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Early View

Original article

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Serum levels of hyaluronic acid are associated with COPD severity and predict survival

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Take-Home Message:. Serum hyaluronic acid (HA) is associated with COPD severity, outcome and predicts overall survival. Enzymatic degradation of HA is associated with airflow limitation and impairment of lung function.

Abstract

Hyaluronic acid (HA) and its degradation products play an important role in lung pathophysiology and airway remodeling in COPD.

We investigated if HA and its degrading enzyme hyaluronidase-1 (HYAL-1) are associated with COPD severity and outcome.

Serum HA was assessed in a discovery cohort of 80 COPD patients at stable state and exacerbations. HA, HYAL-1 and HYAL-1 enzymatic activity were evaluated at stable state, exacerbations and 4 weeks after exacerbations, in 638 COPD patients, from the PROMISE validation cohort.

In the discovery cohort, serum HA was higher at exacerbations, compared with stable disease ($p=0.015$). In the validation cohort, HA was higher at moderate and severe exacerbations than at baseline ($p<0.001$) and remained higher after 4 weeks ($p<0.001$). HA was strongly predictive for overall survival since it was associated with time to death ($p<0.001$) independently of adjusted Charlson-score, annual exacerbation rate and BODE-index. Serum HYAL-1 was increased at moderate ($p=0.004$) and severe ($p=0.003$) exacerbations but decreased after 4 weeks ($p<0.001$). HYAL-1 enzymatic activity, at stable state, was inversely correlated with FEV1% predicted ($p=0.034$) and survival time ($p=0.017$).

Serum HA is associated with COPD severity and predicts overall survival. Degradation of HA is associated with airflow limitation and impairment of lung function.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a complex heterogeneous disease characterized by the progressive development of airflow limitation that is largely irreversible. Acute exacerbations of COPD (AECOPD) may occur at any stage of COPD and are associated with patients' mortality and morbidity (1, 2).

A key feature of COPD is airway remodeling, which describes the serious alterations of the extracellular matrix (ECM) affecting airway wall thickness, resistance, and elasticity (3). The ECM consists of a dynamic combination of structural molecules such as collagens and glycosaminoglycans. Hyaluronic acid (HA) is a ubiquitous, naturally occurring glycosaminoglycan that is found in high concentrations in the mammalian connective tissue, including the lung (4, 5). HA is synthesized by the action of HA synthases and it is degraded by hyaluronidases (HYAL) (6, 7). There is emerging evidence that HA and its degradation products play an important role in lung pathophysiology.

The biological functions of HA depend on its molecular size. High-molecular weight HA (>1,000 kDa), which is the physiologically available form, promotes cell survival and has anti-inflammatory, anti- angiogenic and immune-suppressive effects. In contrast, low-molecular weight HA (150-350 kDa) is produced during inflammation, it promotes cell migration and has pro-inflammatory and pro-angiogenic properties (8-12).

Studies in animal models of inflammatory lung conditions and in patients with asthma suggest a potential role for high-molecular weight HA as a treatment for airway diseases (13-15). In mouse models of COPD, aerosolized HA protects endogenous HA in lung tissue against degradation by cigarette smoke-generated reactive oxygen species and reduces air space enlargement and mitigation of elastic fiber injury (13).

We have previously shown that in airway smooth muscle cells from patients with COPD, HA metabolism is impaired and characterized by decreased synthesis of HA, associated with down-regulation of HAS-1 and up-regulation of HYAL-1 (16). Furthermore, acute exacerbations of COPD are associated with increased HYAL activity in BAL and subsequent degradation of HA, which may contribute to airway inflammation and subsequent lung function decline during exacerbations (17). Besides HA, degradation products of other ECM molecules such as collagen and elastin are also associated with clinically relevant outcomes in COPD (18).

In the present study, we analyzed serum levels of HA and of HYAL-1 in two well-matched cohorts of COPD patients. We hypothesize that HA is associated with COPD progression and severity and predicts overall survival in COPD.

METHODS

Study design and participants

A two-stage study design was used to minimize unnecessary waste of patient's samples. Initial analysis was performed in a discovery cohort of 80 COPD patients with either stable disease (n=65) or at exacerbation (n=15), included in the BASCO study (Basel Study on COPD), a cross-sectional, observational, monocentric study (Figure 1). The discovery analysis was limited to assessment of serum levels of HA and was undertaken before the complete detailed analysis of a validation cohort consisting of 638 patients that were included in the PROMISE-COPD cohort (Predicting Outcome using Systemic Markers in Severe Exacerbations of Chronic Obstructive Pulmonary Disease; ISRCTN99586989) (Figure 1). The PROMISE-COPD study was an investigator-driven, multicenter, longitudinal trial, performed at 11 tertiary respiratory centers in 8 European countries. The BASCO and the PROMISE-COPD compiled with the Helsinki Declaration and GCP Guidelines and were approved by the IRB (EKBB05/06 and EKBB295/07). The PROMISE-COPD study was registered at www.controlled-trials.com (identifier ISRCTN99586989). All the patients provided prior written consent for the study assessments.

For the validation cohort, we consecutively recruited 638 patients that, at baseline, appeared with clinically stable, moderate to very severe COPD [forced expiratory volume in 1 second (FEV1) <80% of predicted value and a ratio of FEV1 to forced vital capacity (FVC) ≤0.7], based on physical examination and spirometry, at least 4 weeks after the latest exacerbation was resolved (19-21). All patients were ≥40 years old with a smoking history of ≥10 pack-years. Exclusion criteria were rapid fatal disease with death expected within 6 months, pulmonary condition other than COPD as the main respiratory disease (e.g. bronchiectasis, asthma or pulmonary fibrosis), immunosuppression including organ transplantation or chronic steroid use (>20mg prednisolone equivalent per day), muscle-skeletal or neuromuscular process preventing ambulation.

At baseline, patients underwent clinical evaluation, sputum analysis and standard spirometry after the administration of inhaled short-acting bronchodilator. The severity of obstruction and condition of the patients was graded according to the stages of disease defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) and divided in GOLD Group II-IV. Each patient underwent 6-minute walking testing and provided information on Quality of life questionnaires using the St. George's Respiratory Questionnaire (SGRQ) and the Short Form-36, (SF-36). Furthermore, a modified Medical Research Council dyspnea score

(MMRC), BODE score and age-adjusted Charlson score were performed for each patient. Comorbidities including associated medical therapy were assessed at baseline, at exacerbation and at biannual visits. Non-contrast-enhanced multi-slice CT imaging data sets were acquired in 150 patients in inspiratory breath hold. From these data sets, the lung volume was extracted automatically using an open source software (http://86.119.33.242:2000/) that was developed in house and based on image (22). From these data, lung volume and previously described histogram-based density value distributions including the low attenuation area less than –950 Hounsfield units (HU) (LAA950 [%]) and adjusted lung density (g/L) were extracted and assessed to automatically quantify emphysema (23). After the baseline visit, patients had scheduled visits every 6 months and unscheduled visits at exacerbation and 4 weeks later. The median follow-up was 24 months.

For patients included in the discovery cohort, serum samples, at stable state or at exacerbation were collected at the day of bronchoscopy. In the validation cohort, serum samples were collected at stable state, at exacerbation (moderate and severe) and at follow-up (4 weeks after exacerbation).

The diagnosis of stable COPD and of exacerbations was made according to the GOLD guidelines. According to standard definition exacerbations of COPD were diagnosed as an acute event characterized by a worsening of the patient's respiratory symptoms that is beyond normal day-to-day variations and leads to a change in the medication. Typically, patients reported a deterioration of one or more of the following typical symptoms: increased cough in frequency and severity, increased sputum production, change in sputum color, and/or increased dyspnea. COPD exacerbations were defined as moderate (requiring either antibiotics and/or parenteral corticosteroids) and severe (requiring hospitalization or resulting in death) (2).

Measurement of Hyaluronic acid and Hylauronidase-1 in the serum of COPD patients

HA was measured in aliquots of serum obtained from COPD patients of the discovery cohort at stable state and at exacerbation, and from COPD patients of the validation cohort at stable state, at exacerbation and 4 weeks after an exacerbation using an HA binding protein-based sandwich ELISA (Corgenix, Westminster, CO, USA). Intra-Assay CV:≤7.7%; Inter-Assay CV:≤8.3%.

HYAL-1 was measured in aliquots of serum, obtained from COPD patients of the validation cohort at stable state, at exacerbation and 4 weeks after an exacerbation using a sandwich ELISA (Wuhan EIAab Science, CO, China). Intra-Assay CV:≤4.3%; Inter-Assay CV:≤7.5%.

Hyaluronidase activity in serum

HYAL activity was measured in aliquots of serum (5 µl from diluted serum 1/100), obtained from COPD patients of the validation cohort, at stable state, by HA substrate zymography on 10% polyacrylamide gels containing 0.17 mg/ml HA (Sigma, St Louis, USA). Gels were washed with 0.1M HCOONa buffer, 0.03M NaCl and 2.5% TritonX-100, pH 4.0, for 1 h at room temperature and then incubated in 0.1 M formic acid buffer, 0.03 M NaCl, pH 4.0, for 20 h at 37 °C. After washing with 20% ethanol-10% acetic acid for 20 min, gels were stained with 0.5% Alcian blue in 20% ethanol and 10% acetic acid and distained with the same solution without the dye. The HA-lysis bands were quantified using the computer-assisted image analysis program of Kodak (Eastman Kodak, Rochester, NY).

Determination of markers of extracellular matrix turnover

Serum levels of markers of extracellular matrix turnover such as degradation fragments of collagens type I, type IV (α1 chain and α3 chain), type VI, pro-forms of collagens type III, type V and type VI and neutrophil elastase were measured with Nordic Bioscience assays, as previously described (18, 24).

Statistical analysis

For data analysis Statistical Package for Social Sciences (SPSS®, Chicago, IL) for Windows, version 23.0 was used. Details are described in the on-line supplement.

Results

Patients

The baseline demographic and clinical characteristics of the 80 COPD patients from the BASCO study that were included in the discovery cohort are shown in supplementary Table 1.

This cohort includes patients with relevant smoking history, severe obstruction, mild hyperinflation, marked impairment of the diffusion capacity and significant emphysema. All patients were adequately treated.

From the 638 patients that were recruited in the validation cohort, a total of 506 patients attended a scheduled visit at 6 months and where therefore eligible for inclusion in subsequent analyses (Figure 1). The baseline demographic and clinical characteristics of the patients included in the validation cohort are shown in Table 1. Both cohorts were well-matched. Most patients were male, had a considerable smoking history, clinically relevant disease (at least one exacerbation requiring physician attention in the previous year) and multiple comorbidities (Supplementary Table 2). At the stable state visit, 155 patients (30.6%) were able to produce sputum of good quality for microbiological analysis. From those, 48 samples (30.9%) evidenced potentially pathogenic bacteria growth. Patients were followed during a median of 722 [395-762] days. During the follow-up, 308 patients (60.9%) suffered from at least one exacerbation and 38 patients (7.5%) deceased.

Patients that had an exacerbation performed an unscheduled visit and were sampled within 2 [1-5] days after the event (21.8% of the patients were sampled on the same day, 16.1% of patients were sampled 1 day after the AECOPD; 14.0% of the patients were sampled the second day; 10.1% of patients were sampled the third day, 8.3% of the patients were sampled 4 days after the event and 5.7% of patients were sampled the 5th day).

The clinical outcomes within the 2 years follow up period, are shown in supplementary Table 3. From baseline, median time to exacerbation was 338 days and to severe exacerbation was 701 days.

Hyaluronic acid is increased during COPD exacerbations

Serum levels of HA were measured in the discovery cohort of 80 patients with COPD. Among these patients, 65 patients had stable disease and 15 patients had an exacerbation. Concentration of HA in serum was significantly higher in COPD patients at exacerbation as compared with patients at stable state ($p=0.015$) (Figure 2A).

Subsequently, we evaluated HA serum concentrations in the validation cohort of 506 patients with COPD. HA was measured at stable state, at exacerbations (moderate and severe) and at follow-up (4 weeks after exacerbation). HA was significantly increased at moderate and severe exacerbations, as compared with the stable state of the disease ($p<0.001$, for both

comparisons) (Figure 2B). At follow-up, serum levels of HA remained significantly higher as compared with serum levels at stable state ($p<0.001$).

Correlation analysis of HA serum levels and patient's baseline characteristics, markers of inflammation and disease outcomes

HA levels in the serum of COPD patients at stable state were positively correlated with the age of patients ($p<0.001$), with post bronchodilatation FEV_1% predicted ($p=0.002$), with FEV_1/FVC ($p=0.025$) and with the age-adjusted Charlson score ($p=0.001$). Regarding markers of inflammation, HA serum levels were significantly correlated with adrenomedullin ($p=0.001$) and with atrial natriuretic peptide ($p<0.001$) (Table 2). There was no significant correlation of serum levels of HA with body mass index, smoking history, Borg dyspnea scale or walking distance. Furthermore, there was no significant correlation of HA with diffusion capacity or with any of the quantitative CT scan parameters, indicating that HA is not related to emphysema.

Furthermore, HA was significantly associated ($p<0.001$) with time to death (HR: 1.019; 95% CI: 1.009-1.029). Cox-regression multivariate analysis revealed that this association was independent of adjusted Charlson score, annual exacerbation rate and BODE index components (Table 3).

Subsequently, in an exploratory analysis, we used linear mixed models to test the association between various clinical characteristics at baseline and the change of HA serum levels between stable state and moderate or severe exacerbations. Among 102 baseline predictors tested, post bronchodilation FEV_1% predicted, was a significant predictor ($p=0.0186$) for HA increase at moderate exacerbations (Supplementary Table 4). Adjusted and unadjusted Charlson scores were significant predictors ($p=0.0275$ and $p=0.0328$, respectively) for HA increase at severe exacerbations (Supplementary Table 4).

At exacerbation, HA serum levels were not significantly correlated with markers of systemic inflammation such as leucocyte counts and CRP (Supplementary Table 5).

HA serum levels and sputum bacteriology

At stable state, 155 patients could produce sputum of good quality for microbiological analysis that revealed potential pathogenic bacteria growth in 48 patients. Serum HA levels were similar in patients with positive cultures of potential pathogenic bacteria in sputum (21.3

ng/ml [13.8-39.9], median [IQR]) compared with patients without growth of potential pathogenic bacteria in sputum (19.0 ng/ml [6.5-35.3], median [IQR]) (p=0.076). Similarly, there were no significant differences in HA serum levels at exacerbation between patients with positive sputum cultures and patients with negative sputum cultures at moderate exacerbations (26.1 ng/ml [20.8-46.3] vs 34.3 ng/ml [21.6-59.1], median [IQR], p=0.575) and at severe exacerbations (44.5 ng/ml [28.8-83.6] vs 83.2 ng/ml [44.3-104.2], median [IQR], p=0.362).

Hyaluronidase-1 in the serum of patients with COPD

Since the biological effects of HA are determined by its molecular weight, we further investigated the enzymatic activity, as well as protein levels of HYAL-1, which is the main enzyme that degrades HA, in serum. Enzymatic activity of HYAL in the serum of COPD patients, at stable state, was evident as a single lysis band that migrated with a molecular mass of 57 kDa corresponding to HYAL-1 (Figure 3A). Quantification of the enzymatic lysis bands from serum of COPD patients from the validation cohort (n=506) revealed that HYAL-1 enzymatic activity was inversely correlated with post bronchodilation FEV_1% predicted (rho=-0.109, p=0.034) and with survival time (rho=-0.118, p=0.017) (Figure 3B-C). There was no significant correlation of HYAL-1 activity with age, body mass index, smoking history, FEV_1/FVC, Borg Dyspnea scale or walking distance (Supplementary Table 6).

HYAL-1 levels in serum of COPD patients at stable state were significantly correlated with age, with adjusted and unadjusted Charlson score (p<0.001 for all) and inversely correlated with walking distance (p=0.001). Regarding markers of inflammation, HYAL-1 serum levels were significantly correlated with procalcitonin (p=0.011), with adrenomedullin (p<0.001), with copeptin (p=0.004) and with atrial natriuretic peptide (p<0.001) (Table 4). There was no significant correlation of HYAL-1 with diffusion capacity or with any of the quantitative CT scan parameters, indicating that enzymatic degradation of HA is not related to emphysema.

HYAL-1 serum levels were significantly increased at moderate exacerbations (p=0.004) and severe exacerbations (p=0.003) (Figure 4). At follow-up, 4 weeks after exacerbations, HYAL-1 levels were significantly decreased to control levels (p<0.001 for moderate and for severe exacerbations).

Effect of treatment on serum levels of HA and HYAL-1

We have further explored whether various treatments may alter serum levels of HA and HYAL-1 in COPD patients at stable state. As shown in Table 5, HA serum levels did not differ between patients under treatment with inhaled corticosteroids (ICS) (21.7 [10.1-38.8] ng/ml) and patients that were not receiving ICS (22.6 [13.1-44.5] ng/ml) (p=0.437). Furthermore, there were no significant differences in HA serum levels between patients that were receiving and patients that were not receiving treatment with systemic corticosteroids (20.0 [9.0-46.9] ng/ml vs 22.3 [10.3-38.8] ng/ml) (p=0.846) or with the combination of long acting β2 agonists and corticosteroids (21.2 [9.4-38.1] ng/ml vs 23.3 [13.6-44.2] ng/ml (p=0.093). Similarly, long acting muscarinic antagonists did not affect HA serum levels (21.2 [9.7-38.0] ng/ml vs 25.2 [12.1-41.2] ng/ml) (p=0.324).

Even when we adjusted for FEV1 % of predicted value, we were not able to detect any significant difference in HA serum levels between patients that were receiving or not receiving any of the above treatments. In addition, the use of systemic corticosteroids at moderate exacerbations was not associated significantly with HA levels after 4 weeks follow-up as revealed by analysis in paired samples (p=0.458).

Similarly, HYAL-1 serum levels did not differ between patients under treatment with inhaled corticosteroids (ICS) (1.7 [1.2-2.4] ng/ml) and patients that were not receiving ICS (1.6 [1.1-2.4] ng/ml) (p=0.632) (Table 5). Furthermore, there were no significant differences in HYAL-1 serum levels between patients that were receiving and patients that were not receiving treatment with systemic corticosteroids (1.7 [1.4-2.6] ng/ml vs 1.7 [1.2-2.4] ng/ml) (p=0.637) or with the combination of long acting β2 agonists and corticosteroids (1.7 [1.2-2.5] ng/ml vs 1.7 [1.1-2.4] ng/ml) (p=0.640). Similarly, long acting muscarinic antagonists did not affect HYAL-1 serum levels (1.7 [1.2-2.4] ng/ml vs 1.7 [1.1-2.5] ng/ml) (p=0.936).

Even when we adjusted for FEV1 % of predicted value, we were not able to detect any significant difference in HYAL-1 serum levels between patients that were receiving or not receiving any of the above treatments.

Association of HA and HYAL-1 with biomarkers of extracellular matrix turnover

We further investigated the association of HA and HYAL-1 with molecules reflecting ECM turnover, including fragments of collagens type I, III, IV, and VI, pro-forms of collagens type III, V and VI and neutrophil elastase-generated fragments of elastin (Table 6). Serum levels of HA were significantly correlated with serum levels of pro-forms of collagen type III and type VI ($p<0.001$ for both).

HYAL-1 levels were significantly associated with the pro-form of collagen type III and the degradation fragments of collagen type 6 ($p=0.012$) and the α3 chain of collagen type IV ($p=0.020$).

DISCUSSION

In the present study, we investigated serum levels of HA in two independent, well-matched cohorts of COPD patients and we report that HA is associated with COPD severity and outcome. To our knowledge, this is the first study to show that HA and its degradation products resulting from the action of HYAL-1, may play a role in COPD and that disease activity and subsequent outcome can be envisaged through detection of circulating HA. The current analysis provides new insights into the pathophysiology of COPD.

HA is a naturally occurring component of the ECM and it participates in a number of biological processes such as cell migration and proliferation, water homeostasis, tissue repair, inflammation, cell-matrix signalling and angiogenesis (25). HA has exceptional hydrophilic characteristics and can bind approximately a thousand times its weight in water. Therefore, in the ECM, HA produces highly viscous gels that play an essential role in tissue homeostasis and biomechanical integrity (26). HA can bind to specific cell-surface receptors and induce intracellular signal transduction but also creates a matrix coat around the cells protecting them from the environment.

In the present study, we provide evidence that serum levels of HA, at stable state, were significantly associated with time to death independently of adjusted Charlson score, annual exacerbation rate and BODE index components. It has been shown that HA serum levels are elevated in patients with dyslipidaemia and high cardiovascular risk (27). In our study, patients included in the validation cohort had numerous comorbidities among which arterial hypertension and coronary arterial disease. The fact, however, that the association of HA with time to death was independent of age-adjusted Charlson score warrants that the observed association of HA with COPD outcome is not an epiphenomenon and that circulating HA in COPD patients can serve as an independent predictor for survival.

Accurate prediction of mortality is very important to identify COPD patients who would benefit by the implementation of intensified therapeutic measures, such as lung transplantation (28). Several studies have attempted to find predictors of survival for COPD patients and explored the association of clinical characteristics with specific biomarkers of disease activity and/or progression (29, 30). Several serum biomarkers have been described to be

independently associated with increased risk of death (31, 32). A few biomarkers have been shown to further contribute to predict mortality when added to known clinical scores such as the BODE index (29, 33, 34). Since COPD is a complex and heterogeneous disease with various phenotypes, a panel of prognostic biomarkers reflecting the associated comorbidities and different pathobiological pathways that may be altered in this disease has been proposed (30, 32, 35). Simultaneously elevated levels of adrenomedullin, arginine vasopressin and atrial natriuretic peptide have been shown to be associated with increased risk of death in patients with stable COPD (35). In the present study, we provide evidence that HA is significantly corelated with adrenomedullin and atrial natriuretic peptide, and that HYAL-1 is corelated with adrenomedullin, copeptin, procalcitonin and atrial natriuretic peptide. To our knowledge, this is the first study showing a correlation of HA and its degrading enzymes with these COPD prognostic biomarkers. It remains to be elucidated whether the concomitant estimation of HA and HYAL-1 together with well-established mortality predictors would contribute to increase the accuracy for the prediction of mortality.

We have previously shown that increased HA degradation in the BAL of COPD patients is associated with airway obstruction and not with emphysema (17). The lack of a significant association between serum levels of HA and HYAL-1 with diffusion capacity or with quantitative CT scan parameters representing the status of lung parenchyma that we observe in the present study, is a further indication that HA and HYAL-1 are not related to emphysema. It remains to be elucidated whether HA degradation is associated with destruction of the terminal and transitional bronchioles that has been recently shown to represent the initial site of injury in COPD even at an early stage of the disease (36, 37). However, such an analysis would require a novel multiresolution CT imaging protocol, not available in the present study.

In the lung, HA is a dynamic molecule with physiological functions that are closely related to its molecular weight. There is evidence that low-molecular-weight HA that is produced during tissue injury, increases inflammatory responses whereas the physiologically occurring high-molecular weight HA is protective and has anti-inflammatory effects (10-12). Here, we provide evidence, for the first time, that HYAL activity in the serum of 506 COPD patients was negatively correlated with FEV_1% predicted and with survival time. These results indicate that enzymatic degradation of physiologically occurring high-molecular weight HA in COPD patients is associated with airflow limitation, impairment of lung function and time to death. In agreement with these data, previous studies have shown that short fragments of HA were detected in the bronchoalveolar lavage fluid (7, 17, 38-40) and in the parenchyma (7) in a number of lung diseases, indicating that HA turnover is a major component for the development, progression and resolution of inflammatory lung diseases (7, 17).

Besides HA, degradation products of other extracellular matrix molecules are also associated with clinically relevant outcomes in COPD. Pro-forms and fragments of collagens have been shown to play a significant role in COPD severity and disease outcome (18, 24). In the present study, we show that serum levels of HA were significantly associated with pro-collagens type III and VI, the major collagens found in the lung interstitium and in large bronchi. HYAL-1 serum levels were associated with pro-collagen type VI as well as with the degradation products of collagens type VI and collagen type VI that is mainly found in the basement membranes. These results indicate that HA, in line with collagens, is a part of the increased extracellular matrix turnover in COPD, a process that determines disease severity and clinically relevant outcomes.

Exacerbations of COPD are important events in the natural history of the disease as they impact on disease progression, health status and survival. Therefore, there is an unmet need to understand the pathophysiological mechanisms involved in exacerbations of COPD and to identify molecules that are associated with airflow limitation during exacerbations. In the present study, we provide evidence that serum levels of HA are significantly increased at moderate and severe exacerbations of COPD, as compared to the stable state of the disease. HA levels at exacerbation were not related to bacterial infections or to markers of acute inflammation such as leucocyte counts and c-reactive protein at exacerbations. Concomitantly, HYAL-1 serum levels are also increased at exacerbation, indicating that COPD exacerbations are associated with increased degradation of HA leading to the formation of short HA fragments. In the last 2 decades extensive research has shown that signalling by short HA fragments contributes to the accumulation of immune cells in inflammatory sites (41-43) and subsequent release of proinflammatory cytokines and metalloelastases (44, 45).

Another interesting finding in our study was the fact that 4 weeks after exacerbations, HA serum levels remained significantly higher compared to the stable state of the disease, even though HYAL-1 levels were decreased to stable state levels. Therefore, it is tempting to hypothesise that the termination of the action of HYAL-1 precedes the removal of the short fragments of HA from the circulation.

Our study has a few limitations. Measurements of HA serum concentration by ELISA do not provide information about the size of HA. Unfortunately, up to date, there is no appropriate methodology to estimate the size of such a large glycosaminoglycan in serum samples. Thus, parallel measurements of HYAL-1, which is the main hyaluronidase detected in human serum, provide the best indication for the size of HA. Another limitation of our study is that HA serum levels could be modified by treatment since it has been shown that HA turnover is altered by

glucocorticoids and LABA (46-48) and the majority of COPD patients that participated in the present study were under treatment with ICS (81% of the patients) or with the combination of ICS and LABA (61.3% of the patients). However, when we stratified the analysis of HA and HYAL-1 according to treatment, even if the groups that were receiving treatment and the groups that were not receiving treatment, were not balanced for the number of patients, we could not observe any significant differences in HA or HYAL-1 levels between the groups receiving ICS, systemic corticosteroids or ICS and LABA and the groups that were not receiving any treatment.

A major strength of our study is the large sample size and the comprehensive clinical characterization of the patients that enabled us to establish that the association of HA with the time to death was independent of adjusted Charlson score, annual exacerbation rate and BODE index components. A further strength relates to the two-stage design with two well-matched cohorts. The discovery cohort was designed to maximize the chances to establish HA as a potential molecule associated with disease severity and outcome and to minimize unnecessary waste of patient samples. Additional strengths are the longitudinal sampling undertaken at three points in time (at stable state, at exacerbation and at follow-up) and the longitudinal clinical data that enabled us to determine disease outcomes.

In conclusion, the results of our study indicate that HA is associated with COPD severity and outcome and predicts overall survival. During COPD exacerbations, enzymatic degradation of HA is associated with airflow limitation and impairment of lung function. Thus, HA and its degrading enzyme HYAL-1 may be proved to serve as potential targets to control airway inflammation and remodeling in COPD.

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Figure legends

Figure 1. Study design. A discovery cohort of 80 patients was drawn from the cohort of the BASCO study. The validation cohort comprised of 638 patients that were included in the PROMISE study. COPD: chronic obstructive pulmonary disease; GOLD: Global Initiative for Chronic Obstructive Lung Disease; GOLD classification II to IV: moderate to severe COPD.

Figure 2. Hyaluronic acid in the serum of COPD patients. Serum concentration of HA was measured by ELISA in [A] 80 participants of the discovery cohort; 65 patients were at stable state and 15 patients had an exacerbation of COPD; [B] 506 participants of the validation cohort at stable state (n=506), at moderate exacerbation (n=219), at severe exacerbation (n=88) and at 4 weeks follow-up after an exacerbation (n=92). All measurements were made in duplicates.

Figure 3. Hyaluronidase activity in serum samples of COPD patients. Enzymatic activity of hyaluronidase (HYAL) was assessed in serum samples (5 µl from diluted serum 1:100) of COPD patients at stable state (n=506) from the validation cohort by hyaluronic acid substrate zymography. [A] Representative substrate (HA) zymography of serum samples obtained from 9 patients with COPD at stable state. Lysis band migrated at 57 kDa corresponds to HYAL-1 activity. [B-C] Correlation (Spearman's rho) between values of HYAL-1 activity at stable state quantitated by the computer-assisted image analysis program of Kodak and FEV_1% of predicted value [B] and survival time [C].

Figure 4. Hyaluronidase-1 in the serum of patients from the validation cohort. Serum concentration of hyaluronidase-1 was measured by ELISA in participants of the validation cohort at stable state (n=506), at moderate exacerbation (n=219), at severe exacerbation (n=88) and at 4 weeks follow-up after an exacerbation (n=92). All measurements were made in duplicates.

Table 1. Baseline demographic and clinical characteristics of 506 patients included in the validation cohort (PROMISE study)

Characteristics	
Age, years (mean ± SD)	66.8 ± 10.5
Male, n (%)	366 (71.9)
Weight, kg (mean ± SD)	74.9 ± 17.0
Height, cm (mean ± SD)	169.0 ± 8.0
BMI, kg/m^2 (mean ± SD)	26.2 ± 5.5
COPD history	
Current smokers, n (%)	150 (29.6)
Pack-years (mean ± SD)	51.5 ± 30.9
Duration of COPD symptoms, months (mean ± SD)	102.7 ± 89.1
Time elapse since diagnosis, months (mean ± SD)	80.2 ± 74.6
Number of exacerbations in previous year (median IQR)	1 [0-1]
Number of severe exacerbations in previous year (median IQR)	0 [0-1]
MMRC grade (median IQR)	1 [1-2]
6-minute walking distance, m (mean ± SD)	380.3 ± 104.2
BODE index (median IQR)	3 [1-4]
Borg Score	4 [3-6]
SaO_2 at rest, % (mean ± SD)	94.5 ± 2.7
Lowest SaO_2 at exercise, % (mean ± SD)	89.5 ± 5.7
Age-adjusted Charlson score (mean ± SD)	4 ± 1.8
SGRQ	
Symptoms score (mean ± SD)	49.0 ± 22.7
Activity score (mean ± SD)	57.4 ± 22.8
Impact score (mean ± SD)	32.2 ± 18.7
Total Score (mean ± SD)	42.4 ± 18.1
SF-36	
Physical function (mean ± SD)	51.4 ± 25.9
Role Physical (mean ± SD)	51.7 ± 43.5
Role emotional (mean ± SD)	67.5 ± 42.9
Social functioning (mean ± SD)	69.8 ± 28.2
Mental health (mean ± SD)	65.4 ± 19.8
Body pain (mean ± SD)	73.9 ± 27.6
Vitality (mean ± SD)	51.9 ± 20.9
General Health (mean ± SD)	48.2 ± 23.1
GOLD grade, n (%)	
II	254 (50.2)
III	177 (34.9)
IV	75 (14.8)

Lung function parameters	
FVC, post-brd, % predicted (mean ± SD)	77.8 ± 24.8
FEV_1, post-brd, % predicted (mean ± SD)	48.6 ± 18.2
FEV_1/FVC post-brd % (mean ± SD)	48.2 ± 14.1
Residual volume (RV), % (mean ± SD)	157.4 ± 45.6
Total lung capacity (TLC), % (mean ± SD)	118.8 ± 20.2
RV/TLC % (mean ± SD)	53.7 ± 9.6
Diffusion capacity (D_{LCO}), % (mean ± SD)	55.6 ± 20.7
Quantitative CT scan parameters (n=150)	
Low-attenuation area less than -950 HU, % (median, 25%-75% percentile)	13.8 (5.0 - 26.1)
Adjusted lung density, g/L (median, 25%-75% percentile)	53.5 (29.0 – 76.0)
Lung volume, L (median, 25%-75% percentile)	5.4 (4.5 – 6.5)
Positive sputum bacteriology n (%)	50/155 (32.2%)

Abbreviations: COPD: chronic obstructive pulmonary disease; SD: standard deviation; BMI: body mass index; MMRC: Modified Medical Research Council; BODE index: body mass, airflow obstruction, dyspnea, exercise capacity index; GOLD: Global Initiative for Chronic Obstructive Lung Disease; FEV_1: forced expiratory volume in 1 sec; FVC: forced vital capacity; post-brd: post-bronchodilated; IQR = Interquartile Range; mMRC = modified Medical Research Council; SaO_2 = Peripheral oxygen saturation; SF-36 = Short Form-36; SGRQ = St. George's Respiratory Questionnaire; ABGA= arterial blood gas analysis; HU = Hounsfield units. [a] GOLD grades are based on FEV_1 % predicted: II, ≥ 50% <80%; III ≥ 30%<50%; IV, ≤ 30%.

Table 2. Correlation analysis (Spearman's rho) of hyaluronic acid in serum of COPD patients at stable state (n=506), included in the validation cohort, with baseline parameters

Parameter	**rho**	**p value**
Age	0.233	<0.001
Body mass index	0.055	0.216
Packs per year	-0.078	0.086
FEV_1 % predicted, post-brd	0.143	0.002
FEV_1/FVC post-brd	0.103	0.025
Adjusted Charlson score	0.149	0.001
Borg Dyspnea Scale	0.012	0.800
6-minute walking distance	-0.027	0.556
Survival time	-0.139	<0.001
Diffusion capacity	0.112	0.135
CT scan quantitative parameters		
LAA-950 HU, %	-0.037	0.687
Adjusted lung density, g/L	0.012	0.899
Lung volume, L	-0.016	0.865
Inflammation markers		
Procalcitonin, μg/L	0.023	0.610
Adrenomedullin, nmol/L	0.143	0.001
Copeptin, pmol/L	0.053	0.239
Atrial natriuretic peptide, pmol/L	0.173	<0.001

Abbreviations: FEV_1: forced expiratory volume in 1 sec; FVC: forced vital capacity; post-brd: post-bronchodilatation; LAA-950 HU: low-attenuation area less than -950; HU: Hounsfield units.

Table 3. Multivariate cox regression analysis for time to death among the study population included in the validation cohort

Variable	HR (95% CI)	p value
Hyaluronic acid	1.019 (1.009-1.029)	<0.001
Adjusted Charlson score	1.207 (0.961-1.547)	0.105
Annual exacerbation rate	1.306 (1.037-1.644)	0.023
BODE index	1.268 (1.086-1.482)	0.003

Table 4. Correlation analysis (Spearman's rho) of hyaluronidase-1 in serum of COPD patients at stable state (n=506), included in the validation cohort, with baseline parameters

Parameter	**rho**	**p value**
Age	0.298	<0.001
Body mass index	0.068	0.385
Packs per year	0.028	0.724
FEV_1 % predicted, post-brd	0.087	0.278
Adjusted Charlson score	0.370	<0.001
Charlson score	0.311	<0.001
Borg Dyspnea Scale	0.054	0.510
6-minute walking distance	-0.275	0.001
Survival time	-0.137	0.080
Diffusion capacity	0.068	0.687
CT scan quantitative parameters		
LAA-950 HU, %	-0.048	0.799
Adjusted lung density, g/L	0.114	0.541
Lung volume, L	0.263	0.152
Inflammation markers		
Procalcitonin, µg/L	0.200	0.011
Adrenomedullin, nmol/L	0.298	<0.001
Copeptin, pmol/L	0.227	0.004
Atrial natriuretic peptide, pmol/L	0.298	<0.001

Abbreviations: FEV_1: forced expiratory volume in 1 sec; post-brd: post-bronchodilatation; LAA-950 HU: low-attenuation area less than -950; HU: Hounsfield units.

Table 5. Serum levels of hyaluronic acid and hyaluronidase-1 dichotomized according to treatment at baseline

Medication at baseline		Hyaluronic acid (ng/ml) Median [IQR]	P value	Hyaluronidase-1 (ng/ml) Median [IQR]	P value
Inhaled corticosteroids:	Yes	21.7 [10.1 – 38.8]	0.437	1.7 [1.2 – 2.4]	0.632
	No	22.6 [13.1 – 44.5]		1.6 [1.1 – 2.43]	
Systemic corticosteroids:	Yes	20.0 [9.0 – 46.9]	0.846	1.7 [1.4 – 2.6]	0.637
	No	22.3 [10.3 – 38.8]		1.7 [1.2 – 2.4]	
Long acting β2 agonists + corticosteroids:	Yes	21.2 [9.4 – 38.1]	0.093	1.7 [1.2 – 2.5]	0.640
	No	23.3 [13.6 – 44.2]		1.7 [1.1 – 2.4]	
Long acting muscarinic antagonists:	Yes	21.2 [9.7 – 38.0]	0.324	1.7 [1.2 – 2.4]	0.936
	No	25.2 [12.1 – 41.2]		1.7 [1.1 – 2.5]	

IQR: Interquartile range (25^{th}-75^{th} percentile)

Table 6. Correlation analysis (Spearman's rho) of hyaluronic acid and hyaluronidase-1 in serum of COPD patients at stable state (n=506), included in the validation cohort, with biomarkers of extracellular matrix turnover

Parameter	Hyaluronic acid		Hyaluronidase-1	
	rho	p value	rho	p value
Pro-collagen type III	0.204	<0.001	0.131	0.104
Pro-collagen type V	-0.057	0.251	0.060	0.490
Pro-collagen type VI	0.182	<0.001	0.270	0.001
Collagen type I degradation products	-0.047	0.349	0.002	0.979
Collagen type III degradation products	-0.001	0.979	0.120	0.136
Collagen type IV degradation products (α1 chain)	-0.035	0.482	0.053	0.550
Collagen type IV degradation products (α3 chain)	-0.063	0.206	0.203	0.020
Collagen type VI degradation products	-0.015	0.738	0.201	0.012
Neutrophil Elastase-generated fragments of elastin	-0.004	0.932	0.114	0.158

Figure 1

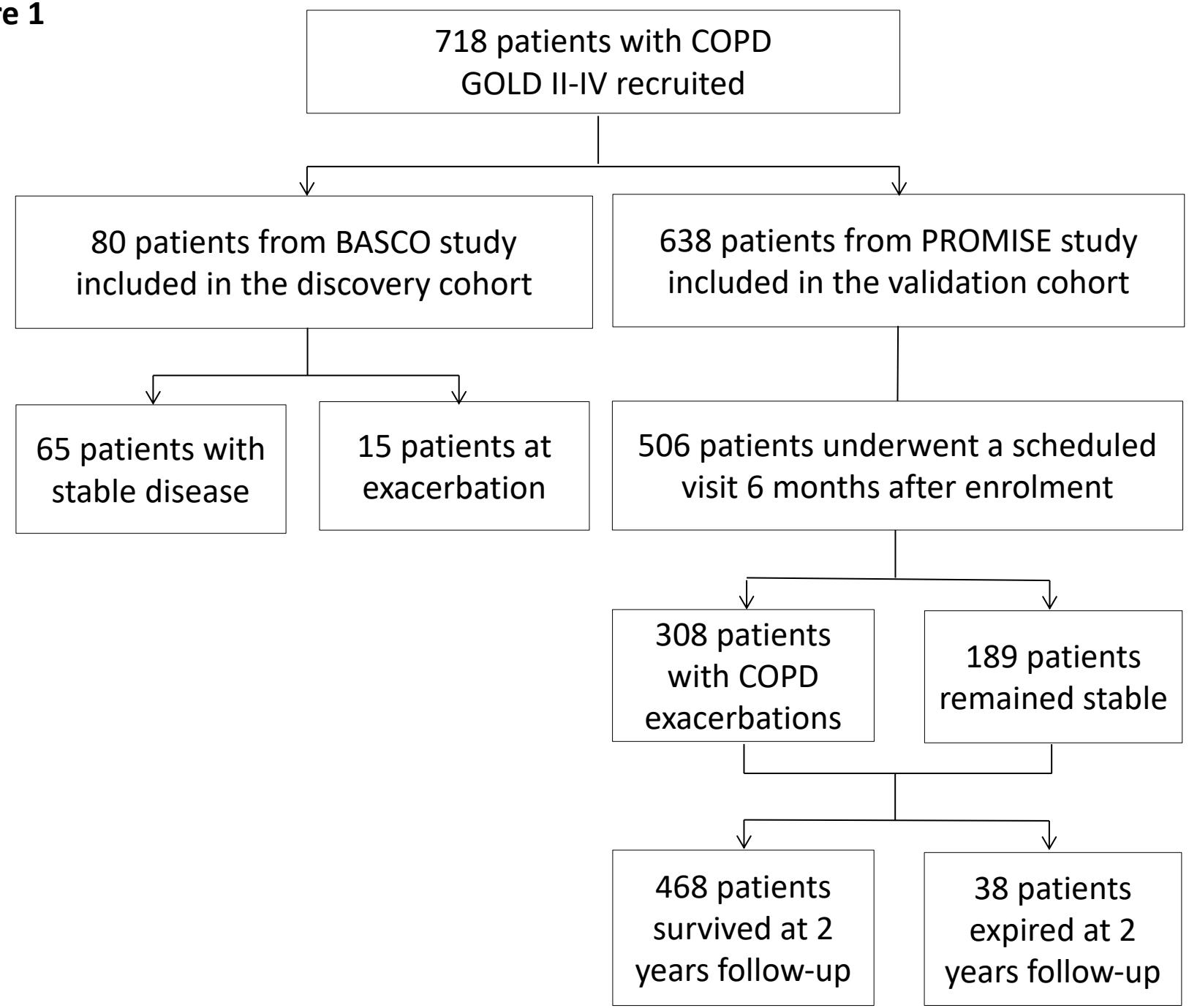
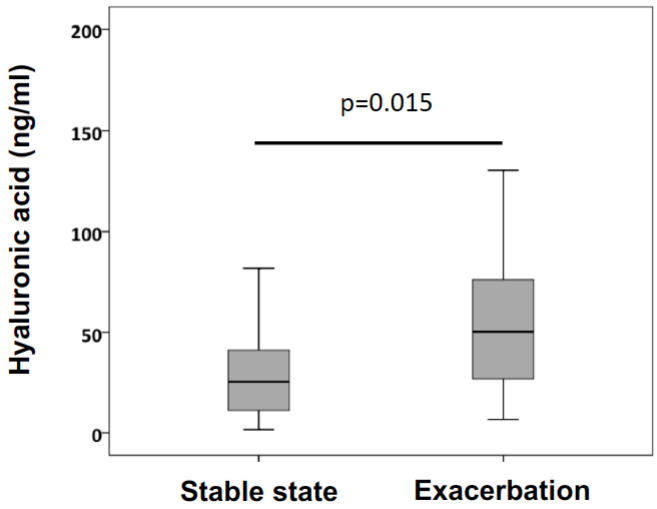

718 patients with COPD GOLD II-IV recruited
80 patients from BASCO study included in the discovery cohort
638 patients from PROMISE study included in the validation cohort
65 patients with stable disease
15 patients at exacerbation
506 patients underwent a scheduled visit 6 months after enrolment
308 patients with COPD exacerbations
189 patients remained stable
468 patients survived at 2 years follow-up
38 patients expired at 2 years follow-up

Figure 2

[A]


Hyaluronic acid (ng/ml)
200
150
100
50
0
p=0.015
Stable state
Exacerbation

[B]

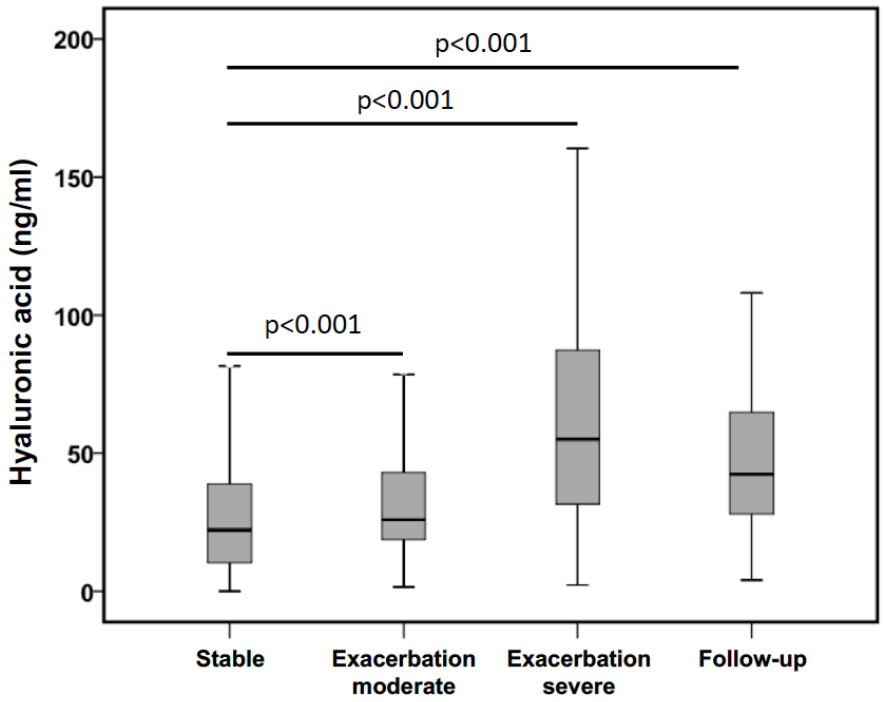

Hyaluronic acid (ng/ml)
200
150
100
50
0
p<0.001
p<0.001
p<0.001
Stable
Exacerbation moderate
Exacerbation severe
Follow-up

Figure 3

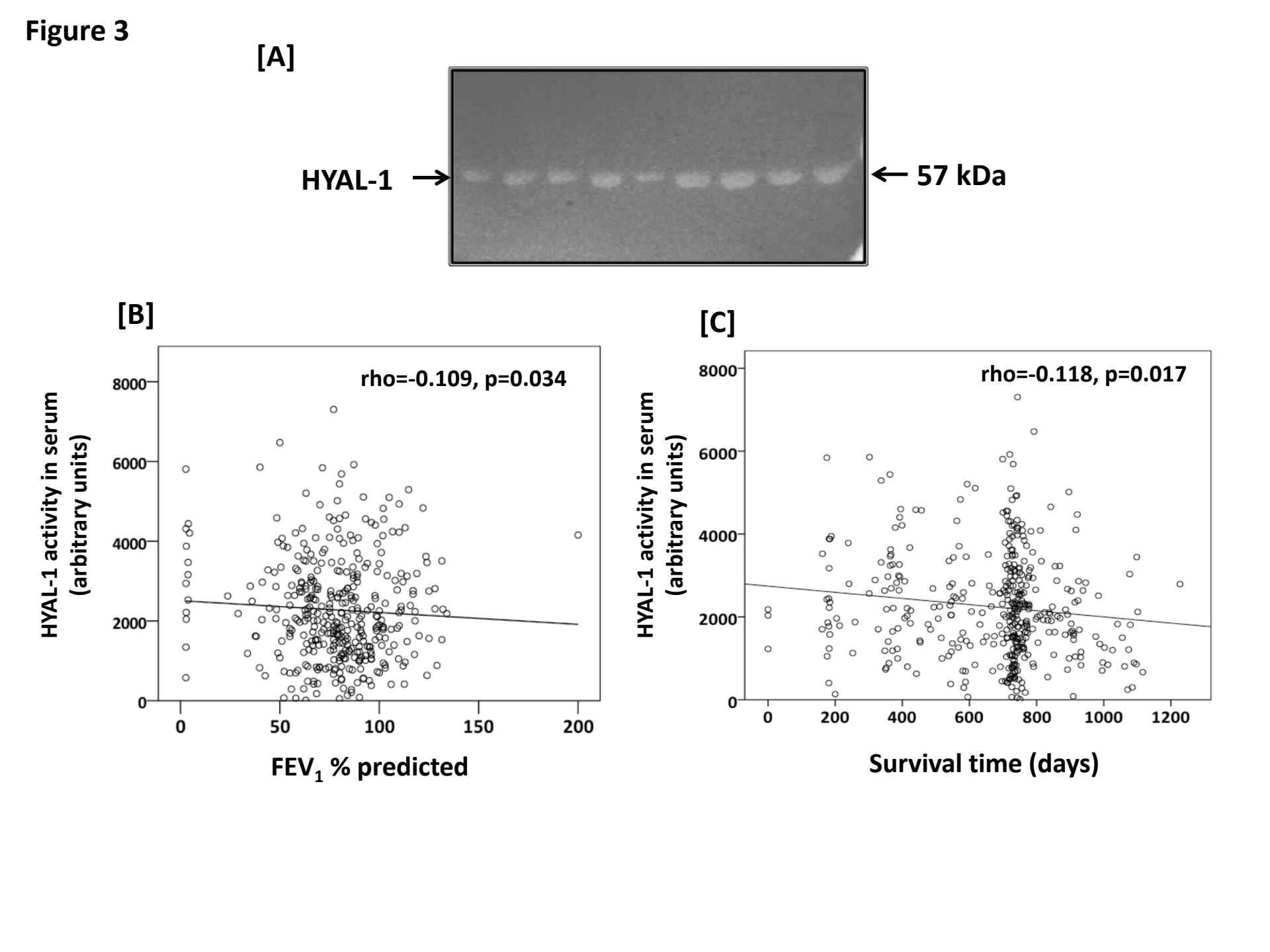
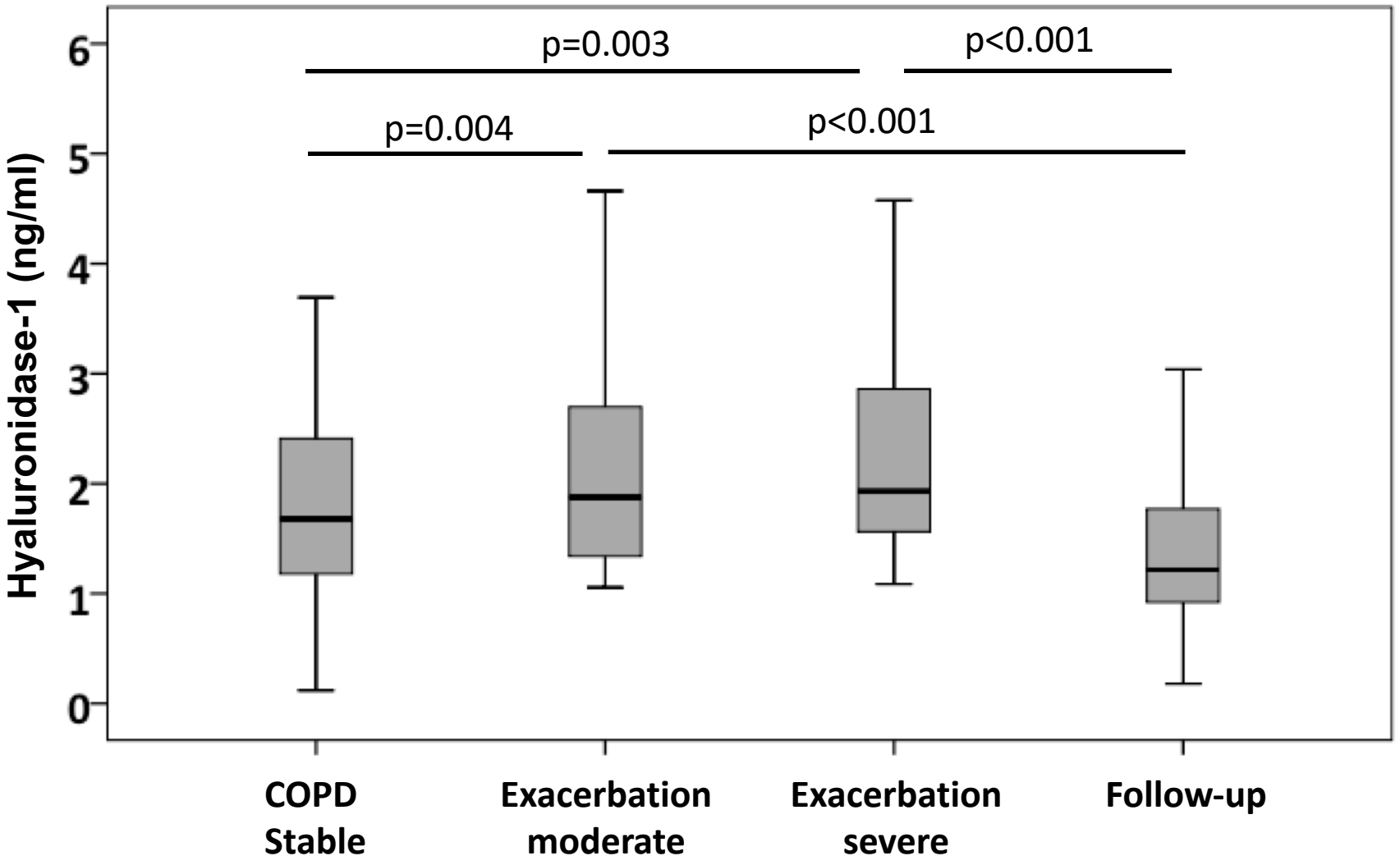

[A]
HYAL-1
57 kDa
[B]
rho=-0.109, p=0.034
HYAL-1 activity in serum (arbitrary units)
FEV1 % predicted
[C]
rho=-0.118, p=0.017
HYAL-1 activity in serum (arbitrary units)
Survival time (days)

Figure 4


p=0.003
p<0.001
p=0.004
p<0.001
Hyaluronidase-1 (ng/ml)
6
5
4
3
2
1
0
COPD Stable
Exacerbation moderate
Exacerbation severe
Follow-up

Statistical analysis

For data analysis Statistical Package for Social Sciences (SPSS®, Chicago, IL) for Windows, version 23.0 was used. Normal distribution of all dependent variables was checked using Shapiro-Wilk analysis (for samples <50 participants) or Kolmogorov-Smirnov analysis (for samples >50 participants). Chi-square test was used for the comparison of percentages, whereas means were compared with the use of independent samples *t*-test or Mann-Whitney *U* test for non-parametric data. Two-tailed levels of significance were used in all statistical calculations. One-way ANOVA or Kruskal-Wallis analysis was used for the comparison of means of dependent continuous variables when data were divided in ≥3 groups. To evaluate the relationship among different parameters we used a multivariate Cox regression model analysis. Correlation analyses were performed with Pearson product-moment correlation coefficient or Spearman's Rank Order Correlation for non-parametric data. Putative baseline predictors of a change in the level of HA and HYAL-1 after moderate or severe exacerbations were assessed using linear mixed-effects models. This exploratory analysis was performed using the R statistical software version 3.4.2 with the extension package 'lme4'.

Supplementary Table 1. Baseline demographic and clinical characteristics of 80 patients with COPD included in the discovery cohort (BASCO study)

Characteristics	**n=80**
Age, years (mean ± SD)	68.9 ± 8.9
Male, n (%)	49 (61.2)
Current smokers, n (%)	30 (37.5)
Pack-years (mean ± SD)	57.2 ± 28.5
GOLD stage, n (%)	
II	42 (52.5)
III	24 (30.0)
IV	14 (17.5)
Lung function parameters	
FVC, post-brd, % predicted (mean ± SD)	85.8 ± 16.3
FEV_1, post-brd, % predicted (mean ± SD)	50.1 ± 19.4
FEV_1/FVC post-brd % (mean ± SD)	40.9 ± 12.4
Residual volume (RV), % (mean ± SD)	166.4 ± 50.4
Total lung capacity (TLC), % (mean ± SD)	117.1 ± 20.8
RV/TLC % (mean ± SD)	55.3 ± 10.1
Diffusion capacity (D_{LCO}), % (mean ± SD)	52.3 ± 20.1

Abbreviations: SD: standard deviation; GOLD: Global Initiative for Chronic Obstructive Lung Disease; FEV_1: forced expiratory volume in 1 sec; FVC: forced vital capacity; post-brd: post-bronchodilation; FVC= forced vital capacity; ABGA= arterial blood gas analysis . [a] GOLD stages are based on FEV_1 % predicted: II: ≥ 50% <80%; III: ≥ 30% <50%; IV: ≤ 30%.

Supplementary Table 2. Comorbidities of the 506 patients included in the validation cohort

Comorbidities	**N=506, number (%)**
Arterial Hypertension	256 (50.6)
Coronary arterial disease	114 (22.5)
Congestive heart failure	76 (15.0)
Diabetes mellitus	62 (12.3)
Dementia	8 (1.6)
Depression	71 (14.0)
Malignancy	31 (6.1)
Myocardial infarction	49 (9.7)
Pulmonary hypertension	50 (9.9)
Renal failure	43 (8.5)

Supplementary Table 3. Clinical outcomes within the 2 years follow up period for the COPD patients in the validation cohort

Clinical outcome	**Median (interquartile range)**
Exacerbations per year	0.72 (0-1.8)
Severe exacerbations per year	0 (0-0)
Time to exacerbation, days	338 (135-697)
Time to severe exacerbation, days	701 (342-748)
Time to death, days	722 (395-762)

Supplementary Table 4. Association of various predictors at baseline with HA changes between stable state and moderate or severe exacerbations

Parameter	Estimate	Std. Error	z value	Pr(>|z|)
Moderate exacerbations				
Age	0.2999	0.3340	0.8979	0.3993
FEV_1 % predicted	0.3220	0.1303	2.4707	0.0186
Charlson score	0.2889	3.1617	0.0914	0.9892
Adjusted Charlson score	1.1065	2.2549	0.4907	0.7173
Severe exacerbations				
Age	0.6153	0.5567	1.1053	0.2912
FEV_1 % predicted	0.0532	0.4391	0.1212	0.9729
Charlson score	10.1173	4.4064	2.2960	0.0328
Adjusted Charlson score	7.7463	3.3490	2.3130	0.0275

Putative baseline predictors (n=102) of a change in serum levels of HA between baseline and moderate or severe exacerbations were assessed using linear mixed-effects models. Among parameters tested were: symptoms (dyspnea, fatigue, cough, color of sputum); physical examination (blood pressure, heart rate, respiratory rate, peripheral oxygen saturation, 6-minute walking distance); comorbidities (adjusted and unadjusted Charlson score, medication for comorbidities); lung function (FEV1 % predicted, FEV1/FVC).

Supplementary Table 5. Correlation analysis (Spearman's rho) of hyaluronic acid in serum of COPD patients at moderate and severe exacerbations with biomarkers of inflammation

Hyaluronic acid	Leucocytes		CRP	
	rho	p value	rho	p value
Moderate exacerbations	-0.246	0.052	-0.143	0.292
Severe exacerbations	0.063	0.564	-0.037	0.747
All exacerbations	0.090	0.273	0.052	0.553

Supplementary Table 6. Correlation analysis (Spearman's rho) of hyaluronidase activity in serum of COPD patients (n=506)

Parameter	Rho	p value
Age	-0.005	0.914
Body mass index	0.011	0.824
Packs per year	0.052	0.305
FEV_1 % predicted	-0.109	0.034
FEV_1/FVC	-0.067	0.194
Adjusted Charlson score	-0.031	0.528
Borg Dyspnea Scale	0.016	0.765
6-minute walking distance	-0.005	0.922
Survival time	-0.118	0.017

Abbreviations: FEV_1: forced expiratory volume in 1 sec; post-brd: post-bronchodilatation