THE ROLE OF THE NITRIC OXIDE PATHWAY AND EOSINOPHILIC INFLAMMATION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

PhD thesis

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1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a pressing global health concern, affecting over 200 million individuals worldwide and contributing to more than 3 million deaths annually, making it the third leading cause of mortality. This multifactorial disease results from a complex interplay of genetic susceptibility, environmental exposures, and defects of normal lung development or accelerated aging, leading to chronic airway inflammation and structural lung alterations. The disease is punctuated by episodes of acute worsening of inflammation and symptoms, called acute exacerbations (AE or ECOPD). Preventing and predicting them is paramount as they have long-term impact on quality of life and serve as the primary cause of COPD-related mortality.

The airway inflammation seen in COPD is a complex process involving the recruitment and activation of different white blood cells, alongside the amplification of cytotoxic and destructive mechanisms, including oxidative and nitrative stress.

The dysfunction of nitric oxide synthases (NOS) is observed in COPD. It is linked with the development of airway nitrative stress and the decreased production of vascular NO, a hallmark of endothelial dysfunction. Endothelial dysfunction may lead to the development of cardiovascular diseases, the most common comorbidities of COPD. However, the exact link between airway

inflammation and systemic endothelial dysfunction is not well understood. Assessment of NO pathway functionality via markers such as L-arginine, asymmetric and symmetric dimethylarginine (ADMA, SDMA), and nitrite/nitrate levels in sputum and peripheral blood offers insights into endothelial dysfunction. L-arginine is the substrate of nitric oxide production, nitrite and nitrate are the degradation products, while ADMA and SDMA are inhibitors of NO synthesis.

The airway inflammation seen in COPD is mostly neutrophil associated; however, during an AE, a subset of patients exhibit elevated eosinophil counts in both systemic and airway compartments. This eosinophilic phenotype presents a distinct clinical profile, however, the association between eosinophilic AEs and the risk of developing subsequent events remains inconclusive.

The overall aim of this doctoral thesis was to assess the complexities of the airway inflammation in COPD from a clinical perspective, and to relate it to endothelial dysfunction. In the first study included in this thesis, our hypothesis was that COPD is associated with a higher degree of endothelial dysfunction due to chronic airway inflammation, and that markers of NO synthase dysfunctionality further increase during AE. In the second study, we hypothesised that severe AEs characterized by eosinophilic inflammation correlate with earlier recurrence of subsequent events.

2. OBJECTIVES

Our aims were to:

1. To assess markers of NO production (serum concentrations of L-arginine, ADMA, SDMA, nitrite and nitrate) in patients with stable and exacerbated COPD and smoking control subjects.
2. To compare the markers of NO production at hospital admission and in the convalescence of the AE at hospital discharge.
3. To investigate the associations of NO production with markers of airway (exhaled NO level, sputum cellularity, sputum nitrite and nitrate concentration) and systemic inflammation (serum C-reactive protein concentration, blood leukocyte counts), lung function variables and blood gas values.
4. To investigate whether the eosinophil-type severe ECOPD (defined by ≥2% of blood leukocytes or ≥200 cells/μL and also ≥3% of blood leukocytes or ≥300 cells/μL) is related to earlier re-exacerbations in the 12 months after the index event.
5. To analyse the associations of clinical factors with the occurrence of subsequent events.
6. To assess the stability of the eosinophil-type severe ECOPD between different events

3. METHODS

In both studies, patients were enrolled in the Department of Pulmonology, Semmelweis University, Budapest, Hungary. The diagnosis of COPD was confirmed prior to enrolment by a respiratory specialist, following the relevant Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. Exclusion criteria included the need for invasive ventilation, concurrent pneumonia, and administration of systemic corticosteroid or antibiotic treatment four weeks before recruitment.

In the first study, which assessed the relationship between markers of the nitric oxide pathway and clinical characteristics of COPD (nitric oxide pathway study), we included patients with stable COPD and those experiencing an acute severe exacerbation requiring hospital admission, with symptoms appearing within the past 72 hours. Additionally, we recruited control subjects (C) who were smokers with no respiratory symptoms for at least four weeks before inclusion. In the second study, which assessed the relationship between eosinophilic exacerbations and subsequent relapses (eosinophil study), we enrolled only hospitalized patients due to AE.

The study design of both studies was longitudinal observational trial. In the nitric oxide pathway study, control subjects and patients with stable COPD attended a single visit and no follow-up measurements were performed, while ECOPD patients were evaluated at two time points: within 24 h of hospital admission and, when

feasible, during convalescence. In the eosinophil study, patients were enrolled during hospitalisation and were followed up for 12 months or until the first moderate or severe ECOPD.

In the eosinophil study, follow-up was carried out by regular standardised telephone interviews every three months or by personal interviews during readmission to our department to assess the time to the first re-occurrence and severity of ECOPD. Patients were grouped based on their admission blood eosinophil count and blood eosinophil percentage of the total leukocyte count (eosinophilic group: ≥2% of leukocytes and/or ≥200 eosinophils/μL).

In both studies, we recorded baseline demographic parameters, routine qualitative and quantitative blood parameters, and blood gas and lung function parameters. In the nitric oxide pathway study, fractional exhaled NO concentration (F_{ENO}), serum ADMA, SDMA, L-arginine, nitrite and nitrate levels, and sputum nitrate and nitrite concentrations were measured. In the eosinophil study, no additional measurements were performed besides clinical routine.

The primary outcome of the nitric oxide pathway study was the differences in markers of NO pathway functionality between COPD patients and control subjects, and their changes between hospital admission and convalescence. The primary outcomes of the eosinophil study were the rate and time to the first moderate or severe

exacerbation. Additionally, we explored the risk factors associated with the occurrence of relapses. A further secondary outcome was the stability of relapse phenotypes, i.e., the proportion of patients experiencing a subsequent eosinophilic exacerbation after an eosinophilic index exacerbation.

In both studies, continuous variables were presented as mean±sd or median (interquartile range (IQR)) according to the distribution of the variables. Categorical variables were presented as numbers and percentages and were compared with Chi-squared tests or Fisher-exact tests.

In the nitric oxide pathway study, demographic data and serum parameters were compared between the groups using ANOVA tests, and pairwise post hoc comparisons were made with Bonferroni correction. Paired t-tests were used to compare variables in ECOPD between admission and convalescence. Correlations were tested using Pearson's or Spearman's correlation.

In the eosinophil study, continuous variables were compared using the t-test and Mann–Whitney U-test. Cox proportional hazards models, log-rank tests, and generalised linear regression models with a negative binomial distribution were employed to assess risk for an earlier relapse.

4. RESULTS

We enrolled a total number of 76 (53% male) participants in the nitric oxide pathway study, of which 32 patients presented during an ECOPD, 29 in stable state, and 15 of them were controls. The control subjects were younger and had a less extensive smoking history than the COPD patients, and the prevalence of cardiovascular comorbidities were higher among the patients than among the control participants.

The ratio of L-arginine and ADMA, which indicates the availability of L-arginine for NOS, was decreased in stable COPD ($p < 0.01$) and during ECOPD ($p < 0.05$) when compared to controls ($p < 0.01$) and it did not change at convalescence ($p = 0.89$). Serum ADMA levels correlated with age ($r = 0.25$, $p = 0.04$), blood neutrophil percentage ($r = 0.36$, $p < 0.01$), and exhaled F_{ENO} ($r = 0.42$, $p < 0.01$).

The levels of SDMA were increased only during an exacerbation ($p<0.001$ and $p<0.01$) and it decreased upon recovery ($p < 0.05$). Admission serum SDMA showed correlation with age ($r = 0.67$, $p < 0.001$), total sputum inflammatory cell count ($r = 0.61$, $p < 0.01$), and sputum neutrophil count ($r = 0.62$, $p < 0.01$) in ECOPD.

There were no differences between any of the groups in serum nitrate concentrations ($p = 0.36$), and it was also similar at hospital admission and discharge in patients with ECOPD ($p = 0.87$). On the other hand, serum nitrite levels were elevated in COPD compared to control

participants (both $p < 0.001$); however, there were no differences between admission and recovery concentrations in ECOPD ($p = 0.26$). Serum nitrite levels in COPD patients did not correlate with sputum nitrate or nitrite concentrations, F_{ENO}, age, smoking history, lung function, or blood parameters ($p > 0.1$ for all variables).

Sputum nitrate concentration was elevated in ECOPD compared to that in stable COPD ($p < 0.05$) and declined at hospital discharge. In contrast, there was no difference in the levels of sputum nitrite between ECOPD and S-COPD patients ($p = 0.17$), and at admission and discharge in ECOPD, either ($p = 0.88$).

In the eosinophil study, we included 152 patients, of whom 51 had eosinophil-high exacerbations as an index event. Patients in the eosinophilic group had higher FEV_1/FVC values and lower serum CRP concentrations than those in the non-eosinophilic relapse group.

During follow-up, 118 patients (78%) had at least one exacerbation. We observed more severe exacerbations than moderate exacerbations as the first subsequent event. The distribution of patients regarding relapse severity did not differ between the groups (p=0.84).

The time to first relapse was not different between the eosinophilic (≥2% and/or ≥200 eosinophils/μL) and non-eosinophilic groups (p=0.48). We also found no difference in this outcome when separated the groups with different eosinophil cut-offs (≥3% of total leukocytes and/or ≥300

eosinophils/μL, p=0.53). Moreover, we did not detect differences in the outcomes by dividing patients into tertiles of the eosinophil ratios (p=0.21).

Using logistic regression models, we found that the number of previous annual exacerbations, the degree of airflow limitation (% predicted post-bronchodilator FEV_1 at discharge) and a higher cigarette exposure burden were associated with a shorter time to relapse. Furthermore, according to the regression coefficients, the best predictor of future AE was the number of previous events, which was also confirmed by forward stepwise regression modelling.

Eosinophil data of subsequent exacerbations were available for 73 patients. Of these patients, 27 (37%) had an index eosinophilic exacerbation (≥2% of total leukocytes and/or ≥200 eosinophils/μL), and 46 (63%) had a non-eosinophilic event. During follow-up, more than three-quarters (75.3%) of the patients experienced a similar type of exacerbation as the initial event. However, almost half of the patients (48.1%) who had an eosinophilic index AE experienced a non-eosinophilic subsequent event, while only around 1 of 10 patients (10.9%) moved categories in the other direction, i.e., from non-eosinophilic index AE to eosinophilic subsequent AE. Furthermore, the overall proportion of eosinophil-type relapses was lower during the follow-up than at baseline (p<0.001).

5. CONCLUSIONS

1. In COPD, vascular NO substrate availability is decreased, and suppression of NO production is enhanced.
2. Impairment in the vascular NO pathway during COPD flare-ups is partially reversed after systemic corticosteroid therapy and convalescence.
3. Serum levels of ADMA and SDMA correlate with airway nitrosative stress and neutrophilic inflammation, suggesting an association between endothelial dysfunction and airway inflammation in COPD.
4. Patients presenting with an eosinophil-type severe ECOPD do not have an increased risk of earlier recurrences of moderate or severe relapses in 12 months after the index event.
5. Number of severe AEs in the past 12 months, degree of airflow limitation and higher exposure to cigarette smoke are related to a shorter time to a subsequent severe or moderate AE.
6. Upon recurrent severe exacerbations within 12 months, the eosinophilic phenotype is less consistent than the non-eosinophilic phenotype.

The findings of this doctoral thesis contribute to a better understanding of the inflammatory processes in COPD and highlight the need for comprehensive approaches that address both pulmonary and cardiovascular aspects of the disease, recognizing that COPD is more than a localized respiratory disease.

6. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

1. **Csoma Balázs**, Bikov András, Nagy Lajos, Tóth Bence, Tábi Tamás, Szűcs Gergő, Komlósi Zsolt István, Müller Veronika, Losonczy György, Lázár Zsófia

   Dysregulation of the endothelial nitric oxide pathway is associated with airway inflammation in COPD

   RESPIRATORY RESEARCH 20: 1 Paper: 156, 10 p. (2019)

   Scopus - Pulmonary and Respiratory Medicine SJR indicator: Q1

   IF: 3.924

2. **Csoma Balázs**, Bikov András, Tóth Ferenc, Losonczy György, Müller Veronika, Lázár Zsófia

   Blood eosinophils on hospital admission for COPD exacerbation do not predict the recurrence of moderate and severe relapses

   ERJ OPEN RESEARCH 7: 1 Paper: 00543-2020, 8 p. (2021)

   Scopus - Pulmonary and Respiratory Medicine SJR indicator: Q2

   IF: 4.239