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Alpha-1 antitrypsin is elevated in exhaled breath condensate and serum in exacerbated COPD patients

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Summary
Background: Exacerbations of chronic obstructive pulmonary disease (COPD) significantly contribute to COPD-related morbidity. Diagnosis of COPD exacerbations may be improved by analyzing biomarkers such as alpha-1 antitrypsin (AAT). AAT is an acute-phase protein and inhibitor of neutrophil elastase. Deficiency of AAT may result in early-onset respiratory symptoms. Measurement of exhaled breath condensate (EBC) is a noninvasive method to investigate biomarkers present in the epithelial lining fluid, such as AAT.
Objective: To investigate whether AAT can be detected and quantified in EBC and to compare AAT levels in the EBC of healthy controls, patients with COPD, and during exacerbations of COPD.
Methods: EBC from 10 healthy controls, 17 subjects with COPD, and 18 subjects with exacerbations of COPD was collected with the RTube™ device. AAT from EBC and serum were quantified by ELISA.
Results: AAT in EBC was detectable in every individual. Patients with exacerbations of COPD had significantly increased AAT values (mean, 514.33 pg/mL, [SD 279.41]) compared with healthy controls (mean, 251.32 pg/mL, [SD 44.71]) and stable COPD patients (mean, 242.01 pg/mL [SD

Abbreviations: AAT, alpha-1 antitrypsin; ANOVA, one-way analysis of variance; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; EBC, exhaled breath condensate; ELF, epithelial lining fluid; GOLD, Global initiative for chronic Obstructive Lung Disease; IL, interleukin; PBS, phosphate-buffered saline; PCT, procalcitonin; WBC, white blood cell; CI, confidence interval; NS, no significant; HC, healthy control.

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65.74]) ($P = 0.0003$; $P = 0.00003$). EBC AAT showed only a correlation trend with serum AAT ($r = 0.3$, $P = 0.054$).
Conclusions: AAT in EBC was detectable and quantifiable. AAT measured in EBC was significantly increased during exacerbations of COPD and can potentially be used as a biomarker in exacerbations.

Introduction

One important factor in the pathogenesis of chronic obstructive pulmonary disease (COPD) is believed to be inflammation of the small airways caused by inhalation of particles and gases.[1] Two main mechanisms are thought to lead to inflammation which causes pathological changes in lung tissue: production of reactive oxygen metabolites and disturbance of the physiological protease-antiprotease balance. One of the principal antiproteases is alpha-1 antitrypsin (AAT).

The main function of AAT is to protect the lungs against the protease neutrophil elastase, which is produced in response to infection and inflammation, and when not neutralized by AAT, destroys lung tissue. Furthermore, AAT is an acute-phase protein and an anti-inflammatory mediator.[2–4] It has been shown that AAT is elevated in the serum of patients with acute systemic and pulmonary infections.[5,6]

Recently, there has been increasing interest in using exhaled breath condensate (EBC) as a simple, noninvasive method to sample the lower respiratory tract for markers of inflammation.[15,16,20] The measurement of EBC is an easy and inexpensive method, however, there are still problems regarding standardization of the procedure.[7]

The diagnosis and classification of COPD exacerbations may be improved by the analysis of biomarkers. During acute COPD exacerbations, changes in the levels of chemical and inflammatory markers have been found. A number of potential markers have been investigated for use as markers for exacerbation of COPD, such as H_2O_2 in exhaled air or interleukin (IL)-8 and the soluble cell adhesion molecule sICAM in serum.[8,9] None of these markers have yet been used in routine clinical practice.

Initial investigations indicate that AAT can be detected in EBC, which may reflect AAT levels in the epithelial lining fluid (ELF).[10] AAT has not yet been sufficiently quantified in the EBC of patients with acute exacerbations of COPD. However, AAT may have potential as a marker in exacerbations because of its role in the disease as a chemoattractant for neutrophils and as an anti-inflammatory and antimicrobial protein. The aims of this study were to detect and compare AAT in the EBC of healthy controls, in patients with COPD, and in patients with acute exacerbations of COPD, and, to determine whether levels of AAT in serum and EBC are correlated.

Methods

Design

This observational cohort study aimed to test whether AAT is detectable in the EBC of patients with acute exacerbations of COPD. As controls, healthy subjects and COPD patients during stable disease were enrolled.

Subjects

EBC was collected from 10 healthy nonsmoking controls, 17 stable COPD patients, and 18 acute exacerbated COPD patients of comparable age. COPD patients were categorized according to Global initiative for chronic Obstructive Lung Disease (GOLD) criteria[11,12] and graded as Stages I–IV.[13] Acute exacerbated COPD patients were chosen according to criteria described by Cazzola et al.[14] No subjects approached to take part in the study declined.

All patients underwent a single study visit where EBC was collected once. Acutely exacerbated COPD patients, who had not received systemic steroids, were recruited and their EBC collected in the hospital emergency area during the first 60 min within presentation to the hospital. Unstable patients who needed respiratory ventilation support, or who were referred to intensive care, were not included. Every patient received a chest X-ray. Patients with pneumonia were excluded. All participants gave informed consent and the study (No. 0.59/06) was approved by the Philipps University Marburg local ethics committee. The study was planned as an observational cohort study with a limited number of subjects and was not registered as a clinical trial.

EBC collection

EBC samples were collected during 10 min of quiet breathing through a single-use disposable RTube™ collector (Respiratory Research, Inc.; Charlottesville, VA), while subjects were wearing a nose clip. The aluminum sleeve of the device had been cooled to an initial temperature of −20 °C prior to collection.[15,16]

ELISA assay of AAT in EBC

A total of 1 mL of EBC was lyophilized using a speed vac (Bachofer; Reutlingen, Germany) and resuspended as 100 μl/well using 0.1% Tween in phosphate-buffered saline (PBS). ELISA was performed using anti-human AAT rabbit antibody (Sigma–Aldrich; Taufkirchen, Germany) (1:200 dilution), followed by horseradish-marked goat anti-AAT (Bethyl Laboratories, Inc.; Montgomery, TX) (1:2000 dilution). Absorption was detected with a Tecan Ultra 384 Reader (Tecan; Crailsheim, Germany) at 450 nm.

Western blot assay of AAT

Western blots were performed using anti-human AAT antibody (Sigma–Aldrich; Taufkirchen, Germany) (1:5000 dilution), followed by anti-rabbit (Pierce; Bonn, Germany) (1:1000 dilution). The membrane was incubated using a chemiluminescence kit (Super Signal® West Femto Maximum Sensitivity Substrate, Pierce, Rockford).

Serum analysis and white blood cell (WBC) count

Serum AAT measurements were performed using a routine ELISA kit for all samples. Serum C-reactive protein (CRP) levels, WBC count, and procalcitonin (PCT) were measured for every subject.

Data analysis

Statistical analysis was performed using Sigma stat® and Graphpad® 5.0 software. Data are presented as mean ± SD. The Wilcoxon matched-pairs signed-ranks test was employed. Differences between values of groups were explored by one-way analysis of variance (ANOVA). Correlation analysis was performed using Spearman's correlation coefficient.

Results

Subjects

Patient characteristics are reported in Table 1. Healthy subjects were 36.1 ± 11.5 years old, patients with stable COPD were 66.6 ± 7.8 years old, and patients with acute exacerbated COPD were 72.2 ± 11.8 years old (Table 1).

Levels of AAT in EBC of exacerbated COPD patients measured by ELISA assay

It was possible to obtain EBC in stable COPD and also in exacerbated COPD patients. AAT mean values were 251.32 pg/mL (SD 44.71) for healthy controls, 242.01 pg/mL (SD 65.74) for stable COPD, and 514.33 pg/mL (SD 279.41) for exacerbated COPD subjects (Fig. 1a). Exacerbated COPD patients had significantly elevated AAT levels compared with healthy controls ($P = 0.0003$) and stable COPD patients ($P = 0.00003$). One clinically stable patient was excluded from statistical analysis because of serum signs of systemic inflammation with clearly elevated CRP, PCT, and WBC values and radiological signs of pneumonia.

Western blot detection of AAT in EBC

AAT spiking experiments showed that the assay detected the added AAT. In Western blot experiments, AAT was visible at 54 kD (data not shown).

Levels of AAT in the serum of exacerbated COPD patients

Serum levels of AAT were analyzed to determine whether these levels were increased during acute COPD exacerbations. Serum levels of AAT, for healthy controls were 1.29 g/L (SD 0.13), for COPD subjects 1.49 g/L (SD 0.22), and for subjects with acute exacerbations of COPD 1.85 g/L (SD 0.53). Patients with acute exacerbated COPD had significantly elevated AAT serum levels ($P = 0.00015$) compared with healthy controls and stable COPD patients (Fig. 1b).

Correlation between serum AAT and EBC AAT

To answer the question whether AAT levels in serum and EBC were correlated, we calculated the Spearman's correlation coefficient for the EBC and the serum AAT levels of the study subjects ($r = 0.3$), which did not reach statistical significance ($P = 0.054$) (Fig. 2).

Levels of CRP, PCT, and WBC in patients with acute exacerbated COPD

To find evidence for a systemic component of the COPD exacerbation, serum CRP, WBC count, and serum PCT were measured (Fig. 3). Mean CRP was <5 mg/L (SD 0) in healthy controls, 6.59 mg/L (SD 3.55) in stable COPD patients, and 31.72 mg/L (SD 44.9) in exacerbated COPD patients (Fig. 3a). The differences reached statistical significance (healthy controls vs stable COPD; $P = 0.035$, healthy controls vs acute exacerbated COPD; $P = 0.0006$, stable COPD vs acute exacerbated COPD; $P = 0.003$).

Mean PCT was 0.12 (SD 0.04) in healthy controls, 0.1 (SD 0) in stable COPD patients, and 0.21 (SD 0.27) in patients

Table 1 Patient characteristics. No significant differences for lung function, age and smoking were found, comparing stable COPD and exacerbated COPD patients (Wilcoxon rank sum test).

	Gender	COPD (GOLD grade)				FEV_1/VC % pred.	Age (years)	Smoking history (py)	Current/ ex-smoker	Inhaled steroids	β-Mimetics	Tiotropium
		I	II	III	IV							
Control	6 M/4 F	–	–	–	–	103.5	36.1	4	0/3	–	–	–
SD	–	–	–	–	–	6.8	11.5	7,4	–	–	–	–
COPD	11 M/6 F	2	8	7	–	56.3	66.6	38.8	3/14	13/17	13/17	12/17
SD	–	–	–	–	–	17.5	7.8	21.5	–	–	–	–
COPD ex	13 M/5 F	1	4	6	7	59.2	72.2	43.9	1/17	11/18	11/18	16/18
SD	–	–	–	–	–	14.5	11.8	26.4	–	–	–	–
P-value (Wilcoxon)	–	–	–	–	–	0.632	0.07	0.987	–	–	–	–

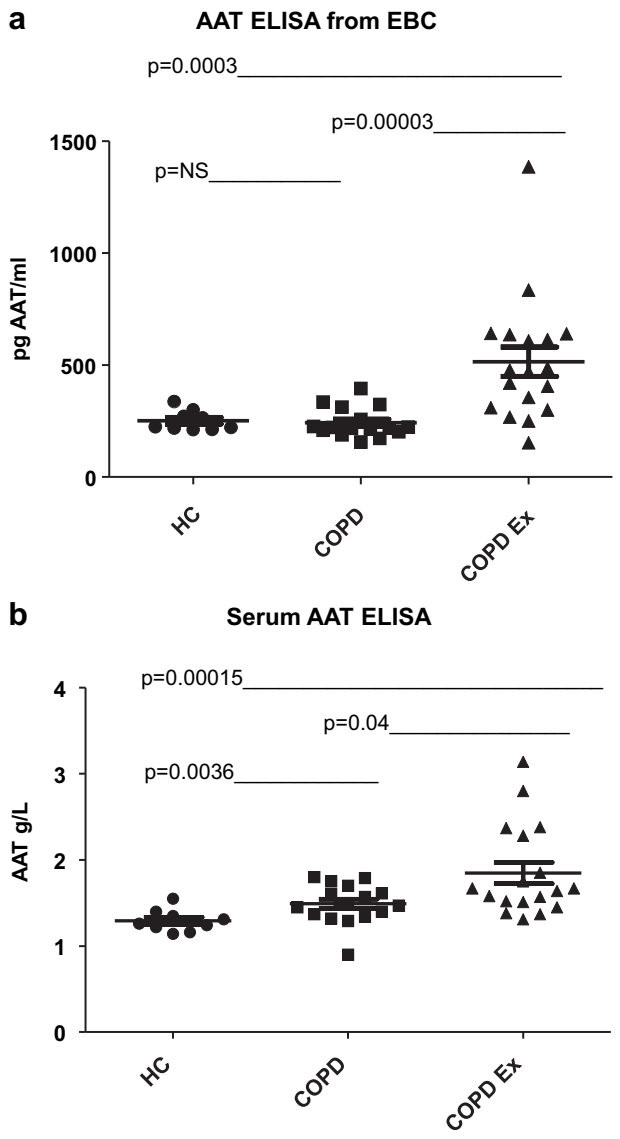

Fig. 1 (a). Comparison of EBC AAT values (with standard errors of mean) obtained by RTube™ in healthy controls, in patients with COPD, and in patients with an acute exacerbation of COPD AAT values in patients with an acute exacerbation of COPD were significantly higher than in healthy controls ($P = 0.0003$) and stable COPD ($P = 0.00003$) patients. (b) Comparison of serum AAT values in healthy controls, patients with COPD, and patients with an acute exacerbation of COPD AAT values (with standard errors of mean) in patients with an acute exacerbation of COPD were significantly higher than in healthy controls ($P = 0.00015$) and stable COPD patients ($P = 0.04$).

with acute exacerbations of COPD (Fig. 3b). No significance was found when comparing the PCT values in the healthy controls, COPD stable, and acute exacerbated COPD groups. Mean WBC count was 6.62 g/L (SD 2.02) in healthy controls, 7.44 g/L (SD 1.63) in stable COPD patients, and 11.83 g/L (SD 3.95) in exacerbated COPD patients (Fig. 3c). In terms of the WBC count, we found significant differences by comparing healthy controls with exacerbated COPD patients ($P = 0.0006$), stable COPD patients with

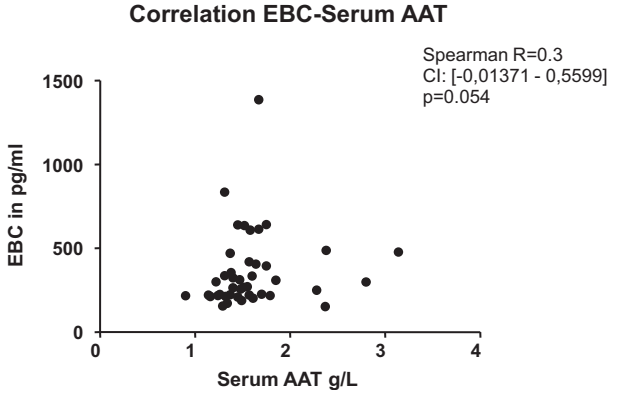

Fig. 2 Serum and EBC AAT values show a correlation trend ($P = 0.05$). The Spearman's correlation coefficient is $r = 0.3$.

exacerbated COPD patients ($P = 0.0007$) and stable COPD patients with healthy controls ($P = 0.03$).

The levels of AAT in the serum of patients with acute exacerbated COPD were significantly correlated with CRP in serum; Spearman's r was 0.87 ($P = 0.000003$) (Fig. 3d). Acute exacerbated COPD serum AAT was also observed to correlate with PCT (Spearman's $r = 0.56$, $P = 0.017$; data not shown). No significant correlation was found between serum AAT and WBC (Spearman's $r = 0.24$, $P = 0.13$).

Moreover for all included subjects there was no correlation found between EBC AAT and serum CRP (Spearman's $r = 0.24$, $P = 0.13$), PCT (Spearman's $r = -0.135$, $P = 0.4$) but for WBC (Spearman's $r = 0.48$, $P = 0.0013$) data not shown.

Discussion

The main findings of this paper are that AAT is significantly elevated in the EBC and serum of exacerbated COPD patients, and that AAT levels in serum and EBC have a trend to be positively correlated. Significant differences were observed when WBC counts and CRP in healthy controls and stable COPD patients were compared with the count in exacerbated COPD patients. Regarding PCT, the differences were not statistically significant but correlations were found between serum AAT with PCT and AAT with CRP in serum.

A previous EBC study showed that AAT is detectable in EBC, but AAT concentrations reached the lower limit of detection or quantification (0.8 ng/mL) in only 19.6% of subjects.[10] In our assay the limit of detection was less, and the difference between the two studies may have been that we used different antibodies. AAT spiking experiments showed that the assay indeed detected the added AAT. In Western blot experiments a positive band was visualized at 54 kD, the size of AAT. We were able to improve the ELISA reaching down to a sensitivity range below 0.1 ng/mL. At a threshold of 0.3 ng/ml there is a sensitivity of 0.89 and a specificity of 0.78. Find further description of the ELISA in the provided supplement.

The observation that EBC AAT was significantly elevated in exacerbated COPD patients is likely to be explained by the acute-phase response of AAT. In EBC, exacerbated

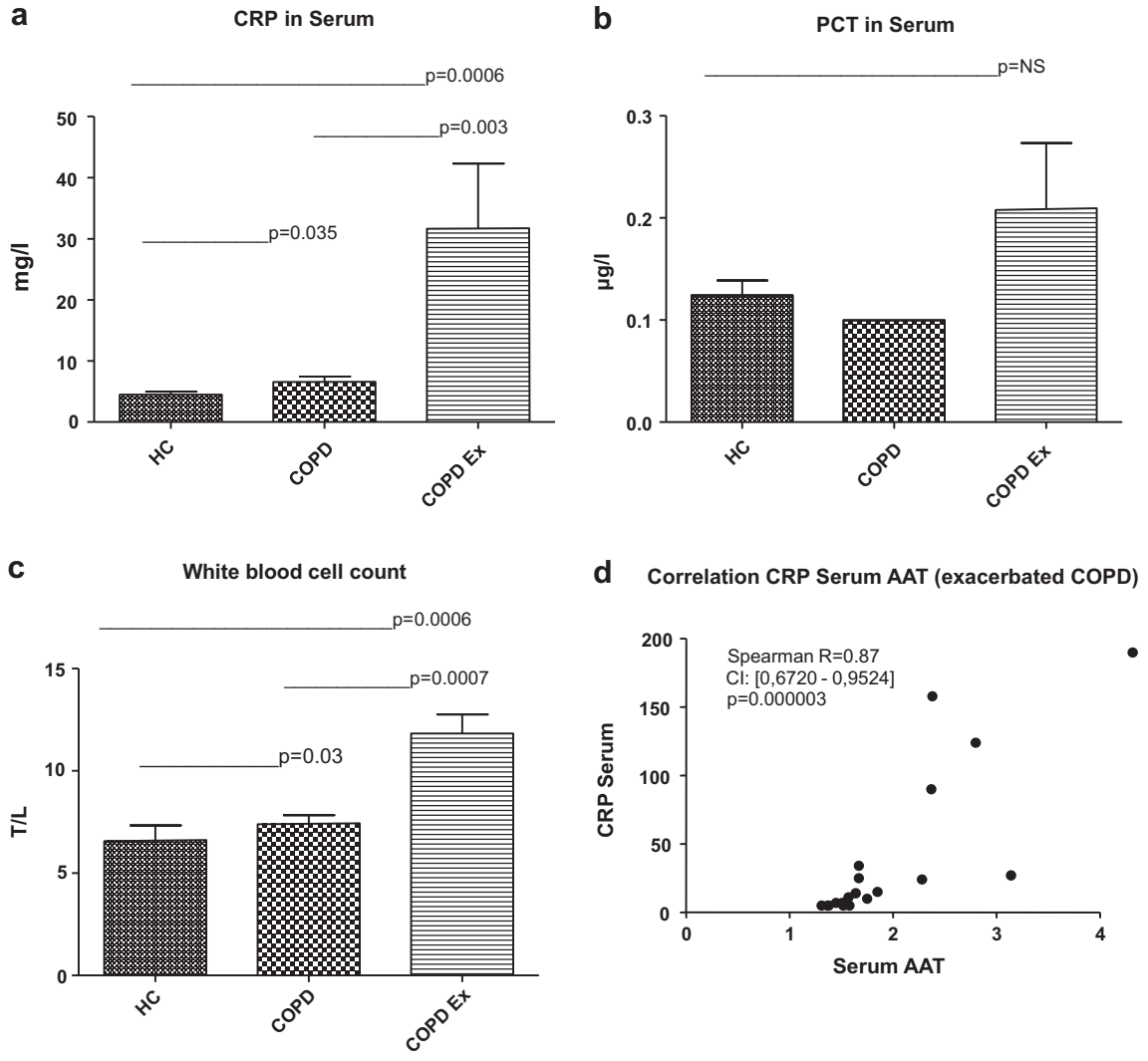

Fig. 3 (a) Comparison of serum CRP in healthy controls, patients with COPD, and patients with an acute exacerbation of COPD. CRP values (with standard errors of mean) in patients with an acute exacerbation of COPD were significantly higher ($P = 0.0006$) compared with healthy controls and stable COPD patients ($P = 0.003$). (b) Comparison of serum PCT (with standard errors of mean) in healthy controls, in patients with COPD, and in patients with an acute exacerbation of COPD. Differences did not reach the level of significance. (c) Comparison of WBC (with standard errors of mean) in healthy controls, in patients with COPD, and in patients with an acute exacerbation of COPD. WBC levels in patients with an acute exacerbation of COPD were higher compared with healthy controls ($P = 0.0006$) and stable COPD patients ($P = 0.0007$). (d) Correlation between CRP and AAT in serum of patients with an acute exacerbation of COPD. Spearman's correlation reached 0.87 ($P = 0.000003$).

COPD patients had significantly elevated AAT levels compared with healthy controls.

One might speculate that stable COPD patients might have higher AAT EBC levels compared with healthy controls because of neutrophil burden. Another idea might be an interaction of AAT with neutrophils like described before.[26]

Further experiments are necessary to prove these speculations.

WBC count, PCT, and CRP all showed elevated values in the exacerbated COPD group however, only WBC and CRP values reached significance compared with healthy controls and stable COPD patients.

By standard clinical measures patients with pneumonia were excluded. The large range observed for CRP and WBC was considered as a reflection of random scatter in a small sample.

An increase in inflammation markers like CRP has also been shown in other studies for acute exacerbated COPD patients.[17–19] Although CRP was the most useful biomarker of the 36 plasma biomarkers assessed in the study reported by Hurst et al,[17] plasma biomarkers were not found to be useful in predicting the clinical severity of exacerbation. One important point is that only serum parameters were analyzed in the latter study. Since COPD exacerbations are difficult to diagnose, AAT might have the potential to serve as an additional marker of COPD exacerbations in the future, especially when considering both local and systemic approaches, as COPD exacerbations are likely to have a systemic component. Since AAT is an acute-phase protein and a regulator of inflammation, it may also be elevated in other inflammatory conditions and be present as a non-specific inflammatory marker. As yet, local and systemic

levels of AAT have not been investigated in patients with non-COPD inflammatory disease.

This study showed that AAT is elevated in serum of exacerbated COPD patients. There are limited data on the serum elevation of AAT in COPD patients.[21] CRP and PCT are commonly used serum parameters to measure inflammation. PCT is elevated in sepsis and bacterial infection.[22,23] CRP is elevated in bacterial and autoimmune inflammation and non-specifically reflects systemic inflammation. Recent data suggest that CRP is elevated during an acute exacerbation of COPD, but CRP alone is neither sensitive nor specific in predicting clinical severity or outcome. Copeptin increases during acute exacerbation of COPD and may correlate with disease severity.[24] In our study, serum PCT and CRP correlated with elevated serum AAT, in exacerbated COPD subjects but the local increase of AAT in the EBC of patients with acute exacerbations of COPD was not associated with PCT, CRP, and WBC count. A correlation for EBC AAT with WBC was found calculating for all included subjects.

This study showed that, when using a suitable assay, AAT could be measured in the EBC of all subjects, including healthy controls, patients with COPD, and patients with acute exacerbations of COPD. The consistency of the data also indicated that the method was reproducible. The study has some limitations; only a small number of patients were included and the design of the study was cross-sectional with only one time point. Although only a small number of patients were included in the experiments, we were able to demonstrate in that explorative study that AAT was elevated in COPD exacerbations. The small number of patients included was of course more prone to be influenced by outliers in the data, which also cause larger standard deviations. We therefore consider the correlations observed only as confirmations of the consistency of the data and would not consider these as clinically relevant at the current state of knowledge.

As we only studied one time point directly after the patients were admitted to the emergency room, it would be interesting to determine if and when the level of AAT decreases, and if this decrease is correlated with clinical improvements.

The group of healthy controls included in the study was younger than the patients with COPD and not matched for age, which might have resulted in a bias. However, it could be shown from the levels of nitrate and nitrite in EBC that age was not a major confounder.[25] Furthermore, we could also detect a significant difference in AAT levels (both local AAT levels and systemic AAT levels being increased) in exacerbated COPD patients compared with the age-matched group of stable COPD patients which and were the major focus of that proof of concept study.

Regarding the content of proteins in the EBC, the ATS/ERS task force has highlighted that one current limitation of EBC measurement is the low concentration of many biomarkers, and therefore their measurement may be limited by the sensitivity of the assays.[7] The development of highly sensitive, reproducible ELISA assays aims to improve this limitation.

Another point of criticism regarding the use of EBC is the lack of standardization of the condensate measurements. Both a time-dependent measurement and a volume-controlled measurement are possible. It is not yet known whether airflow obstruction has a major effect on the levels of protein in EBC and a validation factor for EBC measurements is not yet available.

Conclusion

In conclusion, we found in that explorative study that collection of EBC is simple to perform in stable COPD and in patients with acute exacerbations of COPD. AAT could prove to be an important marker protein in the exacerbation of COPD. The source of the measured AAT must be more clearly defined in future experiments as it has not been defined whether it is of local origin or is part of the systemic increase. The sensitivity and specificity for AAT as a biomarker for detecting acute exacerbated COPD appears to be worthy of further examination.

Author contributions

A.R. Koczulla contributed to the design and conception of the study. He performed the experiments, analyzed and interpreted the data and drafted the manuscript. Sarah Noeske performed the experiments. Christian Herr performed the experiments. Severin Schmid performed the experiments. Janine Koepke performed the experiments. Christoph Nell did the biostatistics. Rudolf A. Jörres contributed to the design, conception, analysis and interpretation of the study. Claus Vogelmeier contributed to the design, conception, analysis and interpretation of the study. Robert Bals contributed to the design, conception, analysis and interpretation of the study. All authors critically reviewed and approved the final manuscript.

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Conflict of interest

A.R. Koczulla has received speaker fees and travel support from Talecris Biotherapeutics. Severin Schmid, Janine Koepke and Christoph Nell have no financial and non-financial disclosures. Sarah Noeske has received travel support from Talecris Biotherapeutics. Christian Herr has received travel support from Talecris Biotherapeutics. Rudolf A. Jörres has received travel grants from GlaxoSmithKline and speaker fees from AstraZeneca. Claus Vogelmeier has received speaker fees, research grants and consultancy fees from Talecris Biotherapeutics. Robert Bals has received speaker fees, travel support and research grants from Talecris Biotherapeutics.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2011.06.015.

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