



## Exhaled nitric oxide and carbon monoxide in lung transplanted patients<sup>☆</sup>



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### ABSTRACT

**Background:** Exhaled nitric oxide (eNO) and carbon monoxide (eCO) are markers of pulmonary inflammation associated with acute graft rejection and lung infections in lung transplant (LTX) recipients. Regarding eNO and eCO levels in LTX patients affected by bronchiolitis obliterans syndrome (BOS), published data are discordant.

**Objectives:** We aim to evaluate eNO at multiple flows, alveolar concentration of nitric oxide (Calv<sub>NO</sub>), maximum conducting airway wall flux (J'aw<sub>NO</sub>) and eCO levels in LTX patients to assess the potential role of these parameters in BOS evaluation.

**Methods:** Fractional exhaled nitric oxide (FeNO), Calv<sub>NO</sub> and J'aw<sub>NO</sub> were analysed in 30 healthy subjects and 27 stable LTX patients (12 BOS patients). Pulmonary function tests were performed after eNO and eCO assessment. Receiver operating characteristic (ROC) curves were conducted to evaluate diagnostic accuracy for BOS of eNO parameters.

**Results:** LTX patients reported higher values of FeNO at flow rates of 50 ( $p < 0.01$ ), 150 ( $p < 0.05$ ), 350 ml/s ( $p < 0.001$ ), and Calv<sub>NO</sub> ( $p < 0.0001$ ) than healthy controls. BOS patients showed higher FeNO at flow rates of 150 ( $p < 0.05$ ) and 350 ml/s ( $p < 0.01$ ) and Calv<sub>NO</sub> ( $p < 0.001$ ) than non-BOS patients. Calv<sub>NO</sub> reported a remarkable diagnostic accuracy for BOS (AUC: 0.82). There were no significant differences of eCO levels between LTX patients and healthy controls.

**Conclusion:** LTX patients affected by BOS showed higher levels of FeNO 150 and 350, and Calv<sub>NO</sub> than non-BOS LTX patients, probably due to chronic airway inflammation and fibrotic remodelling. Calv<sub>NO</sub> may be a potential biomarker of BOS in LTX patients.

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**Abbreviations:** LTX, lung transplant; SLTX, single lung transplant; SSLTX, sequential single lung transplant; NO, nitric oxide; eNO, exhaled nitric oxide; FeNO, fractional exhaled nitric oxide; Calv<sub>NO</sub>, alveolar concentration of NO; eCO, exhaled carbon monoxide; J'aw<sub>NO</sub>, maximum conducting airway wall flux; IPF, idiopathic pulmonary fibrosis; BAL, bronchoalveolar lavage; CF, cystic fibrosis; PFT, pulmonary function tests; TLC, total lung capacity; FVC, forced vital capacity; TLCO, transfer factor of the lung for carbon monoxide; COPD, chronic obstructive pulmonary disease.

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## 1. Introduction

Lung transplant (LTX) is a valid therapeutic option for patients with life-threatening pulmonary diseases, who are refractory to conventional therapy and are acceptable candidates. Many advances in pre- and post-transplant management have led to improved survival and quality of life outcomes for lung recipients in the last two decades, especially in the early post-operative period. However, LTX is still a challenge and long-term survival is mainly dependent on the development of chronic lung allograft dysfunction (CLAD). CLAD is an overarching term that embraces all forms of chronic lung dysfunction after transplant [1]. The most common phenotype of CLAD is bronchiolitis obliterans syndrome

(BOS), that affects more than 50% of the recipients at 5 years from LTx, and it is the leading cause of death beyond 1-year post transplant [2,3]. BOS is generally equated with the term chronic rejection of LTx, and both immune and non-immunological factors have been implicated in its pathogenesis. A persistent inflammatory reaction and fibrotic remodelling of small airways lead to the typical picture of obliterative bronchiolitis (OB) and a persistent airflow obstruction. Recent ISHLT/ATS/ERS guidelines propose as BOS diagnostic criteria the decrease of forced expiratory volume in 1 s (FEV<sub>1</sub>) and the careful exclusion of other post-transplant complications that can cause delayed lung allograft dysfunction [1]. Other biomarkers have not been validated for the management and the diagnostic work-up of BOS yet, although several non-invasive potential bioindicators have been proposed in the last decade.

Nitric oxide (NO) and carbon monoxide (CO) have been investigated as potential biomarkers for several chronic inflammatory lung disorders such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and pulmonary arterial hypertension [4] and their clinical utility has been largely validated. At pulmonary level, NO acts as a vasodilator, bronchodilator, neurotransmitter and inflammatory mediator. The anatomical origin of NO production can be identified using a two-compartment model (2CM) of the lung (airway and alveolar compartments) [5] or a trumpet model with axial diffusion (TMAD) [6]. These models can partition NO into alveolar concentration of NO (Calv<sub>NO</sub>) and maximum conducting airway wall flux (J'aw<sub>NO</sub>) which expresses bronchial NO flux. Thanks to the reproducibility and non-invasiveness of fractional exhaled NO (FeNO), some studies have investigated its potential role in the management of LTx patients. Higher levels of exhaled nitric oxide have been reported in LTx patients with acute rejection [7–9], infection [10,11] and lymphocytic bronchiolitis [10]. In CLAD results are contradictory: Gabbay et al. found increased FeNO at flow of 250 ml/s in LTx patients with BOS or bacterial infection (with a significant positive correlation between FeNO 250 and BAL neutrophilia and inducible nitric oxide synthase (iNOS) expression in the bronchial epithelium) [12]. On the other side, a raised FeNO at the flow of 50 ml/s has been described in LTx patients with unstable BOS or with developing BOS, but not in stable BOS, underlining the possible role of FeNO 50 as negative predictive marker of CLAD [13,14].

Another interesting potential biomarker of inflammation and oxidative stress in asthma [15,16], COPD [17] or bronchiectasis [18] is exhaled CO (eCO), the principal product of organic combustion. In LTx patients, eCO has been reported as a useful tool for the early detection of CLAD and it can enhance the negative predictive value of eNO for BOS [19]. Vos et al. reported, analogously to FeNO, a direct correlation between eCO levels, neutrophilic count and BAL pro-inflammatory cytokine levels [20].

The present study aimed to evaluate FeNO and eCO concentrations in LTx patients (BOS and non-BOS), compared to a healthy sex- and age-matched controls, to assess their potential role as BOS biomarkers. To our knowledge, this is the first study that analyses the potential role of FeNO at multiple flows in BOS patients, after the publication of ATS guidelines for FeNO standardization [4] and interpretation [21]. It also examines in depth eNO kinetics for evaluation of flow-independent parameters like Calv<sub>NO</sub> and J'aw<sub>NO</sub> in LTx patients.

## 2. Materials and methods

### 2.1. Study population and study design

Twenty-seven LTx patients were enrolled in the study from March to December 2014. Eight patients underwent single-lung

transplantation (SLTx) (7 males, mean age 66 ± 3.9 years), and nineteen patients received sequential-single lung transplantation (SSLTx) (7 males, mean age 50 ± 12.8 years). The underlying pulmonary diseases were idiopathic pulmonary fibrosis (n = 12), cystic fibrosis (n = 5), COPD (n = 4), alveolar microlithiasis (n = 2) and non-specific interstitial pneumonia (n = 1), chronic hypersensitivity pneumonia (n = 1), systemic sclerosis with interstitial lung disease (n = 1) and rheumatoid arthritis with interstitial lung disease (n = 1). A single patient underwent pulmonary retransplantation because of graft loss for BOS (24 months after the first transplant). The average time after lung transplantation was 35 ± 34.9 months. All patients received lung transplantation between 2004 and 2013 at Siena Lung Transplant Program and were followed at the Respiratory Diseases and Lung Transplant Unit, “Le Scotte” Hospital, Siena, Italy. An accurate medical history was obtained from all patients in order to evaluate risk for lung diseases from professional exposure, smoking and medication history. None of the patients was on oxygen therapy at the moment of the analysis. Thirteen LTx patients were never-smokers and 14 were ex-smokers (mean 12.5 ± 15.5 packs/year). None of LTx patients was a current smoker. All LTx patients had no history of allergy, concomitant asthma or cancer; patients with respiratory infections and/or acute deterioration in the last 4 weeks were excluded.

Twelve LTx patients (8 males, mean age 61 ± 8.1 years) were diagnosed with bronchiolitis obliterans syndrome (BOS) (16.7 ± 12.5 months prior to study procedures). Diagnosis and grading of BOS were performed according to international guidelines [22]. Eight patients were BOS-1, 2 patients BOS-2 and 2 patients BOS-3.

The control group included 30 healthy volunteers (16 males, mean age 62 ± 4.73 years), 15 non-smokers and 15 ex-smokers (mean 5.25 ± 7.2 packs/year). Subjects with a history of allergies or taking phosphodiesterase-5 inhibitors were excluded. All healthy volunteers had normal lung function parameters, they had no respiratory symptoms or infections in the last 4 weeks and they were not taking any drug that could affect the test. All patients and controls gave their informed consent to the study, which was approved by the local ethical committee.

### 2.2. Study protocol

Exhaled nitric oxide and carbon monoxide measurements were performed in the morning of the expected follow-up visit for LTx patients. All participants had fasted for at least 8 h prior to the moment of the examination. They also had abstained from foods containing nitrates (lettuce, spinach, cabbage, sausages) for at least 12 h before the examination. Patients on bronchodilators had to suspend therapy 12 h before the assay. All participants had a mouthwash with water just before the test. eNO and eCO measurements were taken after 10 min of rest in a comfortable environment.

### 2.3. PFTs

Lung function measurements were recorded according to ATS/ERS standards [23], using a Jaeger Body Plethysmograph with corrections for temperature and barometric pressure. In particular forced expiratory volume in the first second (FEV<sub>1</sub>), forced vital capacity (FVC), FEV<sub>1</sub>/FVC, total lung capacity (TLC), residual volume (RV), carbon monoxide lung transfer factor (T<sub>LCO</sub>) and capacity carbon monoxide lung transfer factor/alveolar volume (T<sub>LCO</sub>/VA) were recorded. All parameters were expressed as percentages of predicted reference values. In the same day PFTs were performed after eNO and eCO measurements.

#### 2.4. eNO measurements

FeNO was measured according to American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines for online measurement of FeNO in adults, using a chemiluminescence analyzer (model Hypair FeNO Medisoft Cardioline Exp'air, 2010) [4] with adequate sensitivity to NO from 1 to 500 ppb and a resolution of 1 ppb. All measurements were undertaken at ambient NO levels of <10 ppb. Subjects were studied in sitting position. FeNO was measured during slow exhalation from total lung capacity against a positive pressure kept constantly between 5 and 20 cm H<sub>2</sub>O to generate exhalation flow rates of 50, 100, 150 and 350 ml s<sup>-1</sup>. The exhalation flow rate was kept as constant as possible using a biofeedback visual display. For each flow rate, at least two technically adequate measurements were performed. The flow-independent NO parameters, Calv<sub>NO</sub> and J'aw<sub>NO</sub> were calculated using both the Tsoukias 2CM of NO exchange [5] and the TMAD by Condorelli [6]. A linear relationship between the four points (50, 100, 150 and 350 ml/s) of the NO flux against the flow was evaluated for each subject by a linearity test. Each measurement was considered acceptable with a confidence rate >95% and a flow stability >90%. All measurements were made by a single investigator, to maximize inter- and intra-observer agreement.

#### 2.5. eCO measurements

CO concentrations were evaluated using a chemiluminescence analyser (Smokerlyzer). Subjects were studied in sitting position and were asked about smoking status and passive smoking exposure. eCO was measured following exhaled NO evaluation, after 30 min of rest. Subjects were asked to hold breath to total lung capacity for 15 s: eCO and COHb% were measured during slow exhalation and reported on display. At least two technically adequate measurements were performed.

#### 2.6. Statistical analysis

Data is presented as mean ± standard deviation (SD). Mann–Whitney U test was performed to compare the LTX patient group

with healthy controls and BOS and non-BOS groups. BOS, non-BOS and control groups were compared using one-way ANOVA test. Correlations between Calv<sub>NO</sub> and PFT parameters were performed through Spearman's test. A p-value < 0.05 was considered statistically significant. Receiver-operating characteristic (ROC) curves were made using SPSS Statistics 22.0 while all the other statistical analyses were performed using Graph Pad Prism 5.

### 3. Results

#### 3.1. Clinical and functional parameters

Clinical and demographic parameters of LTX patients and controls, together with PFTs, including T<sub>1CO</sub> percentages, and current therapy from all transplanted patients were reported in Table 1. No significant differences were evidenced between SSLTX and SLTX patients for all these parameters (p > 0.05). Globally, LTX patients showed mild impairment of FEV1 and TLCO, and BOS group reported significantly lower functional parameters than non-BOS group (FVC, FEV1, TLC and TLCO, all p < 0.05). No significant differences were observed between BOS and non-BOS patients regarding the use and the dosage of corticosteroid and immunosuppressive therapies.

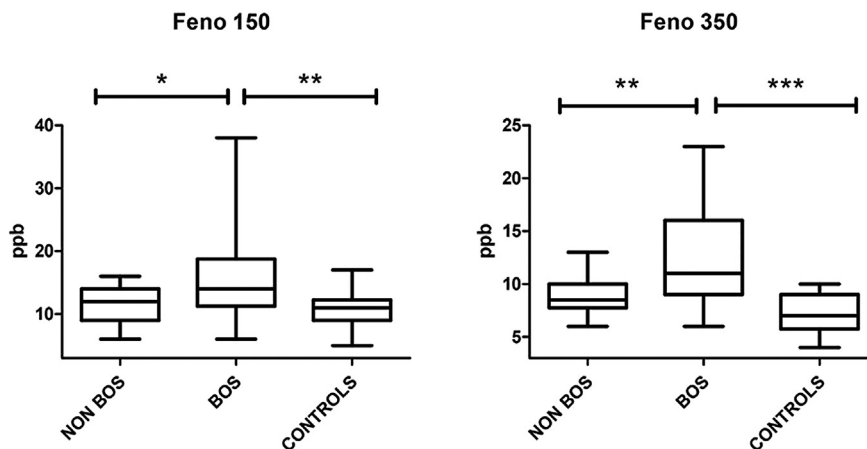
#### 3.2. eNO and eCO between LTX and controls

FeNO values at flow rates of 50, 150 and 350 ml/s were significantly higher in LTX patients than in healthy controls (FeNO 50: 22.6 ± 10.5 vs 15.8 ± 4.1 ppb, p < 0.01; FeNO 150: 13.4 ± 6.1 vs 10.8 ± 2.9 ppb, p < 0.05; FeNO 350: 10.5 ± 4.2 vs 7.2 ± 1.9 ppb, p < 0.001). Differences of FeNO at flow rate of 100 ml/s between LTX patients and healthy controls were not significant. Calv<sub>NO</sub> was significantly higher in LTX patients than in healthy controls (10.4 ± 6.9 vs 4.6 ± 4.5 ppb, p < 0.0001), while J'aw<sub>NO</sub> levels were not statistically different between the two groups (p > 0.05). These results were obtained applying both 2CM and TMAD models.

There were significant correlations between Calv<sub>NO</sub> and TLCO as well as between Calv<sub>NO</sub> and TLC (in both cases, r = -0.44, p = 0.01).

**Table 1**  
Demographic findings, clinical features, current therapy and lung function parameters in patients with BOS, compared with non-BOS patients and healthy controls. Lung function parameters are reported as percentages of predicted values. Results were expressed as mean ± SD. BOS: bronchiolitis obliterans syndrome. LTX: lung transplantation; SSLTX: sequential-single lung transplantation, FVC: forced volume capacity; FEV1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity; TLCO: transfer factor of the lung for carbon monoxide; VA: alveolar volume. ns: not significant.

Parameters	BOS	Non-BOS	Controls	p-value
N°	12	15	30	Ns
Age (yrs)	61 ± 9.1	49.5 ± 14.4	57.8 ± 11	Ns
Male (%)	8 (75%)	6 (40%)	14 (46%)	Ns
Current smokers	0	0	0	
Tobacco use (p/y)	19.5 ± 16.8	7.1 ± 12.4	6 ± 7.3	Ns
SSLTX (%)	8 (66%)	11 (73%)		Ns
Time from LTX (months)	45.9 ± 34.7	26.2 ± 32.5		Ns
PFT				
FVC %	72.7 ± 12.1	84.5 ± 13.5		<0.05
FEV1 %	62.6 ± 14.1	79.3 ± 16.5		0.01
FEV1/FVC	68.7 ± 14.5	78 ± 8.4		Ns
RV %	111.8 ± 31.2	129.4 ± 40.2		Ns
TLC %	83.7 ± 15	97 ± 16.2		<0.05
TLCO %	49.2 ± 11.8	58 ± 12.5		<0.05
TLCO/VA %	81.6 ± 16.2	80.1 ± 22.3		Ns
Therapy				
Prednisone (mg/day)	12 (17.5 ± 21)	15 (15.7 ± 18.6)		Ns
Mycophenolate mofetil (mg/day)	4 (875 ± 250)	6 (750 ± 273.8)		Ns
Tacrolimus (mg/day)	5 (2.2 ± 0.2)	11 (4.6 ± 5.2)		Ns
Everolimus (mg/day)	3 (2 ± 1.2)	1 (1.25)		Ns
Cyclosporin A (mg/day)	2 (75)	2 (112.5)		Ns
Azithromycin (mg/day)	7 (125 ± 62.5)	8 (156 ± 81)		Ns



**Fig. 1.** Comparison of FeNO at flow rates of 150 and 350 ml/s between BOS, non-BOS patients and healthy controls. BOS patients reported higher FeNO 150 and 350 values than non-BOS patients and healthy controls (ppb. Pars per billion; FeNO. Fractional exhaled nitric oxide; BOS. Bronchiolitis obliterans syndrome). \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

eCO and COHb% values did not differ significantly between LTX patients and healthy controls.

No statistically significant differences were observed between SSLTX and SLTX groups for any parameter.

### 3.3. eNO and eCO measurements between BOS, non-BOS and controls

Values of FeNO 50 and 100 were not significantly different between BOS and non-BOS groups (*p* > 0.05), while non-BOS patients reported significantly higher FeNO 50 values than healthy controls (*p* < 0.05). BOS patients presented significantly increased FeNO 150 and 350 values compared to non-BOS group (*p* < 0.05 and *p* < 0.01, respectively) and healthy controls (*p* < 0.01 and *p* < 0.001, respectively) (Fig. 1, Table 2).

Regarding flow-independent parameters, CalvNO was significantly higher in BOS patients than in non-BOS patients (*p* < 0.001) and healthy controls (*p* < 0.001) (Fig. 2, Table 2), while there were

**Table 2**

eNO and eCO parameters in LTX patients affected by BOS, compared with LTX patients free from BOS and healthy controls. BOS: bronchiolitis obliterans syndrome; FeNO: fraction of exhaled nitric oxide; CalvNO: alveolar concentration of nitric oxide; J'awNO: maximum conducting airways wall flux; eCO: exhaled carbon monoxide; ns: not significant.

Parameters	BOS	Non-BOS	Controls	p-value <sup>c</sup>
Nitric Oxide				
FeNO 50 ml/s (ppb)	20.4 ± 7.8	22.2 ± 9.7	14.9 ± 4.2 <sup>d</sup>	<0.05
FeNO 100 ml/s (ppb)	17.7 ± 9	15.1 ± 6.9	13.4 ± 2.9	Ns
FeNO 150 ml/s (ppb)	15.9 ± 8	11.4 ± 3.1 <sup>e</sup>	10.4 ± 2.4 <sup>f</sup>	<0.05
FeNO 350 ml/s (ppb)	12.8 ± 5.3	8.7 ± 1.8 <sup>g</sup>	6.2 ± 5.4 <sup>f</sup>	<0.001
CalvNO (ppb) <sup>a</sup>	14.7 ± 9.3	6.9 ± 3.3 <sup>g</sup>	4.9 ± 2.4 <sup>f</sup>	<0.0001
J'awNO (nl/min) <sup>a</sup>	34.6 ± 22.2	48 ± 25.1	40.3 ± 18.6	Ns
CalvNO (ppb) <sup>b</sup>	13.4 ± 8.9	6 ± 3.7 <sup>h</sup>	4.3 ± 2.6 <sup>i</sup>	<0.0001
J'awNO(nl/min) <sup>b</sup>	37.1 ± 25.7	57.2 ± 19.5	47.5 ± 25.1	Ns
Carbon monoxide				
eCO (ppb)	1.2 ± 0.4	1.09 ± 0.5	1 ± 0.3	Ns
COHb %	0.2 ± 0.08	0.2 ± 0.09	0.2 ± 0.04	Ns

<sup>a</sup> Two-model compartment by Tsoukias and George [5].

<sup>b</sup> Model of trumpet shape of the airway tree and axial diffusion by Condorelli et al.

[6].

<sup>c</sup> p-value between BOS, non-BOS and controls groups.

<sup>d</sup> *p* < 0.05 between non-BOS group and healthy controls.

<sup>e</sup> *p* < 0.05 between BOS and non-BOS groups.

<sup>f</sup> *p* < 0.01 between BOS group and healthy controls.

<sup>g</sup> *p* < 0.01 between BOS and non-BOS groups.

<sup>h</sup> *p* < 0.001 between BOS and non-BOS groups.

<sup>i</sup> *p* < 0.001 between BOS group and healthy controls.

no statistically significant differences in J'awNO levels among the three groups (*p* > 0.05).

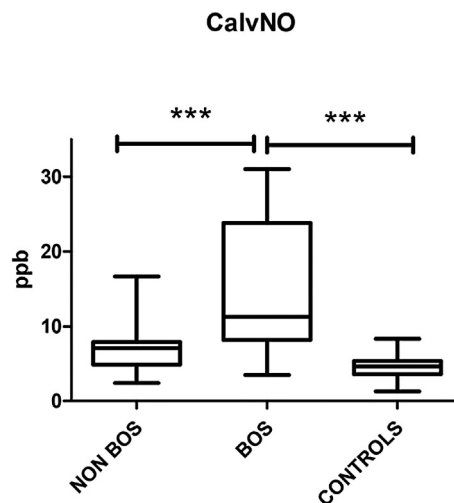
Patients with BOS treated with azithromycin showed no significant differences of eNO levels with untreated BOS patients. (*p* > 0.05).

By applying a cut-off value of 7.9 ppb, CalvNO presented a high predictive diagnostic BOS accuracy (area under the curve (AUC) of 0.82), with a sensitivity of 83% and a specificity of 80%, while FeNO 150 (AUC = 0.67) and 350 (AUC = 0.75) demonstrated a lower but acceptable accuracy (Fig. 3). The positive and negative predictive values of CalvNO were 77% and 86%, respectively.

BOS, non-BOS and healthy control groups showed comparable values of eCO and COHb%.

## 4. Discussion

This study aimed at evaluating exhaled NO and CO parameters in LTX patients, compared with a group of healthy subjects. The results document a statistically significant increase of FeNO values at flow rates of 50, 150 and 350 ml/s and CalvNO levels in LTX patients than controls. LTX patients not affected by BOS showed higher levels of FeNO 50 ml/s, while BOS patients had higher values



**Fig. 2.** Comparison of CalvNO between BOS, non-BOS patients and healthy controls. BOS patients showed higher CalvNO levels than non-BOS patients and healthy controls (ppb. Pars per billion; CalvNO. Alveolar concentration of nitric oxide; BOS. Bronchiolitis obliterans syndrome) \*\*\**p* < 0.001.

of FeNO at 150 and 350 ml/s. Regarding flow-independent parameters, BOS patients showed more elevated Calv<sub>NO</sub> than non-BOS patients, while J'aw<sub>NO</sub> levels were comparable in all populations. No differences were found in carbon monoxide concentrations between LTX patients (BOS and non-BOS) and healthy controls.

In the literature, very few studies have analysed the potential role of FeNO and eCO as biomarkers of LTX complications. Moreover, results are discordant: some authors reported that FeNO and eCO could be used to identify LTX patients affected by bacterial infection or acute rejection [8], others sustained a potential predictive value of these parameters for early detection of BOS [13,19]. These discordances are probably due to the non-uniformity of the applied methods to analyse FeNO and eCO. The present study carried out measurements of FeNO and eCO in BOS, non-BOS and healthy control groups following the recently proposed standard guidelines for measurement of FeNO [4]. Furthermore, an analyser capable of discriminating between alveolar and major airways production of NO was used to investigate the role of these potential biomarkers in BOS and non-BOS LTX patients.

#### 4.1. SLTX vs SSLTX

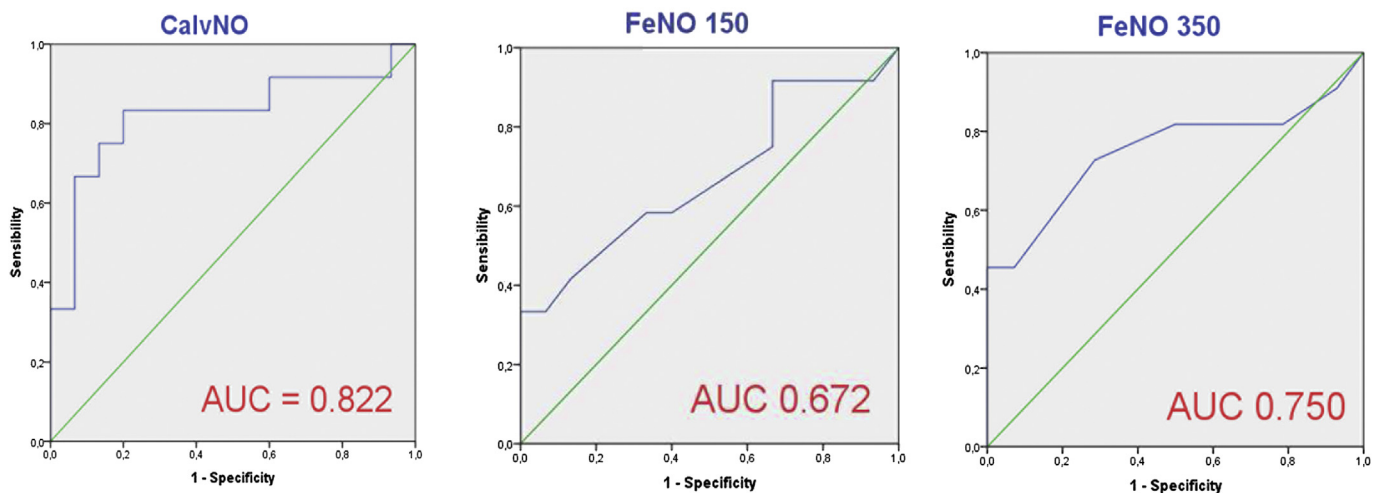
To our knowledge, only one study has focused on the possible impact of native lung diseases on eNO and eCO production in a population of SLTX patients [24]. An irrelevant role of the native lung was suggested and, in agreement with those results, our study shows no statistically significant differences in these biomarkers between SSLTX and SLTX patients. In our population, only eight patients underwent SLTX, and although SLTX patients affected by IPF and NSIP showed higher values of Calv<sub>NO</sub> (in line with previous findings in these diseases [25,26]), the difference was not significant.

#### 4.2. eNO and BOS

We found a significant increase of Calv<sub>NO</sub> and FeNO at flows of 150 and 350 ml/s in LTX patients affected by BOS. In particular, Calv<sub>NO</sub> demonstrated a higher sensibility and specificity than FeNO 150 and 350 in identifying BOS in LTX patients. In order to avoid possible bias due to excessive rigidity of the 2CM model, we have also calculated Calv<sub>NO</sub> and J'aw<sub>NO</sub> levels with the TMAD

model. However, neither Calv<sub>NO</sub> nor J'aw<sub>NO</sub> values were significantly different between the two models. Elevated alveolar concentrations and FeNO 150–350 ml/s levels, associated with not increased J'aw<sub>NO</sub> e FeNO 50–100 values reported in this study in BOS patients, may be consequent to the specific pathogenetic involvement of the peripheral small airways. The production of NO by inducible NO synthase (iNOS) is commonly increased in chronic inflammatory processes, in particular when lymphocytes are involved [27,28], and BOS is traditionally considered the outcome of chronic alloimmune T-cell reactivity [29,30]. These assumptions can justify elevated concentrations of NO in little airways of LTX patients affected by BOS. In agreement with previous studies demonstrating an increase of iNOS expression in bronchial epithelium, especially in LTX patients in the early stage of chronic rejection [12], our BOS population was mainly composed by stage 1 BOS patients. Thus, our results support the evidence that in BOS patients, especially in the early phase (stage 1), the increase of NO production was related with the airway inflammation that leads to an abnormal iNOS activation. Although in BOS stages 2–3, patients reported FeNO150 and 350 and Calv<sub>NO</sub> values higher than non-BOS patients, they do not show different values with respect to BOS stage 1 patients. As expected, our BOS patients had lower FEV1 values than non-BOS patients, but no significant correlations were found between eNO parameters and FEV1 percentages in BOS patients. Although the sample size was insufficient to apply statistical tests, the Calv<sub>NO</sub> values did not change at different stages of BOS and no data was found to support Calv<sub>NO</sub> as a severity marker of disease. Analogously Fisher et al. found higher FeNO 200 values in patients affected by BOS than in healthy controls [10], they considered that the most elevated levels were expressed by BOS stage 1 patients and justified the results by suggesting a reduced iNOS expression in BOS stages 2 and 3, due to replacement of inflammatory status by fibrosis. Although our results are preliminary, the lack of a correlation between functional parameters and Calv<sub>NO</sub> values is in line with the findings by Fisher et al. [10] and suggests that Calv<sub>NO</sub> did not represent a potential biomarker of BOS severity.

However, in LTX population, we found an inverse correlation between Calv<sub>NO</sub> levels and TLCO percentages. According to the kinetics of NO, probably, a TLCO impairment compromises the NO removal from alveoli, because of a increased thickness of alveolar-



**Fig. 3.** Receiving operating characteristic (ROC) curves for Calv<sub>NO</sub>, FeNO at flow rate of 150 ml/s (FeNO 150) and 350 ml/s (FeNO 350) for the detection of bronchiolitis obliterans syndrome (BOS) in stable LTX patients. Calv<sub>NO</sub> showed the best diagnostic accuracy for BOS in stable LTX recipients (Calv<sub>NO</sub>, Alveolar concentration of nitric oxide; FeNO, Fractional exhaled nitric oxide; AUC, Area under the curve).

capillary membrane. The elevated  $\text{CalV}_{\text{NO}}$  levels in LTX patients may be consequent both to the increased expression of iNOS in bronchial epithelium [12] and to an imbalance in the elimination rate of alveolar NO.

#### 4.3. eCO in LTX patients

Regarding eCO measurements, no differences were found between LTX patients and healthy controls, nor between BOS and non-BOS patients. Two previous studies indicated a potential role of eCO as a risk predictor of BOS development, suggesting that higher eCO levels in BOS patients, especially at stage 0p and 1, ensued from an abnormal expression of heme oxygenase-1 activity in response to oxidative stress [19,20]. However, different devices were used to measure eCO and possible factors affecting eCO levels (such as current therapy and active-passive smoking exposure) were not considered. It is known that different methodologies of eCO assessment can lead to significantly different results [31] and, to make reliable comparisons among studies on eCO, there is a need to use standardized instruments and methods [4].

In conclusion, our study shows elevated levels of peripheral eNO and  $\text{CalV}_{\text{NO}}$  in LTX patients affected by BOS, probably due to chronic inflammation and remodeling of small airways. In particular,  $\text{CalV}_{\text{NO}}$  showed the best performance in the detection of BOS, and it may be proposed as a potential non-invasive biomarker in the long-term management of stable LTX patients because of its high reproducibility and accessibility.

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