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Increased Soluble Serum Markers Caspase-Cleaved Cytokeratin-18, Histones, and ST2 Indicate Apoptotic Turnover and Chronic Immune Response in COPD

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Introduction: Chronic obstructive pulmonary disease (COPD) is a worldwide burden and a major cause of death. The disease is accompanied by chronic inflammation and increased cellular turnover that is partly due to an overwhelming induction of apoptosis. In this study, we hypothesized that systemic markers of apoptosis are altered in patients with mild-to-severe COPD.

Materials and Methods: A total number of 64 patients and controls were enrolled in the study. Lung function parameters of all groups (nonsmoker, healthy smoker, COPD GOLD I&II, COPD GOLD III&IV) were evaluated at the time of inclusion. Enzyme-linked immunosorbent assays were used to quantify protein levels in serum samples.

Results: Serum contents of apoptotic end-products caspase-cleaved cytokeratin-18 and histone-associated-DNA-fragments were increased in patients with COPD, whereas anti-inflammatory soluble ST2 showed a peak in patients with COPD I&II ($P = 0.031$) compared to healthy smokers. Levels of pro-inflammatory caspase-1/ ICE correlated significantly with the number of pack years ($R = 0.337$; $P = 0.007$).

Discussion: Our results indicate a systemic release of apoptosis-specific proteins as markers for increased cellular turnover accompanied by progression of COPD. Furthermore, soluble ST2 seems to have a critical role in the anti-inflammatory regulatory mechanism at early stages of the disease. J. Clin. Lab. Anal. 23:372–379, 2009. © 2009 Wiley-Liss, Inc.

Key words: COPD; GOLD; apoptosis; cytokeratin; smoking; immune system; serum

INTRODUCTION

Prevalence and hospitalization rates caused by chronic obstructive pulmonary disease (COPD) have inclined dramatically over the past years (1). Several studies have shown a strong correlation between tobacco smoking and the development of COPD (2). The progression of this disease is accompanied by an increasing airflow

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obstruction due to constant airway remodeling. COPD comprises both chronic bronchitis and emphysema—conditions characterized by an augmented immune response and massive tissue destruction (3). The persistent activation of the immune system is thought to be the key factor in disease progression (4). Oxidative stress on lung parenchyma seems to be involved in initiating the inflammatory response to tobacco smoke exposure (5). The remodeling process is a consequence of chronic apoptosis induction in lungs of patients with COPD or smokers (6,7).

Apoptosis describes a form of cell death that is energy-dependent and active. Controlled apoptosis is a wanted and constant process and is a critical parameter in cellular homeostasis. Morphological alterations exhibited by actively dying cells are manifold and include cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. Apoptosis induction in COPD includes alveolar and bronchial epithelial cells in the airways as well as endothelial cells in the parenchyma (8). However, excessive triggering of apoptosis and increased turnover of alveolar cells leads to an impaired cell homeostasis, which ultimately results in tissue destruction (7). Therefore, increased levels of apoptosis have a crucial role in the development of COPD, affecting structural cells and immune cells in the lungs of patients. Induction of apoptosis seems to be the common final path of inflammation/direct cytotoxicity, an imbalanced protease/anti-protease system, and decreased VEGF production due to oxidative stress (9). The deranged cellular homeostasis caused by an altered balance of apoptosis and proliferation may therefore explain the tissue alterations in COPD.

Impairment of the immune system is not restricted to the lungs, as COPD patients are also at higher risk for systemic failures including cardiovascular diseases (10). Increased levels of circulating tumor-necrosis-factor α (TNFα)—a potent inducer of apoptosis—and soluble TNF receptors R55 and R75 were described in patients with COPD (11). These findings suggest an extension of increased apoptosis induction to other organ systems as a result of chronic immune activation in the lung. Besides being a target for apoptosis induction, the airway epithelium is a critical component of the immune reaction, producing pro-inflammatory cytokines, including TNFα, interleukin-1β (IL-1β), and chemokines (12). Epithelial cells of the lung also modulate acute exacerbations and reactions to pathogens that are common in patients suffering from COPD. Epithelial cells have a key role in the immune response to exogenous antigens and their cytokine response may also affect other organ systems.

In this study, we hypothesized that concentrations of end-products of apoptosis and inflammatory cytokines and enzymes are altered in the systemic blood flow of patients with COPD. Previous results of our group showed elevated serum levels of apoptosis-specific caspase-cleaved cytokeratin-18 (ccCK-18) in patients with acute myocardial infarction, ischemic cardiomyopathy, sepsis, liver degeneration, and after on-pump coronary artery bypass grafting (13–17). ST2 is a member of the interleukin-1 receptor family. A soluble isoform of the protein is expressed in type-2 helper T-cells and can have a role in effector functions (18). Increased concentrations of the soluble form of the ST2 protein were found in patients with acute heart failure, acute exacerbation of idiopathic pulmonary fibrosis, sepsis, and after cardiac surgery (19–22). This mechanism may serve as a "switch-off" signal of the interleukin-33—ST2 pathway (19). Additionally, soluble ST2 serves as negative feedback-loop to control excessive pro-inflammatory signaling (23). The cytokines IL-1β and IL-18 can actively induce apoptosis pathways. Both proteins require the caspase-1/ IL-1β-converting enzyme (ICE) to be converted from their respective precursors into their active forms (24). Therefore, ICE has an essential role in the exogenous triggering of apoptosis.

Based on these previous results, we decided to investigate whether levels of circulating serum products of increased apoptotic turnover (histone-associated-DNA-fragments and ccCK-18) (25,26), pro-inflammatory ICE (27) and anti-inflammatory soluble ST2 (28), are altered in 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 Scientific Practice of the Medical University of Vienna. All patients and controls provided written, informed consent before entering the study. Healthy 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. Demographic 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 (e.g. corticosteroids) within the past 14 days, history of asthma, autoimmune diseases, other relevant lung diseases (e.g. lung cancer, known α1-antitrypsin deficiency), or any known cardiopulmonary co-morbidity. Height and weight (Seca, Vogel and Halke, Hamburg,

TABLE 1. Demographic Data

Subject category	Healthy nonsmoker	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)

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. Abbrevations 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.

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 min after inhaling of 200 μg salbutamol. Arterial blood gases (PaO_2 and $PaCO_2$) 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°C until further testing.

Quantification of Caspase-Cleaved Cytokeratin-18 (ccCK-18)

Levels of cytokeratin-18 neo-epitope M30 were measured in samples using the M30-Apoptosense enzyme-linked immunosorbent assay (ELISA) (Peviva, Bromma, Sweden). In short, this ELISA uses an antibody recognizing a neo-epitope exposed after apoptosis-induced cleavage of cytokeratin-18. M30 antigen levels can be measured in units per liter (U/L)—1 U/L is equivalent to 1.24 pmol of a synthesized peptide of the M30 recognition motif—according to the manufacturer. The sensitivity of the ELISA was stated to be 25 U/L. Within assay and between assay reproducibility were <10%. Serum concentrations were calculated by comparing optical density (OD) values of the samples to OD values of the standard dilutions.

Evaluation of Serum Histone-Associated-DNA-Fragments

A commercial ELISA kit was used (Roche Applied Science, Mannheim, Germany) to quantify serum content of histone–DNA complexes. Samples were co-incubated in 96-well Microtitration plates with biotin-conjugated mouse monoclonal anti-histone antibody (clone H11-4) and peroxidase-conjugated mouse monoclonal anti-DNA antibody (clone MCA-33) for 2 hr at room temperature. After washing the wells, ABTS solution was added to each well. Enzyme reaction was monitored until sufficient color development was achieved and plates were read at 405 nm. OD values were calculated by subtracting OD values of blank wells from the mean of the sample wells.

Quantification of Serum Caspase-1/ICE

A commercially available (ELISA BenderMedSystems, Vienna, Austria) was used to determine serum contents of soluble caspase-1/ICE in serum samples. Microtitration plates precoated with monoclonal antibody to human ICE were incubated with sample material or diluted standard concentrations (400–6.25 pg/ml, seven dilution steps) at room temperature for

1.5 hr. Plates were washed three times with wash buffer and polyclonal rabbit ICE antiserum was added for 30 min. After another washing step, horseradish-peroxidase-conjugate was applied for 30 min. Wells were washed, and TMB substrate solution was used for the detection of enzyme activity. The reaction was stopped using sulphuric acid (2 N). Plates were read at 450 nm on a Wallac Multilabel counter 1420 (PerkinElmer, Waltham, MA). Concentrations were calculated by comparing OD values of samples with OD values of known concentrations of the standards.

Quantification of Soluble ST2

A commercially available ELISA (R&D Systems, Minneapolis, MN) was used to determine serum contents of soluble Interleukin-1 Receptor 4/ST2 (ST2) in our samples. Microtitration plates were precoated with a capture antibody against human ST2. Plates were then washed and blocked. Samples and standard dilutions (2,000–31.25 pg/ml) were incubated at room temperature. A biotinylated goat anti-human IL-1 R4 antibody was used for detection. TMB substrate solution was used for the detection of enzyme activity after addition of streptavidin conjugated to horseradish-peroxidase. The reaction was stopped using sulphuric acid (1 N). Plates were read at 450 nm on a Wallac Multilabel counter 1420 (PerkinElmer). Concentrations were calculated by comparing OD values of samples with OD values of known concentrations of the standards.

Quantification of Interleukin-10

ELISA technique (BenderMedSystems) was used to quantify levels of interleukin-10 (IL-10) in serum samples obtained after centrifugation of whole blood. Ninety-six-well plates were coated with a monoclonal antibody directed against the specific antigen and incubated over night at 4°C. After a washing step, plates were blocked with assay buffer for 2 hr. Following another washing step, samples and standards with defined concentrations of antigen were incubated as described by the manufacturer. Plates were then washed and incubated with enzyme-linked polyclonal antibodies. TMB substrate solution was applied after the appropriate time of incubation and another washing step. Color development was then monitored using a Wallac Multilabel counter 1420 (PerkinElmer). The OD values obtained were compared to the standard curve calculated from OD values of standards with known concentrations of antigen.

Statistical Methods

SPSS Software (SPSS Inc., Chicago, IL) 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. A univariate logistic regression model in a subgroup excluding healthy nonsmokers was calculated for ccCK-18. Receiver operating characteristic (ROC) curve was plotted to demonstrate sensitivity and specificity of the evaluated serum protein.

RESULTS

Apoptosis-Induced Caspase-Cleaved Cytokeratin-18 was Increased in Patients with COPD

We were able to show elevated serum levels of M30 neoepitope in patients with COPD I&II (294.96 U/L [87.81–502.11], mean [95% confidence interval], $P = 0.008$) and COPD III&IV (464.86 U/L [79.69–850.03], $P = 0.001$) compared to serum contents of smokers without COPD (119.67 U/L [93.85–145.49]). Healthy controls showed slightly increased levels of M30 (284.08 U/L [64.26–503.90]), but this difference was not statistically significant (Fig. 1).

Content of Histone-Associated-DNA-Fragments was Augmented in Patients with Severe or Very Severe COPD

The mean OD value in patients with COPD III&IV was determined at 0.43 [0.26–0.60]. This level was statistically significant different to histone levels in sera of healthy controls (0.27 [0.12–0.42], $P = 0.037$) and smokers without COPD (0.26 [0.08–0.45], $P = 0.022$). Patients with COPD I&II had elevated levels of serum histone-associated-DNA-fragments (0.40 [0.22–0.59]),

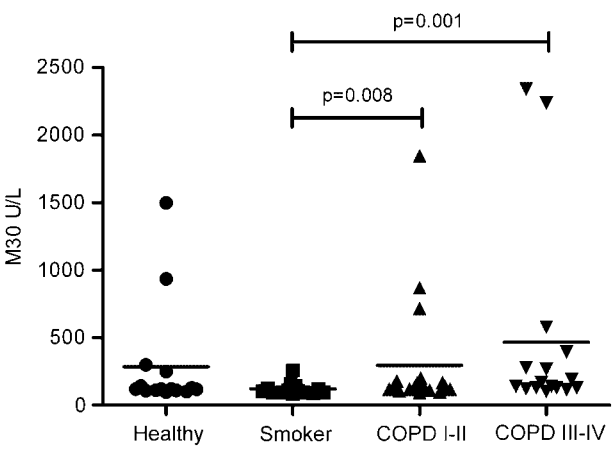

Fig. 1. Dot plot showing serum concentrations of soluble apoptosis-specific caspase-cleaved cytokeratin-18, detected using antibody M30. The bars indicate the mean value of each study group.

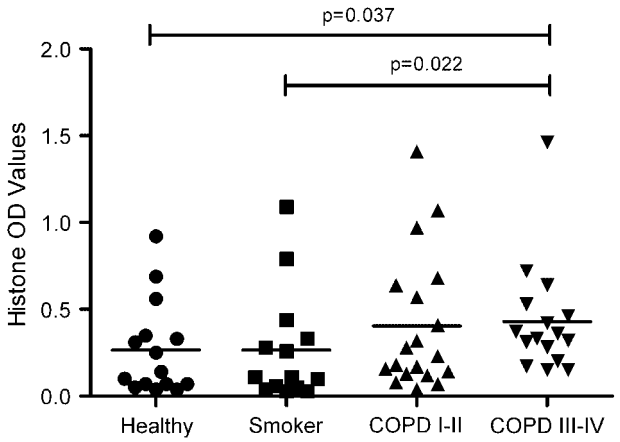

Fig. 2. Measured optical density (OD) values of circulating histone-associated-DNA-fragments in serum samples are illustrated. The bars indicate the mean value of each study group.

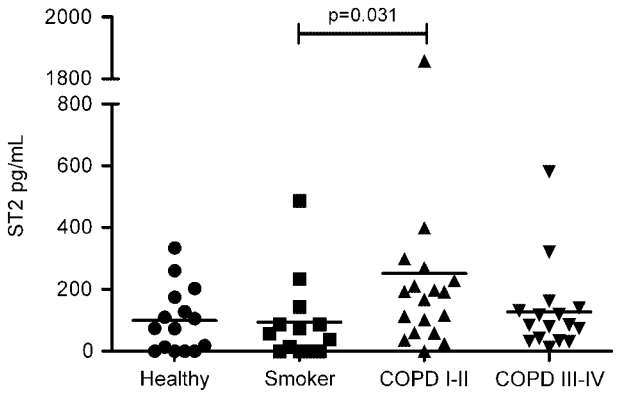

Fig. 3. The dot plot shows concentrations of soluble ST2 in serum samples of patients and controls. Serum concentrations of soluble ST2 peaked in patients with COPD I&II. The bars indicate the mean value of each study group.

though there was no statistical effect compared to other groups (Fig. 2).

Serum Caspase-1/ ICE Levels Showed No Statistically Significant Difference

Concentrations of ICE in serum samples were 84.86 pg/ml [64.85–104.87] for healthy controls and 102.76 pg/ml [56.59–148.93] for smokers without COPD. Patients with COPD GOLD stage I&II showed a moderately higher mean content of 114.56 pg/ml [78.98–150.14], and patients with COPD III&IV had a mean serum level of 111.87 pg/ml [82.21–141.53] comparable to COPD I&II. There was no significant difference between any groups.

Serum Levels of ST2 are Heightened in Patients with COPD

We were able to show twofold increased serum levels of ST2 in patients with COPD I&II (251.80 pg/ml [45.74–457.85], $P = 0.031$) compared to serum contents of healthy smokers (94.01 pg/ml [11.57–176.46]). All other group comparisons showed no significant differences; healthy controls (99.86 pg/ml [41.86–157.86]), COPD III&IV (127.24 pg/ml [51.72–202.75]) (Fig. 3).

Serum IL-10 Levels Showed No Statistically Significant Difference

Concentrations of IL-10 in serum samples were 8.84 pg/ml ([−6.14–23.81]) for healthy controls and 10.25 pg/ml [−3.84–24.34] for smokers without COPD. Patients with COPD GOLD stage I&II showed a content of 11.57 pg/ml [−8.49–31.62], and patients with COPD III&IV had a lower mean serum level of 0.63 pg/ml [−0.71–1.97]. There was no significant difference between any groups.

DISCUSSION

COPD is characterized by chronic inflammation of lung tissue and concomitant affection of other organ systems. This process is thought to be mainly triggered through numerous years of tobacco smoking. We were able to show a strong correlation between the number of pack-years and serum concentration of pro-inflammatory ICE, indicating a direct effect of smoking habits on the systemic inflammatory response (Fig. 4A). According to our primary hypothesis we found elevated levels of apoptosis-specific soluble ccCK-18 and histone-associated-DNA-fragments (histones) in patients with COPD GOLD I&II and GOLD III&IV. Serum contents of biological active protein ICE correlated significantly with levels of histones (Fig. 4B). In apoptotic cells, endonucleosome activation results in cleavage of DNA and generates long chromatin fragments, H1-rich short oligonucleosomes, and mono-nucleosomes (termed histones), which are not attached to the nucleus (26). The release of apoptosis-specific histone–DNA–complexes seems to be a result of constant tissue remodeling due to an impaired induction of apoptosis.

Pathogenesis of COPD is associated with degeneration of lung tissue due to increased activation and pro-apoptotic signaling of the innate and adaptive immune system in disease susceptible patients. Chronic inflammation leads to increased levels of ICE, a protease with Cys285 serving as the catalytic residue, that cleaves the 21 kDa biological inactive IL-1β precursor at Asp116-Ala117 to generate the 17.5 kDa mature form of IL-1β (24). The active enzyme consists of two non-identical subunits (p10 and p20), both of which are essential for enzymatic activity, and therefore have a

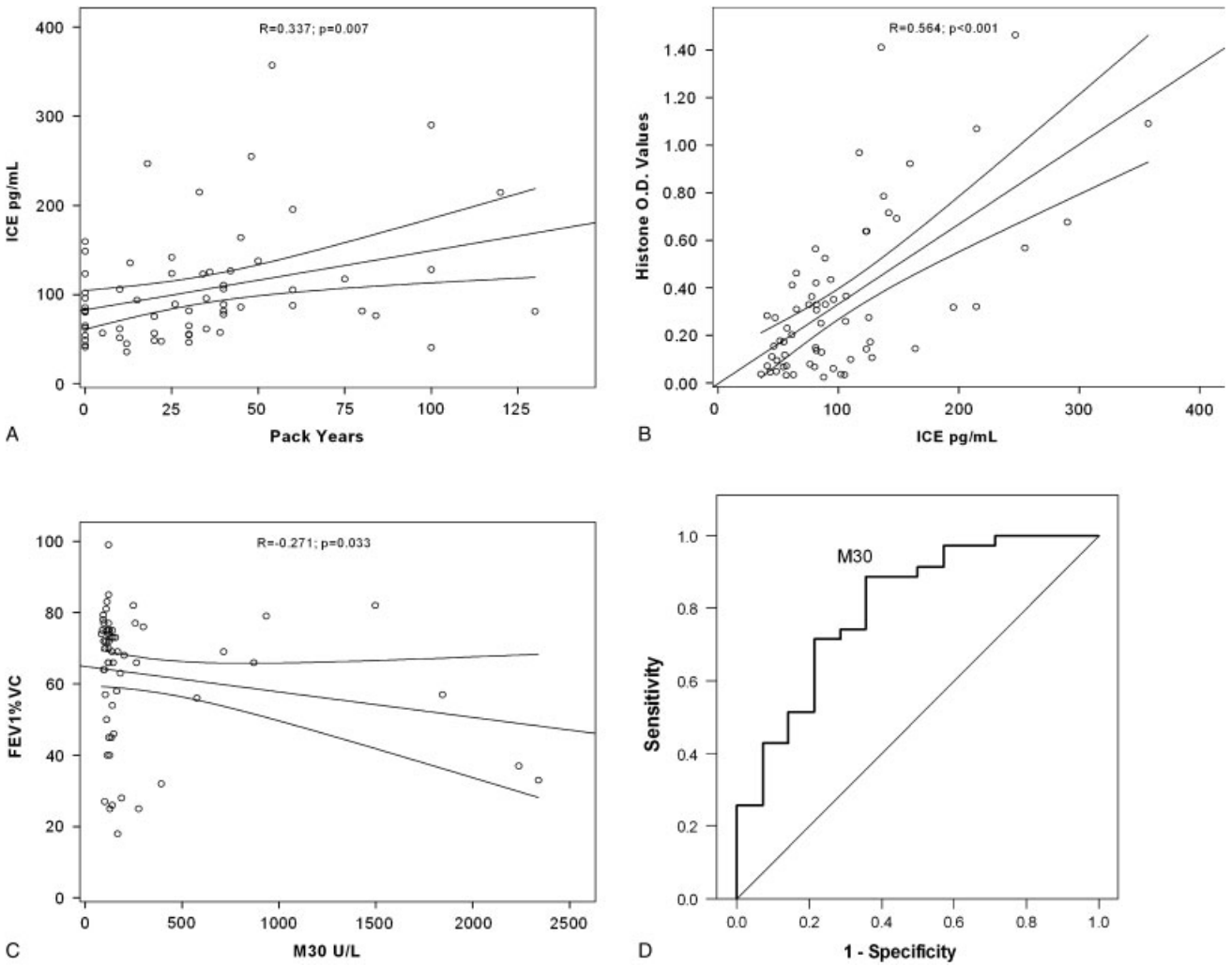

Fig. 4. Correlations between serum concentrations of ICE and number of patients' pack years (**A**), circulating histone-associated-DNA-fragments and serum ICE levels (**B**), and FEV1% of vital capacity (VC) and serum caspase-cleaved cytokeratin-18 (**C**), respectively. *R*, spearman's rank correlation coefficient. The ROC curve for ccCK-18 (M30) indicates potential of this serum protein to serve as diagnostic marker in patients with stable COPD (**D**).

pivotal role in the apoptosis of various cells, including endothelial and epithelial cells (29,30). Apoptotic turnover results in the liberation of cellular intermediate filaments such as cytokeratin-18 (CK-18) (15). CK-18 is a major component of intermediate filaments and is widely expressed by epithelial and endothelial tissues (31). In apoptotic cells, CK-18 is phosphorylated and microfilaments aggregate rapidly (32). Different conditions of cellular stress increase cytoplasmatic microfilament reorganization as well as altered CK-18 polymerization (33). Consequently, caspase-mediated cleavage of CK-18 occurs during apoptosis and leads to the formation of a specific neo-epitope, recognized by the antibody M30 (34). The binding-specificity of this antibody is restricted to apoptotic CK-18 degeneration products (caspase-cleaved CK-18 [ccCK-18]). Increased serum levels of apoptotic ccCK-18 were also present in acute myocardial infarction (13). Interestingly, the presence of soluble ccCK-18 was associated with impairment of lung function according to classification of COPD using the GOLD guidelines (Fig. 4C). The ROC curve analysis revealed an area under the curve of 0.806 ($P = 0.001$), indicating a high sensitivity and specificity of ccCK-18 as diagnostic marker in patients with stable COPD (Fig. 4D).

In line with the above data we found strong evidence for apoptosis-dependent lung degeneration in serum samples. We suppose that cleavage of cytokeratin-18 and release of ccCK-18 into the blood flow occurs with progression of the disease and can be used to monitor morbidity of the patient.

Previous studies have demonstrated a predominance of TH1 cytokine milieu in COPD patients (35). Since inflammation is always accompanied by secretion of

proteins known to have anti-inflammatory properties, such as IL-10 (36), we sought to investigate another protein known to be related to the innate immune system named ST2 (37). This soluble molecule is thought to modulate the TH1/TH2-associated immune responses. We evidenced a significant increment of anti-inflammatory ST2 serum levels in patients with COPD I&II, but not in severe COPD. This observations indicate that COPD evidences increased makers of innate immune activation and regulation similar to autoimmune diseases, sepsis, and lung-specific diseases like fibrosis and asthma (38,39). We believe that noxious substances, e.g. smoke, trigger Toll-like receptors (TLRs), so-called pattern recognition receptors that normally sense presence of microbial components to the innate immune system (40,41). Binding of activation ligands, e.g. lipopolysaccharide (LPS), to TLRs induces activation of the NF-κB, leading to the production and release of pro-inflammatory cytokines. It is commonly accepted that pre-exposure to LPS reduces the sensitivity to a second challenge with LPS, resulting in a diminished production of numerous cytokines both in rodents and humans (42). The molecular mechanisms of TLR-induced tolerance may involve the down regulation of the TLR4-MD2 complex (43), which may lead to diminished TLR signaling (44). Several studies suppose an important role for the Toll/IL-1 receptor (TIR) family member ST2 in the induction of endotoxin tolerance (28). ST2 is expressed by TH2 cells, mast cells, and macrophages. It was shown to be up-regulated after LPS stimulation and regulates cytokine expression in a model of acute lung injury (20). We therefore believe that secretion of ST2 in the initiation of COPD might act as negative regulator of TLR4 and IL-1R signaling. In the clinical context this may mean that systemic soluble ST2 is functioning as protective serum protein in order to prohibit lung destruction due to over-active adaptive and innate immune system.

Our results indicate that patients with COPD evidence increased serum levels of apoptotic turnover (ccCK-18, histones) in the systemic blood flow. Detection of serum ccCK-18 has the potential to serve as diagnostic marker in patients with stable COPD. Interestingly, we were able to evidence that soluble ST2 in patients with COPD I&II compared to healthy smokers was significantly increased in the study cohort. We believe that this increment of anti-inflammatory ST2 at early stages of COPD may be a possible negative feedback-loop to control the chronic inflammatory response set by inhaled noxious substances. Finally, we were able to evidence that soluble ICE correlated significantly with history of smoking (pack years), indicating triggering of inflammation and apoptosis due to noxious inhalants. The identified serum markers might serve to identify patients at risk for development and progression of COPD.

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The Medical University of Vienna claims financial interest.

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