection [8]. There is lot of evidence on the role of chlamydial 60-kD heat shock protein (cHsp60) in the development of immunopathological damage following *C. trachomatis* infections [9], but the role of Hsp60 in the pathogenesis of *C. pneumoniae* infections has not been clarified yet. However, the presence of antibodies against cHsp60 has been demonstrated in adult patients with asthma who developed symptoms after an acute respiratory illness [10]. Additionally, we have previously shown that IgA antibodies to Hsp60 of *C. pneumoniae* (CpHsp60) are associated with decreased pulmonary function in asthma patients [11].

The typical feature in asthma is airway inflammation and accumulation of inflammatory cells in the mucosa and submucosa of the airways [12]. Chronic inflammation, in turn, is associated with epithelial cell injury and deposition of fibrous material in the subepithelial basement membrane [13, 14]. There are patients who do not fulfill the functional criteria for asthma, though they suffer from asthma-like symptoms, and who may have eosinophilic airway inflammation [15]. It is also known that the risk for airway remodeling increases even with mild asthma during persistent inflammation [16]. *C. pneumoniae* infection may be one factor promoting the development of persistent airflow limitation in asthma patients [17]. Therefore, more information is needed about the risk factors underlying airway inflammation and about the sensitive markers that can be used to detect these changes and hence to prevent the progression of inflammatory airway diseases.

C-reactive protein (CRP) is a typical acute-phase reactant, whose concentration increases within hours of any tissue-damaging, inflammatory event, including infection [18]. Slightly elevated CRP levels, measured by high-sensitivity assays, have been shown to predict cardiovascular events [19], and we have shown earlier that risk is particularly high when slightly elevated CRP levels are present together with markers of chronic *C. pneumoniae* infection and autoimmunity to Hsp60 [20]. We can thus hypothesize that chronic low-grade inflammation measured as slightly elevated CRP is associated with asthma even in

the absence of major asthma symptoms. One potential agent maintaining persistent airway inflammation and moderately elevated levels of CRP in asthma patient may be chronic infection.

As *C. pneumoniae* infection and immune response to CpHsp60, in particular, have been suggested to be associated with asthma, our aim now was to study whether a slightly elevated CRP level as a marker of low-grade systemic inflammation has a role in this association.

## Methods

### Study Subjects

Altogether 103 asthma patients with disease severity ranging from mild to moderate and 30 healthy controls were included. The clinical severity of asthma was classified according to the Global Initiative for Asthma guidelines [21]. The asthma patients were recruited between November 1998 and December 1999 from the outpatient clinic of Oulu University Hospital and the Department of Allergy, Helsinki University Central Hospital, Finland. The moderate asthma group included 50 patients with a functional asthma diagnosis, permanently diminished lung function, hospitalization due to asthma or daily symptoms. The mild asthma group included 33 patients fulfilling the American Thoracic Society asthma criteria and 20 patients with mild intermittent asthma: asthma symptoms, bronchial hyperreactivity, exclusion of other lung diseases, but less than 15% FEV<sub>1</sub> reversibility. The two asthma groups differed from each other in the use of inhaled corticosteroids: the median (interquartile range) was 500 (400–800) µg/day among the patients with mild asthma and 900 (475–1,600) µg/day among the patients with moderate asthma ( $p = 0.006$ ). The 30 healthy controls were volunteers with no lung disease and normal lung function. They were not health care workers and none had a history of respiratory infection for at least 4 weeks. The demographic data of the asthma patients and the healthy controls are presented in table 1.

### Sputum Induction

Sputum was induced by inhalation of 5 ml of 3% NaCl solution from an ultrasonic nebulizer (Omron U1; Omron Healthcare GmbH, Hamburg, Germany). Both the healthy controls and the asthma patients were given salbutamol 0.2 mg as dry powder inhalation 15 min before the sputum induction (Buventol easyhaler 0.1 mg/dose; Orion Pharma, Espoo, Finland). The sputum samples were processed by the method of Pizzichini and colleagues modified according to Efthimiadis et al. [22]. The filtered suspensions were centrifuged, and the cell pellets and supernatants were stored at  $-20^{\circ}\text{C}$  for later assays.

### Antibody Measurements

Serum IgG and IgA antibodies against *C. pneumoniae* elementary bodies (CpEB) were measured with commercial enzyme immunoassay (EIA) kits (Labsystems, Helsinki, Finland). IgG and IgA antibodies against the CpHsp60 protein of *C. pneumoniae* were measured with EIA as described in detail elsewhere [11]. Briefly, microtiter plates were coated with a recombinant CpHsp60 protein produced in *Bacillus subtilis* [23] at a concentration of 5 µg/ml in PBS (pH 7.4) overnight at  $37^{\circ}\text{C}$ . After coating, the plates were incubated

**Table 1.** Characteristics of study subjects

Variable	Healthy controls (n = 30)	Mild asthma (n = 53)	Moderate asthma (n = 50)	p value
Age, years	39 ± 14	43 ± 13	45 ± 12	0.075
Sex (male/female)	10/20	14/39	17/33	0.668
Current smoking	10%	9%	24%	0.080
BMI, kg/m <sup>2</sup>	24 ± 4	27 ± 5	28 ± 6	0.012
FEV <sub>1</sub> , % pred.	98 ± 11	101 ± 11	81 ± 13	<0.001

Values for continuous variables are presented as means ± SD. p value: Analysis of variance for continuous variables (age, BMI, FEV<sub>1</sub>) and the  $\chi^2$  test for categorical variables (sex, smoking) between three groups.

for 2 h at 37°C with duplicate samples diluted 1:50 for serum IgA, 1:200 for serum IgG, and 1:5 for sputum IgA antibodies in PBS containing 10% FBS. Sputum IgA antibodies were measured against plates coated with PBS only. The plates were then incubated for 2 h at 37°C with alkaline phosphatase-conjugated anti-human IgA (Caltag Laboratories, Burlingame, Calif., USA) and anti-human IgG (Sigma, St. Louis, Mo., USA). Following a 30-min incubation at 37°C with a substrate solution containing 1 mg of *p*-nitrophenyl phosphate disodium in 1 ml of carbonate MgCl<sub>2</sub> buffer, absorbance was measured at 405 nm. The results were expressed as EIA units (EIU) by multiplying the optical densities by 100.

#### Measurement of CRP Concentration

Serum CRP levels were measured with a rapid two-site ultra-sensitive assay based on time-resolved immunofluorometry [24] using the prototype reagents from InnoTrac Diagnostics Oy (Turku, Finland). The dynamic range is from 0.1 to 150 mg/l with a within-assay coefficient of variation of <6% over the whole range.

#### Statistical Analysis

Analysis of variance was used to compare age, body mass index (BMI), and FEV<sub>1</sub> between the three study groups. In the case of the categorical variables (sex and smoking), the groups were compared using the  $\chi^2$  test. The variables representing the use of inhaled corticosteroids and the laboratory results were skewed, and nonparametric testing was therefore used. The possible trend across three groups was tested by the nonparametric test for trend across ordered groups. Pairwise comparisons between two groups were done by the Mann-Whitney U test. The upper quartile for CRP in the healthy controls (1.8 mg/l) was used as cut-off value when the subjects were divided into two groups on the basis of CRP concentration. Relationship of higher CRP values with ordered study groups was tested by the  $\chi^2$  test for trend.

#### Ethical Considerations

The study was approved by the Ethics Committee of the University of Oulu and Oulu University Hospital and by the Ethics Committee of Helsinki University Central Hospital. Informed consents were obtained from all participants.

## Results

Serum IgA antibody levels against CpHsp60 were higher in both asthma groups than in the control group (test for trend,  $p = 0.05$ ), but only the patients with moderate asthma differed statistically significantly from the healthy controls ( $p = 0.039$ ; table 2). Also sputum IgA antibody levels to CpHsp60 were higher in the asthma groups compared to the control group, but the differences did not reach statistical significance. In contrast, serum IgG antibody levels against CpHsp60 did not differ between the asthma patients and the healthy controls. The levels of serum IgA or IgG antibodies against CpEB antigen in either of the two asthma groups did not differ from the controls.

Serum CRP levels were considerably higher in the asthma patients suffering from either mild asthma ( $p = 0.009$ ) or moderate asthma ( $p < 0.001$ ) compared to the healthy controls (table 2). Moreover, the patients with moderate asthma had higher CRP levels than the patients with mild asthma (test for trend,  $p < 0.01$ ). A comparison of the CRP levels of the two asthma groups showed borderline significance ( $p = 0.051$ ). The difference in CRP levels was statistically significant between the healthy controls and the patients with moderate asthma both among the subjects with BMI <25 kg/m<sup>2</sup> ( $p = 0.021$ ) and BMI  $\geq 25$  kg/m<sup>2</sup> ( $p = 0.014$ ) as well as among non-smokers ( $p = 0.001$ ) and smokers ( $p = 0.018$ ). A slightly elevated CRP concentration, defined as  $\geq 1.8$  mg/l cut-off level, was found in 27% of the healthy controls, but in 49% of the subjects with mild asthma and in 62% of the subjects with moderate asthma (test for trend,  $p = 0.003$ ).

The subjects were then examined on the basis of CRP concentration. The subjects with a CRP level  $\geq 1.8$  mg/l had higher levels of both CpEB IgA antibodies ( $p = 0.001$ )

**Table 2.** Levels of C-reactive protein (CRP) and IgA and IgG antibodies to *Chlamydia pneumoniae* elementary bodies (CpEB) and *Chlamydia pneumoniae* heat shock protein 60 (CpHsp60) in the healthy controls and the asthma patients

Variable	Healthy controls (n = 30)	Mild asthma (n = 53)	Moderate asthma (n = 50)	p for trend	p value	
					mild asthma vs. controls	moderate asthma vs. controls
CRP, mg/l	0.85 (0.32–1.84)	1.79 (0.63–3.35)	2.53 (1.05–5.95)	<0.01	0.009	<0.001
CpEB serum IgA, EIU	13.5 (6.8–24.0)	14.0 (8.5–28.5)	21.5 (6.0–30.0)	0.45	0.569	0.453
CpEB serum IgG, EIU	65.5 (22.0–139.0)	67.0 (30.5–107.5)	77.5 (42.3–110.0)	0.37	0.722	0.407
CpHsp60 serum IgA, EIU	15.6 (13.6–18.2)	17.6 (14.7–22.8)	16.8 (15.4–26.5)	0.05	0.099	0.039
CpHsp60 serum IgG, EIU	38.9 (26.3–58.2)	36.6 (22.7–55.5)	36.5 (29.0–49.4)	0.96	0.407	0.901
CpHsp60 sputum IgA, EIU	1.9 (0.0–9.8)	7.3 (0.9–23.0)	5.6 (0.0–22.5)	0.56	0.056	0.441

All values are presented as medians (interquartile range). EIU: Enzyme immunoassay unit, i.e. optical density multiplied by 100. p value: Nonparametric test for trend across ordered groups and Mann-Whitney U test between two groups.

**Table 3.** Levels of IgA and IgG antibodies to *Chlamydia pneumoniae* elementary bodies (CpEB) and *Chlamydia pneumoniae* heat shock protein 60 (CpHsp60) among subjects with and without C-reactive protein (CRP) concentration  $\geq 1.8$  mg/l cut-off level

Variable	CRP < 1.8 mg/l (n = 68)	CRP $\geq 1.8$ mg/l (n = 65)	p value
CpEB serum IgA, EIU	11.0 (6.0–21.0)	23.0 (10.0–33.5)	0.001
CpEB serum IgG, EIU	57.0 (23.3–95.5)	79.0 (44.0–146.5)	0.008
CpHsp60 serum IgA, EIU	16.2 (13.9–21.4)	18.0 (15.3–25.3)	0.023
CpHsp60 serum IgG, EIU	40.2 (27.0–56.0)	34.6 (24.5–53.9)	0.427
CpHsp60 sputum IgA, EIU	4.3 (0.0–15.2)	6.6 (0.6–27.4)	0.139

All values are presented as medians (interquartile range). EIU = Enzyme immunoassay unit, i.e. optical density multiplied by 100. p value: Mann-Whitney U test between two groups.

and CpEB IgG antibodies ( $p = 0.008$ ) and also serum IgA antibodies to CpHsp60 ( $p = 0.028$ ) than the subjects with a CRP level < 1.8 mg/l (table 3). The sputum IgA or serum IgG antibodies to CpHsp60 did not differ between these two groups.

## Discussion

Our study showed that slightly elevated serum CRP levels as possible markers of low-grade systemic inflammation are frequently present in patients with stable asthma, despite the therapy with inhaled corticosteroids. Interestingly, it has also been suggested recently that sensitive systemic inflammations markers, including CRP and serum amyloid A, are associated to bronchial asthma in cross-sectional population-based study [25]. The measurement of CRP with sensitized methods might thus

help to identify asthmatics with ongoing inflammation and a risk for airway remodeling.

In agreement with our previous study [11], elevated levels of serum IgA antibodies to the CpHsp60 protein were associated with asthma, especially with moderate asthma (no severe asthma cases were included in the present study) and they were also associated with slightly elevated CRP concentration. These results point to the possibility that CpHsp60 may participate in the immunopathology of asthma just like Hsp60 protein has been shown to be associated to the infertility and trachoma, chronic sequelae of *C. trachomatis* infection [9]. In addition to serum samples, we further analyzed induced sputum samples for the presence of mucosal CpHsp60 IgA antibodies as a marker of local antibody synthesis and found them to be somewhat, though not statistically significantly, higher among the asthma patients than among the healthy controls. Heat shock proteins are highly con-

served in nature, and in our previous study, a correlation was found between serum IgA antibodies to *C. pneumoniae*-specific and human-specific Hsp60 proteins [11]. However, we could not find any association between human Hsp60 antibodies and asthma, neither in our previous study nor in the present study (data not shown) suggesting that the development of autoimmunity to Hsp60 protein does not play any major role in the pathogenesis of asthma.

In the present study, only antibodies to CpHsp60, and not those against CpEB antigen, were associated with asthma. Hsp60 proteins are usually very good immunogens [9], but they are mainly exposed on the surface of *C. pneumoniae* reticulate bodies and not elementary bodies [26], which were used as antigen in the measurement of CpEB antibodies. This may explain the discrepancy seen between CpHsp60 and CpEB antibodies in the present study. However, the both antibodies were significantly associated with slightly elevated CRP concentrations. These findings suggest that low-grade inflammation at least in some of the asthma cases may be associated with chronic *C. pneumoniae* infection. Most of the studies linking *C. pneumoniae* infection to asthma, especially in adults, have been serological, and evidence of a causal relationship or even of the presence of *C. pneumoniae* in bronchial or lung tissue is thus still lacking.

The limitations of the present study were that the asthma patients were more obese than the healthy controls and there were slightly more current smokers in the moderate asthma group than in the other two groups. The mean age was also somewhat higher in the asthma groups than in the control group. These confounding factors may affect the relationships studied.

Higher BMI is associated with higher CRP concentrations, even among young adults aged 17–39 years. These findings suggest a state of low-grade systemic inflammation in overweight and obese persons [27, 28]. Even though the asthma patients of the present study were more obese than the controls, the difference in CRP levels between the healthy controls and the patients with moderate asthma remained statistically significant also among the subjects with normal BMI ( $<25 \text{ kg/m}^2$ ). The association between asthma and CRP remained significant both among smokers and non-smokers, too. Yudkin et al. [28] found that smoking did not affect CRP levels in a healthy population. On the other hand, *C. pneumoniae* infection seems to be more common in smokers than in non-smokers [29, 30], suggesting that smoking may predispose to the development of a chronic *C. pneumoniae* infection, and the immune response of smokers is directed towards

a Th2 response [31]. In our earlier study, we showed that smoking, when associated with markers of chronic *C. pneumoniae* infection, significantly increases the risk for a future cardiac event [32]. None of the subjects in the present study had, however, a clinical diagnosis of coronary heart disease. Furthermore, even though there is an association between age and CRP, and the mean age in our study was somewhat higher in the moderate asthma group than in the control group, the difference in CRP levels between the healthy controls and the patients with moderate asthma remained statistically significant both among people under and over the age of 40 years.

To conclude, especially slightly elevated CRP concentrations but also elevated levels of serum IgA antibodies to CpHsp60 were associated with asthma. Elevated levels of serum IgA and IgG antibodies to CpEB, measured by EIA in the present study, were not associated directly with asthma, but similarly to elevated levels of serum IgA antibodies to CpHsp60, they were associated with slightly elevated CRP concentration. These findings support the theory that, at least in some asthmatics, inflammation might be a consequence of a chronic *C. pneumoniae* infection and immunopathological response to this infectious agent. However, as the found associations were rather weak, further studies with more patients and controls are needed to elucidate the clinical significance of the findings. Moreover, a follow-up study is needed to evaluate the role of CRP as an inflammatory marker in predicting the outcome of asthma both for monitoring the sufficiency of anti-inflammatory medication and as part of poor outcome risk assessment. Elevated CRP levels may also reflect longstanding tissue damage and airway remodeling in chronic airway inflammation. Furthermore, if *C. pneumoniae* infection and subsequent inflammation have a role in the pathogenesis of asthma, intervention trials with antibiotics effective against *C. pneumoniae* are needed to elucidate the causal relationship.

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