Increased Serum Levels of HSP27 as a Marker for Incipient Chronic Obstructive Pulmonary Disease in Young Smokers

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Key Words
Chronic obstructive pulmonary disease · Air trapping · Emphysema · Heat shock protein 27 · High-resolution computed tomography scan

Abstract
Background: Although chronic obstructive pulmonary disease (COPD) is amongst the leading causes of morbidity and mortality, no biomarkers for its early detection are known. We have recently demonstrated that COPD is accompanied by elevated serum heat shock protein (HSP) 27 levels as compared to a control population. Objectives: In an open prospective study, we investigated whether elevated HSP27 levels are associated with the early radiological signs of COPD, i.e. air trapping (AT), emphysema (E) and impaired lung function. Methods: In total, 120 apparently healthy smokers underwent lung function testing and serum sampling. Serum levels of HSP27, phospho-HSP27, CXCR2 chemokines and proteins related to inflammation, tissue remodeling and apoptosis were evaluated by ELISA. Of these 120 subjects, 94 voluntarily underwent a high-resolution computed tomography scan. Results: AT or AT and E were detected in 57.45%. Subjects with AT and E (n = 23) showed significantly higher HSP27 levels than those without any pathology [i.e. nothing abnormal detected (NAD)] (4,618 ± 1,677 vs. 3,282 ± 1,607 pg/ml; p = 0.0081). In a univariate logistic regression model including NAD and AT and E, the area under the curve of HSP27 in the receiver-operating-characteristic curve was 0.724, (0.594–0.854, 95% CI; p = 0.0033). Interestingly, proinflammatory IL-8 was elevated in those subjects with evidence of AT and E compared to those with AT and NAD. Lung function did not correlate with increased HSP27 levels or pathological radiological findings. Conclusions: HSP27 serum levels correlated with the early radiological signs of COPD, whereas lung function did not match

H.J.A. and S.N. contributed equally to this work.
with radiological findings or HSP27 serum levels. Serum HSP27 levels may serve as a potential marker to identify the early signs of COPD independent of lung function in young smokers.

Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of disability and death and its prevalence is expected to rise worldwide. Overall, the prevalence in the general population is estimated to be about 8–10% or higher in individuals aged 40 years or older [1].

Two distinct parenchymal changes are associated with COPD: parenchymal destruction leading to the enlargement of alveolar spaces (emphysema) and/or inflammation of the small airways, resulting in airway wall thickening and occlusion of these airways with inflammatory exudates and mucus. These two phenotypes usually co-exist and contribute to total airflow limitation [2, 3]. There is growing recognition that increased resistance in the small airways plays a greater role in airflow limitation in COPD than decreased elastic recoil due to emphysema (E). The narrowing of small airways traps air in the alveoli, resulting in the incomplete emptying of the lung during expiration [4]. Focal end-expiratory air trapping (AT), a hallmark of obstruction or collapse of small airways [5, 6], and E can be assessed semiquantitatively based on the comparison of inspiratory and expiratory high-resolution computed tomography (HR-CT) scans at comparable anatomical levels [7–9].

The recognition that inflammation plays a key role in the pathogenesis of COPD is considered so important that it has led to the inclusion of the term ‘abnormal inflammatory response’ [10, 11]. Cellular inflammatory infiltrates throughout the wall are the earliest and most constant pathological finding in the airway of smokers. Along with neutrophils and macrophages, CD4+ and CD8+ T lymphocytes are prominent components in the inflammatory reaction of the airways [12]. T cell-mediated inflammation persists for years after the cessation of smoking, suggesting that the immune response is involved in the pathogenesis of COPD [13]. An immunological reaction developing in some patients exposed to inhalation toxins might be the turning point from early nonspecific inflammation to subclinical and later clinically manifest COPD, indicating that COPD could be an autoimmune disease triggered by chronic antigen exposure [14]. Lambers et al. [15] recently described that the level of a clonally expanded cytotoxic T cell subset with natural killer-cell features is significantly increased in COPD patients compared to controls. Furthermore, Hacker et al. [16, 17] have shown that increased serum levels of heat shock protein (HSP) 27, HSP70, soluble ST2, caspase-cleaved cytokeratin 18 (ccCK-18) and histones correlate with COPD disease severity.

In terms of a definite preclinical phase with no observable lung obstruction by current standards, the initial stage of COPD is now recognized to cause damage to lung tissue, including emphysema, air trapping and airway inflammation [18]. Subjects with this initial stage of COPD, formerly termed ‘GOLD 0’, have pathological changes in lung structure that cannot be confirmed by spirometry [19, 20]. Of importance are reports demonstrating that by the time medical advice is sought because of symptoms, ventilatory reserves are already irreparably lost in many patients [21].

Additional diagnostic tools that would allow the early identification of patients at risk for developing COPD, such as serum biomarkers, are warranted independently of spirometry analysis [22]. We therefore designed a prospective open cohort study in order to investigate whether proteins known to be related to cellular stress, inflammation and angiogenesis are related to HR-CT-verified lung pathology in subjectively healthy smokers with a mean age younger than 45 years.

Methods

Study Subjects

The study protocol, including serum sampling and a voluntary HR-CT scan, was approved by the local Ethics Committee review board (No. 091/2006). All tests were performed in accordance with the Declaration of Helsinki and the guidelines for good scientific practice of the Medical University of Vienna. All study subjects gave informed and written consent.

A total number of 120 subjectively healthy smokers (53 males and 67 females) with a mean age of 43.1 ± 9.7 [mean ± standard deviation (SD)] years and a smoking history of 17.0 (10.5/31.0) [median (1st quartile/3rd quartile or Q1/Q3)] pack years participated initially in this open cohort study. Pulmonary function parameters (FVC as a %, FEV1 as a % and FEV1/FVC ratio) were measured using a portable lung function testing device (PC Spirometry, SDS 104, Schiller AG, Linz, Austria). Due to the ambulatory setting of this study, no body plethysmography evaluation could be performed. All study subjects were asked to complete a questionnaire regarding smoking habits and health-related behavior (modified Austrian Health Interview Survey and Fagerström Test for Nicotine Dependence). The exact smoking habits of each enrolled study subject were investigated by an occupational health practitioner. Blood samples were collected at the time of pulmonary evaluation, serum was obtained and aliquots...
were stored at −80°C until further workup. Exclusion criteria in our middle-aged study population were: known lung diseases (e.g., COPD, lung cancer, asthma and α1-antitrypsin deficiency) as determined by past medical history (all study subjects were routinely examined by physicians at the Work Health Center Erste Bank), relevant cardiopulmonary morbidities, autoimmune diseases and the use of immunomodulatory drugs within the previous 14 days.

Quantification of Soluble Serum Parameters
Commercially available ELISA kits (R&D Systems, Minneapolis, Minn., USA) were used to determine serum contents of HSP27, phospho-HSP27, HSP60 and HSP70, CXCR2 cytokines (IL-8, GRO-α, ENA-78 and RANTES), the metalloproteinases MMP-1, MMP-7 and MMP-9 and other proteins related to inflammation (IL-1β, IL-6, TNF-α, sST2 and sRAGE). We used commercially available ELISA kits from IBL International, Hamburg, Germany for the detection of HMGB1 and acute-phase protein hsCRP.

In short, microtitration plates were precoated with a capture antibody and incubated at room temperature according to the manufacturer. Plates were then washed and blocked with block buffer for 2 h. Following another washing step, samples and standards with defined concentrations of antigen were incubated. Plates were then washed and incubated with enzyme-linked polyclonal antibodies. After 2 h and another washing step, horseradish-peroxidase-conjugate was applied. Wells were washed, tetramethylbenzidine (TMB; Sigma-Aldrich Corp., St. Louis, Mo., USA) substrate solution was used for the detection of enzyme activity, and the reaction was stopped using sulphuric acid (1N). Color development was then monitored using a Wallac Multilabel Counter 1420 (PerkinElmer, Waltham, Mass., USA) substrate solution was used for the detection of enzyme activity hsCRP.

Statistical Methods
Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, Ill., USA) and GraphPad Prism5 (GraphPad Software, La Jolla, Calif., USA). Gaussian-distributed data are given as mean ± SD. Variables with a skewed distribution are present as medians with the 1st and 3rd quartiles (Q1 and Q3). The D’Agostino and Pearson omnibus normality test was carried out to determine Gaussian distribution. One-way ANOVA was used for normally distributed data and 2-sided Student’s t tests was used for pair-wise comparisons; if data were not Gaussian-distributed, Kruskal-Wallis and (for pair-wise comparisons) Mann-Whitney U tests were used. Categorical variables were compared using the χ² test. To assess the predictive capacity of HSP27 serum values, receiver-operating-characteristic (ROC) curves with its area under the curve (AUC) were computed. Bonferroni-Holm correction was used to adjust p values for multiple testing. p values <0.05 were considered statistically significant.

Results
Evaluation of Lung Function Parameters and HSP27 Serum Values
The mean FVC(%) of all the initial 120 study subjects was 90.2 ± 11.5, the mean FEV1(%) was 83.6 ± 11.8 and FEV1/FVC was 0.78 ± 0.08. In lung function testing, 15 out of 120 study subjects (12.5%) presented with signs of obstruction (FEV1/FVC <0.7). Mean HSP27 values were 3,623 ± 1,552 pg/ml (3,343–3,904, 95% CI). Out of 120 study subjects, 94 voluntarily underwent an HR-CT scan. Lung function parameters of this group were as follows: FVC(%) 90.6 ± 11.7, FEV1(%) 83.5 ± 12.9 and FEV1/FVC 0.77 ± 0.08. According to lung function results, 13 of them (13.8%) were diagnosed with signs of obstruction by a specialist. Mean HSP27 values were 3,761 ± 1,582 pg/
There were no significant differences in lung function testing results or HSP27 serum values between subjects who underwent HR-CT and those who did not.

Diagnosis of Air Trapping or Air Trapping with Emphysema

We were able to detect AT (n = 31; 33%) or both AT and E (n = 23; 24.5%) in 54 out of 94 study subjects (57.5%); E without AT could not be detected in any of them. HR-CT scan of 40 (42.5%) subjects revealed no evidence [nothing abnormal detected (NAD)] of airway pathology. FVC(%), FEV1(%) and FEV1/FVC were not statistically different between groups. Study subjects with AT and E presented with significantly more pack years than NAD (p = 0.019) and AT (p = 0.004) (table 1).

HSP27 Levels Detected by HR-CT Scan

Concentrations of HSP27 in serum samples of subjects without any pathology (NAD subjects) were 3,282 ± 1,607 pg/ml (2,768–3,796, 95% CI); those from subjects with AT were slightly, but not significantly, increased at 3,744 ± 1,210 pg/ml (3,300–4,187) compared to those of NAD subjects. In contrast, subjects with AT and E presented with significantly increased HSP27 serum levels at 4,618 ± 1,677 pg/ml (3,893–5,344) compared to NAD subjects (p = 0.0081) and clearly higher amounts of HSP27 than those with AT (p = 0.0604) (fig. 1). HSP27 serum values also correlated with phospho-HSP27 serum levels (r = 0.6163, p < 0.0001; fig. 2).

HSP27 serum values neither correlated with age nor with pack years smoked (age: r = –0.056, p = 0.595; pack years: r = –0.028, p = 0.801).

A Univariate Logistic Regression Model Demonstrated a High HSP27 Sensitivity and Specificity

ROC curve analysis for AT revealed an AUC of 0.607 (0.475–0.738, 95% CI; p = 0.126). In a univariate logistic regression model including NAD and AT and E, HSP27 had an AUC of 0.724 (0.594–0.854, 95% CI; p = 0.003), indicating a high sensitivity and specificity of HSP27 as diagnostic marker in patients with both AT and E (fig. 3).

Serum Levels of IL-8 Detected by HR-CT Scan

With HR-CT, we were able to show elevated serum levels of IL-8 in study subjects with AT and E 20.17 (8.73/27.60) [median (Q1/Q3)] pg/ml compared to the serum contents of NAD subjects [7.85 (4.81/16.72) pg/ml; AT and E vs. NAD: p = 0.0294] and those with AT alone [7.22 (5.39/16.05) pg/ml; AT and E vs. AT: p = 0.0448].

No Other Soluble Parameters Were Altered in Subjects with Air Trapping or Air Trapping with Emphysema

Serum concentrations of phospho-HSP27, MMP-1, MMP-7, MMP-9 and HSP70 were slightly increased in study subjects with AT and E compared to NAD and AT subjects. ENA-78 serum levels differed significantly from one another in 1-way ANOVA, but pair-wise Bonferroni-Holm-corrected comparisons did not show any significant results (table 2).
**Fig. 1.** HSP27 serum levels are significantly elevated in study subjects with both AT and E (AT+E) compared to NAD subjects. A Student t test was used to calculate significances, and p values were adjusted using a Bonferroni-Holm correction.

**Fig. 2.** Correlation of HSP27 serum levels with phospho-HSP27 serum levels (Pearson’s correlation coefficient $r = 0.6163$; $p < 0.0001$).

**Fig. 3.** ROC curve indicating sensitivity and specificity of HSP27 to diagnose AT (a; AUC 0.607; $p = 0.126$) or both AT and E (b; AUC 0.724; $p = 0.003$) in our smoking study population.
than FEV1, but the value of FEV1 as a surrogate marker is of COPD or its exacerbation has been identified otherwise. Recent studies confirm that COPD continues to be severely underdiagnosed [23, 24]. Therefore, a generally accepted and simple diagnostic procedure is needed, identifying patients early in the disease process and counseling them toward the required lifestyle changes and therapy. The international research community has recognized the need for predictive biomarkers for COPD. The American Thoracic Society and the European Respiratory Society have created a task force on ‘Outcomes for COPD pharmacological trials: from lung function to biomarkers’ [25]. Based on the review of the public literature, they have compiled a comprehensive set of reviews on all aspects of the disease. The task force concluded that ‘currently, no well validated biomarker or surrogate marker of COPD or its exacerbation has been identified other than FEV1, but the value of FEV1 as a surrogate marker is limited’. They further concluded that a biomarker should be defined as the measurement of any molecule that reflects the disease process. Based on current knowledge of the increased serum levels of HSP27 and HSP70 in patients with manifest COPD, we hypothesized that serum HSP27 may serve as a prognosticator for COPD development in smokers perceived to be healthy. According to our primary hypothesis, we proved that elevated serum levels of HSP27 in smokers identified HR-CT-verified pathology independently of spirometry analysis. To the best of our knowledge, this is the first report in the literature that aims to correlate serum HSP27 levels with HSP27 are an independent prognosticator of AT and E in smokers perceived to be healthy. ROC curve analysis revealed an AUC of 0.724 (p = 0.003), indicating a high sensitivity and specificity of HSP27 as diagnostic marker for COPD development. Like other HSPs, HSP27 is a chaperone of the HSP group, to which also HSP20 belong. The protein usually expressed when cells are exposed to cellular stresses such as infection, inflammation and cell toxins. HSP27 is two fold. We suggest that increased levels of serum HSP27 are an independent prognosticator of AT and E in a study cohort of young smokers subjectively perceived to be healthy. ROC curve analysis revealed an AUC of 0.724 (p = 0.003), indicating a high sensitivity and specificity of HSP27 as diagnostic marker for lung pathology. Since the only ‘deviation’ of all our study patients was the inhalation of noxious substances, leakage of HSP27 into the circulation must mirror an immunological activation process. Like other HSPs, HSP27 is an acute-phase protein usually expressed when cells are exposed to cellular stresses such as infection, inflammation and cell toxins. HSP27 is a chaperone of the HSP group, to which also ubiquitin, α-crystalline and HSP20 belong. The protein

### Table 2. ELISA results of serum samples obtained from study subjects who underwent an HR-CT examination

<table>
<thead>
<tr>
<th>HR-CT findings</th>
<th>NAD</th>
<th>AT</th>
<th>AT + E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (Q1/Q3)</td>
<td>min-max</td>
<td>median (Q1/Q3)</td>
</tr>
<tr>
<td>hsCRP, μg/ml</td>
<td>1.07 (0.56/2.20)</td>
<td>0.08–22.02</td>
<td>1.62 (0.64/5.09)</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>0.0 (0.0/2.67)</td>
<td>0.08–31.6</td>
<td>0.0 (0.0/0.08)</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>0.0 (0.0/1.08)</td>
<td>0.0–76.26</td>
<td>0.0 (0.0/0.65)</td>
</tr>
<tr>
<td>TNFα, pg/ml</td>
<td>0.0 (0.0/0.0)</td>
<td>0.0–9.37</td>
<td>0.0 (0.0/0.0)</td>
</tr>
<tr>
<td>sST2, pg/ml</td>
<td>67.24 (41.19/137.1)</td>
<td>3.16–810.4</td>
<td>60.24 (32.36/125.7)</td>
</tr>
<tr>
<td>IL-8, pg/ml</td>
<td>7.95 (4.81/16.72)</td>
<td>1.51–106.5</td>
<td>7.22 (5.39/16.05)</td>
</tr>
<tr>
<td>GROα, pg/ml</td>
<td>24.79 (8.74/45.54)</td>
<td>0.0–116.9</td>
<td>27.39 (18.76/44.70)</td>
</tr>
<tr>
<td>M30, U/l</td>
<td>147.1 (108.6/223.8)</td>
<td>59.13–519.0</td>
<td>115.5 (96.55/164.3)</td>
</tr>
<tr>
<td>HSP60, pg/ml</td>
<td>2.21 (1.53/3.52)</td>
<td>0.0–7.79</td>
<td>2.09 (1.36/2.87)</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>0.0 (0.0/1.180)</td>
<td>0.0–20.228</td>
<td>0.0 (0.0/0.0)</td>
</tr>
</tbody>
</table>

One-way ANOVA or, for skewed data, Kruskal-Wallis tests were utilized to calculate significances. max = Maximum; min = minimum.
Elevated Serum HSP27 in Incipient COPD

has a domain structure with several distinct features. The C-terminus is a highly conserved so-called α-crystalline domain, whereas the N-terminus consists of a so-called WD/EPF domain, which is essential for the formation of high-molecular-weight oligomers. This oligomerization is required for the chaperone function. The formation of large aggregates takes place under heat shock. The protein is mainly located in cytosol, but also in the perinuclear region, endoplasmatic reticulum and nucleus, and is the target of a number of kinases. Its degree of phosphorylation is related to its biological activity. Besides known intracellular chaperoning, HSPs may also be released into the extracellular space following massive trauma or stress [26, 27]. This spillage of proteins serves as a ‘danger signal’ leading to cytokine transcription and release [28]. Furthermore, extracellular stress proteins are able to induce the adaptive immune system through the binding of antigenic peptides. These HSP-peptide complexes are then processed by antigen-presenting cells via MHC-class-I molecules and this leads to the activation of cytotoxic T lymphocytes [29], hence inducing an immune response. HSP27 is also involved in the apoptotic signaling pathway. It interacts with the outer mitochondrial membranes and interferes with the activation of the cytochrome c/Apaf-1/dATP complex and therefore inhibits the activation of procaspase-9 [30]. The phosphorylated form of HSP27 inhibits Daxx apoptotic protein and prevents the association of Daxx with Fas and Ask1 [31]. A well-documented function of HSP27 is the interaction with actin and intermediate filaments. It prevents the formation of noncovalent interactions of the intermediate filaments and protects actin filaments from fragmentation. It also preserves the focal contacts fixed at the cell membrane [30]. Another of its functions is the activation of the proteasome. It speeds up the degradation of irreversible denatured proteins and junk proteins by binding to ubiquitinated proteins and to the 26S proteasome. It enhances the activation of the NF-κB pathway, that controls a variety of processes, such as cell growth and inflammatory and stress responses [32]. The cytoprotective properties of HSP27 result from its ability to modulate reactive oxygen species and to raise glutathione levels [33]. By interpreting accepted knowledge pertaining to HSP27, we conclude that toxins inhaled by smokers lead to an immune response that causes HR-CT-detectable pulmonary changes and a spillage of HSP27 into the pulmonary vascular network in COPD-susceptible subjects. Most intriguingly, this radiologically verified lung pathology was not associated with impaired lung function. However, most probably these early and minor morphological changes accompanied by the release of HSP27 will be followed by spirometric impairment and, subsequently, the development of clinically manifest COPD in some of the studied individuals.

In line with the above data, we found a near-to-statistical significance (p = 0.062) for apoptosis-dependent lung degeneration, as evidenced by ccCK-18 in serum samples of patients with manifest AT and E compared to the controls. Also of interest was our finding that the CXCR2 cytokine IL-8 evidenced a 2-fold increase in the AT and E group compared to the controls. These observations are supported by recent literature that advocated IL-8 in the development of COPD [34–36]. A further insight was that proangiogenic factors (ENA-78, GRO-α and RANTES), inflammatory cytokines (IL-1β, IL-6, TNF-α), matrix metalloproteinases (MMP-1, MMP-7, MMP-9) and sST2 failed to demonstrate any correlation with HR-CT-verified lung pathology or lung function in our study cohort. A further investigated proinflammatory pathway, namely the sRAGE/SHMGB1 axis, did not show any significance in predicting lung pathology in the early stage of COPD development [37, 38].

If we interpret our data correctly, a detailed picture emerges. We hypothesize that chronic antigen exposure (e.g. smoking) leads to the secretion of proteins related to the inflammation, apoptosis and remodeling of lung tissue. In this study, including 94 study subjects, we have only identified serum HSP27 to be related to early signs of COPD. A further important finding of this study was that lung function did not correlate with manifest radiological pathologies. Based on the results of our study, we are confident in claiming that serum HSP27 could be a potential marker for incipient COPD. The Departments of Thoracic Surgery (H.J.A., S.N., M.Z. and W.K.), Pneumology (Dr. Petkov and C.L.), and Radiology (Dr. Prosch and M.T.) are currently designing a clinical study to further validate the above-presented data.

Limitations of the Study

We identified >50% pathological HR-CT scans in 94 study subjects with COPD risk behavior. Serum HSP27 correlated with AT and E but not with lung function testing.

We can therefore only claim that increased HSP27 is associated with incipient COPD (AT and E) irrespective of lung function. We are presently monitoring our study group in order to answer the question whether these study subjects with increased serum HSP27 and pathological lung CT scan are in the process of developing manifest COPD.
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References

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