Association of Plasma Heat Shock Protein 70 with Disease Severity, Smoking and Lung Function of Patients with Chronic Obstructive Pulmonary Disease

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Research

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Abstract

Background: Extracellular heat shock protein 70 (eHsp70) acts like a damage-associated molecular pattern (DAMP) and it might modulate immune responses in patients with chronic obstructive pulmonary disease (COPD). The aim of the study was to explore plasma eHsp70 concentration in patients with stable COPD, its association with disease severity and smoking status as well as its diagnostic performance in COPD assessment.

Methods: Blood samples were collected from 137 COPD patients and 95 healthy individuals. COPD patients were subdivided into GOLD 2-4 stages based on airflow obstruction severity and GOLD A-D groups regarding symptoms and exacerbations. Concentration of eHsp70 was assessed in EDTA plasma by the commercially available ELISA kit. Statistic analysis was performed by MedCalc statistical software.

Results: eHsp70 concentration was increased in COPD patients when compared to controls and was increasing with the severity of airflow limitation as well as symptoms burden and exacerbation history. There were no differences in eHsp70 concentrations among COPD patients based on smoking status, yet eHsp70 was increased in healthy smokers compared to healthy non-smokers. Interestingly, healthy smokers had similar eHsp70 level as COPD patients in GOLD 2 stage and those in GOLD A group. In addition, eHsp70 showed significant negative correlation with lung function parameters FEV_1 and FEV_1/FVC and positive correlation with COPD multicomponent indices BODCAT, BODEx, CODEx and DOSE. Finally, eHsp70 showed great predictive value (OR=7.63) and correctly classified 76% of cases.

Conclusions: Plasma eHsp70 is associated with COPD prediction and disease severity and might have a potential of becoming an additional biomarker in COPD assessment.

Background

Heat shock proteins (Hsps) are highly conserved and ubiquitously expressed proteins that normally acts as molecular chaperones which help in maintenance of protein homeostasis by assisting their folding processes (1, 2). Their involvement in proper protein folding and prevention of protein aggregation as well as apoptosis is of great importance for cellular function, especially when the cells are exposed to stressful conditions (3–5). The 72-kDa Hsp (Hsp70 in the following refers to this protein) is located in cytosol and nucleus, and its expression is induced as a part of response to different stressors like heat, bacterial or viral infections (6, 7). However, apart from being an intracellular protein, Hsp70 can be released from cells passively following cellular lysis i.e. necrotic death and/or actively through non-classical exocytotic pathways (8–10). In extracellular milieu, Hsp70 becomes a damage associated molecular pattern (DAMP) and represent a “danger signal” to the immune system (11). Extracellular Hsp70 (eHsp70) acts mainly pro-inflammatory and activate immune responses by engaging appropriate receptors (Toll-like receptors (TLRs) 2 and 4, cluster of differentiation (CD) 14, CD40, CD91, lectin-like oxidized low-density lipoprotein-1 (LOX-1), receptor for advanced glycation endproducts (RAGE)) (9, 12,

13). On the other hand, eHsp70 might also modulate adaptive immune response via binding to antigenic peptides and presenting them to antigen presenting cells (14). The sources of Hsp70 in peripheral circulation has not been fully elucidated, with various viable cells of both haematopoietic (e.g. peripheral blood mononuclear cells) and non-haematopoietic origin (e.g. epithelial cells) being potential candidates (8, 15).

Chronic obstructive pulmonary disease (COPD) is an inflammatory syndrome characterized by permanent airflow limitation. It is a multicomponent condition with both pulmonary and extra-pulmonary effects (16). Chronic respiratory inflammation involves activation and infiltration of macrophages and neutrophils and leads to abnormal immune responses, mucus hypersecretion, oxidant-antioxidant imbalance and apoptosis (17). The role of Hsp70 in COPD pathogenesis is still unclear, despite the effort of some researchers. Hsp70 was increased in sputum of COPD patients compared to both healthy smokers and non-smokers (18). In addition, increased expression of Hsp70 at both mRNA and protein level was detected in lung tissue of COPD patients (17). On the contrary, lower numbers of Hsp70 immunoreactive cells in bronchial tissue of COPD patients compared to healthy control subjects were detected (19). In our previous research, Hsp70 expression was significantly decreased in leukocytes of COPD patients, especially in COPD smokers, but also in healthy smokers in comparison to never smoking individuals, and we suggested suppressed Hsp70 transcription or its increased release from cells as potential underlying mechanisms that could explain observed phenomenon (20). Later on, we have explored effects of extracellular Hsp70 by employing recombinant human Hsp70 protein (rhHsp70) on human monocytic and bronchial epithelial cellular models (primary cells and cell lines), and we confirmed that rhHsp70 alone and in combination with cigarette smoke stimulate TLR2 and/or TLR4 receptors, mitogen-activated protein kinase (MAPK) and/or nuclear factor kappa B (NF-κB) signalling pathways and pro-inflammatory cytokines release (21–23). Positive associations between eHsp70 and cytokines as well as other inflammatory markers in circulation were reported (24, 25), and we have established presence of systemic inflammation in our group of COPD patients (26).

Concentration of eHsp70 was assessed in peripheral circulation of COPD patients in only few studies, but with inconsistent results in comparison to healthy subjects (increased or similar values) (4, 16, 27). However, data about eHsp70 predictive value and its association with disease severity are lacking. Therefore, the aim of the study was to investigate if there are any alterations in concentration of eHsp70 in patients with stable COPD when compared to healthy individuals, and to evaluate eHsp70 concentration in patients at different stages of the disease regarding airflow obstruction as well as symptoms and history of exacerbations. Moreover, association between eHsp70 concentration and smoking status was investigated. We also explored diagnostic performances of eHsp70 and its associations with COPD multicomponent indices (BODCAT, BODEx, CODEx, DOSE) and lung function parameters.

Methods

Participants

There were 137 patients at stable phase of COPD and 95 healthy individuals matched by age and gender. COPD was diagnosed by a specialist pulmonologist at Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia), in 2017 and 2018. Patients were in stable phase of the disease without exacerbations during the last three months, without changes in therapy regime and without infections in a lower respiratory tract. Health state of control subjects was established based on anamnestic data and normal spirometry test results. Both patients and healthy individuals had to be older than 40 years, could not have any lung disease (except COPD for COPD patients), could not have inflammatory diseases, manifest cardiovascular diseases, acute infections, diabetes with severe complications, severe liver diseases, severe kidney insufficiencies, malignant diseases, transplantations or other ongoing inflammations. All of them signed an informed consent for scientific research they volunteer for and were introduced to the aims of the research. Ethics Committee of University Hospital Centre Zagreb and Ethics Committee for Experimentation of Faculty of Pharmacy and Biochemistry, University of Zagreb (Zagreb, Croatia) approved the research. Except spirometry diagnosis criterion ($FEV_1/FVC < 0.70$) by the Global Initiative for COPD (GOLD), there were also disease severity classifications based on airflow limitation assessed by FEV_1 measurements (GOLD 1–4 stages) as well as symptoms and exacerbations history assessed by score from COPD Assessment Test (CAT) (GOLD A-D groups) (28). Also, all participants reported data about smoking status, so groups of healthy non-smokers (n = 48), healthy smokers (n = 47), COPD non-smokers (n = 10), COPD former smokers (n = 90) and COPD smokers (n = 37) were formed. For calculation of multicomponent indices related to COPD assessment, following data were collected for COPD patients: body mass index (BMI), score obtained from modified Medical Research Council (mMRC) Dyspnoea Scale, number of previous exacerbations and Charlson's comorbidity index. Afterwards, BODCAT (BMI, airflow obstruction, dyspnoea, CAT score), BODEx (BMI, airflow obstruction, dyspnoea, previous exacerbations), CODEx (Charlson's comorbidity index, airflow obstruction, dyspnoea, previous exacerbations) and DOSE (dyspnoea, airflow obstruction, smoking status, previous exacerbations) were calculated (29).

Assessment of lung function

Airflow limitation was diagnosed by spirometry on a Master-Screen Pneumo spirometer (Jaeger, Germany), and airflow obstruction was confirmed if FEV_1/FVC was lower than 0.70 after three acceptable measurements. Furthermore, diffusing capacity for carbon monoxide (DLCO) was measured three times on Master-Screen PFT Pro (Jaeger, Wurzburg, Germany), as described before (29).

Measurement of eHsp70

Blood samples were collected between 7 and 9 a.m. into the tubes with ethylenediaminetetraacetic acid (EDTA) as anticoagulant (Greiner Bio-One, GmbH, Kremsmünster, Austria) by venepuncture of a large antecubital vein after overnight fasting (26). Plasma was separated after centrifugation at 1000 x g for 15 minutes at 4 °C and stored immediately at 80 °C until eHsp70 determination. eHsp70 concentration

was measured using The AMP'D HSP70 high sensitivity ELISA kit (Enzo Life Science, Farmingdale, New York, USA). All experiments were performed following the manufacturer's protocol and recommendations, including minimal 1:4 dilution of EDTA plasma samples with assay buffer due to the matrix interference removal. Calculation of eHsp70 concentration in samples was performed by 4-parameter logistic curve fitting program within Origin software (OriginLab Corporation, Northampton, Massachusetts, USA). The sensitivity or limit of detection of the assay was 0.007 ng/ml, as determined by the manufacturer.

Statistical analysis

Data were tested for normality by Kolmogorov-Smirnov test, and all data failed it. Therefore, nonparametric Mann-Whitney test was performed for analysis between controls and COPD patients. When more than two groups were compared based on different classifications, Kruskal-Wallis One Way Analysis of Variance on Ranks with post-hoc analysis was used. Categorical variables were tested by Chi-squared test. Spearman Rank Order was performed for testing the correlations between investigated parameters, while assessment of predictive value of eHsp70 was obtained by univariate logistic regression analysis. Statistical tests were run in MedCalc statistical software version 17. 9. 2. (Ostend, Belgium), and results were considered statistically significant if $P < 0.05$.

Results

Association of eHsp70 with COPD severity

Patients with stable COPD were of similar age as control subjects and gender distribution was also similar between patients and healthy individuals, while lung function was decreased in COPD group as expected (Table 1). Concentration of eHsp70 was increased in plasma of COPD patients (0.98 (0.63–1.29) ng/ml) in comparison to controls (0.37 (0.25–0.63) ng/ml) ($P < 0.001$).

Table 1. Basic characteristics and spirometry parameters of all participants. Age was shown as median with minimum and maximum, and gender as absolute numbers. All other data were presented as median with interquartile range. Data were tested by Chi-squared or Mann-Whitney test.

parameter	controls n = 95	COPD patients n = 137	P-value
age	64 (46–83)	65 (44–86)	0.073
gender male/female	49/46	86/51	0.118
FEV_1 (L)	2.60 (2.12–3.19)	1.08 (0.78–1.57)	**< 0.001**
FEV_1 (% pred.)	93 (86–104)	39 (28–60)	**< 0.001**
FVC (L)	3.35 (2.77–4.16)	2.28 (1.81–2.77)	**< 0.001**
FEV_1/FVC (%)	81 (77–88)	48 (41–58)	**< 0.001**
FEV_1 – forced expiratory volume in one second; FVC – forced vital capacity.			

Moreover, it was associated with disease severity when COPD patients were subdivided regarding FEV_1-based airflow limitation (Fig. 1A) as well as symptoms severity and history of exacerbations (Fig. 1B). eHsp70 showed statistically significant differences regarding GOLD 2–4 stages in comparison to controls ($P < 0.001$) and throughout GOLD A-D groups in comparison to controls ($P < 0.001$). Increasing concentration of eHsp70 successfully distinguished each group of the patients regarding both subdivisions.

Influence of smoking status on plasma eHsp70 concentrations

When comparison of all participants based on self-reported smoking history was performed, it was observed that there were significant differences in eHsp70 levels between controls and COPD patients ($P < 0.001$). More precisely, COPD patients had increased eHsp70 compared to both healthy non-smokers and healthy smokers, yet there was no difference between COPD patients according to their smoking status. However, healthy smokers had higher values of plasma eHsp70 concentrations in comparison to healthy non-smokers (Fig. 2).

Additionally, when GOLD 2–4 stages (Fig. 3A) and GOLD A-D groups (Fig. 3B) were compared to healthy individuals subdivided according to their smoking status, significant difference in eHsp70 concentration

was observed (P < 0.001). Healthy smokers had similar levels of eHsp70 in plasma as COPD patients at GOLD 2 stage and those in GOLD A group, while patients with more advanced disease stages showed to have increasing levels of plasma Hsp70 (Fig. 3).

Associations of lung function parameters and COPD multicomponent indices with eHsp70

eHsp70 showed moderate to good positive correlation with COPD multicomponent indices BODCAT, BODEx, CODEx and DOSE as well as moderate to good negative correlation with lung function parameters FEV_1 and FEV_1/FVC. DLCO and eHsp70 showed to be poorly negatively correlated (P < 0.001 for all correlations) (Table 2).

Table 2
Spearman Rank Order analysis was performed between eHsp70 and COPD multicomponent indices as well as lung function parameters.

parameter	Spearman's correlation coefficient, r	P-value
BODCAT	0.712	< 0.001
BODEx	0.715	< 0.001
CODEx	0.710	< 0.001
DOSE	0.672	< 0.001
FEV_1	-0.708	< 0.001
FEV_1/FVC	-0.644	< 0.001
DLCO	-0.479	< 0.001

BODCAT – BMI, airflow obstruction, dyspnoea, score from COPD Assessment Test (CAT); BODEx – BMI, airflow obstruction, dyspnoea, previous exacerbations; CODEx – comorbidities (Charlson's index), airflow obstruction, dyspnoea, previous exacerbations; DOSE – dyspnoea, airflow obstruction, smoking status, previous exacerbations; FEV_1 – forced expiratory volume in one second; FVC – forced vital capacity; DLCO – diffusing capacity for carbon monoxide.

Previous exacerbations are defined as the number of exacerbations in the previous year.

Predictive performance of eHsp70

Univariate logistic regression analysis showed that eHsp70 had a great predictive value with its odds ratio (OR) of 7.63, 95% confidence interval (CI) = 3.68–15.82, and there were 76% cases correctly classified (P < 0.001).

Discussion

COPD is a highly prevalent yet underdiagnosed disease, with increasing morbidity and mortality rates. Complex underlying mechanisms that are reflected by diverse clinical presentation are making this disease challenging for specific diagnosis and therapy. Ideally, due to COPD heterogeneity, a particular endotype and phenotype should be recognised for each patient to personalise its treatment. It is now recognised that inflammation is present in COPD not just at the local level i.e. in lungs and airways but also at the whole-body level, with persistent systemic inflammome being demonstrated in some patients (30). Blood is a very accessible sample obtained by quite non-invasive way. Therefore, searching for a good peripheral blood diagnostic, prognostic, predictive biomarker and/or biomarker of disease severity in any disease is recommendable, and especially in complex and heterogeneous diseases. In this study, we assessed concentration of Hsp70 in peripheral blood of patients with stable phase of COPD and its association with disease characteristics defined by spirometry and clinical presentation. We found significantly elevated eHsp70 in overall COPD patients compared to healthy subjects. This increase was related to degree of airflow limitations as well as symptoms burden and history of exacerbation, and eHsp70 concentrations were the highest in patients with GOLD 4 stage and GOLD D group. It is also important to emphasize that eHsp70 was elevated even in patients classified as GOLD A and GOLD 2 (which is in clinical practice often the lowest GOLD stage) compared to overall control group. To the best of our knowledge, this is the first study that assessed eHsp70 concentrations in COPD patients subdivided by GOLD ABCD classification, and the first one to show GOLD stages dependent differences in eHsp70 levels. In addition, when participants of the control group were subdivided according to their smoking status, patients belonging to GOLD 2 and GOLD A subgroups had higher eHsp70 levels than never smoking individuals, but similar eHsp70 levels as smokers with normal lung function. Therefore, it could be suggested that so-called healthy smokers might be more susceptible to altered inflammatory responses provoked by eHsp70 being a danger signal to immune system, and some of them might even develop COPD in time to come. This assumption should be tested in the future studies. In addition, as only 20% of smokers develop COPD, a specific individual genetic makeup seems to be also decisive.

By searching the literature, we found only three studies that assessed Hsp70 concentration in the blood of COPD patients so far (4, 16, 27). However, in addition to being performed on significantly lower number of participants compared to our study, there are some concerns regarding their sample and ELISA kit selection. Reported concentrations on eHsp70 are very dependent on the matrix in which it was measured. Whitham and Fortes showed that eHsp70 concentrations were the highest in EDTA plasma, its values in heparinized plasma were somewhat lower, while the lowest eHsp70 levels were measured in serum, and they hypothesized that this was due to the binding of eHsp70 to aggregated clotting proteins in serum. Therefore, they recommended EDTA plasma as a sample of choice in the future investigations (8). This is especially important at least for the studies with healthy participants at rest as their eHsp70 values tend to be very low as well as for the studies with elderly subjects as their eHsp70 concentrations are significantly lower than in young individuals (25). Still, in previous researches assessing eHsp70 in COPD patients that were mostly older subjects, serum (4, 27) or heparinized plasma (16) were used. In addition, there are several concerns regarding ELISA kit applied in previous studies. Ünver et al. used an

adopted ELISA kit that is not entirely appropriate for blood as a matrix, and this could be the reason for extremely high eHsp70 values they obtained in the study although they used serum as the sample (4). Hacker et al. also used an adopted ELISA kit that was specific for intracellular Hsp70 determination in cell lysates (27). It is important to recognise limitations of those assays, as they are not optimised for biological fluids such as blood. On the other hand, Cui et al. selected a proper ELISA kit validated for serum and plasma with EDTA used as an anticoagulant, but not with citrate and heparin. However, they used heparinized plasma as the sample for eHsp70 measurement, and this could be the reason for higher eHsp70 values they obtained (16). In our study, we used EDTA plasma and ELISA kit that was more sensitive and could detect lower eHsp70 concentrations compared to other ELISA kit also validated for EDTA plasma and serum (31). With this choice of sample and kit we were able to detect eHsp70 in each study participant (controls and patients), and, as already mentioned, we were also able to distinguish patients by their disease severity. Dong et al. reported that the expression of intracellular Hsp70 was closely related with COPD disease severity (17). However, an association of extracellular Hsp70 with disease severity was not shown for COPD yet, but was demonstrated for some other diseases including asthma, chronic heart failure and rheumatoid arthritis (3, 32, 33).

In this study, we obtained positive associations between eHsp70 and multicomponent COPD indices (BODCAT, BODEx, CODEx, DOSE) that reflect airflow obstruction, smoking status, symptoms and history of exacerbations, which are all important in assessing patients' overall condition. We also obtained significant negative associations between eHsp70 and lung function parameters, and this was only reported for the expression of intracellular Hsp70 in COPD patients (17). Finally, eHsp70 showed to have a good predictive characteristic with its OR of 7.63 (95% CI = 3.68–15.82).

Although we presented some novel and interesting results, our study has some limitations. It did not include COPD patients in GOLD C group or in GOLD 1 stage. However, in clinical practice COPD patients belonging to GOLD 1 group rarely contact their physician due to very mild symptoms, and GOLD C category of patients is also very rare as the patients that do not have many symptoms are not usually frequent exacerbators. In addition, larger number of participants should be recruited in the further studies and longitudinal study design should be considered.

Conclusions

We showed that eHsp70 concentrations are increased in EDTA plasma of COPD patients in stable phase of the disease when compared to healthy subjects, and its levels were associated with airflow limitation as well as symptoms burden and history of exacerbations. Smokers with normal lung function had significantly higher eHsp70 values than healthy never smokers, and chronically elevated eHsp70 might contribute to development of some pathologies in the future, including COPD, in some genetically or otherwise susceptible healthy smokers. We suggest that eHsp70 has a potential to become a new biomarker in COPD assessment, and its evaluation in healthy smokers might also merit further investigation.

Abbreviations

BMI - body mass index;

BODCAT- BMI, airflow obstruction, dyspnoea, CAT score;

BODEx - BMI, airflow obstruction, dyspnoea, previous exacerbations;

CAT – COPD Assessment Test;

CD – cluster of differentiation;

CODEx - Charlson's comorbidity index, airflow obstruction, dyspnoea, previous exacerbations;

COPD – chronic obstructive pulmonary disease;

DAMP – damage associated molecular pattern;

DOSE - dyspnoea, airflow obstruction, smoking status, previous exacerbations;

EDTA - ethylenediaminetetraacetic acid eHsp70 – extracellular heat shock protein 70;

eHsp – extracellular heat shock protein;

FEV_1 – forced expiratory volume in one second;

FVC – forced vital capacity;

GOLD – Global Initiative for chronic obstructive pulmonary disease;

LOX-1 - lectin-like oxidized low-density lipoprotein-1;

MAPK - mitogen-activated protein kinase;

mMRC - modified Medical Research Council;

NF-kB - nuclear factor kappa B;

RAGE – receptor for advanced glycation endproducts;

rhHsp70 – recombinant human heat shock protein 70;

TLR – Toll like receptor.

Declarations

Ethics approval and consent to participate

Ethics Committee of University Hospital Centre Zagreb and Ethics Committee for Experimentation of Faculty of Pharmacy and Biochemistry, University of Zagreb (Zagreb, Croatia) approved the research (Approval Protocol Numbers: 02/21/JG and 251-62-03-14-78, respectively).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

IH and LR wrote the main manuscript text, and all authors contributed to the design of the work. AHT, MGR and IH performed the experiments. AVG and SPG were responsible for collecting the samples, performing spirometry and DLCO analysis, and collecting data about participants. LR performed statistical analysis and interpreted it with IH. All authors agreed to be personally accountable for the author's own contributions and to ensure that the accuracy or integrity of any part of the work are appropriately investigated, resolved, and the resolution documented in the literature.

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Figures

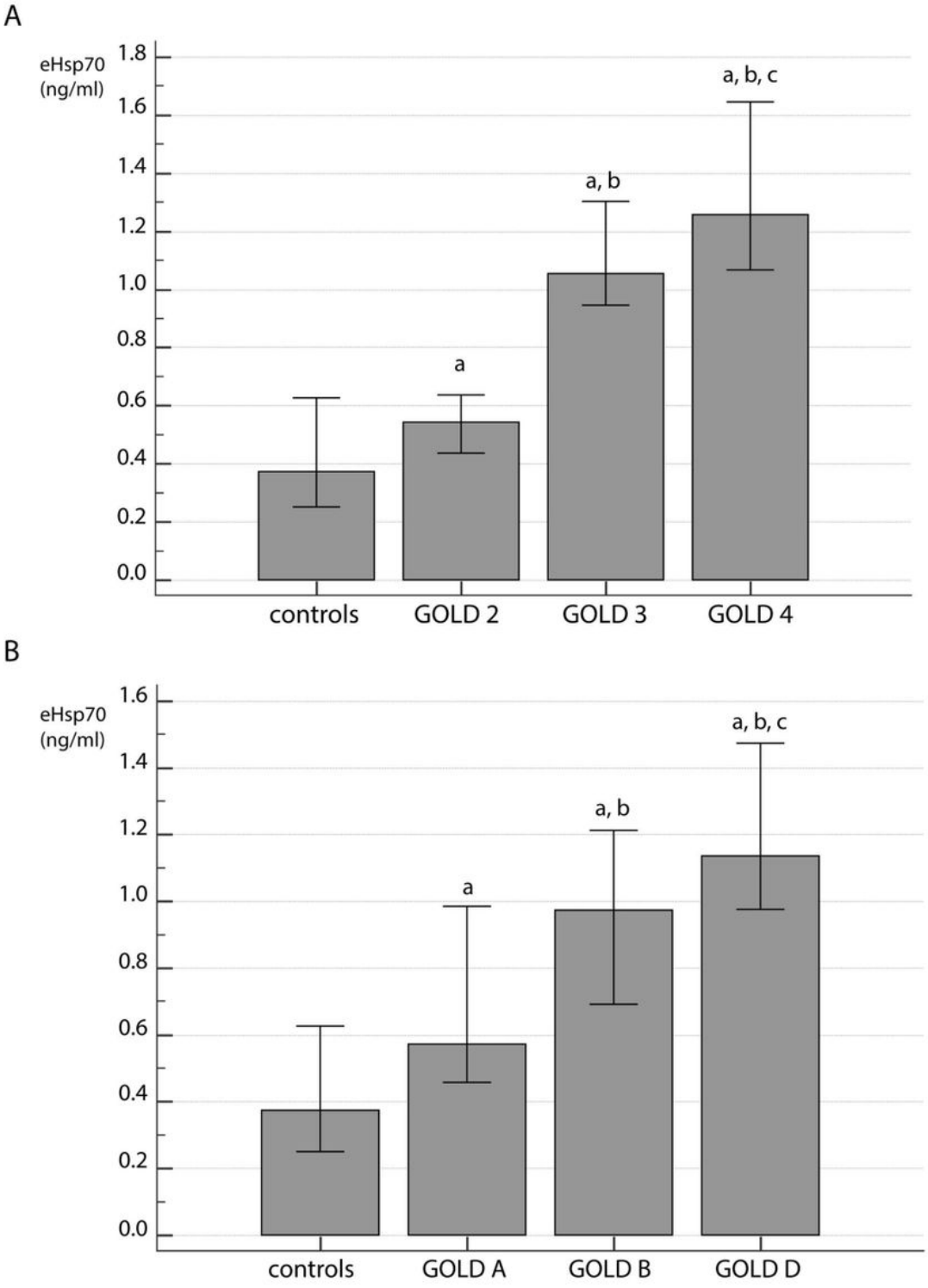

Figure 1

Concentration of eHsp70 in plasma of COPD patients regarding FEV1-based GOLD classification (A) and ABCD classification based on symptoms severity and history of exacerbations (B). All data were presented as median with interquartile range. Statistical analysis was performed by Kruskal-Wallis One Way Analysis of Variance on Ranks. a statistically significant increase in eHsp70 concentration in comparison to controls; b statistically significant increase in eHsp70 concentration in comparison to

GOLD 2 (A) or GOLD A (B); c statistically significant increase in eHsp70 concentration in comparison to GOLD 3 (A) or GOLD B (B).

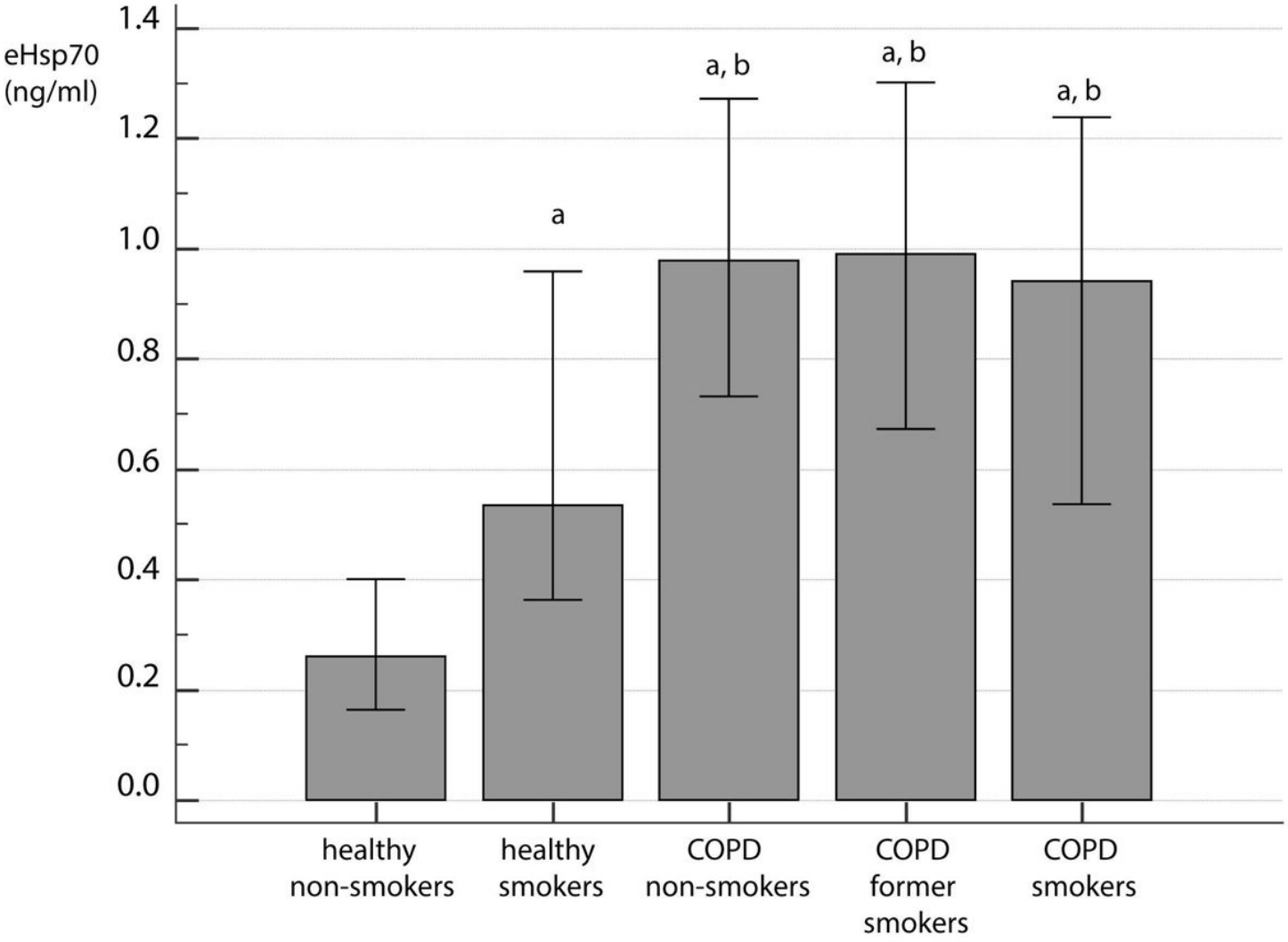

Figure 2

eHsp70 in healthy individuals and COPD patients regarding smoking status. All data were presented as median with interquartile range. Statistical analysis was performed by Kruskal-Wallis One Way Analysis of Variance on Ranks. a statistically significant increase in eHsp70 concentration in comparison to healthy non-smokers; b statistically significant increase in eHsp70 concentration in comparison to healthy smokers.

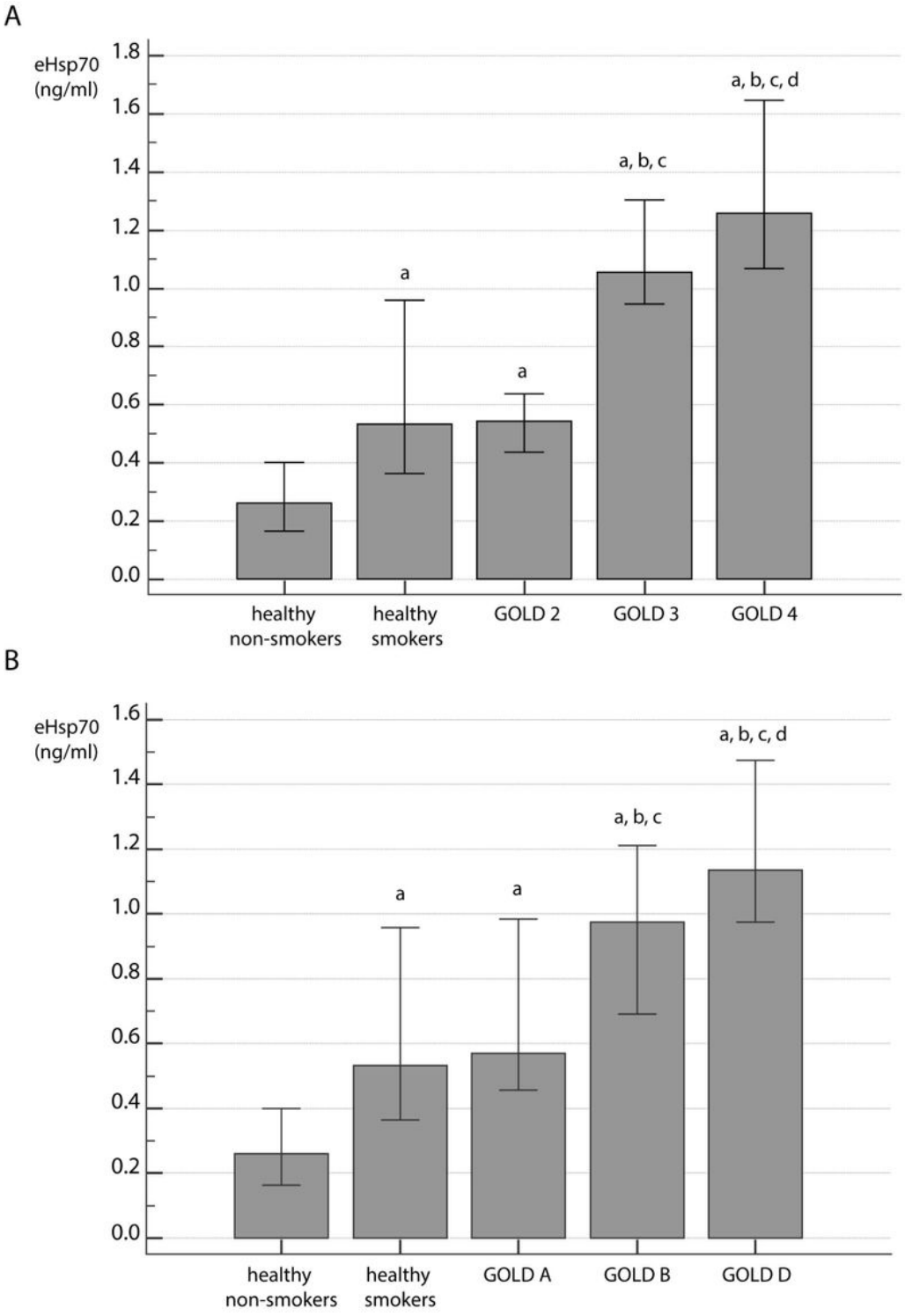

Figure 3

eHsp70 concentration in COPD patients at different stages of FEV1-based airflow limitation (A) and with based on symptoms severity (B) compared to healthy subjects regarding their smoking status. All data were presented as median with interquartile range. Statistical analysis between five groups of participants was performed by Kruskal-Wallis One Way Analysis of Variance on Ranks. Significant difference in eHsp70 concentration was observed throughout GOLD 2-4 stages and GOLD A-D groups

when healthy individuals were subdivided based on their smoking status ($P<0.001$). Afterwards, post-hoc analysis was performed. a statistically significant increase in eHsp70 concentration in comparison to healthy non-smokers; b statistically significant increase in eHsp70 concentration in comparison to healthy smokers; c statistically significant increase in eHsp70 concentration in comparison to GOLD 2 (A) or GOLD A (B); d statistically significant increase in eHsp70 concentration in comparison to GOLD 3 (A) or GOLD B (B).