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

PhD thesis

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List of abbreviations

ADMA	asymmetric dimethylarginine
AECOPD	acute exacerbation of chronic obstructive pulmonary disease
Akt	protein kinase B
BAL	bronchoalveolar lavage
BEC	blood eosinophil count
BH_4	tetrahydrobiopterin
CAT	COPD Assessment Test
CCI	Charlson Comorbidity Index
CCL	CC-motif ligand
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CT	computed tomography
CXCL	CXC-chemokine ligand
DNA	deoxyribonucleic acid
EBC	exhaled breath condensate
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EPO	eosinophil peroxidase
EU	European Union
FAD	flavin adenine dinucleotide
F_{ENO}	fractional exhaled nitric oxide

FEV_1	forced expiratory volume in 1 second
FMN	flavin mononucleotide
FVC	forced vital capacity
GM-CSF	granulocyte-macrophage colony-stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
ICD	International Classification of Diseases
ICS	inhaled corticosteroids
IFN-γ	interferon-γ
IL	interleukin
LABA	long-acting beta2-agonist
LAMA	long-acting muscarinic antagonist
LTOT	long-term oxygen therapy
MBP	major basic protein
mmHg	millimetre of mercury
MMP-9	matrix metalloproteinase-9
mMRC	modified Medical Research Council
NADPH	nicotinamide adenine dinucleotide phosphate
NIV	non-invasive ventilation
NLRP3	nucleotide-binding oligomerization domain-like receptor pyrin domain 3
NO	nitric oxide
NOS	nitric oxide synthase
OSA	obstructive sleep apnoea
$PaCO_2$	arterial carbon dioxide tension

PaO_2	arterial oxygen tension
PI3K	phosphatidylinositol 3-kinase
RCT	randomized clinical trial
RNS	reactive nitrogen species
ROS	reactive oxygen species
SABA	short-acting beta2-agonist
SAMA	short-acting muscarinic antagonist
SDMA	symmetric dimethylarginine
SERPINA1	Serpin Family A Member 1
TGF-β	transforming growth factor-beta
Th	T-helper cell
TNF-α	tumour necrosis factor-alpha
US	United States
V'_A/Q'	ventilation / perfusion ratio
VCAM-1	vascular cell adhesion molecule-1
WBC	white blood cell
WHO	World Health Organization

1. Introduction

1.1. Chronic obstructive pulmonary disease

1.1.1. Definition

According to the Global Initiative for Chronic Obstructive Lung Disease, "Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous lung condition characterized by chronic respiratory symptoms (dyspnoea, cough, sputum production and/or exacerbations) due to abnormalities of the airways (bronchitis, bronchiolitis) and/or alveoli (emphysema) that cause persistent, often progressive, airflow obstruction" [1]. The most prevalent cause of COPD is chronic cigarette smoke exposure, however, other genetic and environmental factors, such as α_1-antitrypsin deficiency, premature birth, repeated childhood infections, and indoor and outdoor air pollution are also contributing factors [2]. The chronic, and in most cases, progressive nature of the disease is punctuated by episodes of acute worsening, which are called acute exacerbations (AEs) [1]. AEs are important events from the perspective of the disease course as they worsen lung function [3], contribute to the accumulating debilitating effects of the disease [4], and increase the frequency of hospitalizations [5], and disease-associated mortality [6, 7].

1.1.2. Epidemiology

According to the data of the World Health Organization (WHO), COPD affected over 200 million people worldwide in 2019, and was responsible for more than 3 million deaths, becoming the 3rd most common cause of death [8]. Furthermore, the estimated global prevalence of COPD in 2019 was between 10 to 15% among people aged 30-79 years, with 4 of every 5 people with COPD living in low- and middle-income countries [9, 10].

Nevertheless, COPD is a global health crisis that affects high-income, developed countries as well, especially in aging populations. For example, the overall self-reported age-adjusted prevalence of COPD in the United States (US) was 5.6% in 2020, affecting at least 18.5 million people [11]. According to estimates based on a systematic review and modelling analysis, the prevalence of COPD including the undiagnosed cases in

Europe was 10.1% in 2019 in the population between age 30-79, which translates to 56.1 million people [9]. In Hungary, around 185 000 people were reported to have COPD in 2022 [12]. However, due to the high rate of underdiagnosis reported in international literature [13] and high number of smokers [14], the true prevalence may be around 5-600 000 people.

The treatment of this enormous number of patients is associated with significant healthcare costs, too. In the European Union (EU), COPD accounts for more than half of the total healthcare costs of respiratory diseases, achieving almost 40 billion euros per year [15]. The majority of these costs are associated with the hospital treatment of AEs, and the treatment costs per patient are also related to the severity of the disease [1]. Besides the direct expenditures on medical treatment, COPD is also associated with a high burden of indirect costs, such as early retirement from work, costs of social support, and the potential need of a family member also leaving the workplace to care for the disabled patient. Considering the indirect costs as well, the total yearly expenses for COPD are estimated to be more than 140 billion euros in the EU [16]. In addition, the incidence of COPD in countries with low sociodemographic status is still increasing due to high prevalence of smoking, indoor and outdoor air pollution, and low accessibility to healthcare; therefore, the economic burden of the disease is also expected to rise [8].

1.1.3. Pathogenesis

The pathogenesis of COPD is primarily driven by an abnormal inflammation of the airways, leading to an increase in airway resistance and loss of pulmonary parenchyma and consequently restricted expiratory flow [1]. Previously, the development of COPD has been almost exclusively associated with tobacco smoking, mainly because the majority of the studies investigating COPD were performed in developed countries [17]. However, it is increasingly recognised that although smoking is undoubtedly one of the most important risk factors, there are other determinants of disease development, which can be categorised into 3 distinct groups: genetic susceptibility, environmental exposures, and defects of normal lung development or accelerated aging [18].

The pathobiology starts in most cases with repeated exposure to smoke from cigarette or other organic combustion (e.g., indoor air pollution from wood-burning heating). The

chronic smoke inhalation triggers an abnormal, a more intense and longer-lasting inflammatory immune response in the airways of genetically susceptible people [18]. The way in which cigarette smoke induces inflammation is not fully understood, but the activation of innate immune Toll-like receptors is likely to be involved [19, 20]. Activated respiratory and resident airway inflammatory cells produce various chemotactic factors that attract additional inflammatory cells to the lung, e.g. CC-motif ligand 2 (CCL2) attracts monocytes, CXC-chemokine ligand 1 (CXCL1) and CXCL8 induce the influx of neutrophil granulocytes and CXCL9, -10, -11 CD4+ attract Th1 cells [21]. These cells then collectively secrete proteases (mainly matrix metalloproteinase 9 (MMP-9)), elastases, and other enzymes that degrade the extracellular matrix and alveolar walls, leading to parenchymal damage and emphysema [22]. Certain cytokines, e.g., interleukin (IL)-4, IL-13, IL-17, or IL-23, also cause increased production of mucus by stimulating goblet cells [1, 23]. Transforming growth factor-β (TGF-β), produced by epithelial cells for regeneration, is responsible for the development of small airway fibrosis through fibroblast activation [24]. Reactive oxygen and nitrogen derivatives, which are contained in smoke and are also released from activated immune cells are partly responsible for the maintenance of inflammation [21]. Increased levels of oxidative and nitrosative stress biomarkers (such as hydrogen peroxide, 8-isoprostanes, 3-nitrotyrosine, malondialdehyde, exhaled nitric oxide) in patients with COPD are also indicative of this [25-28]. However, the airway inflammation can persist even after giving up smoking. The reason behind this is not fully understood, however it is most likely driven by autoimmunity and disruptions in airway microbiome [29, 30].

The processes described above induce the abnormalities and symptoms characteristic of COPD in the long term. Inflammation, small airway fibrosis and increased mucus production lead to narrowing of the airways and result in increased airway resistance and therefore decreased expiratory flow [31]. As the parenchymal damage progresses, the elastic recoil of the lungs deteriorates, leading to static, and during exertion, also to dynamic hyperinflation [32]. Furthermore, the parenchymal destruction results in the decrease in the surface area of the lungs involved in gas exchange. Reduced gas exchange capacity then manifests in breathlessness, at first on exertion and later even at rest [33]. As the emphysema worsens, the dead space ventilation also increases and the ventilation/perfusion (V'_A/Q') ratio deteriorates, further complicating the exchange of

oxygen and carbon dioxide and leading to hypoxaemia and hypercapnia [34, 35]. In patients with advanced COPD, small vessel hypertension may develop, partly due to hypoxia-induced vasoconstriction and partly due to reduced capillary blood flow caused by alveolar destruction, which over time may lead to heart failure via increased right heart workload [36, 37].

The factors leading to the pathological processes described above involve chronic inhalation of noxious particles, originating either from tobacco smoking, indoor air pollution through biomass burning, outdoor air pollution, and occupational exposure, or from a mixture of these. Smoking is the primary risk factor for COPD in developed countries [2], although less than half of all heavy smokers develop COPD [38], highlighting the importance of genetic susceptibility and interaction between these factors in the pathogenesis. Furthermore, smoking patients with COPD experience a higher decline in lung function parameters and higher mortality rate than non-smokers [39]. Nevertheless, around 50% of all COPD cases worldwide are due to environmental factors other than smoking, which can be as high as 70% in certain low-income countries [17]. These individuals are generally younger, have a more marked small airway obstruction but with less annual decline in lung function, less emphysema, higher sputum eosinophil count, but have similar respiratory symptoms and quality of life impairment compared to smoking-induced COPD [17]. The optimal pharmacological therapy of this phenotype has not been studied extensively and therefore is not established [17].

Furthermore, besides being chronically exposed to the above agents, a genetical susceptibility is also required to develop COPD. The most obvious link between genetic susceptibility and COPD is the mutation of Serpin Family A Member 1 (*SERPINA1)* gene, which in homozygote form leads to α1-antitrypsine deficiency and severe emphysema in young age [40]. However, the interplay between other several genetic variants which alone do not have marked effect are more likely to increase the likelihood of the disease [18, 41].

Moreover, every aspect that affects the normal lung development (e.g., premature birth, repeated childhood pulmonary infections, early exposure to cigarette or environmental smoke), or accelerates lung aging, also contribute to the development of COPD [2].

Consequently, a new classification has been proposed recently to classify COPD into five distinct categories according to the main risk factors [2]. The types are the following: genetically determined COPD, COPD related to early life events, infection-related COPD, COPD related to smoking or vaping, and environmental exposure-related COPD. This new distinction and the emphasis of risk factors other than smoking may shed light on the importance of early intervention and prevention and may also help explain the inter-individual differences in disease course.

1.1.4. Co-morbidities

COPD is frequently accompanied by other diseases, which are called comorbidities that can significantly affect the patient's prognosis [42, 43]. Comorbidities can occur because of a shared risk factor with COPD, such as cigarette smoking, and COPD itself can also increase the likelihood of developing certain diseases [1, 44]. Furthermore, COPD can worsen the comorbidities, for example the low-grade systemic inflammation and prolonged hypoxia negatively impact cardiomyocyte function [45].

The most common comorbidities include cardiovascular diseases (CVD) [46], lung cancer [47], obstructive sleep apnoea (OSA) [48], metabolic diseases [46], and affective [49] and cognitive disorders [50].

CVDs, such as atherosclerosis, arrhythmias, coronary artery disease or congestive heart failure are the most common comorbidities of COPD. The cumulative prevalence of CVDs can be as high as 70% in COPD [51]. CVDs and COPD share several common risk factors such as smoking and other environmental exposures or aging. Moreover, these diseases also interact with each other through various pathways mostly involving systemic inflammation and endothelial dysfunction [52]. However, the exact link between COPD and endothelial dysfunction is not yet fully understood.

1.1.5. Symptoms and diagnostics

The characteristic symptoms of COPD include dyspnoea, chronic productive or dry cough, recurrent wheezing, and in some cases, recurrent AEs and lower airway infections.

Chronic cough is the most common symptom of COPD, but considering the risk factors, it can be present many years prior to the development of the disease [53]. The cough can be either productive or unproductive, and there are also significant intraindividual and daily variations in the severity of this symptom [54]. Based on the viscosity and volume of the sputum and the ability of the patient to expectorate, the sputum may be retained in the airways which can lead to complications such as airway obstruction and consequent atelectasis, or lower airway infections [55, 56]. On the other hand, the sudden worsening of the cough and/or the newly onset of sputum production can be indicative of an acute exacerbation [1].

Breathlessness is a characteristic symptom of COPD, and it can be present in all stages of the disease, usually occurring early as an exercise-induced dyspnoe and later progressing to be present even at rest [57]. Dyspnoe is one of the most important symptoms of COPD from a patient perspective as it causes significant subjective suffering and leads to anxiety and is the frequent cause of disability [58]. The severity of this symptom can be measured by the modified Medical Research Council test (mMRC), which grades the level of breathlessness in a scale from 0 to 4 [59]. The score of this test is also included in the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification of COPD alongside with the results of the COPD Assessment Test (CAT) results (see later) [1, 60].

In patients with symptoms indicative of COPD, the diagnostic process should include a thorough clinical assessment and history taking, with a particular emphasis on the above-detailed risk factors and symptom severity, and lung function measurements. Additional investigations, such as computed tomography scans to confirm structural changes in the lungs, or blood gas analysis to assess gas exchange abnormalities can be helpful in differential diagnosis and may aid the diagnosis, but the definitive diagnosis lies on forced spirometry post-bronchodilation [1, 61].

Spirometry is a widely accessible, non-invasive, relatively cheap, and easily interpretable test of the lung functions [62]. Its measurement should be performed with forced manoeuvres, i.e., with maximal patient effort during inhalation and exhalation. The initial test should be carried out before and after the administration of short-acting

bronchodilators, such as 400 μg of short-acting beta2-agonist (e.g., salbutamol) to evaluate the reversibility of the airway obstruction. The diagnosis of COPD can be confirmed if in a patient with relevant history, risk factors, and symptoms, the ratio of forced expiratory volume in 1 second of exhalation (FEV_1) and forced vital capacity (FVC) is below 0.7 [1, 62]. Four categories of severity (GOLD1-4) are distinguished based on the predicted values of post-bronchodilator FEV_1, with respective cut-offs of ≥80, 50-79, 30-49, and <30%.

Furthermore, the GOLD document and international guidelines also recommend the use of standardized questionnaires to assess symptom severity and the quality of life [1, 63]. GOLD suggests the use of the mMRC and CAT tests to distinguish between patients with and without a high burden of COPD-associated symptoms. The mMRC test quantifies breathlessness, while the CAT test provides a more comprehensive assessment of COPD symptoms, including dyspnoea, cough, sputum production, chest tightness, the level of physical disability and sleep quality [60].

In addition, considering the above-mentioned symptom scores and the history of AEs, a further classification (the ABE tool) is also possible to guide the initial maintenance pharmacological therapy (**Table 1**).

Table 1. GOLD ABE assessment tool

≥2 moderate exacerbations or ≥1 severe exacerbations in the last year	**E** LABA+LAMA Consider adding ICS if BEC ≥300/μL while stable	
<2 moderate exacerbations and <1 severe exacerbation in the last year	**A** Any kind of bronchodilators	**B** LABA+LAMA
	mMRC <2 CAT <10	mMRC ≥2 CAT ≥10

A, B, and E are the names of the categories, where E stands for “exacerbations”. Abbreviations: BEC: blood eosinophil granulocyte count, CAT: COPD Assessment Test, LABA: long-acting β_2-agonist, LAMA: long-acting muscarinic antagonist, ICS: inhaled corticosteroid, mMRC: modified Medical Research Council. Produced based on www.goldcopd.org [1].

1.1.6. Treatment of stable COPD

The goal of the treatment of COPD is to reduce symptoms and prevent disease progression and the occurrence of AEs [1]. The treatment consists of pharmacological and non-pharmacological measures. The basis of the treatment is the inhaled maintenance pharmacological therapy, however, it must always be supplemented with the appropriate non-pharmacological interventions, such as smoking cessation, vaccination against common respiratory pathogens, pulmonary rehabilitation, introduction of long-term oxygen therapy (LTOT), domiciliary non-invasive ventilation (NIV), surgical interventions, or palliative care [1].

The initial pharmacological therapy should be based on the clinical assessment of symptoms and history of AEs, as illustrated in Table 1. Patients should be followed-up regularly and the treatment should be reviewed, reassessed, and adjusted when necessary. The adjustment is aimed at two treatable traits: breathlessness and exacerbations. Patients developing or continuing to experience severe dyspnoea should be prescribed LAMA+LABA dual therapy if they were treated with a monotherapy alone. If they were receiving dual therapy, the switching of inhaler device (e.g., dry powder inhaler vs. metered dose inhaler) or molecules, or the escalation of non-pharmacological therapies can be considered. Patients having AEs should be escalated to LAMA+LABA dual therapy from monotherapy if their BEC is <300/µL, or to LAMA+LABA+ICS triple therapy if they have an elevated BEC (≥300/µl). If they were already on LAMA+LABA, stepping up to triple therapy may be beneficial if the BEC is ≥100/µL. Patients continuing to exacerbate on triple therapy can be offered roflumilast (a phosphodiesterase-4 inhibitor) if they have a FEV_1 <50% of predicted value and a chronic bronchitis phenotype, or long-term azithromycin (a type of macrolide antibiotics) if they stopped smoking. Moreover, the stepping down and therefore the withdrawal of the ICS should

also be assessed during follow-up reviews if the patient is no longer having exacerbations and the BEC is below the above-mentioned threshold [64].

1.2. Acute exacerbations of COPD

1.2.1. Definition

According to the recent consensus Rome proposal, an AECOPD is "an event characterized by dyspnoea and/or cough and sputum that worsen over ≤14 days, which may be accompanied by tachypnoea and/or tachycardia and is often associated with increased local and systemic inflammation caused by airway infection, pollution, or other insult to the airways" [65].

These events are especially important as they accelerate disease progression, increase the likelihood of subsequent events, and are a leading cause of mortality in COPD [65]. Therefore, their prevention, prediction, and management are of paramount importance.

1.2.2. Pathogenesis

AEs are usually induced by respiratory infections, especially viral infections (although bacterial infections are also common causes), and by environmental factors such as air pollution or exposure to excess heat [66, 67]. The most common viral pathogens associated with AEs are human rhinovirus, influenzavirus, para-influenzavirus and metapneumovirus [1]. Viral AEs are more likely to occur during the winter months, are usually more severe and last longer, and more likely to necessitate hospitalisation [68]. Exposure to high levels of particulate matter for a short time can also increase the risk of hospitalisations and even mortality due to AECOPD [69].

Nevertheless, independent of the provoking factor, the downward steps in the evolution of an AE are similar. A stimulus exacerbates the inflammation already present in the airways, which leads to the recruitment of neutrophils, eosinophils, macrophages and CD4+ T-cells [70]. The immune cells produce a plethora of inflammatory cytokines and other substances that increase mucus production, cause bronchial contraction, induce airway wall oedema, and increase smooth muscle proliferation [71]. Excess mucus production, bronchospasm, and airway oedema reduce the airway calibre, thus increasing

the resistance and worsening the airway obstruction. The expiratory flow limitation makes it more difficult to expectorate the sputum, which in turn causes mucus plugging and further decreasing the expiration and promoting hyperinflation. Furthermore, these processes also worsen the V'_A/Q' ratio, deteriorating the gas exchange capacity of the lungs and leading to ventilatory failure [68]. These pathophysiological phenomena manifest in the classical symptoms of AECOPD: progressive breathlessness, cough, sputum production and purulence, and fatigue [72].

1.2.3. Clinical manifestations and classification

Previously, two classifications have been used to grade the severity of AEs. According to the Anthonisen criteria, an exacerbation is considered type 1 if all three of the three main symptoms (increased breathlessness, sputum expectoration, purulent sputum) are present, type 2 if the patient has only two of the three symptoms, and type 3 if the patient has only one of the three main symptoms and at least one of the secondary symptoms. Secondary symptoms are signs of upper respiratory tract infection (sore throat, nasal congestion), fever, wheezing, increased cough, and increased respiratory or heart rate (at least 20% increase from baseline) in the last 5 days [72]. The other classification, proposed by the GOLD committee, was based on the need for treatment rather than symptoms. A mild exacerbation was defined as one whose symptoms can be controlled with short-acting bronchodilators, moderate if bronchodilators are accompanied by antibiotics and/or systemic steroids, and severe if the patient requires hospital admission or emergency care [73].

Nowadays, the severity classification of the Rome proposal is recommended by the GOLD document as well [1]. The proposal categorises patients based on widely accessible measurements that can be performed in primary care settings: visual analogue scale of breathlessness (cut-off: ≥5), respiratory rate (cut-off: ≥24/min), heart rate (cut-off: ≥95/min), resting arterial oxygen saturation (cut-off: <92% and >3% reduction to baseline), and, if available, C-reactive protein (CRP). If three of these five criteria are met, the AE is classified as moderate, and the patient needs emergency or hospital care. To determine whether the patient has a severe AE and therefore needs ventilatory support,

an arterial blood gas measurement is needed which shows hypercapnic respiratory failure (partial arterial pressure of carbon dioxide ($PaCO_2$) >45 mmHg and pH <7.35) [65].

1.2.4. Treatment of exacerbations

The goal of the treatment of AECOPDs is to improve and maintain a satisfactory ventilation, suppress inflammation, and to cure the underlying cause, e.g., a bacterial infection.

The ventilatory capacity can be improved by relieving the bronchoconstriction with the use of short- or long-term inhaled bronchodilators, for example with short-acting inhaled beta2-agonists (SABA) with or without short-acting muscarinic antagonists (SAMA). Indeed, bronchodilation is an absolute necessary part of the treatment and is the first step in the initial therapy [74]. The inhaled form of bronchodilators should always be preferred over the intravenous form, and they can be delivered via pressurized metered dose inhaler (pMDI) with or without spacer, or via nebulization [1]. In addition, in case of respiratory failure and hypoxia, supplemental oxygen should also be given and titrated to a target saturation of 88-92% [75]. There is a wide range of interfaces for oxygen therapy, e.g., nasal cannula, simple face mask, reservoir mask, Venturi mask [75]. Furthermore, in acute hypercapnic respiratory failure, external ventilatory support should also be provided [76]. There are two major forms of mechanical ventilation, invasive and non-invasive ventilation [34]. NIV should be the first choice in hypercapnic AECOPD if the patient is able and willing to comply with the therapy [76] as it is beneficial in terms of complications, length of hospital stay, treatment costs and mortality compared to invasive ventilation [34]. However, there are several contraindications for NIV therapy (most important are the lack of spontaneous breathing and cardiac arrest) and in these cases the patient should be intubated and ventilated invasively [76].

The acute-on-chronic airway and systemic inflammation seen during AECOPDs can be mitigated, at least partially, by systemic glucocorticosteroids. Systemic steroids improve lung function and gas exchange parameters, reduce the length of hospital stay, and the risk of treatment failures and improve survival in AECOPD [77-79]. However, steroids have a wide range of acute and chronic side effects (for example hyperglycaemia, myopathy, psychiatric disturbances, osteoporosis, suppression of endogenous hormone

production, etc.), therefore, they should be used sparingly [80]. The recommended regime is daily 40 mg prednisolone-equivalent for 5 days [1]. Courses longer than this are also associated with adverse events, e.g., pneumonia and mortality [81].

In AECOPDs associated with bacterial infections, antibiotic therapy is indicated [74]. However, it is not always evident whether the infection is bacterial or viral and bacterial infections are responsible for only around 50% of all cases [82]. Clinical markers that can guide towards antibiotics are increased sputum volume and purulence, elevated CRP and/or procalcitonin levels, recent history of certain bacterial infections (e.g., Pseudomonas aeruginosa) and the need for invasive ventilation [1, 83]. The choice of medication should be based on local epidemiologic data and antibiotic resistance patterns, but the empirical initial therapy is most frequently an aminopenicillin with or without beta-lactamase inhibitor, or macrolides, tetracycline, or fluoroquinolone [1]. In case of lack of clinical improvement, in frequent exacerbating, and in invasively ventilated patients, a sputum culture should also be obtained to guide the therapy [1].

1.2.5. The frequent exacerbator phenotype

The number of yearly AEs show individual variation; however, a subgroup of patients experiences recurring AEs [5, 84, 85]. Patients having more than 2 moderate to severe AEs per year are called frequent exacerbators [84]. This phenotype is relatively stable over time and has distinct clinical characteristics. Frequent exacerbators have more respiratory symptoms, poorer quality of life, more severe airflow limitation, faster disease progression, and higher mortality rate [84-86]. However, it is not fully understood what makes these patients prone to AEs. Nevertheless, the best predictor for future AEs is the history of AEs in the past year [5], although other factors, such as annual FEV_1 decline, increasing age, and chronic cough have also been associated with AE risk [87].

1.3. Nitric oxide in health and COPD

Nitric oxide (NO) is a soluble small gaseous molecule which serves as a signal transmitter in mammals [88]. The primary function of NO is relaxation of smooth muscle cells in a variety of tissues, e.g., in vascular or bronchial wall [88]. However, excess NO can react

with oxygen radicals very quickly and together they form very strong pro-inflammatory molecules called reactive nitrogen species (RNS) [25, 89].

Inflammatory diseases may be associated with endothelial dysfunction [90] due to downregulation of endothelial NO production and pathologic production of RNS and reactive oxygen species (ROS) by one of the NO producing enzymes, and thus they may lead to the development of cardiovascular diseases [91]. However, an exact relationship between airway inflammation and systemic endothelial dysfunction has not been established.

1.3.1. Physiology of nitric oxide

1.3.1.1. Production of nitric oxide

NO is mainly produced by enzymatic processes of three isoforms of the haem containing homodimer enzyme, the nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) (see **Table 2**) [92]. The enzymatic production contains a number of redox reactions while performing the degradation of L-arginine into NO and L-citrulline by using molecular oxygen as substrate and nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4) as cofactors (see **Figure 1**) [93, 94]. L-citrulline can then be converted back to L-arginin by the arginosuccinate lyase enzyme and thus maintaining intracellular arginine balance [95]. However, arginin is used for other processes besides NO production (such as proline, glutamate, ornithine, and creatin synthesis or ammonia detoxification) and therefore exogenous arginin uptake or arginin synthesis is also necessary [96].

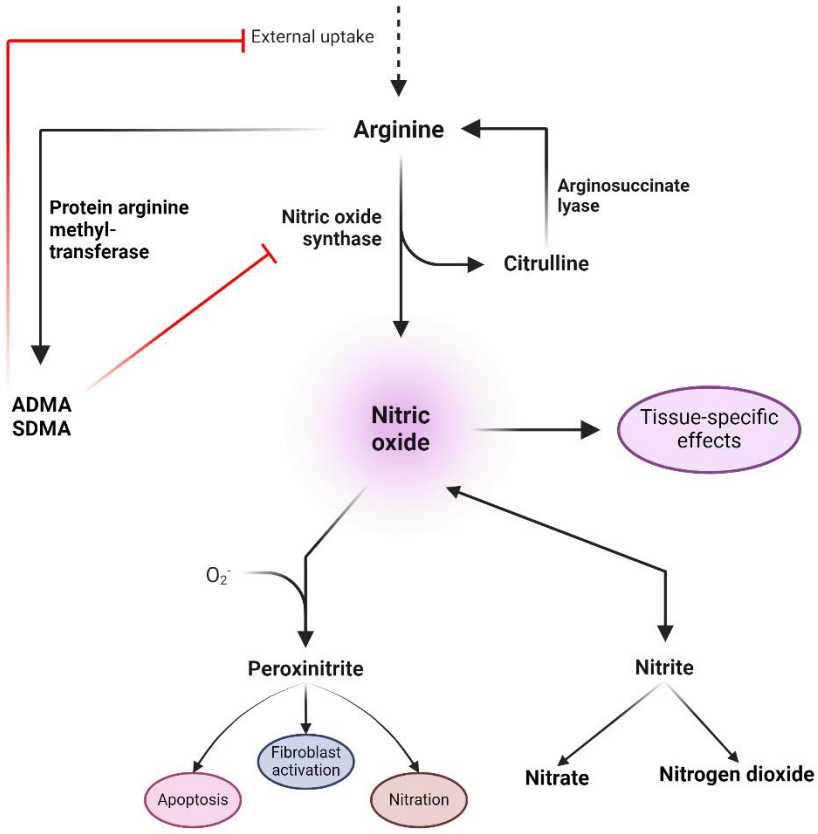

Figure 1. Synthesis and metabolism of nitric oxide. Nitric oxide is produced by the nitric oxide synthase enzyme from arginine while generating citrulline, which can be converted back into arginine by the arginosuccinate lyase enzyme. Nitric oxide can be degraded by forming nitrite and then nitrate or nitrogen dioxide in a reversible process, or by forming peroxynitrite while reacting with a superoxide anion. Methylated arginine products can block the production of nitric oxide by inhibiting either external arginine uptake or nitric oxide synthase. Black arrows indicate the direction of syntheses, red arrows represent inhibition. Abbreviations: ADMA: asymmetric dimethylarginine, O2-: superoxide anion, SDMA: symmetric dimethylarginine. Original work of the author.

The production of intracellular NO is mostly limited by L-arginin availability and intracellular calcium levels [96]. Calcium is needed for calmodulin to bind to NOS and therefore to facilitate the electron flow between NADPH and the oxygenase domain of the enzyme. While eNOS and nNOS are sensitive to intracellular calcium levels, iNOS has a high affinity for calmodulin even at low calcium concentrations [94]. On the other

hand, L-arginin availability and NOS function can be influenced by other products of arginine metabolism, especially symmetric dimethyl-L-arginine (SDMA) and asymmetric dimethyl-L-arginine (ADMA). They are produced by protein arginine methyltransferases that are in competition with NOS for L-arginine. Both ADMA and SDMA can limit L-arginine uptake, while ADMA is also a potent competitive inhibitor of eNOS [94, 97]. Therefore, the ratio of L-arginine and ADMA determines the capacity of eNOS.

Furthermore, besides enzymatic production from L-arginine, nitric oxide can also be produced from nitrite intravascularly and in extravasal tissues under conditions with low pH and oxygen concentrations, thus being an important NO source under ischaemic conditions [98].

Once formed, the breakdown of NO is mediated by different metabolic routes. They can form RNS through reacting with ROS, most importantly, superoxide anion, and be turned into peroxynitrite [99]. Moreover, NO can also form S-nitrosothiols while reacting with redox-activated thiols [100]. However, the most important metabolites are nitrite and nitrate, generated by oxidation [100].

Consequently, although NO has a very short half-life in the circulation, its degradation products, and the by-products of its synthesis, such as ADMA, SDMA, nitrites and nitrates, make it possible to measure the activity of the NO pathway.

Table 2. Localization and main functions of nitric oxide synthase enzymes. Based on [25] and [94].

Isoenzymes	Location	Function
nNOS	Neurons in central and peripheral nervous system	Synaptic plasticity, penile erection
	Airway epithelial cells	Bronchodilation
	Capillary endothelial cells in alveolar septa	Vasodilation in pulmonary circulation
iNOS	Macrophages	Enhancing immune reactions
	Neutrophils	

	Eosinophils	Promoting inflammation
	Mast cells	
	Airway epithelial cells	
	Type II pneumocytes	
	Endothelial cells	
	Fibroblasts	
	Airway and vascular smooth muscle cells	
eNOS	Endothelial cells	Vasodilation in pulmonary and systemic circulation
	Type II pneumocytes	Modulation of ciliary functions and beat frequency
	Airway epithelial cells	
	Macrophages	Initiation of inflammatory response
	Platelets	Inhibiting platelet activation
Abbreviations: eNOS: endothelial nitric oxide synthase, iNOS: inducible nitric oxide synthase, nNOS: neuronal nitric oxide synthase		

1.3.1.2. Physiological roles of nitric oxide

Nitric oxide is a versatile signal transmitter with various functions across the whole body (**Table 2**). NO produced by nNOS acts as a neurotransmitter for inhibitory non-adrenergic non-cholinergic (iNANC) neurons, mediating smooth muscle relaxation and bronchodilation in the respiratory system, vasodilation in the circulatory system, and regulating gut peristalsis [25, 94]. Furthermore, NO has been associated with synaptic plasticity in the central nervous system, having important role in learning and memory [94].

iNOS is upregulated by several inflammatory cytokines, including tumour necrosis factor alpha (TNF-α), IL-1β, or interferon-γ (IFN-γ). Compared to the other isoforms of NOS, iNOS generates a higher amount of NO, which augments the immune defence by activating macrophages and enhancing oxidative and nitrative stress-related cytotoxic

functions and therefore controlling intracellular bacterial and parasitic infections [101, 102].

eNOS is expressed in several types of endothelial and epithelial cells in the circulatory and respiratory systems. NO released into the blood vessels induces vasodilation, inhibits platelet activation and aggregation and adhesion, and inhibits smooth muscle proliferation [94]. Moreover, it also hinders leukocyte adhesion and migration, therefore protecting from atherosclerosis. In the respiratory system, it regulates ciliary function of airway epithelial cells [103] and modulates host defence [104].

1.3.2. Pathophysiology of nitric oxide

As outlined above, NO plays a pivotal role in several physiological functions. Therefore, disturbances in its synthesis cause a variety of pathological changes.

During NO synthesis, NOS must be dimerized (coupled) in the presence of haem and BH_4 to catalyse the production of NO from L-arginine. Disruption of the dimerization can cause NOS to function as an NADPH oxidase and generate superoxide anions instead of NO, which is known as NOS uncoupling. One factor that can contribute to NOS uncoupling is the presence of peroxynitrite, which is generated by an initial ROS and NO. Peroxynitrite can convert BH_4 into an inactive form, leading to the production of more ROS and starting a vicious cycle [105]. Furthermore, a reduction in the bioavailability of L-arginine and an increase in ADMA can also lead to the uncoupling of NOS. In addition, the production of ADMA by protein arginine N-methyltransferase type 1 is increased and the degradation by dimethylarginine dimethylaminohydrolase is decreased under oxidative stress, which also contributes to the vicious circle [106].

The increased production of peroxynitrite and other forms of ROS and RNS due to the disruption of the NO pathway can lead to a variety of single and multiorgan diseases, such as septic shock, atherosclerosis, thrombosis formation, or airway hyperresponsiveness and airway remodelling seen in asthma and COPD [107-109]. The pathophysiological changes that lead to the diseases involve the reduction of NO levels and the consequences of peroxynitrite production.

1.3.2.1. Nitric oxide and COPD

Alterations in the NO pathway are involved in the pathogenesis of COPD [25, 90]. Oxidative stress, mediated by peroxynitrite plays a pivotal role in MMP activation and parenchymal destruction and remodelling seen in COPD [110]. Moreover, peroxynitrite can also cause apoptosis [111] by activating MAP kinases and inhibiting phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway [112], further worsening the architectural changes in the lung. Furthermore, peroxynitrite can also cause programmed cell death by nitrating proteins leading to increased levels of misfolded proteins and thus triggering unfolded protein response induced cell death [112]. Peroxynitrite can also lead to necrosis by nitrating the deoxyribonucleic acid (DNA) and critical proteins of cell organelles [113]. In addition, it also activates the nucleotide-binding oligomerization domain-like receptor pyrin domain 3 (NLRP3) inflammasome [114], which has an important role in fibroblast activation and extracellular matrix depletion [115].

The levels of NO produced in the respiratory system can be measured non-invasively by the analysis of exhaled breath or the sputum. NO can be directly measured from the exhaled breath and its most frequently used parameter is the fractional exhaled nitric oxide concentration at 50 mL/s exhalation flow (F_{ENO50}) [27, 116, 117]. The downstream products of NO metabolism, such as nitrite, nitrate, and nitrosothiols can be measured in the exhaled breath condensate (EBC) and in the sputum [118].

Interestingly, although the pathobiological background and the expression studies of NOS suggest an elevated NO production in COPD, F_{ENO50} seems not to be elevated in stable COPD [27]. It may be due to that the superoxide anion production is also increased, and NO is consumed in the reaction [25]. However, NO concentration of the small airways and alveoli, which can be calculated using NO values measured at multiple expiratory flows, is elevated in stable and exacerbated disease [116]. Measurements of markers of NO metabolism in sputum and EBC produced conflicting results in stable COPD [119, 120]. However, the level of fractional exhaled nitric oxide (F_{ENO}) and other NO markers elevates during AE, indicating a further increase in iNOS expression as suggested by

studies measuring the iNOS protein expression in lung tissue [27, 121, 122] and highlighting the role of oxidative and nitrative stress in the pathobiology of AEs [27, 123].

Consequently, it is well established that dysregulation of NO production plays an important role in airway inflammation and in COPD. However, the relationship between airway oxidative and nitrative stress and systemic changes in the NO pathway and vascular eNOS functionality is not well understood. Our hypothesis was that airway inflammation and nitrosative stress are elevated and linked with changes in endothelial NO signalling in stable COPD, and even more marked during an exacerbation. Nonetheless, human studies investigating airway and systemic NO signalling in COPD are lacking.

1.4. Eosinophil granulocytes in COPD

1.4.1. Physiology of eosinophil granulocytes

Eosinophil granulocytes are cells of the innate immune system. They are predominantly formed in the bone marrow from pluripotent CD34+ progenitor stem cells, and they account for less than 5% of all circulating white blood cells [124]. After maturation, they are released from the bone marrow into the circulation and then they exit within 18 hours on average to the peripheral tissues, predominantly to the digestive tract and thymus [125]. However, in response to chemokines produced during inflammatory processes, e.g., C-C motif ligand (CCL) 5, 7, 11 (also named as eotaxin), 13, 15, IL-5, and IL-1β, eosinophils also migrate to bronchial and vascular endothelium, where they can exit the blood stream and migrate to lung tissue if the endothelial cells produce surface adhesion receptors (e.g., vascular cell adhesion molecule 1 (VCAM-1) and P-selectin). If the cytokine milieu is favourable in the lung tissue (granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 in abundance), they can survive for long periods of time [126].

Eosinophils contain four types of basic proteins that mediate cytotoxic effects, namely, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) [124]. Furthermore, eosinophils can also store and produce a wide range of chemotactic and pro-inflammatory agents, such as

TNF-α, TGF-β, IL-2 to IL-5, IL-10, IL-12, IL-13, IL-16, and IL-25 [127]. Overall, this suggests that eosinophils have an immunoregulatory role as well.

The role of eosinophils in the pathomechanism of allergic diseases and in the protective response to parasitic infections is well studied [128]. However, their role in the lung is not fully understood, it is likely that they have complex and overall important functions [128]. Animal models in mice have shown that they are involved in the defence against bacteria and viruses, recognise pathogenic agents by Toll-like receptors expressed on their cell surface, enhance the humoral immune response by stimulating B cells, and are involved in the regulation of the Th1-Th2 cell balance by secreting IL-4, -5 and -13 and by their antigen-presenting capacity [125, 129-131] (**Figure 2**). However, not all these functions are performed at the same time, and the exact function depends on the stimulus, tissue type, and cellular and cytokine environment. It is likely that there are several phenotypes of eosinophil granulocytes, and that a specific, regulatory type is found in the lung [132].

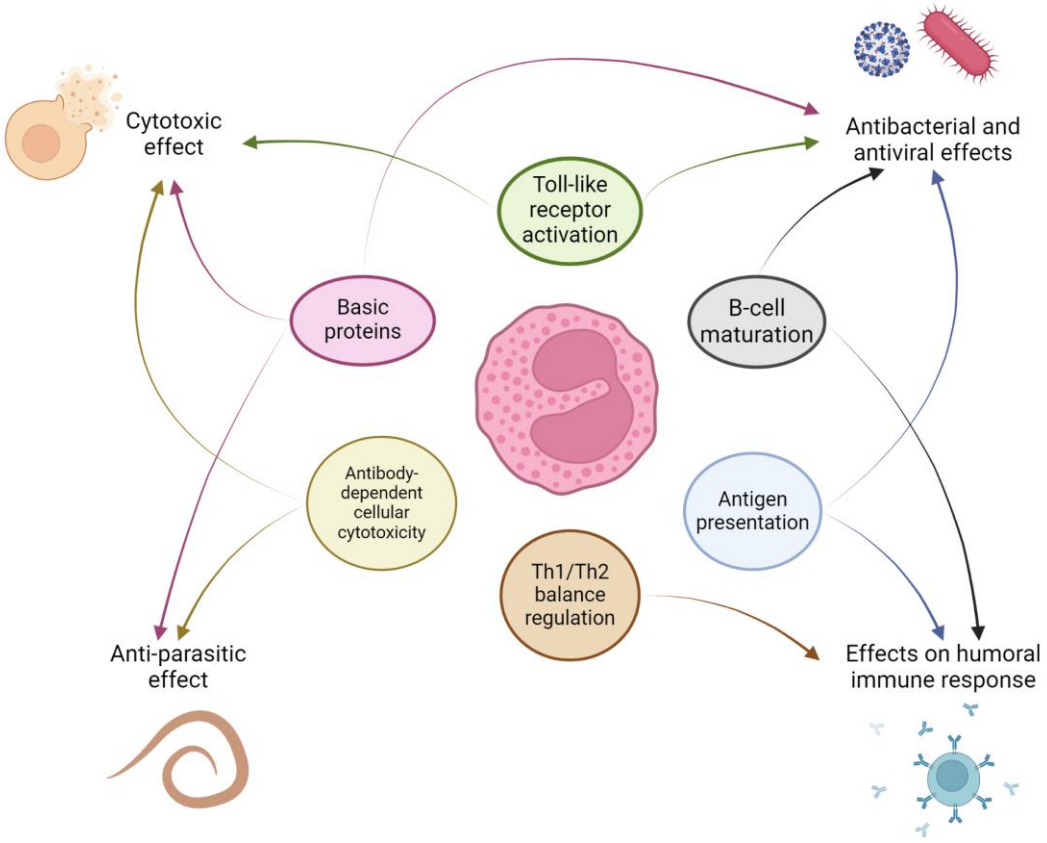

Figure 2. Physiologic roles of eosinophil granulocytes. Arrows represent functional relations. Original work of the author. References are found in the main text.

1.4.2. Eosinophilic inflammation in COPD

Airway inflammation in COPD is usually characterised by the abundance of neutrophil granulocyte and CD8+ T cells [133]. However, in 20-40% of patients, an elevated eosinophil cell count (>3%) can be observed in sputum and bronchoalveolar lavage fluid, either in stable phase or during exacerbations [126]. Nevertheless, the role of eosinophils in the pathophysiology of the disease is not well understood. It is likely that their levels in the lung increase in response to epithelial injury, viral infection, or even to abnormal changes in the airway microbiome [21, 30]. Therefore, the eosinophilic inflammation may be a consequence of the disease rather than a cause. Furthermore, macrophage dysfunction may also play a role in the development through an impaired clearance of eosinophils [134].

1.4.3. The biomarker role of eosinophil number and percentage in COPD

The WHO defines a biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [135]. Airway and blood eosinophil parameters have been investigated as potential disease biomarkers in COPD.

Airway eosinophil count, such as bronchoalveolar lavage (BAL) or induced sputum eosinophil counts serve as an important biomarker for higher clinical improvement following systemic corticosteroids. Brightling et al. found in a crossover RCT that following inhaled momethason therapy, a higher sputum eosinophil count is associated with a more marked improvement in post-bronchodilator FEV_1 in COPD [136]. Similarly, the same research team found in another crossover RCT that the greatest response to a short-term course of systemic prednisolone in terms of FEV_1, quality of life scores and functional test is seen in patients with the highest sputum eosinophil counts [137]. Moreover, Siva et al. found that by aiming to minimise eosinophil counts in induced sputum, a higher reduction in the annual rate of AEs can be achieved than compared to standard therapy [138]. In addition, Ho et al. found that the presence of BAL eosinophilia was associated with an increased 12-month risk of AEs in their secondary analysis of the DISARM RCT [139].

However, the application of airway samples to assess the biomarker role of eosinophils requires laboratory expertise and the invasive or semi-invasive collection of samples can be burdensome both to the patient and to the healthcare provider.

Meanwhile, using BEC as an alternative to sputum eosinophil count is advantageous as the measurement of blood eosinophils is widely available and relatively cheap. However, its levels show intra- and interindividual variability. In healthy people, BEC is reduced by diet, exercise, systemic corticosteroid use and show diurnal variability of up to 21% [140]. According to the ECLIPSE study, measured once a year for 3 years, 51% of patients with COPD remain below or above a threshold of 2% blood eosinophil ratio after the index measurement [141]. Furthermore, Bafadhel et al. found that 65% of patients remained persistently below or above 400 cells/μL by measuring BEC trimonthly for 12 months [142]. Similarly, a meta-analysis also found that 51% of patients remain in the

eosinophil predominant (>2%) or eosinopenic (<2%) category [143]. However, it is important to note that the commonly used 2% cut-off of eosinophil granulocyte ratio is well within the normal range of 5%, although this does not affect its applicability as a biomarker [144]. However, as shown by the contradictory results, there is a gap in the knowledge about the clinical applicability of BEC and blood eosinophil ratios in predicting AECOPDs.

Nonetheless, irrespective of the cause of eosinophilic inflammation, several retrospective epidemiological studies in large populations have shown an association between blood eosinophil number and adverse clinical outcomes. A Dutch study spanning over more than 30 years found that BEC > 275 cells/μL was associated with increased mortality in the general population, and they have observed a 4.8-fold increase in the risk of COPD-associated mortality [145, 146]. Similarly, a Danish study with a median follow-up of 3.3 years demonstrated a 1.76-fold increased risk of exacerbation in patients with a cell count above 340 cells/μL, although the predictive value of BEC was not robust (ROC AUC: 0.63) [147]. However, a Spanish prospective clinical trial published in 2018 with a mean follow-up of 9.6 years found no association between steady-state BEC and exacerbation frequency in COPD patients, and even showed a positive effect on survival [148]. Other studies have also described other characteristics in the eosinophilic patient group, such as higher FEV_1, fewer respiratory symptoms, and better quality of life as measured by disease-specific questionnaires [142, 143, 149]. Moreover, Pascoe et al. reported that BEC is a sensitive predictor of treatment success of ICS in preventing exacerbations [150]. Their results show that patients with a blood eosinophil percentage above >2% have at least a 24% reduction in exacerbations compared to patients receiving a LABA alone, and the reduction has a direct relationship with the eosinophil percentage, reaching a reduction of 42% for eosinophil percentages above 6%.

The prediction of AEs with easy-to-measure markers holds clinical significance and can aid the tailoring of preventive measures. A few studies (both retrospective and prospective) have focused on the potential of blood eosinophil count and percentage as predictors of recurrent AEs providing conflicting data on the clinical importance of the eosinophil-type of AE [142, 151]. As blood eosinophil measurements are directly available during hospitalization with AE, the clarification of the role of blood eosinophil

percentages and counts as biomarkers for recurrent AE should be further pursued. Therefore, in a well-planned prospective cohort study we aimed to study the ability of blood eosinophil count/percentage at the onset of an AE to predict future moderate-severe relapses.

2. Objectives

2.1. Aims to study the association between markers of the nitric oxide pathway and clinical characteristics in COPD

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.

2.2. Aim to study the relationship between eosinophilic exacerbations and subsequent relapses in COPD

1. To investigate whether the eosinophil-type severe AECOPD (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.
2. To analyse the associations of clinical factors with the occurrence of subsequent events.
3. To assess the stability of the eosinophil-type severe AECOPD between different events

3. Methods

3.1. Selection criteria of participants

Participants were recruited from the Department of Pulmonology, Semmelweis University, Budapest, Hungary, across two studies conducted between March 2016 and August 2018. The diagnosis of COPD was confirmed by a respiratory specialist according to the relevant GOLD criteria [1] before enrolment in both studies.

In the study which assessed the endothelial NO pathway in COPD, we enrolled patients with stable COPD (S-COPD, N = 29), those experiencing an acute severe exacerbation requiring hospital admission (AECOPD, N = 32), and control subjects (C, N=15). The study assessing the relationship between eosinophils during AE and later AEs, included only patients during severe AECOPD requiring hospitalization. AECOPD was defined as a sudden worsening of respiratory symptoms within the last 72 hours prior to admission. The control group consisted of smokers with no respiratory symptoms for at least four weeks before inclusion, aged over 40 years, and with a smoking history of more than 10 pack-years.

In both studies, exclusion criteria included history of asthma or positive airway reversibility test (>200 mL or 12% increase in post-bronchodilator FEV_1), chronic lung conditions other than COPD, lung cancer within the last three years, concurrent pneumonia, or the need for invasive ventilation. Furthermore, exclusion applied to those who had received systemic corticosteroid or antibiotic treatment in the past four weeks before admission (the study assessing the predictive role of eosinophils allowed a single dose of systemic corticosteroids administered during emergency care not more than four hours before blood sample collection).

The treatment of the AE was left to the discretion of the treating physician, but all patients received oxygen supplementation, inhaled short-acting bronchodilators, and systemic steroids during their hospital stay according to the international guidelines and recommendations [1].

In both studies, ethical standards were strictly adhered to in compliance with the 1964 Declaration of Helsinki and its subsequent amendments. The studies were approved by the relevant ethics committees of Semmelweis University (approval No. 34/2015 and 191/2017), and all participants provided written informed consent.

3.2. Study designs and outcomes

The study assessing the role of nitric oxide pathway in COPD was an observational prospective clinical cohort study. AECOPD patients were evaluated at two time points: within 24 h of hospital admission and, when feasible, during convalescence (N = 20). The control smokers and patients with S-COPD attended a single visit and no follow-up measurements were performed.

We collected demographic and clinical data, including smoking history, use of inhaled medications, cardiovascular comorbidities, and respiratory symptom severity (COPD Assessment Test [60]).

Furthermore, several measurements were conducted, including white blood cell (WBC) count, serum CRP concentration, arterial blood gas, lung function, and F_{ENO} measurements. Additionally, spontaneous sputum samples were collected from patients (S-COPD N = 13; AECOPD N = 17) to determine differential cell counts and nitrate and nitrite concentrations. Serum samples were collected from all subjects and stored at -80°C for later analysis of L-arginine, ADMA, SDMA, nitrite, and nitrate concentrations.

Moreover, patients with AECOPD underwent repeated lung function and F_{ENO} assessments during convalescence (within 24 h before hospital discharge), completed the CAT, and provided additional serum (N = 19) and sputum (N = 9) samples.

The primary outcome of this study were the levels of nitric oxide synthesis markers (serum L-arginine, ADMA, SDMA, nitrite, and nitrate) in S-COPD, AECOPD, and C subjects, and the correlation of them with markers of airway and systemic inflammation.

The study assessing the relationship between BEC and later AEs was also an observational prospective clinical cohort study. Patients were enrolled during hospitalisation and were followed up for 12 months or until the first moderate or severe

AECOPD. Baseline measurements and data collection were performed <48h of hospital admission. Follow-up was carried out by regular standardised telephone interviews every three months or by personal interviews during readmission to our department. During the follow-up, we noted the time to the first occurrence and severity of AECOPD. Severe AECOPD required hospitalization, while moderate exacerbations were treated with systemic corticosteroids and/or antibiotics in an outpatient setting [73]. Hospital readmissions within four weeks of discharge after the index event were considered treatment failure and were not counted as new relapses. Patients who did not complete the first follow-up review were deemed lost to follow-up and were not included in the analysis.

The primary outcomes of this 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.

Patients were grouped based on their admission blood eosinophil count and blood eosinophil percentage of the total leukocyte count. During our primary analysis, subjects were classified into two groups: eosinophilic (≥2% of leukocytes and/or ≥200 eosinophils/μL) and non-eosinophilic groups [152]. Furthermore, subgroup analyses were also performed using other thresholds, such as ≥3% eosinophils or leukocytes and/or ≥300 eosinophils/μL [153], and grouping patients based on tertiles of blood eosinophil percentage. The flow chart of the enrolment process can be seen on **Figure 3**.

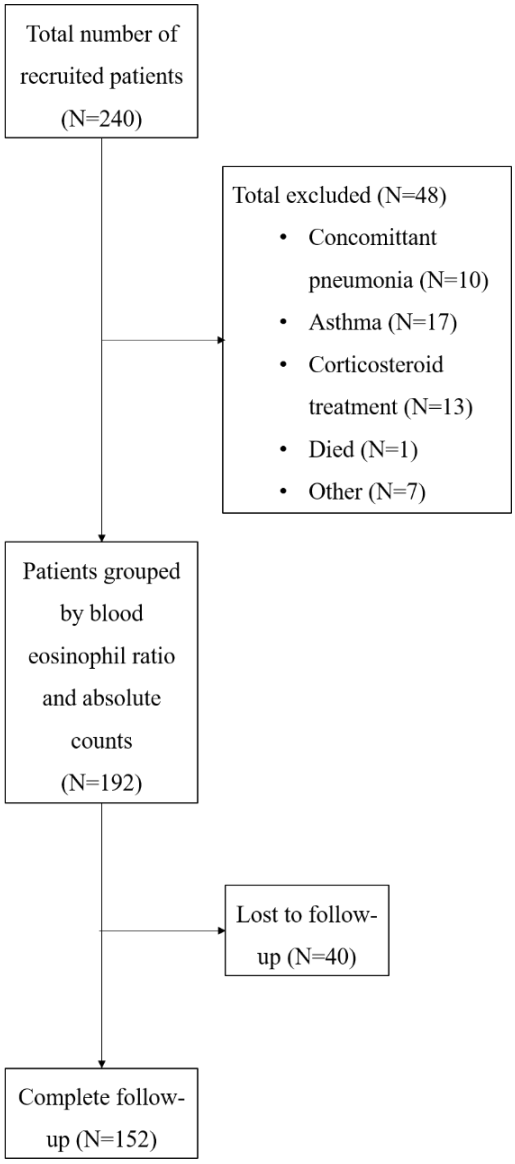

Figure 3. Flow chart of patient enrolment in the eosinophil study. Abbreviation: N: number

3.3. Measurements

Various clinical and laboratory measurements were performed on participants in both studies to assess their health status and physiological responses.

3.3.1. Routine blood tests

WBC and CRP concentrations were measured in venous blood samples from all participants using Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan) and Beckman Coulter AU680 (Beckman Coulter Inc., Indianapolis, IN, USA) analyzers. Blood gases and pH levels were also measured in arterial blood samples from patients using a Cobas b 21 analyzer (Roche, Switzerland).

3.3.2. Respiratory function tests

All subjects underwent spirometry according to current guidelines, and a subset of patients additionally had plethysmography performed (PDT-111, Piston, Budapest, Hungary) [62].

The fractional exhaled nitric oxide (F_{ENO}) concentration was measured at a constant expiratory flow rate of 50 mL/s using the Sievers Nitric Oxide Analyzer i280 (GE Analytical Instruments, Boulder, CO, USA) [118]. Each participant performed at least two F_{ENO} measurements, with the mean values of readings differing by less than 10% being recorded (C: N=15, S-COPD: N=23, AECOPD: N=25).

3.3.3. Sputum and serum analyses

Sputum samples were collected from patients in the morning and processed within two hours according to established protocols [154, 155]. The samples were homogenized in dithiothreitol, filtered, and the supernatant was stored at -80°C for subsequent analysis. Cell counts and differential cell counts of non-squamous cells were determined using a hemocytometer and Diff-Quik staining of cytospins, respectively. Amino acids in blood serum samples were extracted via solid-phase extraction [156], followed by derivatization [157], and analyzed through high-performance liquid chromatography as described by Erdelyi-Botor et al. (Waters 2475 fluorescence detector; Milford, MA, USA) [158]. The detection limits were 0.1μmol/L for L-Arginine and 0.05μmol/L for ADMA and SMDA [159].

3.3.4. Nitrite and nitrate concentrations

Nitrite and nitrate levels were measured in both serum samples and sputum supernatants using a protocol based on capillary electrophoresis, employing a fused silica capillary and

sulfate-β-alanine buffer [160]. Detection limits were set at 0.1 μM for nitrite and 3 μM for nitrate in serum, and 1 μM for nitrite and 10 μM for nitrate in sputum supernatant.

3.3.5. Comorbidity assessment

In the second study, the Charlson Comorbidity Index (CCI) was calculated to quantify the burden of comorbidities in patients [161].

3.4. Statistical analysis

For the first study, statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad Software, San Diego, USA), while TIBCO Statistica version 13 (TIBCO Software Inc., Palo Alto, CA, USA) was used for the second study. Continuous variables were presented as mean ± standard deviation (SD) or median (interquartile range, IQR) depending on their distribution, while categorical variables were expressed as counts or percentages. Comparison of continuous variables were performed by ANOVA or Kruskal-Wallis tests with pairwise post hoc comparisons by Bonferroni correction, or t-tests or Mann-Whitney U-tests, depending on the number of groups and the distribution of the variables. Categorical variables were compared using chi-squared tests or two-tailed Fisher exact tests as appropriate. Correlations between outcome parameters and variables were assessed using Pearson's or Spearman's correlation, depending on the distribution of the data.

In the NO pathway study, F_{ENO}, sputum cell counts, and nitrate and nitrite concentrations in sputum and serum were log-transformed before analysis to reduce skewness.

In the eosinophil study, a Cox proportional hazards model was employed to investigate the association between eosinophil granulocyte count or ratio and the time to first re-exacerbation. The time to the next exacerbation between high- and low-eosinophilic subgroups was compared using the log-rank test. A generalized linear regression model with a negative binomial distribution was used to identify risk factors for relapse.

A significance level of $p < 0.05$ was applied throughout.

In the eosinophil study, the required sample size per group was calculated based on previous studies on COPD-related readmission rates [152] and the ECLIPSE study, where 47% of the patients experienced ≥1 moderate or severe exacerbations in the year before recruitment [5]. The minimum sample size per group was calculated to be 39 to achieve a statistical power of 0.80 with a significance level of 0.05.

4. Results

4.1. Study on the markers of the nitric oxide pathway in COPD

4.1.1. Clinical characteristics of the subjects

The control subjects were younger and had a less extensive smoking history than the COPD patients (**Table 3**). Cardiovascular comorbidities were more prevalent among the patients than among the control participants (proportion of subjects with at least one comorbidity: control, 33%; S-COPD, 72%; AECOPD, 75%; $p = 0.01$). WBC and CRP levels were elevated in patients with AECOPD compared with those in stable patients. F_{ENO} was increased in AECOPD but not in S-COPD. There was no difference in inhaled maintenance therapy between the patient groups. The sputum profiles of patients with S-COPD and AECOPD were similar, and there were no significant differences between hospital admission and discharge values (**Table 3**).

Lung function parameters (FEV_1 % pred. 38 ± 13 vs. $49 \pm 19\%$, $p = 0.02$) and symptom severity (CAT score 22 ± 8 vs. 17 ± 9, $p = 0.03$) significantly changed at hospital discharge (9 ± 3 days after admission) compared to the AECOPD onset (**Table 3**).

Table 3. Clinical characteristics of participants in the study assessing the relationship between COPD and NO pathway.

	Control	S-COPD	AECOPD	p-value
		COPD		
Number (male)	15 (6)	29 (13)	32 (21)	(0.15)
Age, years	51±7	63±8***	63±8***	<0.001
Current/ex-smoker, N	12/3	20/9	19/13	0.36
Pack-years	30 (25-40)	50 (40-75)*	50 (31-78)*	<0.01
Systemic hypertension, N	5	18	21	0.10
Heart failure, N	0	4	3	0.32
Cerebrovascular accident, N	1	2	4	0.70

ICS, N	NA	18	25	0.26
LABA, N	NA	28	29	0.61
LAMA, N	NA	27	29	0.99
Oral theophylline, N	NA	5	11	0.15
White blood cell count, G/L	9.4±3.8	8.6±2.9	11.1±3.9$^{\#}$	0.02
CRP, mg/L	4 (2-6)	5 (2-10)	8 (3-14)	0.05
FEV_1, %ref.	101±14	47±14***	39±13***	<0.001
FVC, %ref.	109±13	78±19***	65±18***$^{\#\#}$	<0.001
FEV_1/FVC	0.78±0.08	0.49±0.09***	0.47±0.09***	<0.001
RV/TLC	0.31±0.05	0.56±0.12***	0.58±0.10***	<0.001
Raw, $kPa \cdot s \cdot L^{-1}$	0.24±0.08	0.46±0.15***	0.46±0.12***	<0.001
pH	NA	7.40±0.03	7.41±0.03	0.49
PaO_2, mmHg	NA	60±8	62±10	0.29
$PaCO_2$, mmHg	NA	43±8	42±7	0.61
CAT score	NA	19±7	21±8	0.16
F_{ENO}, ppb	12±2	14±2	26±3**$^{\#}$	<0.01

Data are presented as mean ± SD (geometric mean ± geometric SD for F_{ENO}) and compared with ANOVA and post-hoc test or chi-square test (categorical variables) or shown as median (interquartile range) and analysed with Kruskal Wallis and Dunn's post hoc test. Abbreviations: AECOPD: acute exacerbation of COPD, CAT: COPD Assessment Test, CRP: C-reactive protein, ICS: inhaled corticosteroid, F_{ENO}: fractional exhaled nitric oxide concentration, FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, ICS: inhaled corticosteroid, , LABA: long-acting beta2-agonist, LAMA: long-acting muscarinic antagonist, N: number, NA: not applicable, $PaCO_2$: partial pressure of carbon dioxide in arterial blood, PaO_2: partial pressure of oxygen in arterial blood, Raw: airway resistance, ref.: reference, RV: residual volume, S-COPD stable COPD, TLC: total lung capacity. *p<0.05, ***p<0.001 vs. control, $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. stable COPD.

4.1.2. Serum L-arginine/ADMA and SDMA concentration

The ratio of L-arginine and ADMA, which indicates the availability of L-arginine for NOS, was decreased in S-COPD ($p < 0.01$) and AECOPD ($p < 0.05$) when compared to controls (C: 287 ± 64, S: 214 ± 58, AE: 231 ± 68; ANOVA $p < 0.01$; **Figure 4**a) and it did not change at convalescence (243 ± 123; paired t-test, $p = 0.89$, **Figure 4**b). In COPD, there was a trendwise correlation between serum L-arginine and F_{ENO} levels ($r = 0.28$, $p = 0.05$), but not with other parameters ($p > 0.1$). Notably, serum ADMA levels correlated with age ($r = 0.25$, $p = 0.04$), blood neutrophil percentage ($r = 0.36$, $p < 0.01$; **Figure 4**c), and F_{ENO} ($r = 0.42$, $p < 0.01$; **Figure 4**d).

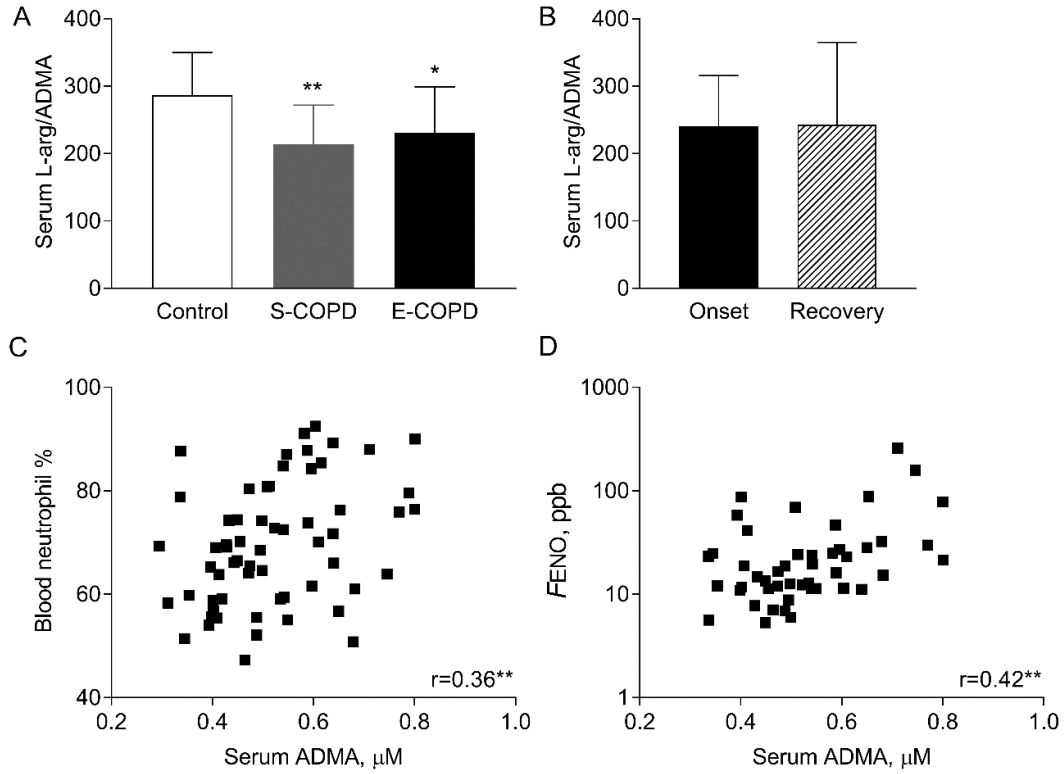

Figure 4. Serum L-arginine/ADMA in smoking controls and patients with COPD. Serum L-arginine/ADMA was analysed among smoking control and patients with stable and exacerbated COPD (A) and between the onset and the recovery of an acute severe exacerbation (B). Correlation between serum ADMA concentration and blood neutrophil percentage and F_{ENO} was also analysed in patients with stable and exacerbated COPD (C and D). Control: smoking control subjects, S-COPD: stable COPD, AECOPD: exacerbation of COPD, F_{ENO}: fractional exhaled nitric oxide concentration. *$p < 0.05$, **$p < 0.01$ vs. Control. Data are shown as mean and standard deviation. Adopted from [162] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

The levels of SDMA were increased only during an exacerbation (0.78 ± 0.39 μM vs. C: 0.45 ± 0.14 μM and S-COPD: 0.53 ± 0.14 μM, $p < 0.001$ and $p < 0.01$; **Figure 5**a) and it decreased upon recovery (0.57 ± 0.42 μM, $p < 0.05$; **Figure 5**b). Admission serum SDMA showed correlation with age ($r = 0.67$, $p < 0.001$), total sputum inflammatory cell count

($r = 0.61$, $p < 0.01$; **Figure 5**c), and sputum neutrophil count ($r = 0.62$, $p < 0.01$; **Figure** 5d) in AECOPD.

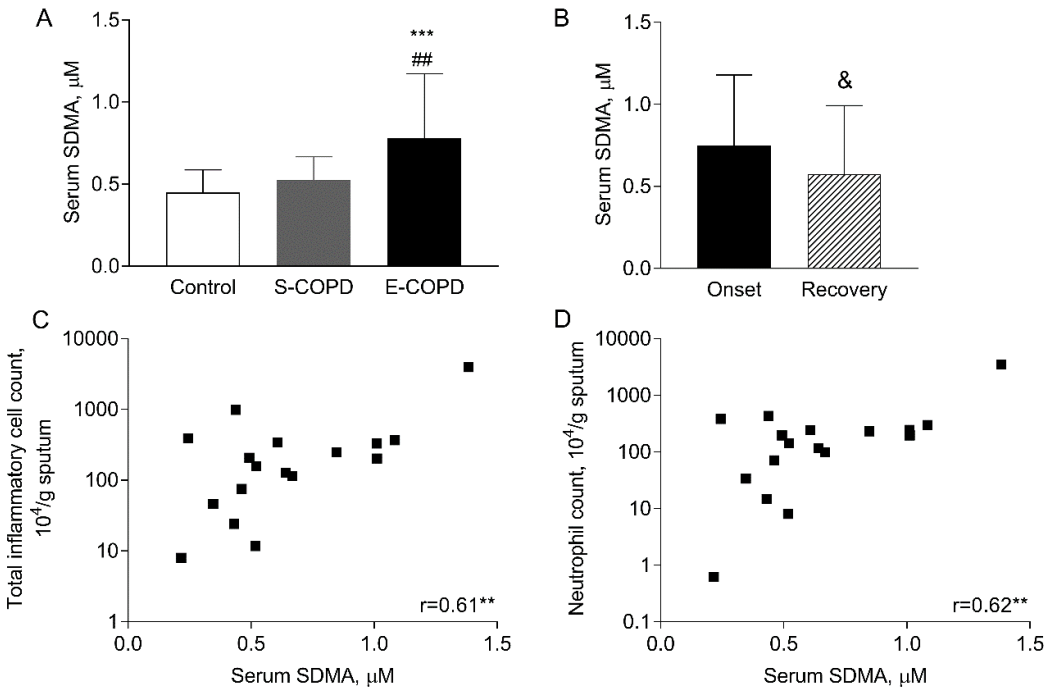

Figure 5. Serum SDMA concentration in smoking controls and patients with COPD. Serum SDMA concentration was compared among smoking controls and patients with stable and exacerbated COPD (A) and between the onset and the recovery of an acute severe exacerbation (B). Correlation between serum SDMA concentration and sputum inflammatory cell count and neutrophil count was also analysed in patients with AECOPD (C and D). Control: smoking control subjects, S-COPD: stable COPD, AECOPD: exacerbation of COPD. **$p < 0.01$, ***$p < 0.001$ vs. Control. ##$p < 0.01$ vs. S-COPD, &$p < 0.05$ vs. Onset. Data are shown as mean and standard deviation. Adopted from [162] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.1.3. Serum nitrate and nitrite concentration

There were no differences between any of the groups in serum nitrate concentrations (C: 121 ± 2 μM, S-COPD: 79 ± 4 μM, AECOPD: 65 ± 5 μM, $p = 0.36$, **Figure 6**a), and it was also similar at hospital admission and discharge in patients with AECOPD (47 ± 9 μM, $p = 0.87$; **Figure 6**b). On the other hand, serum nitrite levels were elevated in COPD compared to control participants (S-COPD: 4.11 ± 2.12 μM and AECOPD: 4.03 ± 1.77 μM vs. 1.61 ± 1.84 μM, both $p < 0.001$, **Figure 6**c); however, there were no differences between admission and recovery concentrations in AECOPD (3.64 ± 1.63 μM $p = 0.26$, **Figure 6**d).

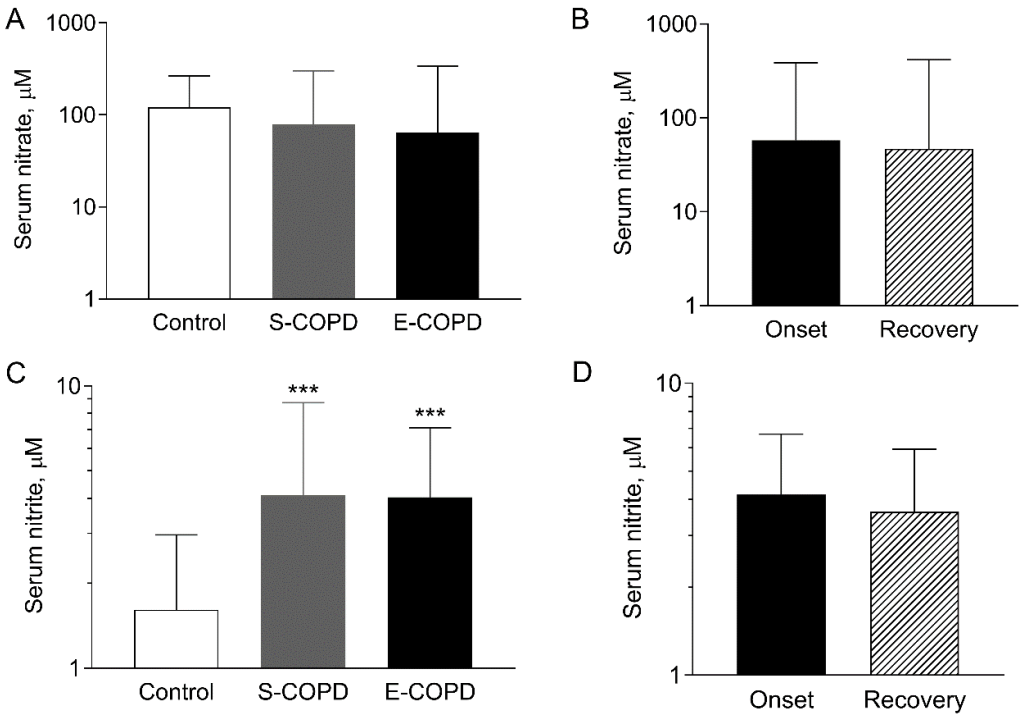

Figure 6. Serum nitrate and nitrite concentration in smoking controls and patients with COPD. Logarithmically transformed serum nitrate and nitrite concentrations were analysed among smoking control and patients with stable and exacerbated COPD (A and C, ***$p < 0.001$) and between the onset and the recovery of an acute severe exacerbation (B and D). Control: smoking control subjects, S-COPD: stable COPD, AECOPD: exacerbation of COPD. Data were analysed after log transformation and are shown as geometric mean and geometric standard deviation. Adopted from [162] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

Serum nitrite levels in COPD patients did not correlate with sputum nitrate or nitrite concentrations, F_{ENO}, age, pack-years, lung function, or blood parameters ($p > 0.1$ for all variables).

4.1.4. Sputum nitrate and nitrite concentration

Sputum nitrate concentration was elevated in AECOPD compared to that in S-COPD (205 ± 2 µM vs. 87 ± 3 µM, $p < 0.05$; **Figure 7**a) and declined at hospital discharge (81 ± 3 µM; **Figure 7**b). In contrast, there was no difference in the levels of sputum nitrite between AECOPD and S-COPD patients (14.59 ± 1.97 µM vs. 21.74 ± 2.41 µM, $p = 0.17$; **Figure 7**c), and at admission and discharge in AECOPD, either (15.02 ± 1.58 µM, $p = 0.88$; **Figure 7**d).

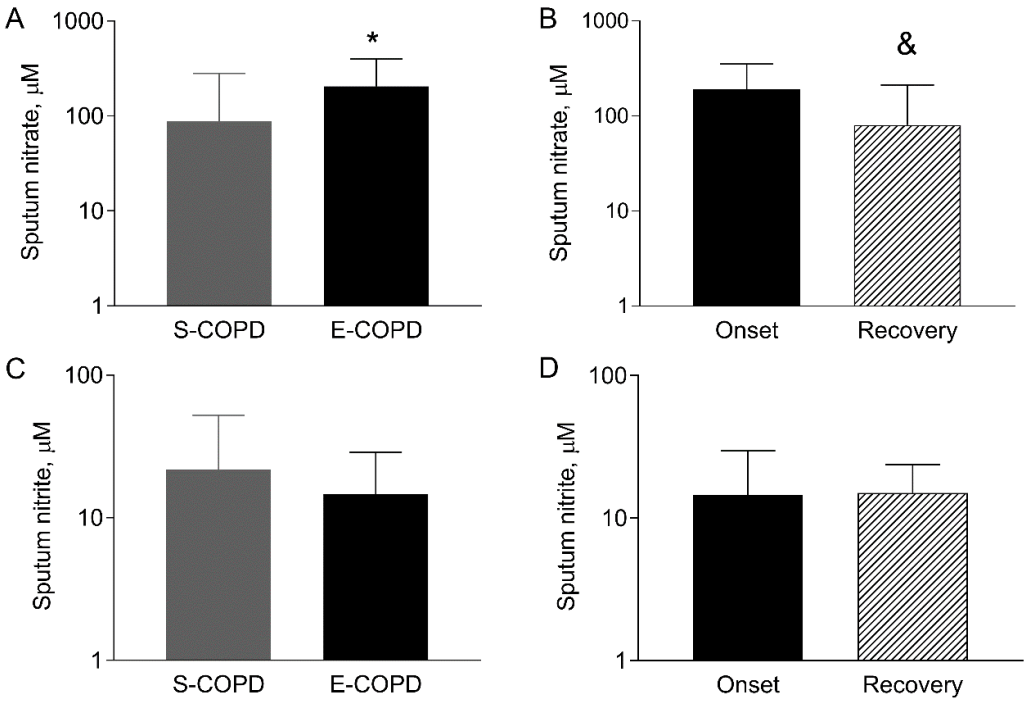

Figure 7. Sputum nitrate and nitrite concentration in COPD. Sputum nitrate and nitrite concentrations were analysed between patients with stable and exacerbated COPD (A and C, *$p < 0.05$), and between the onset and the recovery of an acute severe exacerbation (B and D, $p = 0.06$). S-COPD: stable COPD, AECOPD: exacerbation of COPD. Data were analysed after log transformation and are shown as geometric mean and geometric standard deviation. Adopted from [162] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

4.2. Study on eosinophilic exacerbations and subsequent relapses in COPD

4.2.1. Clinical characteristics of the subjects

A total of 152 patients were included in the study (Error! Reference source not found.). Of these, 51 patients had eosinophil-high exacerbations as an index event (**Table 4**). Patients in the eosinophilic group had higher FEV_1/FVC values and lower serum CRP concentrations than those in the non-eosinophilic relapse group. There were no other differences in baseline clinical characteristics, such as antibiotic therapy, need for non-invasive ventilation, length of hospital stay, or proportion of treatment failure.

Table 4. Patient characteristics of the eosinophilic exacerbation study.

	Total (N=152)	Eosinophilic AECOPD (N=51)	Non-eosinophilic AECOPD (N=101)	P value
Male	71 (46.7)	23 (45.1)	48 (47.5)	0.86
Age, years	66.2 ± 8.7	65.9 ± 9.1	66.3 ± 8.6	0.81
Charlson Comorbidity Index	2 [1-3]	2 [1-3]	2 [1-3]	0.93
Current smoker	98 (64.5)	32 (62.7)	66 (65.3)	0.56
Tobacco smoke exposition, PY	40 [30-50]	40 [30-50]	40 [30-50]	0.94
FEV_1 (postbronchodilator), %predicted	35.52 ± 14.07	35.89 ± 11.59	35.35 ± 15.18	0.85
FEV_1/FVC	0.47 ± 0.11	0.51 ±0.10	0.45 ± 0.11	**<0.001**
Time since COPD diagnosis, years	7 [3-12]	7 [2-11]	6.5 [3.5-12.5]	0.52
Hospital admission due to COPD in the previous year, N	0 [0-1]	0 [0-1]	0 [0-1]	0.67

Admission corticosteroid use, N (%)	43 (37.0)	17 (41.4)	26 (34.7)	0.55
Baseline ICS use, N (%)	93 (61.2)	29 (56.9)	64 (63.4)	0.38
Baseline LAMA use, N (%)	118 (77.6)	38 (74.5)	80 (79.2)	0.40
Baseline LABA use, N (%)	117 (77.0)	38 (74.5)	79 (78.2)	0.53
Post-discharge ICS use, N (%)	105 (69.1)	33 (64.7)	72 (71.3)	0.46
WBC count on admission, 10^9/L	10.88 ± 4.17	11.20 ± 4.10	10.72 ± 4.22	0.50
Eosinophil count, cell/μL	91 [19-261]	362 [251-524]	37 [4-89]	**<0.0001**
Eosinophil count, % of WBC	0.80 [0.20-2.45]	3.50 [2.40-5.20]	0.40 [0.03-0.80]	**<0.0001**
C-reactive protein, mg/L	9.7 [3.0-27.0]	7.8 [2.6-17.5]	14.4 [4.2-37.9]	**0.02**
Antibiotic use, N (%)	116 (77.9)	37 (72.5)	79 (80.6)	0.30
Need for non-invasive ventilation, N (%)	24 (15.8)	5 (9.8)	19 (18.8)	0.24
Length of hospitalization, days	7 [5-10]	7 [5-12]	7 [6-10]	0.89
Treatment failure, N (%)	31 (20.6)	13 (26.0)	18 (18.0)	0.29
Data are shown as mean ± SD and compared with t-test or shown as median [interquartile range] and analysed with Mann-Whitney U-test. Categorical variables were analysed with Fisher exact test. Abbreviations: AECOPD: acute exacerbation of COPD, CRP: C-reactive protein, ICS: inhaled corticosteroid, FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, ICS: inhaled corticosteroid, LABA:				

long-acting beta2-agonist, LAMA: long-acting muscarinic antagonist, N: number, PY: pack-year, WBC: white blood cell

4.2.2. Re-exacerbations in the eosinophilic and non-eosinophilic groups

During follow-up, 118 patients (78% of all patients) 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 (Fisher's exact test: p=0.84, **Figure 8**).

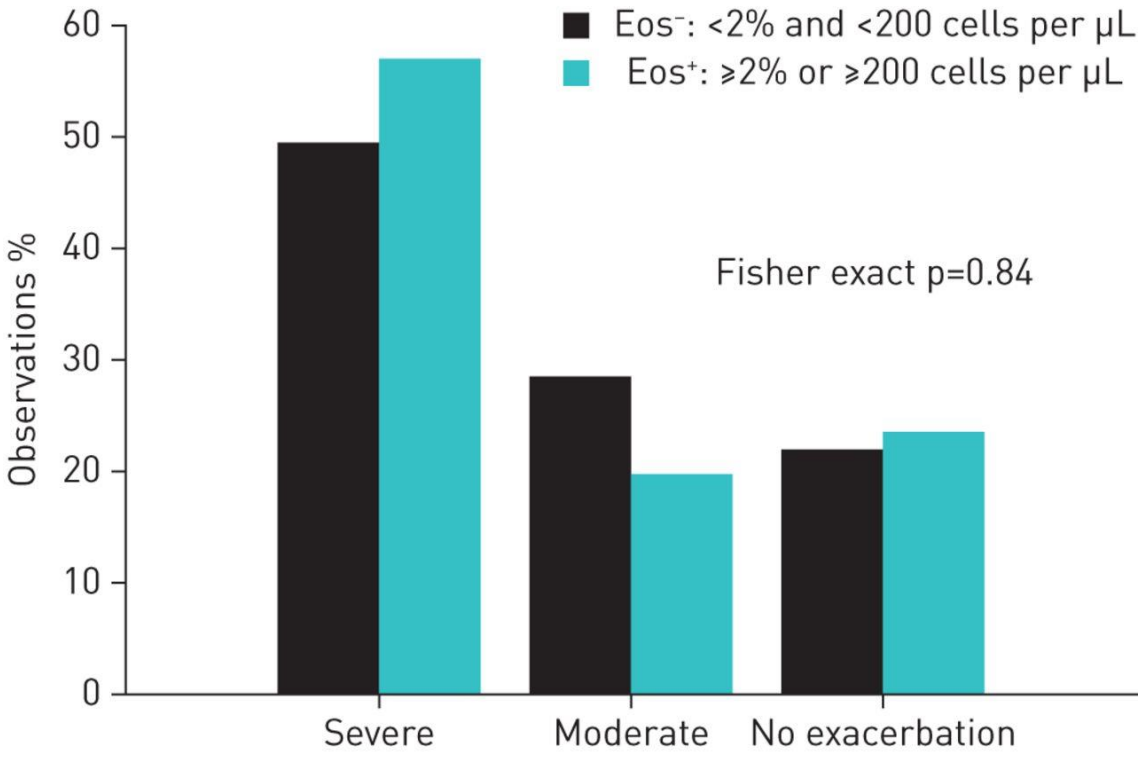

Figure 8. Patients with and without exacerbations in the eosinophilic (Eos^+) and non-eosinophilic (Eos^-) groups during the 12 months of follow-up. Adopted from [163] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

The time to first relapse was not different between the eosinophilic (≥2% and/or ≥200 eosinophils/µL) and non-eosinophilic groups (21 (10–36) weeks vs. 17 (9–36) weeks, p=0.48, log-rank test: p=0.63), as shown by the Kaplan–Meier function plot (**Figure 9**). Similarly, the Cox proportional hazard model did not show an elevated hazard for a shorter time to re-exacerbation in the eosinophil-high group (HR: 1.10, 95% CI 0.75–1.61, p=0.64). Furthermore, no difference was found in the time to the first relapse when separating events based on severity (log-rank test for severe exacerbations: p=0.90; for moderate exacerbations: p=0.51). Moreover, the exclusion of patients who received systemic steroids before blood sampling did not result in any intergroup differences.

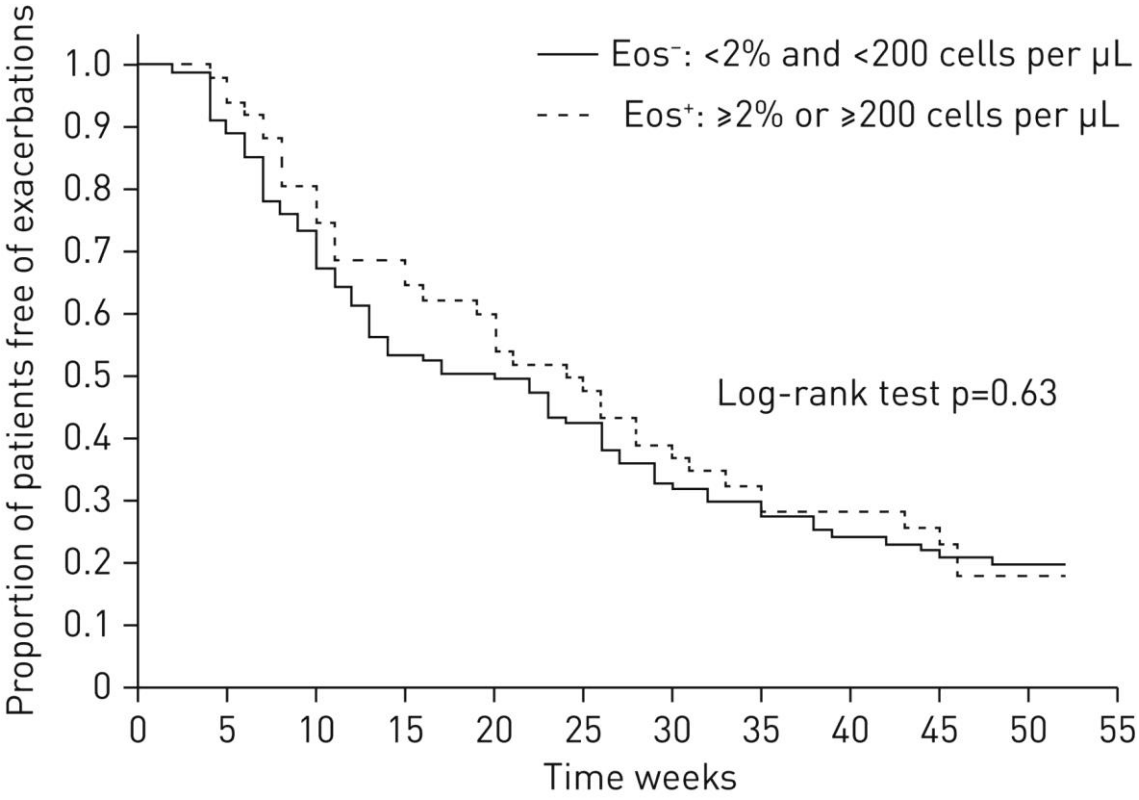

Figure 9. Recurrence of moderate or severe exacerbations in the eosinophilic (Eos+) and non-eosinophilic (Eos−) groups. Adopted from [163] under the terms of the Creative Commons Attribution Non-Commercial Licence 4.0 (http://creativecommons.org/licenses/by/4.0/).

A higher proportion of patients were discharged from the hospital receiving oral corticosteroids on a tapering regime in the eosinophilic group (76% vs. 57%, p=0.03). However, the time to the first re-exacerbation was not different between the subgroups in this regard (log-rank p=0.13).

4.2.3. Other cut-off values for the eosinophilic phenotype

As previously outlined, we also tested our hypothesis using a threshold of blood eosinophil granulocytes ≥3% of total leukocytes and/or ≥300 eosinophils/μL [153]. In this scenario, 39 patients (26% of all patients) belonged to the eosinophil-high group. We did not find any difference in the rate of subsequent AECOPD during follow-up (Eos^+: 76.9%, Eos^-: 77.9%, p=0.53), the severity of the relapse (no relapse/moderate exacerbation/severe exacerbation Eos^+: n=9/7/23; Eos^-: n=25/32/56, p=0.42), the time until the event (Eos^+: 20 (8–33) weeks vs. Eos^-: 22 (10–38) p=0.54; log-rank test p=0.66, Figure 3), or the HR of the eosinophilic group (HR: 0.91, 95% CI 0.60–1.38, p=0.66).

Moreover, we did not detect differences in the outcomes by dividing patients into tertiles of the eosinophil ratios (<0.4% n=50, 0.4–1.8% n=51, >1.8% n=51, time to the first AE Eos low: 23 (10–52) weeks; Eos medium: 14 (7–35) weeks; Eos high: 20 (10–35) weeks, analysis of variance p=0.21).

4.2.4. Risk factors associated with the time to recurrence of exacerbations

We used logistic regression models to assess which clinical parameters were associated with the time until the first subsequent AE. We included in the models either BEC or blood eosinophil percentage. **Table 5** and **Table 6**). We found that the number of past yearly exacerbations, degree of airflow limitation (% predicted post-bronchodilator FEV_1 at discharge), and higher burden of cigarette exposure were associated with a shorter time to relapse. Furthermore, according to the regression coefficients, the best predictor for a future AE was the number of past events, which was also confirmed by the forward stepwise regression model building.

Table 5. Linear regression model of risk factors and time to first re-exacerbation including blood eosinophil percentage.

Variables	Regression coefficient	Standard error	Lower CI (95%)	Upper CI (95%)	P value
Eosinophil (%)	-0.034	0.041	-0.115	0.046	0.40
% Predicted post-bronchodilator FEV_1 at discharge	0.015	0.006	0.003	0.028	**0.01**
Post-bronchodilator FEV_1/FVC ratio in % at discharge	-1.320	0.764	-2.818	0.177	0.08
Charlson-index	0.010	0.068	-0.123	0.143	0.89
Smoking history, PY	-0.008	0.003	-0.014	-0.001	**0.03**
No. of severe AEs in the past 12 months	-0.103	0.047	-0.195	-0.010	**0.03**
Time since COPD diagnosis, years	0.001	0.014	-0.026	0.028	0.92
Age	0.003	0.010	-0.016	0.022	0.74
Gender (male)	0.019	0.084	-0.145	0.183	0.82
Smoking habit (current smoker)	-0.130	0.088	-0.302	0.043	0.14
Need for non-invasive ventilation	-0.011	0.111	-0.229	0.206	0.92
Abbreviations: AE: acute exacerbation, CI: confidence interval, COPD: chronic obstructive pulmonary disease, FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, PY: pack-year					

Table 6. Linear regression model of risk factors and time to first re-exacerbation including blood eosinophil count.

Effect	Regression coefficient	Standard error	Lower CI (95%)	Upper CI (95%)	P value
Absolute eosinophil count	-0.001	0.000	-0.001	0.000	0.12

% Predicted post-bronchodilator FEV_1 at discharge	0.016	0.006	0.004	0.028	**0.01**
Post-bronchodilator FEV_1/FVC ratio in % at discharge	-1.288	0.758	-2.773	0.197	0.09
Charlson-index	0.001	0.067	-0.131	0.133	0.99
Smoking history, PY	-0.008	0.003	-0.014	-0.001	**0.03**
No. of severe exacerbations in the past 12 months	-0.102	0.046	-0.193	-0.011	**0.03**
Time since COPD diagnosis, years	<0.001	0.014	-0.026	0.027	0.98
Age	0.002	0.010	-0.016	0.021	0.80
Gender (male)	0.021	0.083	-0.142	0.183	0.80
Smoking habit (current smoker)	-0.137	0.088	-0.308	0.035	0.12
Need for non-invasive ventilation	-0.020	0.110	-0.235	0.195	0.85
Abbreviations: AE: acute exacerbation, CI: confidence interval, COPD: chronic obstructive pulmonary disease, FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, PY: pack-year					

4.2.5. Phenotypes of the subsequent exacerbation

Eosinophil data of subsequent severe exacerbations were available for 73 patients (**Table 7**). 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 ($p<0.001$).

Table 7. Variability of the rate of eosinophilic (Eos+) and non-eosinophilic (Eos-) exacerbations between the index hospitalization and re-admission by the next relapse

	Re-admission: Eos+	Re-admission: Eos-	Total
Index AE: Eos+	14 (51.9)	13 (48.1)	27
Index AE: Eos-	5 (10.9)	41 (89.1)	46
Total	19 (26.0)	54 (73.0)	73
Data are presented as number of observations (%). Fisher-exact test $p<0.001$. Abbreviations: AE: acute exacerbation, Eos: eosinophil granulocyte			

5. Discussion

Despite significant advances in our understanding of the pathomechanism in COPD, the relationship between airway and systemic inflammation and its impact on disease progression and exacerbation remains poorly understood. Importantly, vascular inflammation in stable and exacerbated COPD may contribute to the development of cardiovascular comorbidities commonly observed in COPD patients [164], and a recent clinical study suggests that triple inhalation therapy including anti-inflammatory inhaled corticosteroids may prevent cardiovascular events [165]. Furthermore, acute exacerbations may have different inflammatory phenotypes linked with therapeutic consequences and different clinical characteristics. Blood eosinophil count, as a surrogate non-invasive readout for airway eosinophil inflammation, has been suggested to delineate the eosinophilic subtype of AEs, which has better short-term outcomes [152], nonetheless, it has not been proven if it helps clinicians to predict subsequent exacerbations, an important clinical endpoint. Therefore, we conducted two studies that investigated the relationship between airway inflammation and markers of endothelial dysfunction, as well as the role of blood eosinophil count as a biomarker of the risk for acute relapses.

The precise mechanism of vascular wall damage in COPD remains unclear. Endothelial dysfunction, which serves as an early indicator of heightened cardiovascular risk, is a component of the vascular damage [166]. Nitric oxide, produced in the endothelium, plays a vital role in regulating vascular tone and local blood flow [93]. Brachial artery flow-mediated dilatation (FMD), a marker of reduced NO-mediated vasodilation and endothelial dysfunction, is impaired in COPD patients [167]. Moreover, in a cohort of patients with diverse COPD severity and prevalent cardiovascular comorbidities, lower FMD was associated with lower FEV_1% predicted and lower daily physical activity [168]. However, our study was the first to simultaneously assess endothelial NO signalling and airway inflammation in COPD.

Endothelial dysfunction links cardiovascular comorbidities and COPD [52, 169], which can be investigated by measuring circulatory markers of the NO pathways [170]. Of note, it has been established that in COPD, the production of NO is reduced in the endothelium

[171], in part due to the increased concentration of ADMA, a competitive eNOS inhibitor [170, 172]. Our study expands upon previous research by demonstrating that the serum L-arginine/ADMA ratio, which indicates the availability of eNOS substrate, is reduced to a similar extent in both stable and exacerbated disease states. According to our findings, serum ADMA levels in patients with COPD were found to be correlated with both systemic and airway inflammatory markers, such as blood neutrophilia, sputum neutrophilia, and exhaled NO. Notably, the treatment of COPD exacerbation did not have any impact on the serum L-arginine/ADMA ratio, suggesting that there is a permanent dysfunction that is not influenced by the clinical state of COPD. The serum ADMA concentration increased by an average of 0.1 and 0.2 μM in patients with stable and exacerbated COPD, respectively, can have circulatory effects [173], and falls within the range associated with an elevated risk of cardiovascular death [174].

During a COPD exacerbation, we observed higher serum levels of SDMA, which suggests that eNOS functionality is further impaired during flare-ups. This is supported by the fact that COPD relapses, particularly those requiring hospitalization, are associated with an increased risk of cardiovascular events [175]. Furthermore, serum SDMA levels were also linked to the severity of airway neutrophilia, indicating a possible connection between eNOS dysfunction and airway processes in COPD.

The demonstrated association between COPD and endothelial dysfunction is of great importance as CVDs are one of the most common comorbidities in COPD, affecting up to 70% of patients, and is a leading cause of mortality in this population [176]. Several studies have previously investigated the role of ADMA on the development of cardiovascular events, including a prospective observational study spanning 24 years, which found that in women, each 0.15 μmol/L increase in ADMA levels increased the risk of fatal CV events by 30%, even after adjustment for traditional risk factors [177]. Less evidence is available for SDMA, but the existing literature consistently describes an increased risk. For example, Schulze et al. in a 7-year follow-up of patients with ischaemic stroke found that serum SDMA levels were a direct risk factor for post-stroke mortality (HR 2.4 95% CI: 1.6-3.7) [178]. These associations highlight the relevance of our results that serum SDMA levels are reduced by treatment of AE, thereby presumably reducing CV risk.

Our findings imply that medications targeting airway inflammation could potentially benefit endothelial dysfunction in COPD patients by enhancing eNOS functionality. This is supported by evidence that ICS treatment might decrease the likelihood of acute myocardial infarction in individuals with COPD [179], and arterial stiffness can be improved by ICS/LABA therapy [180]. In line with this, Singh et al. recently showed that triple combination of LAMA/LABA/ICS reduced the occurrence of cardiovascular events in patients with moderate-severe COPD and a history of exacerbations [165]. We have demonstrated that an increase in serum SDMA concentration during AECOPD is reversed together with a decrease in airway inflammation (as indicated by reduced sputum nitrate concentration and F_{ENO}) due to systemic corticosteroid treatment.

Glucocorticoids can induce eNOS functionality. It was shown that dexamethasone administration to human vascular endothelial cells [181] and also to mice [182] increases eNOS activity via non-transcriptional activation pathways i.e. by inducing phosphoinositide 3-kinase/Akt signalling. This required a high glucocorticoid dose [181], which is presumably achieved by the corticosteroid regimen administered during COPD exacerbations (the usual starting dose at our department is 40-60 mg daily dose of methyl-prednisolone, and then 20-40 mg drug is usually administered daily for 5-7 days). However, the daily and cumulative drug dosages were not registered in our studies.

We did not observe any correlation between ADMA, SDMA, and lung function, blood gas parameters, or serum CRP. This aligns with the results of Clarenbach et al., who did not report an association between endothelial dysfunction and markers of systemic inflammation and oxidative stress, hypoxaemia, age, current smoking, and pack-years in patients with stable COPD [168]. Nevertheless, we found that age correlated with both parameters. This result is consistent with previous research indicating that ADMA and SDMA contribute to endothelial dysfunction in the elderly population [183].

The levels of nitrite in the serum were found to be increased in both stable and exacerbated COPD. Nitrite can act as a reservoir for NO, as it can be converted into nitric oxide by deoxyhemoglobin-mediated reduction under conditions of acidosis or hypoxia, which are commonly present in COPD [184]. This mechanism is supported by the observation that nitrite and then NO production from dietary supplements rich in inorganic nitrate leads

to a decrease in systemic blood pressure in patients with COPD [185]. This mechanism can lead to an increase in serum nitrite levels in COPD which may result in local nitric oxide production in the vasculature to counteract the reduced activity of eNOS.

The activity of the inducible NOS in the airway is elevated in stable COPD [90], which leads to a higher concentration of airway nitrite and nitrate [186]. We have previously demonstrated that during an exacerbation, the heightened nitrosative stress can be measured as raised NO levels in the exhaled breath [27]. The present findings also show that sputum nitrate concentration is higher during exacerbation. Serum ADMA levels showed correlations with F_{ENO} but did not correlate with sputum nitrate or nitrite concentrations. The multi-fold reactions of NO in COPD airways, such as the production of nitrite/nitrate and peroxynitrite or its reaction with tyrosine residues, might explain this phenomenon.

It cannot be excluded that the elevated serum nitrite levels in COPD patients could be connected to increased activity of iNOS in circulating and vascular cells. Additionally, we cannot discount the possibility that the altered concentration of serum ADMA and SDMA may also influence iNOS function in these cells. Blood lymphocytes in individuals with COPD exhibited heightened iNOS activity [187], whereas neutrophil granulocytes, which were present in increased numbers in patients, did not display iNOS activity in healthy humans [188]. Additionally, patients with end-stage disease exhibited a decreased pulmonary artery eNOS expression, but an increased iNOS expression [189]. However, these findings were not confirmed by another study [190].

We found that F_{ENO} levels were elevated only during AEs compared to control subjects, which is consistent with several previous studies dating back to decades [191, 192]. The elevation of F_{ENO} levels during AEs was previously linked to an increase in iNOS expression [121], and it has also been associated with eosinophilic inflammation and blood eosinophilia [191]. Moreover, we also found that serum ADMA concentration was correlated with F_{ENO} levels, possibly linking eosinophilic inflammation during AE to endothelial dysfunction. However, serum SDMA showed correlation only with neutrophilic inflammation, but not to sputum or blood eosinophil counts.

Overall, we demonstrated the dysregulation of the endothelial nitric oxide pathway in COPD and confirmed an elevated F_{ENO} production during AEs, which in part has also been linked to elevation of eosinophil granulocyte production [118, 193], a better response to systemic corticosteroid treatment and an increased risk of future relapses [194-196]. However, F_{ENO} measurements are not easy to access in most settings, thus, we aimed to study eosinophilic exacerbations defined by blood eosinophil counts. As we outlined in detail in the Introduction of this thesis, these relapses may represent a distinct clinical phenotype influencing the risk of AEs and response to therapy [152, 197, 198]. Hence, in the second study included in this thesis, we performed a real-world observational clinical trial assessing the relationship between elevated BEC levels and the risk for later re-exacerbations.

We carried out the first prospective study to evaluate the relationship between the eosinophilic acute severe COPD exacerbation and future relapses. Our results indicate that patients with an eosinophilic exacerbation, do not have an increased risk of earlier recurrences of moderate or severe relapses. In line with the literature, the history of previous severe exacerbation was the strongest predictor for future relapses [199].

We explored the recurrence of both severe and moderate exacerbations [73]. Although moderate relapses do not necessitate hospitalization, they have negative effects on health status with similar magnitude as of severe exacerbations. It has been proven that similarly to reported exacerbations, unreported and moderate exacerbations are associated with increased symptoms, airflow limitation and increased levels of inflammatory markers, although the time to resolution of all symptoms is shorter [200-202].

Blood eosinophil percentage is a surrogate marker of sputum eosinophilia during an exacerbation [197] and it also correlates with small airways inflammation [203]. Strategies directed to normalize sputum eosinophil count improved airflow limitation, reduced symptoms and decreased the number of severe exacerbations [137, 138, 204], also implying a biological role for these cells in COPD.

Increasing evidence suggests that blood eosinophil level (either count or percentage) can be a signal of corticosteroid response, and it can also guide steroid therapy [142, 205-207]. Bafadhel et al. have found that patients with eosinophilic AEs treated with systemic

corticosteroids experienced more significant improvements in symptoms and a better recovery compared to those without eosinophilic inflammation. Furthermore, the multicentre RCT known as Eosinophil Guided Corticosteroid Therapy in Patients Admitted to Hospital with COPD Exacerbation (CORTICO-COP), assessed whether using daily serum eosinophil measurements to guide corticosteroid therapy could influence AE risk. The results showed the eosinophil-guided regimen to be noninferior to the standard care, while also reducing corticosteroid exposure. Moreover, the most recent multicentre double-blind RCT (Studying Acute Exacerbations and Response, STARR2) conducted in primary care setting has led to similar conclusions, namely, blood eosinophil-directed prednisolone therapy is noninferior to usual care [208]. However, the results of these trials should be interpreted with caution. The CORTICO-COP trial was an open-label study in which both the investigators and the patients were aware of the group allocation, which may introduce bias. Furthermore, all patients received a single dose of 80 mg methylprednisolone prior to treatment assignment, which is more than double the dose recommended in the GOLD document for the treatment of AEs. In addition, patients in STARR2 were treated with systemic steroids for 14 days compared with the GOLD recommendation of 5 days, and the study was terminated early due to the COVID-19 pandemic and suffered from significant randomisation errors at the beginning. Furthermore, several observational studies have failed to detect a clear association between BEC and more favourable outcomes [209]. Consequently, the GOLD document does not yet recommends eosinophil-directed systemic corticosteroid treatment in exacerbations and warrants for more clinical trials [1]. Nevertheless, given that even short courses of glucocorticoids increase the risk of severe infections and death [210], steroid-sparing treatment options should be advocated. In our study, all exacerbations were treated with a course of systemic (either intravenously or orally) corticosteroids, however the dose and duration of the treatment was not standardized and decided by the treating physicians on case-based manner.

We found that the distribution of patients with an eosinophilic exacerbation ($\geq$ 2% and /or $\geq$ 200/µl) was similar as reported in other investigations [152, 211]. Like others [211, 212], we also found that patients with an eosinophilic exacerbation present with a lower serum CRP level suggesting lower rate of infections. The eosinophilic type of relapses showed less severe airflow limitation characterized by higher FEV_1/FVC, which is

consistent with the findings of a secondary analysis of the ECLIPSE study, where FEV_1% predicted was higher in the eosinophilic group [141]. Other authors found no difference in baseline lung function, [151, 152, 211] while Ko et al. reported higher improvement in FEV_1 values (both absolute and % predicted) in patients with blood or sputum eosinophilia [213].

The time to first moderate or severe exacerbation was similar after hospital treatment of an eosinophilic and non-eosinophilic exacerbation suggesting that the published cut-off values for blood eosinophil count or percentage are not biomarkers for re-admissions as also shown by others [151, 211]. In contrast, Couillard et al. found in a post-hoc analysis that the number of re-admissions was increased and the time to the first re-admission was shorter in patients after an eosinophilic exacerbation [152]. This discrepancy might be explained by the different outcome parameters (i.e. moderate and severe exacerbations were analysed together in our study), the pre-treatment of patients with systemic corticosteroid before blood tests or the more severe airflow limitation in our patient population compared to the other studies [151, 152, 213]. Of note, in our cohort the rate of corticosteroid therapy on admission was lower than in the study by Bafadhel et al., but higher than in the study of Couillard et al, where eosinophil count was determined before the initiation of a systemic steroid therapy [152, 211].

We did not find a difference in the length of hospital stay or the rate of treatment failure between the eosinophilic and non-eosinophilic groups. This is in line with the results of a recent multicentre observational trial of Martínez-Gestoso et al., who did not observe any relationship between BEC and clinical outcomes, such as time to readmission, proportion of frequent exacerbator phenotype, rate of death, or length of hospital stay during the index hospitalization [214]. Our findings also support the results of Couillard et al., who reported a similar length of hospitalization [152], but our findings contradict other studies showing shorter hospital stay in the biomarker positive group [151, 211, 213]. Furthermore, our data are in line with the report of Prins et al. [151], who showed that the rate of late treatment failure (11-30 days post-discharge) was similar after eosinophilic or non-eosinophilic exacerbations.

Our data demonstrated that a subgroup of patients with an increased number of severe COPD exacerbations in the past have a shorter time till the recurrence of the next moderate or severe relapse. These results corroborate the findings of large-scale studies showing that the best predictor of an exacerbation is the positive history for prior events [5, 215]. We observed that exacerbation-free time also shortens with increasing severity of airflow limitation, which is in line with previous studies [5, 215, 216]. However, there is not enough evidence to use FEV_1 alone as a predictor of exacerbation risk in COPD [217]. Interestingly, the stronger exposure to cigarette smoke negatively affected the time to the next flare-up. This may be explained by the relationship between smoking and FEV_1 decline [218]. In addition, the incidence of lower respiratory infections is higher in current smokers, which can precipitate exacerbations [219, 220] and deteriorates lung function [221].

Our results show that around 25% of patients experience discordant AE type compared to the index event, i.e., having a non-eosinophilic AE after the initial eosinophilic one or vice versa. Interestingly, the stability of non-eosinophilic AE phenotype is almost 90%, while the discordance rate of eosinophilic phenotype is more than 50%. These results corroborate the findings of Schumann et al. who found a 34.5%, 24% and 17.2% discordance rate between two visits using cut-offs of 2%, 3% and 4% of blood eosinophil percentages, of which the discordance rate of eosinophilic phenotype measured during AEs was 45%, 25%, and 16% [222]. Moreover, Citgez et al. also found similar patterns of eosinophilic stability during subsequent severe AEs in a recent trial, namely, 34-45% of patients remained in the eosinophilic group during the first subsequent AE, depending on the used cut-off ($\geq$200 or $\geq$ 300 eosinophils/µL) [223]. These results suggest that there is a considerable variability of AE phenotypes in COPD. The background to whether an AE is associated with elevated BEC may be determined by different triggers. For instance, AEs precipitated by bacterial infection are more likely to be associated with neutrophilic-type inflammation and do not result in elevated eosinophil counts [197]. Consequently, patients who have high BEC in stable state, may experience a low eosinophil-type AE. It is therefore of particular importance to identify the triggers for eosinophil-type exacerbations in order to gain insight into the underlying pathomechanism.

Our studies also have limitations. It would have provided valuable information if we could have assessed endothelial dysfunction by physiological tests and compared the results with markers of the nitric oxide pathway and their changes during an AE. Moreover, despite numerous attempts, we were unable to obtain sputum samples or valid exhaled NO measurements from all the patients. To prevent any potential risks associated with sputum induction, we decided to only collect spontaneous samples and refrained from using sputum induction techniques, and sputum samples were collected from exacerbated patients within the first 24 h of hospitalization. In this timeframe, all patients received at least one dose of systemic corticosteroid, which could potentially influence the results of the serum and sputum analysis. Regarding the study on BEC, approximately one third of patients received a single dose of systemic corticosteroid before blood collection, which could have influenced results on eosinophil count. However, in real life settings treatment is often initiated in severe cases already out of hospital and finding clinically relevant biomarkers is also of importance in this group. In addition, the study has not been powered to analyse the effect of covariates (i.e. previous treatment) on our findings in detail.

6. Conclusions

1. In both stable and exacerbated COPD, serum nitrite levels are increased while serum L-Arginine/ADMA ratio is decreased compared to control subjects. Additionally, serum SDMA levels are similar in controls and patients with stable disease but increased during an AE. These results suggest a decrease in circulatory NO substrate availability and enhanced suppression of vascular NO production in COPD.
2. During a COPD relapse there are no differences between admission and recovery levels in serum nitrite and in serum L-arginine/ADMA ratio, but serum SDMA concentrations are decreased upon convalescence. It indicates that impairment in the vascular NO pathway during COPD flare-ups is partially reversed after systemic corticosteroid therapy.
3. Our results confirm a correlation between serum levels of ADMA and SDMA with airway nitrosative stress and airway neutrophilic inflammation in patients with COPD. This implies a potential association between endothelial dysfunction and airway inflammation in COPD.
4. Patients presenting with an eosinophil-type severe AECOPD (defined by ≥2% of blood leukocytes or ≥200 cells/μL; or ≥3% of blood leukocytes or ≥300 cells/μL) do not have an increased risk of earlier recurrences of moderate or severe relapses in 12 months after the index event.
5. The 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. In patients hospitalized with severe COPDAE within 12 months, the stability of the eosinophilic phenotype is 48%, while the stability of the non-eosinophilic phenotype is 89%.

7. Summary

COPD affects over 200 million people worldwide and is responsible for more than 3 million deaths yearly. Cardiovascular diseases are the most common comorbidities, which can influence COPD progression, but can also be affected by the pathological processes of the airway disease. Exacerbations have long-term unfavourable consequences on patients' quality of life, they accelerate disease progression and are the leading cause of COPD-associated mortality. Easy-to-use biomarkers, predictive of relapses, and the better understanding of the relationship between airway and vascular inflammation are of clinical importance.

We described a connection between airway inflammation and impaired endothelial NO synthase activity, a known mechanism involved in endothelial dysfunction. We found that the substrate availability for endothelial NO synthase (reflected by L-arginine/ADMA) is decreased in stable and exacerbated COPD, while SDMA is transiently elevated during a relapse. ADMA and SDMA showed correlations to airway inflammatory markers including exhaled NO concentration, sputum inflammatory and neutrophil cell counts. Our findings suggest that the suppression of airway inflammation could also modulate vascular NO signalling in COPD and might favourably affect the development of cardiovascular events.

Blood eosinophil granulocyte percentage and number are used to define eosinophilic exacerbations of COPD, which are associated with distinct clinical features. We found that this phenotype is not linked with an increased risk of earlier recurrence of moderate and severe relapses, but the increased number of prior hospitalizations, smoking history and lower FEV_1% predicted are associated with a shorter exacerbation-free time with the strongest parameter being the previous exacerbation history. Importantly, upon recurrent exacerbations, the eosinophilic phenotype is less consistent than the non-eosinophilic phenotype.

Overall, our findings highlight the need for comprehensive treatment approaches that address both pulmonary and cardiovascular aspects of the disease and indicate no role of BEC during exacerbations to predict future relapses.

8. References

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9. Bibliography of the candidate's publications

9.1. Publications related to the subjects of the dissertation

1. **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
2. **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

9.2. Publications not related to the subjects of the dissertation

1. Bikov András, Bentley Andrew, Csoma Balázs, Smith Nicola, Morris Bryn, Bokhari Saba
   Long-Term Adherence to Continuous Positive Airway Pressure in Patients with Obstructive Sleep Apnoea Set Up in a Complete Remote Pathway: A Single-Centre Service Evaluation Project
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11. Supplementary appendix

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