

ORIGINAL ARTICLE

Elevated HSP27, HSP70 and HSP90 α in Chronic Obstructive Pulmonary Disease: Markers for Immune Activation and Tissue Destruction

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death. Although the underlying pathomechanism remains poorly understood, COPD is accompanied by increased cellular stress and inflammation. We investigated serum contents of heat shock proteins (HSP 27, 60, 70, 90 α), 20S proteasomes, C-reactive protein (CRP), and interleukin-6 (IL-6) in patients with mild or severe COPD, healthy smokers and non-smokers. HSP27, HSP70 and HSP90 α were significantly altered in patients suffering from COPD as compared to controls. HSP27 and HSP70 are potential novel serum markers for the diagnosis of COPD in the smoking population. This is the first study to demonstrate elevated serum levels of the described heat shock proteins in patients with COPD. We showed sensitivity and specificity of serum HSP27 and HSP70 as diagnostic markers for COPD. (Clin. Lab. 2009;55:XXX-XXX)

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a significant burden to health care systems worldwide. Despite COPD being one of the leading causes of death and disability (1), well designed epidemiological studies remain vague leading to the impression of COPD as a rare disease (2). Contrary, prevalence has inclined dramatically over the past years (3). Although active tobacco smoking is thought to be the predominant risk factor (4) only a fraction of smokers develop the clinical features of COPD (5). Currently, the diagnostic process requires the assessment of lung function parameters. In several recent studies genetic factors were considered to contribute to the susceptibility to develop COPD (6). Results of these evaluations varied and did not reveal a definite answer. The only accepted genetic cause of emphysema to date is severe α 1-antitrypsin deficiency (AATD).

Like the many possible etiologies triggering the development of COPD, the underlying pathogenetic pathways remain poorly understood. The disease is characterized by irreversible airflow obstruction - the current diagnostic criterion - due to remodelling and an aberrant inflammatory response (7). Chronic bronchitis and lung emphysema are pathologic characteristics of COPD and both conditions result from progressive inflammatory destruction of the lung parenchyma. Airflow limitation is slowly progressive, leading to dyspnoea and limitations of physical exercise capacities (8). However, immune activation is not restricted to the lungs, as patients suffering from COPD are also at higher risk for cardiovascular diseases (9) and autoimmune diseases such as Ulcerative Colitis and Crohn's Disease (10) Interestingly, some authors presented data of persisting inflam-

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matory reactions in COPD patients despite cessation of smoking and without other forms of exogenous triggering of an inflammatory response. These findings are suggesting an autoimmune aspect contributing to the disease progression (11).

Heat shock proteins (HSPs) are a group of highly preserved stress proteins that are ubiquitously expressed in all cells. The main functions of these proteins comprise a process referred to as chaperoning - the conservation of the correct protein structure under stress conditions - as well as the regulation of death pathways (12). The classification of these stress proteins follows their molecular weight, e.g. the HSP70 has an approximate molecular weight of 70 kDa. Under normal physiological conditions HSPs usually account for up to 5% of the total protein content of a cell. Expression of HSPs is up-regulated under stressful events like heat, bacterial or viral infections (13, 14, 15). Newly synthesized HSPs are thought to fold denatured proteins and prevent activation of caspases otherwise leading to active cell death, e.g. apoptosis. Besides known intracellular chaperoning, HSPs may also be released into the extracellular space following massive trauma or stress (16, 17). This spillage of proteins serves as "danger signal" leading to cytokine transcription and release (18). Furthermore, extracellular stress proteins are able to induce the adaptive immune system through binding to antigenic peptides. These HSP-peptide complexes are then processed by antigen presenting cells via MHC class I molecules and lead to activation of cytotoxic T-lymphocytes (19). Extracellular and intravascular HSPs seem to play a key role in the activation of the immune response following stressors like trauma, heat or infection.

The 20S proteasome is a multicatalytic protease complex, localized in the cytosol as well as in the nucleus of all eukaryotic cells. It is crucially involved in the enzymatic degradation of ubiquitinated proteins. The proteasome consists of a cylindrical-shaped core particle, the 20S proteasome, which itself contains two sets of seven different α and β subunits assembled in four heptameric rings. Spillage of intracellular 20S proteasome was recently shown to occur during degradative processes of cells, e.g. sepsis, trauma, acute liver failure, and on-pump coronary artery bypass grafting (20, 21, 22).

The aim of the present study was to evaluate in a well-defined study cohort whether the serum levels of various heat shock proteins and 20S proteasome as markers for immune activation and cellular destruction are elevated in patients with COPD. Furthermore, we tested the ability of serum HSPs and 20S proteasome to serve as diagnostic markers for the detection of COPD. The diagnostic use of serum proteins prior to lung function testing may support the diagnostic process and lead to an earlier treatment of patients with COPD.

MATERIALS AND METHODS

Patients

A total number of 64 patients and controls were included in this case control study. The study protocol was approved by the ethics committee of the Medical University of Vienna (EK Nr.: 091/2006). All clinical and laboratory tests were performed in accordance with the Declaration of Helsinki and the guidelines for Good Clinical Practice of the Medical University of Vienna. All patients and controls provided written, informed consent before entering the study. Healthy non-smoking volunteers (n=15), smokers without COPD (n=14), patients with mild to moderate COPD (n=19) and patients with severe or very severe COPD (n=16) were evaluated in four study groups. Patient characteristics are depicted in Table 1. Exclusion criteria were acute exacerbation as defined by the guidelines of the WHO and the Global Initiative for Chronic Obstructive Lung Disease (GOLD) or use of immunomodulatory drugs - including steroids - within the past 14 days, history of asthma, autoimmune diseases, other relevant lung diseases (e.g., lung cancer, known α 1-antitrypsin deficiency), or any known cardiopulmonary co-morbidity. Height and weight (Seca; Vogel and Halke, Hamburg, Germany) were measured and the body mass index (BMI) was determined. Pulmonary function parameters (FEV1, FVC, FEV1:FVC ratio) were measured using the same model spirometer (AutoboxV6200, SensorMedics, Vienna, Austria). Measurements were made before and - if criteria for airflow obstruction were met - 15-30 minutes after inhaling of 200 μ g salbutamol. Arterial blood gases (PaO₂, PaCO₂) were obtained at rest while breathing room air in a sitting position. Measurement of arterial blood gases was performed with an ABL 510 gas analyzer (Radiometer, Copenhagen, Denmark). Results are expressed as absolute values and as percentages of predicted values for age, sex and height, according to the European Community for Steel and Coal prediction equations. Predicted normal values were derived from the reference values of the Austrian Society of Pulmonary Medicine. Blood samples were collected at the time of pulmonary evaluation. Serum was acquired after centrifugation and aliquots were kept frozen at -20° Celsius until further testing.

Heat Shock Proteins 27, 60, and 70

Levels of HSP27, HSP60, and HSP70 were determined using adapted enzyme-linked immunosorbent assay (ELISA) kits for the quantification of intracellular HSP (Duoset IC; R&D Systems, Minneapolis, Minnesota). Ninety-six-well microtiter plates were coated overnight at 4°C with the capture antibody at a concentration of 1 μ g/mL. After blocking of plates, serum samples and standard protein in different concentrations were added to the wells. After a washing step, a biotin-labelled antibody was added to each well and incubated for 1 hour. Plates were washed and Streptavidin-HRP was added. Color reaction was achieved using tetramethylbenzidine

Table 1: Clinical characteristics (severity of airflow obstruction was determined using lung function test in all subjects; COPD patients meeting the GOLD diagnostic criteria for COPD). Data are given as mean (+/- standard deviation) if not otherwise stated.

Subject Category	Healthy Non-Smoker	Healthy Smoker	COPD GOLD I&II	COPD GOLD III&IV
N	15	14	19	16
Male/Female	10/5	7/7	10/9	10/6
Age	57.20 (12.50)	56.64 (9.17)	60.68 (7.39)	58.31 (8.75)
Body Weight (kg)	71.6 (13.9)	76.4 (8.6)	79.7 (16.7)	81.1 (27.2)
Body Height (cm)	172.7 (10.9)	168.7 (8.1)	167.7 (12.1)	171.2 (7.9)
Lung Function				
FVC (L)	4.55 (0.94)	3.84 (0.66)	3.33 (1.06)	2.14 (0.70)
FEV1 (%)	105.37 (17.11)	94.40 (11.96)	70.21 (13.33)	30.67 (12.66)
FEV1/VC (%)	76.80 (7.85)	75.95 (3.99)	61.74 (8.36)	37.80 (15.33)
MEF 50 (%)	100.67 (28.92)	87.64 (21.45)	39.42 (15.93)	11.93 (6.60)
MEF 25 (%)	103.53 (33.89)	75.71 (31.33)	37.37 (16.19)	20.00 (5.94)
Smoking History				
Never-smoker	15	0	0	0
Ex-smoker	0	3	4	3
Current-smoker	0	11	15	13
Pack Years	0	34 (25.2)	47.3 (29.7)	44.0 (32.6)

Abbreviations used: COPD: Chronic obstructive pulmonary disease, FEV1: Forced expiratory volume in one second, FVC: Forced vital capacity, GOLD: Global Initiative for Chronic Obstructive Lung Disease MEF: Maximum expiratory flow.

(TMB; Sigma, St. Louis, Missouri) and was stopped by an acid stop solution. Optical density was measured at 450 nm on an ELISA reader.

Heat Shock Protein 90alpha

Serum levels of HSP90alpha (HSP90 α) were measured with a commercially available ready-to-use ELISA kit (Stressgen, Ann Arbor, Michigan). In brief, serum samples and standards were incubated in 96-well microtiter plates, precoated with antihuman HSP90 α antibody. After a washing step, anti-HSP90 α :HRP-conjugated antibody was added, and plates were incubated for 24 hours. Plates were washed and TMB substrate was added. Color development was stopped by an acid stop solution, and optical density was determined at 450 nm. The amount of protein in each sample was calculated according to a standard curve of optical density values constructed for known levels of HSP90 α . The sensitivity of the ELISA has been determined to be 50 pg/ mL; the intra-assay variability is stated to be less than 10% by the manufacturer.

20S Proteasome

Microtiter plates were incubated overnight at 4°C with a monoclonal antibody against the C6-subunit of the 20S proteasome (Biomol, Plymouth Meeting, Pennsylvania). Plates were washed and blocked for 1 hour with 1% BSA in phosphate-buffered saline. Serum samples and different concentrations of a standard protein (Biomol) were added, then plates were sealed and incubated for 24 hours at 4°C. A rabbit polyclonal antibody to 20S proteasome α/β subunits (Biomol), serving as the detection antibody, was added; and after a washing step, plates were incubated with a peroxidase-labeled donkey antirabbit IgG (Jackson ImmunoResearch, Soham, United Kingdom) for another 2 hours. Tetramethylbenzidine served as color substrate. The reaction was stopped by adding 1N sulphuric acid. Plates were read at 450 nm using a Wallac Multilabel counter 1420 (PerkinElmer, Boston, Massachusetts).

Interleukin-6

Serum levels of IL-6 were determined by a commercially available ELISA kit (BenderMedSystems, Vienna, Austria). Assays were performed ac-

cording to the manufacturer's instructions. Plates were read at 450 nm on an ELISA reader, and IL-6 contents were calculated comparing optical density values of samples with optical density values of known IL-6 concentrations.

C-reactive Protein

Serum levels of C-reactive protein were routinely analyzed by the Department of Laboratory Medicine at the Medical University of Vienna.

Statistical Methods

SPSS Software (SPSS Inc., Chicago, IL, USA) was used to calculate all results. A p-value <0.05 was considered statistically significant. Pair-wise comparisons between groups were performed using the Mann-Whitney-U-Test. Correlations were calculated using the Spearman-Correlation-Coefficient. Univariate logistic regression models in a subgroup excluding healthy non-smokers were calculated for HSPs and 20S proteasome. Receiver operating characteristic (ROC) curves were plotted to demonstrate sensitivity and specificity of the evaluated serum proteins. Results were not corrected for multiple testing.

RESULTS

HSP27

Serum levels of HSP27 were 2042.57 pg/mL [1599.58 - 2485.57] (mean [95% confidence interval]) in healthy controls, 2199.64 [1641.52 - 2757.75] in healthy smokers, 2862.62 [2280.49 - 3444.74] in COPD GOLD I-II, and 3717.58 [3079.35 - 4355.81] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II ($p=0.025$), healthy controls and COPD III-IV ($p<0.001$), healthy smokers and COPD III-IV ($p=0.001$), and COPD I-II and COPD III-IV ($p=0.026$). Serum levels of HSP27 did not correlate with body weight. Figure 1a.

HSP60

Serum levels of HSP60 were 1836.69 pg/mL [153.30 - 3520.08] in healthy controls, 4378.40 [3851.48 - 12608.28] in healthy smokers, 3497.42 [1561.38 - 8556.23] in COPD GOLD I-II, and 531.81 [132.57-931.05] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of HSP60 showed a weak correlation with body weight ($R=-0.269$; $p=0.034$). Figure 1b.

HSP70

Serum levels of HSP70 were 140.50 pg/mL [67.97 - 213.04] in healthy controls, 108.50 [43.30 - 173.69] in healthy smokers, 454.29 [327.05 - 581.52] in COPD GOLD I-II, and 437.92 [143.41 - 732.42] in COPD GOLD III-IV.

Statistically significant differences were found between healthy controls and COPD I-II ($p<0.001$), healthy

controls and COPD III-IV ($p=0.009$), healthy smokers and COPD I-II ($p<0.001$), and healthy smokers and COPD III-IV ($p<0.001$). Serum levels of HSP70 did not correlate with body weight. Figure 1c.

HSP90alpha

Serum levels of HSP90alpha were 13133.78 pg/mL [9791.40 - 16476.15] in healthy controls, 12827.91 [10838.21 - 14817.62] in healthy smokers, 17884.50 [13307.14 - 22461.85] in COPD GOLD I-II, and 17273.02 [12573.96 - 21972.08] in COPD GOLD III-IV. Statistically significant differences were found between healthy controls and COPD I-II ($p=0.025$), and healthy controls and COPD III-IV ($p=0.049$). Serum levels of HSP90alpha showed a weak correlation with body weight ($R=0.257$; $p=0.044$). Figure 1d.

20S Proteasome

Serum levels of 20S proteasome were 194.78 ng/mL [164.94 - 224.62] in healthy controls, 188.25 [159.26 - 217.25] in healthy smokers, 172.33 [133.78 - 210.88] in COPD GOLD I-II, and 187.50 [145.54 - 229.46] in COPD GOLD III-IV. No statistically significant differences were found between the groups. Serum levels of 20S proteasome did not correlate with body weight. Figure 1e.

Interleukin-6

Serum levels of IL-6 were 5.18 pg/mL [0.17 - 10.19] in healthy controls, 1.65 [0.11 - 3.42] in healthy smokers, 7.14 [1.19 - 13.09] in COPD GOLD I-II, and 2.99 [0.99 - 5.00] in COPD GOLD III-IV. A statistically significant difference was found between healthy smokers and COPD I-II ($p=0.017$). Serum levels of IL-6 did not correlate with body weight.

C-reactive Protein

Serum levels of CRP were 0.19 mg/dL [0.00 - 0.37] in healthy controls, 0.20 [0.11 - 0.29] in healthy smokers, 1.10 [0.54 - 1.66] in COPD GOLD I-II, and 0.71 [0.37 - 1.05] in COPD GOLD III-IV.

Statistically significant differences were found between healthy controls and COPD I-II ($p<0.001$), healthy controls and COPD III-IV ($p=0.001$), healthy smokers and COPD I-II ($p<0.001$), and healthy smokers and COPD III-IV ($p=0.004$). Serum levels of CRP did not correlate with body weight.

Regression Models

In univariate logistic regression models including only healthy smokers and patients with COPD, HSP27 had an area under the curve (AUC) in the receiver operating characteristic (ROC) curve of 0.763 (0.624 - 0.902; 95% CI; $p=0.004$), and HSP70 showed an AUC of 0.885 (0.786 - 0.983; 95% CI; $p<0.001$). All other variables showed no significant result in the univariate logistic regression analysis. Figure 2.

ELEVATED HSP27, HSP70 AND HSP90α IN CHRONIC OBSTRUCTIVE PULMONARY

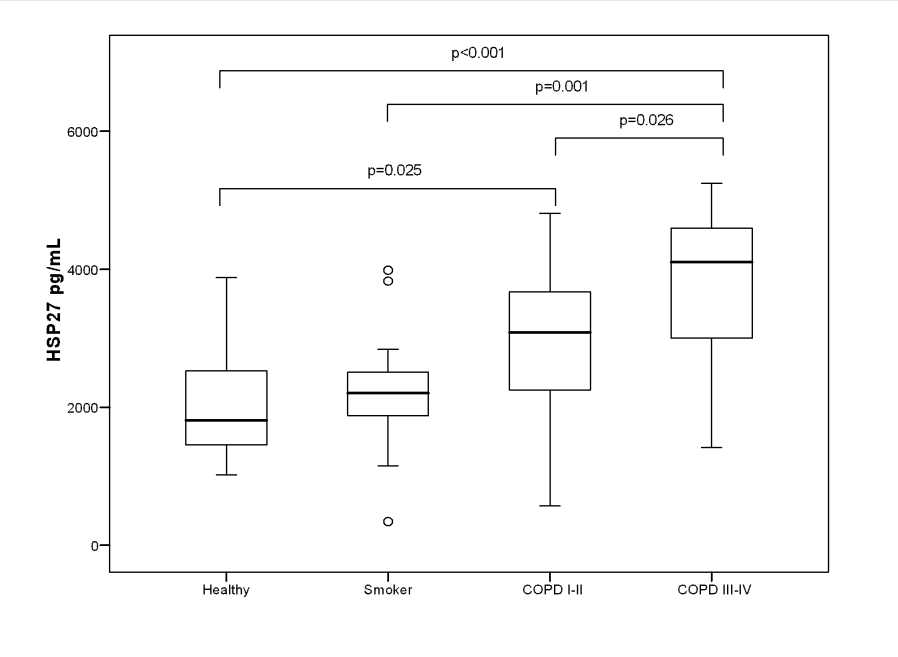


Figure 1a

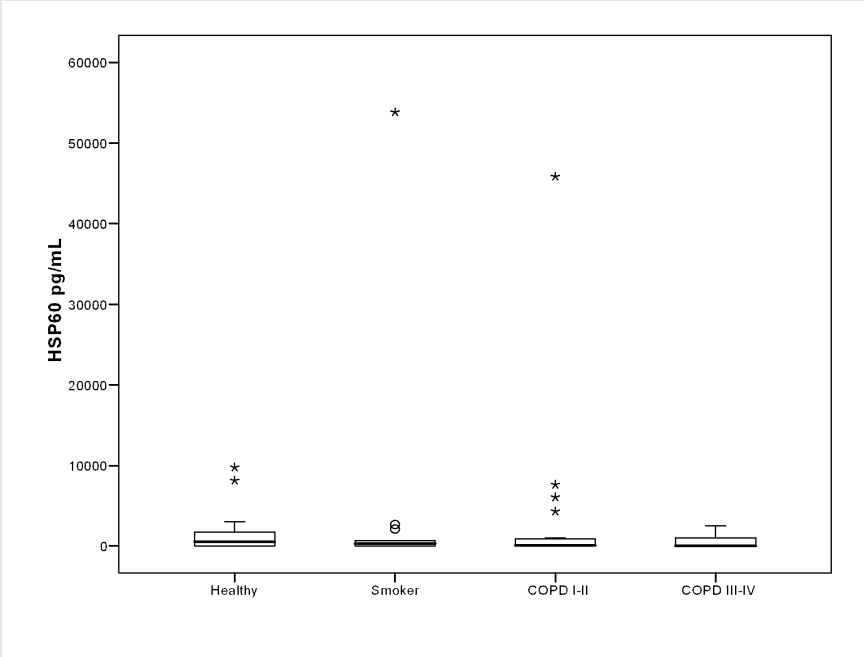


Figure 1b

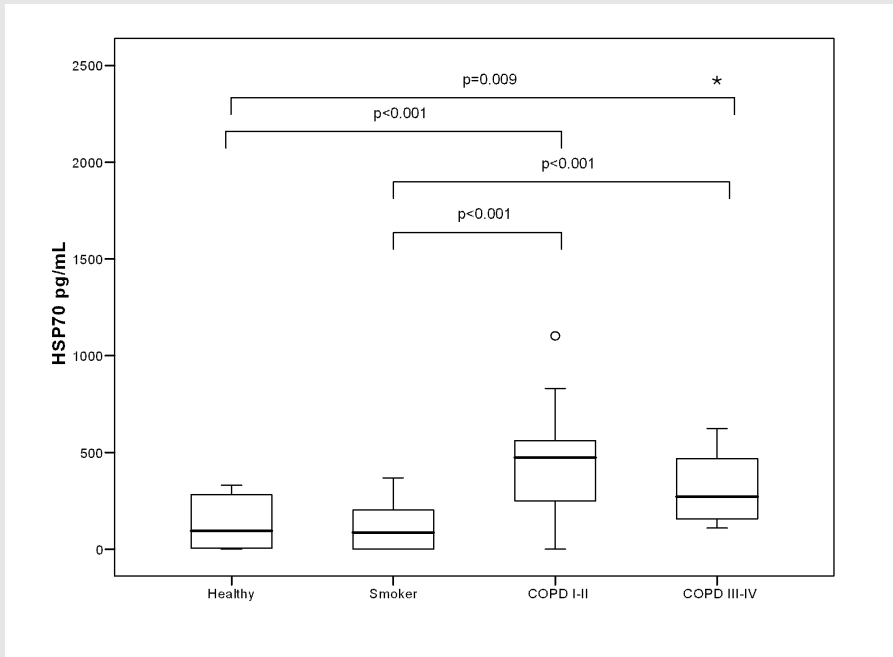


Figure 1c

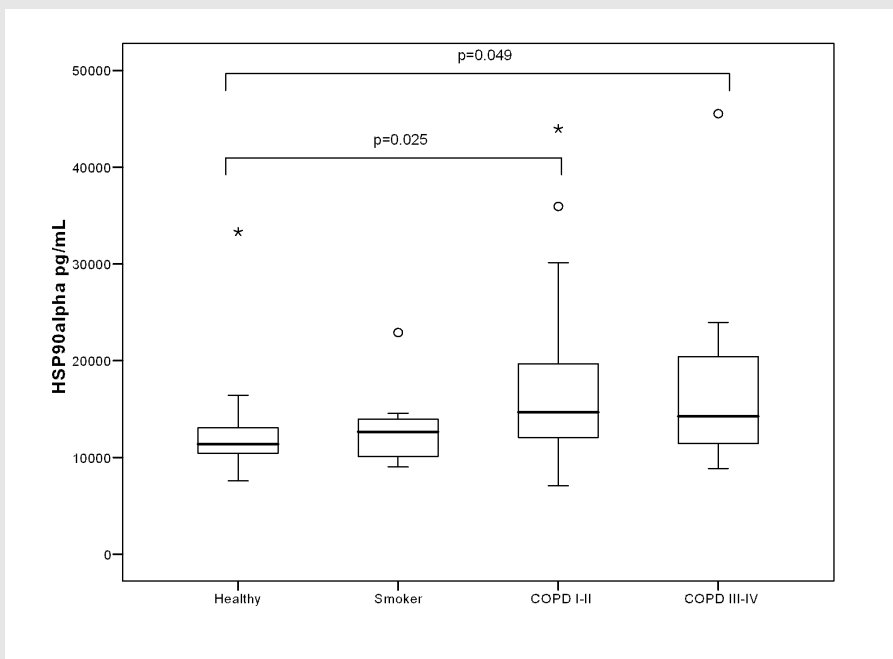


Figure 1d

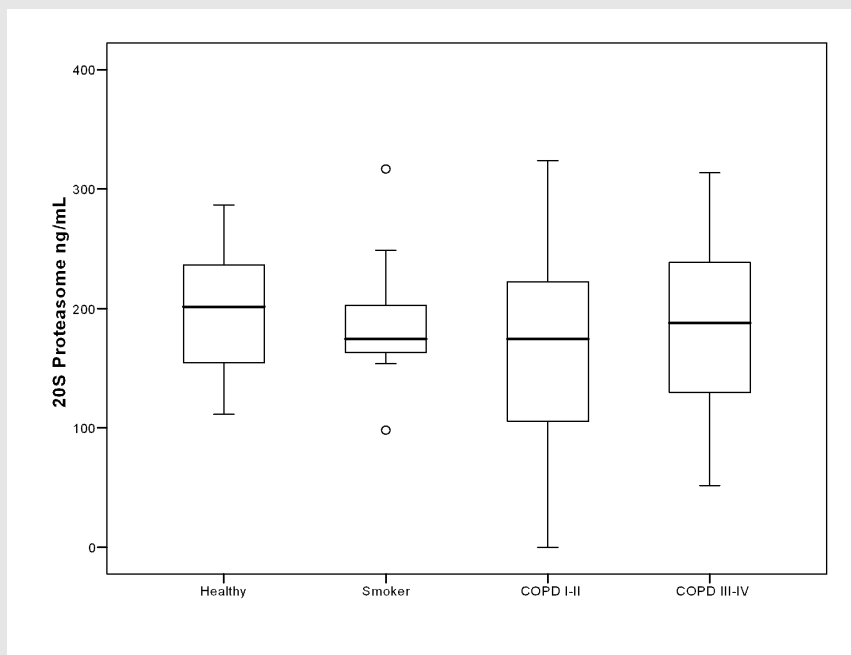


Figure 1e

Figure 1 (a, b, c, d, e): Serum levels of heat shock proteins and 20S proteasome were determined in the systemic blood flow of patients and controls. Bars indicate medians; solid boxes show span between 25th and 75th percentile; whiskers illustrate lowest and highest values; outliers are marked. P-values indicate significant differences.

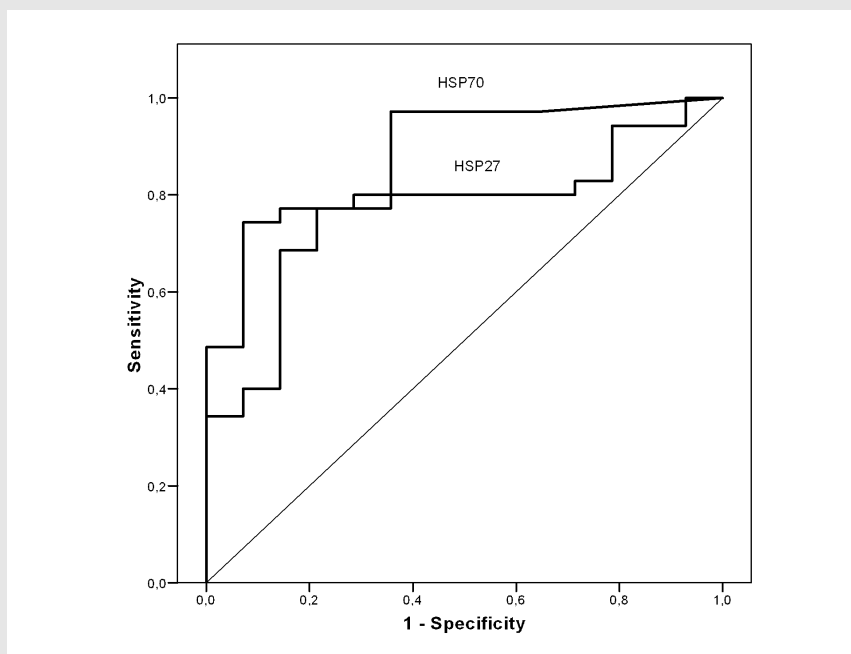


Figure 2: Receiver operating characteristic (ROC) curve indicating sensitivity and specificity of HSP27 and HSP70 to diagnose COPD in the smoking study population.

DISCUSSION

Patients suffering from chronic obstructive pulmonary disease present progressive inflammation of the bronchial airways, small airways, and lung parenchyma. Lung biopsies revealed massive infiltration of the peribronchial tissue with neutrophils, macrophages, and lymphocytes as part of the innate and adaptive immune system (7, 23). Activation of these cells is believed to lead to remodelling of the lung tissue (24). In COPD, both endogenous factors including neutrophils and cytotoxic T-cells (25) as well as exogenous stimuli like tobacco smoke (26) are thought to contribute towards tissue destruction. However, cessation of smoking does not alter impairment of the inflammatory response. The constant induction of inflammatory signals and increased cellular turnover result in upregulation of intracellular heat shock proteins and augmented release into the extracellular environment. We were able to show a significant increase of HSP27 in serum samples taken from the peripheral blood flow of patients suffering from COPD as compared to healthy smokers. HSP27 functions as repair mechanism aiming at the stability and correct posttranslational folding of intracellular proteins as well as the prevention of apoptotic cell death. Elevated serum levels of HSP27 were reported in inflammatory disorders including acute coronary syndrome and chronic allograft nephropathy (27, 28). Expression of HSP27 is transiently induced as a response to stress events. Termination of the acute triggering results in an immediate downregulation of HSP27 concentrations to normal levels. Thus, HSP27 is only upregulated when its cytoprotective properties are required (22). Interestingly, our results demonstrate a continuous increase of serum HSP27 concentrations with disease severity. This effect may be due to increased tissue devastation especially in late stages of COPD and spreading of the inflammatory disease to other organ systems resulting in a systemic spillage of HSP27 into the vascular bed. HSP27 generally acts as antiapoptotic mediator (29) and can be seen as an endogenous immunosuppressive attempt to control excessive inflammation in COPD. Serum contents of HSP27 showed diagnostic potential to determine the occurrence of COPD in a logistic regression model and may serve as marker for diagnosis and prediction of disease severity. Further explorations are needed to determine optimal cut-off values and improve the proposed sensitivity and specificity of serum HSP27 in a clinical setting.

The role of extracellular HSP60 has not been well defined. Some authors have described pro-inflammatory features, primarily in atherosclerosis (12, 30). Our data did not provide any support for HSP60 being a key element in the pathogenesis of COPD. Serum concentrations of HSP60 did not correlate with levels of other HSPs. However, as we did not include any patients with known coronary artery disease in our study, further investigations are needed to define the role of soluble

HSP60 in patients with COPD at increased risk for coronary events.

Serum levels of HSP70 were elevated in patients at early and late stages of COPD. We evidenced a four-fold increase in the GOLD I-II group compared to non-symptomatic smokers. HSP70 is an intracellular chaperone that is released into the extracellular space upon cell death (31) or by means of various secreting pathways (32). Extracellular HSP70 has been reported to activate cells of the innate and adaptive immune system and to stimulate cytokine production (18, 32). We found increased concentrations of soluble HSP70 in COPD samples. Values peaked in the COPD I-II group indicating a state of vast immune activation primarily at the early stages of the disease. Furthermore, serum contents of HSP70 showed high sensitivity and specificity to determine the occurrence of COPD in a logistic regression model and could serve as diagnostic marker. Because there was no significant difference between the COPD groups, HSP70 – unlike HSP27 – might not be suitable as marker for disease progression or response to therapy. Soluble HSP90 α was significantly upregulated in the peripheral blood flow in the COPD groups as compared to healthy non-smokers. Elevated levels of HSP90 α have previously been described in on-pump coronary artery bypass grafting (22) and wound healing after hypoxia (33). Rajagopal et al. characterized HSP90 as central factor in antigen presentation to T lymphocytes via major histocompatibility complex class II molecules (MHCII) (34). In synopsis with our data, we hypothesize that elevated levels of extracellular HSP90 α in COPD are an essential elicitor of the adaptive immune system, triggering a possible autoreactive response to self-antigen. This function of HSP90 α may also change the immunogenicity of the associated antigen. Therefore, HSP90 α has immunomodulatory effects through cross-presentation of associated peptides in the context of major histocompatibility complex molecules. The exact immunological role of increased serum HSP90 α in COPD has to be addressed in further studies. The extracellular content of 20S proteasome was not statistically increased or decreased in the investigated study cohorts. Levels remained under 200 ng/mL in all groups and appear to be of subordinate importance in the progression of COPD.

In conclusion, we were able to demonstrate elevated serum concentrations of soluble heat shock proteins 27, 70 and 90 α in patients with COPD. This spillage into the vascular bed may be caused by continuous activation of the immune system in the deterioration of COPD through endogenous and exogenous trigger mechanisms. This is the first study to demonstrate elevated serum levels of the described HSPs in patients with COPD at stable stages of the disease. Furthermore, HSP27 and HSP70 showed statistical trends to serve as diagnostic markers or markers for disease progression. Further investigations employing higher numbers of patients are needed to establish diagnostic algorithms using serum levels of HSPs.

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