High-sensitivity C-reactive protein in the exhaled breath condensate and serum in stable and unstable asthma

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Summary
Background: Asthma is a chronic airway inflammatory disease. Measurement of serum high-sensitivity C-reactive protein (hs-CRP) levels has suggested the involvement of low-grade systemic inflammation in several disorders, such as cardiovascular disease and diabetes mellitus. In recent years, there have been some reports concerning hs-CRP assessment as a useful tool for detecting systemic inflammation in asthma. The study was undertaken to evaluate hs-CRP levels in the exhaled breath condensate (EBC) of asthmatics with different degrees of asthma severity and their relationship to hs-CRP levels in serum, clinical characteristics, and the intensification of airway inflammation.

Methods: The study group was 62 patients with allergic asthma (20 with steroid-naïve mild asthma, 19 with ICS-treated, stable mild-to-moderate asthma, 23 with ICS-treated unstable, severe asthma) and 15 healthy volunteers.

Results: In the three groups of asthmatics hs-CRP concentrations in EBC and serum were significantly higher than in healthy volunteers. hs-CRP levels both in EBC and serum were significantly higher in patients with unstable asthma than in the two groups with stable disease. hs-CRP concentrations in EBC strongly correlated with those measured in serum. There was a significant correlation between hs-CRP levels both in EBC and serum and exhaled nitric oxide (FENO) in the three groups of asthmatics or serum ECP in the group of patients with steroid-naïve mild asthma and unstable, severe asthma.
Introduction

Asthma is characterized by airway hyper-responsiveness and chronic inflammation in which various cells, cytokines and mediators play a role. It has been shown that levels of acute phase proteins (such as plasma fibrinogen and serum amyloid A) are positively associated with the prevalence of asthma. It has also been demonstrated that increased levels of amyloid A are present in patients with asymptomatic asthma. Therefore, it is very likely that systemic inflammation may exist in asthma.

C-reactive protein is synthesized mainly by hepatocytes and Browicz–Kupffer cells. Monocytes and lymphocytes are also able to produce CRP. The mechanisms of activation of CRP synthesis are still not fully understood. It has been shown that the main stimulators of this process are IL-1, IL-6 and TNFα cytokines with a recognized important role in the pathophysiology of asthma. IL-6 has growth-regulatory effects on many cells and is involved in T-cell activation, growth, and differentiation. It also activates the gene responsible for CRP synthesis through phosphorylation of the transcription factor.

In man, CRP has many important functions, but some of them are still unknown. The main biological role of CRP is the ability to recognize bacteria and damaged human cells and to mediate their elimination through complement and activation of phagocytic cells. CRP is present in the serum of healthy persons; an intensification in CRP synthesis and secretion can be observed in inflammatory processes and malignancies.

CRP is one of the most characteristic markers of the inflammatory process. The monitoring of CRP levels is a good diagnostic tool and is very useful in the assessment of early inflammation, as well as in treatment monitoring and efficacy in acute-phase diseases. The use of high-sensitivity assays for CRP determination (hs-CRP) has made the assessment of low-grade systemic inflammation possible in several disorders, such as cardiovascular disease and diabetes mellitus.

In recent years, there have been some reports concerning the measurement of serum levels of hs-CRP as a useful tool for detecting systemic inflammation in asthma. There were few studies about using exhaled breath condensate (EBC) for the assessment of hs-CRP levels in asthma patients. EBC, collected by cooling exhaled air, is a non-invasive, easily performed, rapid, and effort-independent method for obtaining samples from the lower respiratory tract. There has been increasing interest in measuring EBC in the assessment of inflammatory biomarkers (both local and systemic) in the pathophysiology and treatment monitoring of asthma.

The aim of the study was to assess hs-CRP levels in the EBC of asthmatics with different degrees of disease severity, and to establish the possible correlation of these measurements with hs-CRP serum levels and with other parameters commonly associated with airway inflammation.

Methods

Study population and design

The study was conducted on three groups: 20 steroid-naive mild allergic asthma patients, 19 patients treated with inhaled corticosteroids (ICS) with mild-to-moderate allergic asthma, and 23 ICS-treated patients with severe, unstable allergic asthma. Asthma was diagnosed according to the criteria recommended by the GINA 2006. None of the patients with asthma had complications of other lung diseases or a history suggesting systemic infection, tumours, or autoimmune diseases. Patients with sinus disease were excluded by means of interview. Subjects with heart disease, diabetes mellitus, cancer status, obesity (body mass index BMI >30 kg/m²) and systemic inflammatory disorders (such as collagen vascular diseases) were excluded from the study.

The steroid-naive asthmatics have not been treated with ICS and were free from acute exacerbations and respiratory tract infections during the 3 months prior to the study. Patients with stable, mild-to-moderate asthma had been treated with low- to medium doses of ICS at a constant dose for at least 3 months. Stable asthma was defined as a minimal need for rescue medications (short-acting β2-agonists), no exacerbations, and no use of systemic steroids in the previous 12 months. The patients with severe, unstable asthma had required one or more hospitalizations for asthma and more than three oral steroid bursts in the last year. They had been taking high-doses of ICS and long-acting β2-agonists for at least 6 months. Patients who had respiratory tract infections in the last month before the study were excluded from this study. All the patients were atopic and sensitized to common inhaled allergens, as evaluated by skin prick tests.

Fifteen healthy volunteers were used as a negative control group. They were free of respiratory tract infection within 3 months prior to the study, and from either other significant illnesses known to affect exhaled nitric oxide (FENO) measurements or hs-CRP levels (which have been described previously). Asthma patients and healthy volunteers were non-smokers and during the last year have not been passive smokers.

All of the patients and healthy volunteers were examined by a physician, then underwent EBC collection, FENO measurement, and spirometry. Blood samples were collected to determine serum hs-CRP, ECP, total IgE and blood eosinophil count.

The study protocol was approved by the Ethics of Research Committee of the Medical University of Białystok.

Conclusion: The levels of hs-CRP in EBC are correlated with those measured in serum and may provide another useful diagnostic tool for detecting and monitoring low-grade inflammation in patients with asthma.
number of agreement: R-I-003/80/2006. Informed consent was obtained from every patient entered into the study.

Measurements

Exhaled nitric oxide (FENO) was measured by the chemiluminescence technique using a Sievers 280i NO Analyzer (Boulder, CO, USA). The measurements were performed at an expiratory flow of 50 ml/s according to ATS recommendations for on-line measurement of FENO in adults.\(^1\)

The spirometry (FEV\(_1\)) was performed using a MasterScreen Pneumo PC spirometer (Jaeger, Hoechberg, Germany), according to ATS standards.\(^2\)

EBC was collected by using a commercially available condenser (EcoScreen; Erich Jaeger GmbH, Hoechberg, Germany) according to current ATS/ERS guidelines.\(^3\) All measurements were performed at the same time (between 08:00 and 10:00 h) to avoid possible circadian rhythm of mediator concentrations in EBC. All patients were asked to refrain from eating and drinking before EBC collection. Exhaled air entered and left the chamber through one-way valves and an inlet and outlet, thus keeping the chamber closed. Low temperature inside the condensing chamber throughout the collection time produced a cooling down of the condensate were transferred to Eppendorf tubes and immediately frozen. Samples were stored at −80 °C.\(^4\)

Serum total IgE concentrations and serum ECP were measured using a haematologic analyzer (Coulter Electronics GmbH, Miami, FL, USA). The concentrations of hs-CRP in EBC and serum were determined using a highly sensitive CRP assay (Konelab\(^{TM}\), Waltham, MA, USA). The method is based on the measurement of immunoprecipitation at 540 nm. Microparticles coated with anti-human CRP are added to buffered samples. The increase in absorbance caused by immunoprecipitation is recorded when the reaction has reached its end-point. The change in absorbance is proportional to the amount of antigen (CRP) in the solution. The minimum detectable level of hs-CRP was 0.05 mg/l.

Statistical analysis

Statistical significance was analysed by using analysis of variance (ANOVA) followed by Bonferroni’s t-test post hoc to determine statistical differences. All values were expressed as mean ± SD; p values of <0.05 were considered significant. The relationship between studied parameters was assayed by correlation. Pearson’s linear correlation coefficient was used.

Results

Characteristics of asthmatic patients and healthy volunteers are presented in Table 1.

In the three groups of asthmatics, EBC levels of hs-CRP were significantly higher than those detected in healthy volunteers (steroid-naïve stable asthma: 0.17±0.05 mg/l, p < 0.0001; ICS-treated stable asthma: 0.16±0.06, p < 0.0001; unstable asthma: 0.29±0.04, p < 0.0001; healthy volunteers: 0.08±0.03) (Fig. 1). hs-CRP levels in EBC were significantly elevated in patients with unstable asthma compared with ICS-treated stable asthma patients (p < 0.0001) and steroid-naïve asthma patients (p < 0.0001).

Serum levels of hs-CRP in all groups of asthmatics were significantly higher than those measured in healthy volunteers (steroid-naïve stable asthma: 1.36±0.54 mg/l, p < 0.0001; ICS-treated stable asthma: 1.07±0.28, p < 0.0001; unstable asthma: 2.69±1.04, p < 0.0001; healthy volunteers: 0.30±0.10) (Fig. 2). Serum hs-CRP levels were significantly different from patients with stable, steroid-naïve asthma, p < 0.05.

<table>
<thead>
<tr>
<th>Table 1: Characteristics of study subjects and healthy volunteers.</th>
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<tbody>
<tr>
<td>Characteristics</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Duration of symptoms</td>
</tr>
<tr>
<td>Baseline FEV(_1)</td>
</tr>
<tr>
<td>Serum total IgE concentration</td>
</tr>
<tr>
<td>Blood eosinophil count</td>
</tr>
<tr>
<td>BMI</td>
</tr>
<tr>
<td>FENO</td>
</tr>
<tr>
<td>ECP (serum)</td>
</tr>
</tbody>
</table>

ICS, inhaled corticosteroid; FEV\(_1\), forced expiratory volume in one second; BMI, body mass index; FENO, exhaled nitric oxide; EBC, exhaled breath condensate; ECP, eosinophil cationic protein.

Data are presented as medians (ranges).

\(^{a}\) Values significantly different from patients with stable, steroid-naïve asthma, p < 0.05.

\(^{b}\) Values significantly different from patients with stable, ICS-treated asthma, p < 0.05.

\(^{c}\) Values significantly different from patients with unstable asthma, p < 0.05.
significantly increased in patients with unstable asthma compared with ICS-treated stable asthma patients \((p < 0.0001)\) and steroid-naive asthma patients \((p < 0.001)\).

There were no significant differences in EBC \((p = 0.98)\) and serum \((p = 0.59)\) levels of hs-CRP between stable ICS-treated and steroid-naive asthma patients.

A statistically significant correlation between hs-CRP levels in serum and EBC in all studied groups of asthmatics was revealed. Such a correlation was not observed in healthy volunteers (Fig. 3).

We revealed statistically significant positive correlations between levels of hs-CRP in EBC or serum and \(F_{\text{ENO}}\) in the three studied groups of asthmatics. We discovered a significantly positive correlation between hs-CRP in EBC or serum and serum ECP in the group of steroid-naive asthma patients. A significantly positive correlation between hs-CRP in serum and blood eosinophil count in the group of steroid-naive, stable asthma patients was also revealed. There was no statistically significant correlation between hs-CRP in EBC or serum and other studied parameters in any of the studied groups of asthmatics and healthy volunteers. The results are presented in Tables 2 and 3.

**Discussion**

C-reactive protein is one of the most characteristic markers of the inflammatory process. There are some reports concerning the possibility of using hs-CRP measurements to assess subclinical, systemic inflammation in asthma. However, studies in which no high-sensitivity methods of CRP measurements were used have not revealed any difference in CRP levels between controls and asthmatic patients.\(^3\) On the other hand, a population-based study has shown associations of increased levels of serum hs-CRP with a high frequency of airway hyper-responsiveness and low forced expiratory volume in 1 s \((FEV_1)\), and these results may suggest that systemic inflammation can be associated with respiratory impairment.\(^20\)

Olafsdottir et al. have demonstrated that raised serum levels of hs-CRP are associated with respiratory symptoms in asthmatic patients.\(^21\) The authors also reveal that an increase in serum hs-CRP levels correlated with the degree of airway obstruction. Moreover, they suggest that serum hs-CRP levels may be related to the state of asthma control and chronic airway inflammation and hs-CRP measurements could be useful in the management of asthmatic patients.\(^21\)

Takemura et al. have found that, in patients with steroid-naive asthma, serum levels of hs-CRP were increased compared with healthy volunteers, and correlated negatively with indices of pulmonary function and positively with numbers of sputum eosinophils.\(^12\)

Fujita et al. suggest that serum hs-CRP levels may be a marker of atopic and non-atopic asthma. These authors demonstrate a significant correlation between serum hs-CRP and ECP levels and they suppose that increased hs-CRP levels may be associated with allergic, particularly eosinophilic, inflammation.\(^13\)

Several factors might affect serum levels of hs-CRP. The presence of cardiovascular disease, diabetes mellitus or obesity is associated with increased serum hs-CRP levels, which may be due to adipocyte-derived interleukin-6.\(^10,22\) Smokers also show elevated levels of hs-CRP and smoking cessation leads to their reduction.\(^23\) Ageing is another factor of elevated hs-CRP levels.\(^24\) In our study, patients and healthy volunteers were carefully selected to exclude these confounding factors.

Our results confirmed the presence of low-grade systemic inflammation in asthma as shown previously.\(^2,3,12,13\) We performed the measurement of hs-CRP both in serum and EBC. In previous studies, Rosias et al. were not able to demonstrate the presence of CRP in the EBC of asthmatic children and healthy controls.\(^25\) However, the authors emphasized that high-sensitivity assays for measurement of inflammatory markers in EBC should be used. In the study performed by Ueno et al., in which CRP was measured using ELISA with a high sensitivity, the authors revealed raised CRP levels in the EBC in asthmatics compared with healthy volunteers. However, in this study the correlations between CRP concentrations in EBC and asthma severity or other markers of airway inflammation were not observed.\(^26\)

Our study shows that hs-CRP levels in EBC and serum are higher in asthmatic patients than in healthy controls. In patients with unstable asthma, levels of hs-CRP were significantly higher than in steroid-naive and ICS-treated patients with stable asthma. This observation was confirmed by the previous studies performed by Qian et al., in which the highest serum CRP levels in patients with severe persistent asthma were demonstrated.\(^27\) We
revealed the correlation between hs-CRP levels measured in EBC and serum, as well as between the results of these measurements and other inflammatory markers (such as FENO and ECP).

Reports from the EBC Task Force by the major American and European respiratory societies state that although dilution may be a factor influencing EBC data, it does not appear to improve reproducibility. Because the marker used to correct the difference in the degree of dilution has not yet been established, in our study we have not made any attempt to assess the dilution of airway lining fluid in EBC. One of the main reasons for the difference between EBC and serum level of CRP can be dilution of EBC. However, analysis of study results and significant correlation between serum and EBC levels of CRP suggest that differences in mediator levels observed in various degrees of asthma severity can not be explained by the dilution effect of EBC.

We observed significantly increased levels of hs-CRP in EBC in asthmatic patients compared to healthy volunteers but the difference were not proportional to those observed in serum. In our opinion presence of inflammatory process in asthmatic patients can lead to a change in EBC mediator concentration. Allergic inflammation, increase of vascular permeability but on the other hand thickening and swelling of airway mucus, overproduction of mucus, and forced respiration can also influence EBC composition. In stable, mild- to- moderate asthmatic patients treated with ICS, EBC and serum hs-CRP levels did not significantly differ from those of steroid- naïve mild asthma and did not correlate with serum ECP concentrations. This could suggest the beneficial effect of ICS treatment in down-regulation of CRP. This suggestion is confirmed by a previous study performed by Takemura et al., in which hs-CRP levels in serum were measured. However, more studies are needed to assess the influence of ICS on hs-CRP

**Figure 3** Correlations between hs-CRP levels in serum and exhaled breath condensate in studied groups of asthma patients and healthy volunteers.

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>$F_{\text{END}}$</th>
<th>Serum ECP</th>
<th>Blood eosinophil count</th>
<th>Serum total IgE</th>
<th>Baseline FEV\textsubscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>$r = 0.41$</td>
<td>$r = 0.48$</td>
<td>$r = -0.24$</td>
<td>$r = 0.02$</td>
<td>$r = 0.14$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.12$</td>
<td>$p = 0.07$</td>
<td>$p = 0.38$</td>
<td>$p = 0.93$</td>
<td>$p = 0.60$</td>
</tr>
<tr>
<td>Stable asthma steroid-naïve</td>
<td>$r = 0.52$</td>
<td>$r = 0.69$</td>
<td>$r = 0.50$</td>
<td>$r = -0.36$</td>
<td>$r = -0.22$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.01$</td>
<td>$p = 0.0006$</td>
<td>$p = 0.02$</td>
<td>$p = 0.11$</td>
<td>$p = 0.34$</td>
</tr>
<tr>
<td>Stable asthma ICS-treated</td>
<td>$r = 0.62$</td>
<td>$r = 0.31$</td>
<td>$r = 0.40$</td>
<td>$r = -0.41$</td>
<td>$r = 0.11$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.004$</td>
<td>$p = 0.18$</td>
<td>$p = 0.09$</td>
<td>$p = 0.07$</td>
<td>$p = 0.64$</td>
</tr>
<tr>
<td>Unstable asthma</td>
<td>$r = 0.71$</td>
<td>$r = 0.68$</td>
<td>$r = 0.17$</td>
<td>$r = -0.21$</td>
<td>$r = 0.41$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.0001$</td>
<td>$p = 0.0003$</td>
<td>$p = 0.42$</td>
<td>$p = 0.32$</td>
<td>$p = 0.15$</td>
</tr>
</tbody>
</table>

**Table 2** Correlations between the levels of hs-CRP in serum and other studied parameters in the groups of asthma patients and healthy volunteers.
levels in EBC and serum, as well as the usefulness of hs-CRP measurements in monitoring anti-inflammatory treatment.

In recent studies concerning hs-CRP assessment in asthma, the authors have focused on the possibility of using hs-CRP measurements as a marker of low-grade systemic inflammation in asthma. It is worth noting that CRP could also serve as a mediator of inflammation. CRP could elicit macrophage activation (through interaction with Fc receptors for antibodies)\(^{28}\). CRP inhibits T-lymphocyte production such as monocyte chemoattractant protein-1 (MCP-1), and expression of adhesion molecules in endothelial cells.\(^{33}\)

In recent years, the use of EBC as a non-invasive method for the assessment of samples from the airways has become more and more popular. The analysis of EBC allows for the assessment of mediators for better assessment of the clinical significance of hs-CRP measurements, both in EBC and serum, in diagnostics and in treatment of asthmatic patients.

Conflict of Interest Statement

The authors declare that they have no competing interests in the publication of the manuscript.

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