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REVIEW ARTICLE

Exhaled Nitric Oxide in COPD

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Abstract: Chronic obstructive pulmonary disease (COPD) is a common and progressive disorder which is characterised by pathological abnormalities driven by chronic airway inflammation. The assessment of airway inflammation in routine clinical practice in COPD is limited to surrogate blood markers. Fractional exhaled nitric oxide (FENO) is a marker of eosinophilic airway inflammation in asthma, and it can predict steroid responsiveness and help tailor corticosteroid treatment. The clinical value of FENO in COPD is less evident, but some studies suggest that it may be a marker of the eosinophilic endotype. More importantly, mathematical methods allow investigation of the alveolar/small airway production of NO which potentially better reflects inflammatory changes in anatomical sites, most affected by COPD. This review summarises the pathophysiological role of nitric oxide in COPD, explains the methodology of its measurement in exhaled air and discusses clinical findings of FENO in COPD.

Keywords: Airway inflammation, biomarkers, COPD, lung, nitric oxide, respiratory system.

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the most common chronic disorder of the respiratory system and is currently the 4th leading cause of death worldwide [1]. COPD is characterised by structural and morphological changes in the airways, alveoli and pulmonary vasculature which lead to progressive airflow limitation [1]. Airway abnormalities predominantly concern the small, peripheral bronchioles, but larger airways can also be affected [2]. There is a considerable heterogeneity in the underlying pathophysiological mechanisms and the clinical course of the disease [3]. Not surprisingly, biomarkers identified in population-based studies were not useful in clinical practice [4]. Although COPD is not traditionally viewed as an inflammatory disease, accumulation of inflammatory cells and mediators in the respiratory tract is often seen in this disorder. The increased airway inflammation is related to disease activity, both in terms of the number of exacerbations and the rate of lung function decline [5].

Airway inflammation can be assessed *via* invasive, semi- or non-invasive techniques. Invasive techniques include bronchial biopsies and brushings as well as bronchoalveolar lavage. These provide the most accurate and complex airway samples; however, they hold potential risk for side effects (*i.e*. bleeding, infection, pneumothorax, death) and generate airway inflammation *per se*. Airway cells and mediators can also be analysed in induced or spontaneous sputum samples, however this technique may also induce low-grade inflammation and might be harmful as well.

Exhaled breath analysis is a completely non-invasive method to study the airways, and it can be repeated within short-time intervals. The assessment of human breath has been used since ancient times to detect severe organ dysfunctions, such hepatic or renal failures or diabetic ketoacidosis. Currently, breath analysis is most commonly used for forensic purposes (*i.e*. breath alcohol) and *Helicobacter pylori* detection in medicine [6]. Exhaled breath contains thousands of volatile [7] and non-volatile [8] particles which are related to airway and systemic metabolism, inflammation and oxidative stress. Other modalities, such as exhaled breath temperature can also be used to study the airway inflammation in patients with COPD [9].

Nitric oxide is one of the most widely investigated molecules in exhaled breath. The levels of fractional exhaled nitric oxide (FENO) are elevated in steroid-naïve patients with asthma and its reduction is a good indicator of an effective steroid treatment [10, 11]. Therefore, the recent Global Initiative for Asthma recommendations suggest using FENO as part of the clinical assessment in asthma [10].

The potential of FENO as a biomarker in COPD is less clear. Studies reported not only increased but also similar and even lower FENO levels in COPD [12-14]. Many of the pioneering studies focused on differences between COPD and health. However, we do not need discriminative biomarkers, for this purpose we have lung function. In contrary, biomarkers reflecting disease activity (*i.e*. predicting future exacerbations or the rate of lung function

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decline) or predicting therapeutic response have to be identified. This review will focus on studies investigating the clinical potential of FENO. Of note, FENO can also be used as a non-invasive marker to study the nitrosative stress and eosinophilic inflammation in the airways.

Mathematical models enable the partitioning of the central and distal sources for exhaled nitric oxide. As distal airways are potentially more prominently contribute to the disease pathophysiology, extended nitric oxide analysis deserves special attention.

2. THE BIOLOGY OF NITRIC OXIDE IN COPD

Endogenous nitric oxide (NO) has been implicated in the pathogenesis of various diseases of the respiratory system, such as asthma, COPD, respiratory infections or obstructive sleep apnoea [15]. Nitric oxide is synthesised from L-arginine by a cytosolic enzyme, the nitric oxide synthase (NOS). Three different NOS isoforms have been identified, which play various pathophysiologic roles. The neuronal (nNOS, NOS1) and endothelial isotypes (eNOS, NOS3) are constitutively expressed and their activation depends on calcium/calmodulin resulting NO in relatively low concentrations [16]. The third isoform is the inducible NOS (iNOS, NOS2) which is upregulated by various inflammatory stimuli and oxidative stress and generates NO at high levels [17]. Of note, nNOS may also be induced by nitrosative stress [17] and iNOS may have a constitutive expression in airway epithelium as well [18].

Neuronal NOS is expressed mainly in the cholinergic nerves of the airways and plays a role as a neurotransmitter in neural bronchodilatation [19]. Its expression is found to be both increased [17] and unaffected [20] in COPD. Possible explanations include variances in the sampled populations or differences in COPD phenotypes. However, discordant results also suggest that nNOS has a limited role in COPD compared to iNOS.

Endothelial NOS is expressed in pulmonary endothelial cells [21], alveolar type II cells [22] and alveolar macrophages [23]. Nitric oxide produced by eNOS is a potent endogenous vasodilator which regulates the pulmonary vascular tone. Endothelial dysfunction in patients with COPD has been described long before, which attributes to the inadequate NO production in the lung [24]. This fact is supported by the reduced expression of eNOS in pulmonary arteries [25, 26] and veins [26] of heavy smokers. Smoking-mediated oxidative stress can downregulate eNOS expression in mice and human lungs [27]. Various eNOS gene variants may enhance the susceptibility to COPD by inducing endothelial dysfunction and oxidative stress [28]. Interestingly, decreased [17], similar [20] and elevated [29] eNOS expressions have all been reported in COPD. Moreover, smoking-induced inflammatory processes and oxidative stress may modulate the production of eNOS [29]. Of note, eNOS may contribute to the pathomechanism of COPD *via* uncoupling, meaning that eNOS produces superoxides instead of NO in pathological states. Reduced bioavailability of tetrahydrobiopterin, which is the cofactor of eNOS, results in eNOS uncoupling and consequent ineffective vasodilatation in smokers [30].

Reports on increased iNOS in lung tissue in COPD are more coherent [17, 20, 31]. Inducible NOS has been identified in alveolar macrophages [23], alveolar type II cells [22, 31] and pulmonary arterial smooth muscle cells [32]. This enzyme can be induced by various stimuli, such as lipopolysaccharide (LPS), tumour necrosis factor (TNF) alpha, interleukins (IL) and reactive oxygen species [29, 33]. In humans, a co-stimulation is often required [34]. NF-kB is one of the crucial transcriptional factors, which plays a role in inducing iNOS expression [33].

Nitric oxide has a pluripotent role in physiological circumstances and also in COPD. It regulates endothelium-dependent vasodilatation and bronchodilatation *via* activating the soluble guanylyl cyclase pathway. In COPD, this pathway is damaged resulting in low cyclic-GMP levels and elevated tone of the smooth muscle cells in vessels and bronchi [35]. The insufficient vasodilatation is a key factor in the development of secondary pulmonary hypertension, a feature often complicating COPD [36]. In addition, NO can rapidly react with superoxide anion to form the potent oxidant peroxynitrite (ONOO-), which decreases the bioavailability of endogenous NO in COPD [37]. ONOO- reacts with a wide range of molecules resulting nitration, nitrosylation of different proteins, lipids and RNA [38] and is responsible for an anti-microbial defence [39-41]. However, peroxynitrite also has deleterious effects on human airway cells and corresponds to steroid insensitivity in COPD [42]. Supporting this, the levels of nitrotyrosine, a marker of nitrosative stress are elevated in induced sputum of patients with COPD [29, 37] and correlate with the severity of airway obstruction [29].

Nitric oxide is involved in the cellular response to hypoxia. Hypoxia inducible factor can regulate the expression and activity of NOS isoenzymes. During 24 hours at a hypoxic state, eNOS expression and protein levels are decreased in human endothelial cells *in vitro* [43] leading to vasoconstriction and endothelial dysfunction. *Ex vivo*, human pulmonary arterial tissue eNOS expression is downregulated by the combination of hypoxia and inflammatory mediators, such as LPS, IL-1 and TNF-alpha [44]. The effect of hypoxia on iNOS expression was evaluated by *in vitro* studies [45], where iNOS mRNA and protein levels were increased by chronic hypoxia itself or combined with LPS and cytokine exposure [46-49]. However the biological activity of iNOS is decreased in acute hypoxia because of the impaired function of NOS without oxygen [45]. Neuronal NOS (nNOS) is similarly regulated under acute and chronic hypoxia, as demonstrated by Ward *et al.* [50]. These lines of evidence suggest that acute and chronic hypoxia have different effects on NO biology.

3. PRINCIPLES OF EXHALED NITRIC OXIDE MEASUREMENTS

Nitric oxide is a gaseous and reactive molecule which can be measured in exhaled breath in particles per billion (ppb) concentrations. Of note, nasal passages may contribute significantly to the exhaled NO levels, therefore patients should exhale with a pressure of at least 5 cm H_2O to close the soft palate and avoid nasal contamination. Using

standardised techniques, airway and nasal NO can be measured separately [6].

Exhaled NO can be detected *via* chemiluminescent and electrochemical analysers or laser-spectroscopy [51]. Chemiluminescence is the gold-standard technique, but these devices are expensive, require space and unique investigator skills. Therefore, in clinical practice, less accurate, handheld electrochemical devices are also acceptable. The inter-measurement repeatability for different electrochemical analysers is variable [52, 53]. This variability may attribute to poor NO tracings, which unfortunately cannot be visualised for electrochemical devices. The American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines suggest two subsequent measurements for both the chemiluminescent and electrochemical devices with less than 10% differences between the values provided by the two measurements [54]. The recent ERS technical standard document acknowledges that one measurement can also be accepted due to financial issues with the electrochemical devices [6]. Of note, small but significant inter-device differences were noted, therefore the same device should be used in follow-up studies [53, 55]. It is well known that exhaled NO concentrations decrease with increasing expiratory flow [56], ATS/ERS recommendations set the target flow rate to 50 mL/s [6, 54]. Ideally, subjects should take a deep breath in through a filter and exhale against resistance using a nose-clip and target a constant flow rate for 6-10 seconds. A plateau with less than 10% variation should be used to calculate FENO [54]. Although inhalation to total lung capacity is recommended in the ATS/ERS guidelines [54], this is debated by some authors as lung stretch may alter NO formation [57]. Deep inhalation is suggested by the ERS recent technical standard document [6].

Similarly to lung function, FENO is affected by age, gender and height [58-60], and a number of physiological factors, such as menstrual cycle [61] or exercise [62]. However, correction on these factors is not currently recommended. The exhaled nitric oxide concentration is also influenced by diet [63], therefore it is advised to avoid consuming food with high nitrate levels, such as lettuce or spinach prior to FENO measurements. In clinical practice, mouthwash prior to FENO analysis is not routinely recommended [6]. Of note, due to potential confounding effect, FENO measurements should be performed before lung function testing [6].

4. EXHALED NITRIC OXIDE IN COPD

The measurement of exhaled nitric oxide dates to the early 90s when different workgroups identified this molecule in breath samples and reported significant elevations in asthma [64, 65]. Since then, exhaled nitric oxide has been investigated in various pulmonary diseases, such as cystic fibrosis [66], bronchiectasis [67], respiratory infections [68] and OSA [69]. Unfortunately, FENO has proven far less clinically useful in these disorders than in asthma.

As NOS enzymes are highly expressed in COPD, initial studies expected elevated FENO in this disorder. However, the results are contradictory showing elevated [13, 70, 71], similar [12, 72-74] and even reduced [14] levels. This discrepancy can be explained by numerous factors. First, COPD affects primarily the distal airways, while FENO measured at 50 mL/s represents the conducting bronchi. Second, cigarette smoking reduces exhaled NO levels [75] and even ex-smokers have lower FENO values compared to never-smokers [76]. Third, NO reacts very rapidly with the reactive oxygen species in the airways, and the lack of elevation in FENO may be a result of oxidative burden characterising COPD [77]. Fourth, *cor pulmonale* which frequently accompanies severe COPD is associated with endothelial dysfunction, leading to the impaired production of eNOS [24], and corresponding lower FENO levels [78].

Rather than comparing COPD to health, recent studies focused on the potential role of FENO of differentiating COPD from asthma and identifying asthma COPD overlap (ACO). Despite some promising results in smaller studies [79-81], a recent subgroup analysis of the population-based Copenhagen study showed a large variability of FENO results among patients with airway diseases and did not support the use of FENO either alone or in combination with blood eosinophils to differentiate asthma from COPD or to identify ACO [82]. Studies in COPD reported that a potential cut off levels of 25 ppb can be used to differentiate between ACO and non-ACO COPD [80, 81]. Elevated FENO in ACO is not surprising as high FENO levels were related to other asthma-like characteristics, such as bronchodilator reversibility [13, 83] and sputum eosinophilia [73, 75, 81, 83].

The clinical role of blood eosinophil count, a surrogate marker of airway eosinophilia has been widely recognised in predicting response to inhaled corticosteroids (ICS) in COPD [84]. Theoretically, FENO can serve a similar purpose and the limited number of studies supported this hypothesis [13, 85-87]. Large comparative studies between blood eosinophil count and FENO are warranted to explore if FENO has any additive value to blood eosinophils in COPD. Furthermore, the long-term stability of FENO values in stable COPD needs to be tested similarly to blood eosinophil count [88]. As “low blood eosinophil” endotype proved to be more stable than the “high eosinophil” one [88], clinicians should use blood eosinophil counts to predict no benefit from ICS in patients with low blood eosinophils. In line with this, low FENO values (*i.e.* <25 ppb) may predict no response to ICS in COPD patients in clinical practice, a hypothesis which needs to be tested.

In a recent article, Alcazar-Navarette *et al*. reported that persistently elevated FENO levels may also be predictive for future exacerbations [89], nevertheless this finding should be confirmed by other groups. Of note, FENO levels in COPD patients should be interpreted carefully, as both inhaled corticosteroids [13, 85, 90] and long-acting β2-agonists [91] may result in lower levels in these patients. Interestingly, while inhaling short-acting β_2-agonist increases FENO values in asthmatic subjects, it does not have an effect in COPD patients [92].

Studies are more coherent when FENO was measured during acute exacerbation, showing an elevation compared to stable state [12, 93-96]. In addition, a significant association between sputum eosinophils and FENO during the onset of exacerbation has been reported [93, 97]. Eosinophilic acute exacerbations may be related to viral infections [93], which is supported by virus-induced increase in FENO [68].

Airway eosinophilia is also related to a favourable response to systemic corticosteroids during exacerbations [84] which is supported by a significant association between elevated baseline FENO and good response to systemic corticosteroids in COPD [95]. Similarly to the stable state, a potential additive value of FENO to blood eosinophil count during exacerbation needs to be studied. Of note, although FENO at 50 mL/s can be measured in most stable patients, those with acute dyspnoea may struggle to provide a valid measurement.

5. EXTENDED EXHALED NITRIC OXIDE ANALYSIS IN COPD

Peripheral airways disease is a major disease component of COPD, as the severity of airway inflammation in small airways shows a direct correlation with the degree of airflow limitation [98]. However, the non-invasive measurement of inflammation in the distal lung is an unresolved issue. The extended NO analysis allows the partitioned assessment of pulmonary NO parameters in the conducting airways and in the acinar region, holding promise as a feasible option for quantifying small airways inflammation in COPD.

The extended NO analysis is based on the two-compartment model of pulmonary NO dynamics, where the conducting airways (from trachea to terminal bronchi, airway generations 1-16) release NO to the airway lumen depending on the exhaled flow, while NO is released in the expansible acinar region at a constant rate (from respiratory bronchioles to alveoli). Several mathematical equations have been used so far, which can principally be divided into linear (Fig. **1**) and non-linear models (detailed in other publications including [99-101]). Both models need NO concentrations at several exhaled flow rates as inputs and can give parameters describing inflammatory activity in conductive and small airways as outputs (Table **1**). For the linear model at least two NO concentrations should be measured between 100-500 mL/s flow rates, however, the recent technical standard document of the ERS recommends the detection of NO values at least at three flows [6]. For the non-linear model three data points are required *i.e.* a NO concentration measured at low (<20 mL/s), medium (100 mL/s) and high (≥350 mL/s) flow rates.

Compared to the technical recommendations applied for FENO measurements [54], several issues must be considered when using the extended NO analysis. From the technical point of view, hand-held portable devices are generally not suitable for NO measurements at multiple flows, which can necessitate the application of more expensive instrumentation with higher maintenance costs. Patient-related problems should also be considered. Some subjects especially those with airflow limitation such as patients with severe or uncontrolled COPD and asthma face difficulties holding expiration at slow flow rates (<50 mL/s) and generating fast flows (>200 mL/s) [102]. It is important that exhaled NO plateau is reached over a window longer than 3 seconds at slow flow rates, which may need training for patients. Therefore, we and other authors found that the linear method is more feasible for measurements in patients with COPD [12, 103]. However, the non-linear model was shown to have the least mathematical error in patients with stable COPD [104]. Axial back-diffusion from the bronchial compartment to the acinar region was proposed as an important confounding mechanism of the two-compartment model, but general correction of NO parameters for axial back-diffusion is not recommended as currently used equations do not consider bronchoconstriction and hence could lead to overcorrection [6].

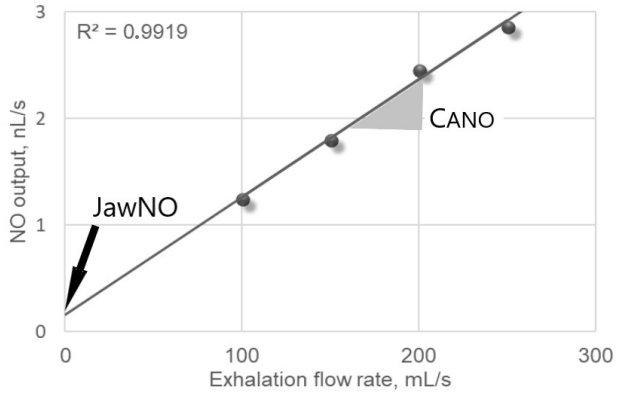

Fig. (1). An example for the calculation of bronchial and alveolar NO parameters in the linear model of the extended analysis. The plateaus of expired NO concentration were detected at four constant exhalation flow rates (100-150-200-250 mL/s). Airway NO output (exhalation flow rate x NO concentration) is shown in relation to the exhalation flow rate. The goodness of linear fitting is demonstrated by the R^2 value. The slope of the line corresponds to the acinar/alveolar NO concentration (*C*ANO, ppb) and the intercept shows the bronchial NO flux (*J*awNO, nL/s). The model is described in detail by previous publications [99-101]. Measurements in a patient with stable COPD are presented from our own database. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The contribution of nitric oxide synthase isoforms to the production of airway NO has been studied. It was shown that compared to *J*awNO, which was significantly suppressed by an iNOS inhibitor, *C*ANO was not modified by iNOS blocking suggesting the involvement of constitutive NOS isoforms in the release of NO in peripheral airways [105].

Similar to FENO, *J*awNO was also lower in control smokers and ex-smokers than in non-smokers [76, 106], but

Table 1. Parameters calculated from the two-compartment models.

-	Linear Modelling	Non-linear Modelling
Small airways	Acinar/alveolar NO (*C*ANO)	Airway wall NO concentration (*C*awNO)
Conducting airways	Total flux of bronchial NO (*J*awNO)	Total maximal flux of bronchial NO (*J'*awNO)
-	-	Diffusing capacity of NO from the airway wall to the lumen (*D*awNO)

no change in stable COPD was noted [106]. Nonetheless, both parameters were increased during exacerbation compared to stable state [12]. *J*awNO and FENO could be decreased by inhaled and systemic steroid treatment in stable and exacerbated COPD, respectively [12, 107]. *J*awNO positively correlated with symptom scores in stable patients [108]. *C*awNO (airway wall NO concentration) was increased in smokers compared to ex-smoking COPD patients with no difference in airway diffusion of NO [109]. In contrast, in another study current smokers but not ex-smokers showed decreased *C*awNO compared to non-smokers [76].

Alveolar NO concentration was shown to be increased in smoking patients with COPD compared to non-smokers [106] and ex-smoking patients and current smokers [109]. A negative association was noted between current smoking and *C*ANO in control subjects [76]. Importantly, *C*ANO was also higher in patients than in smoking-matched controls [12]. Using the non-linear model, no difference in *C*ANO was found among clinical phenotypes of stable COPD including patients with emphysema, chronic bronchitis, frequent exacerbations and disturbed body composition [110]. Additionally, exacerbated patients showed increased *C*ANO compared to smoking controls, but not to stable patients. In stable COPD, a short course of either inhaled or systemic steroid did not change *C*ANO [87, 107, 111], which might be linked to the known corticosteroid resistance of airway inflammation in COPD. In line with this, alveolar NO was not modulated by systemic steroid therapy during an exacerbation [12], either.

To conclude, the extended NO analysis provides insights on the degree of inflammation in central and peripheral airways. It allows the non-invasive monitoring of disease in the distal lung, which is the primary site of pathological processes in COPD, hence enabling the assessment of the efficacy of anti-inflammatory drugs. In contrast to bronchial NO parameters, alveolar NO concentration is not sensitive to steroids. The role of *C*ANO in predicting exacerbations and progression of COPD should be further clarified.

CONCLUSION

Exhaled nitric oxide has been investigated extensively in COPD. Although the results are inconclusive when patients with COPD were compared to asthmatic or healthy controls, FENO has shown some potential when analysing various phenotypes or treatment response. However, these results need to be interpreted very carefully. Former studies usually set a FENO level of 25 ppb as an optional cut off point which separates asthma-like phenotypes (including eosinophilic airway inflammation and corticosteroid response). This is in line with the ATS clinical practice guidelines in asthma [11]; however the same document recommends using a 50 ppb value when determining eosinophilic phenotype, which is based on an upper limit of variability of FENO values in normal population. This implies that FENO values in COPD are within the normal range, therefore the interpretation of exhaled nitric oxide in clinical practice in COPD has crucial limitations (Fig. **2**). Interestingly, recommendations on ICS use in COPD based on blood eosinophilic count also use cut off values of 200-300 cells/μL which are within the normal range of healthy subjects [84].

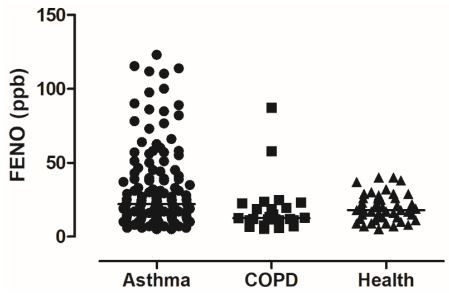

Fig. (2). Comparison of FENO values in asthma, COPD and health. Own data on 133 patients with asthma, 25 patients with COPD and 46 healthy subjects. Please note the large overlap between the three groups which limits the clinical ability of FENO to use as a biomarker in COPD. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Measuring inflammation in the distal airways may be a potential target for drug development and may be more closely related to disease course. However, the potential of the extended nitric oxide analysis has not been tested and validated in large clinical trials.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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