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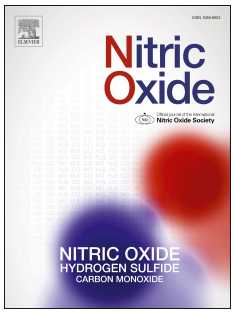

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ALVEOLAR CONCENTRATION OF NITRIC OXIDE AS A PROGNOSTIC BIOMARKER IN IDIOPATHIC PULMONARY FIBROSIS.

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The study was conducted at the Department of Medical and Surgical Sciences and Neurosciences, Respiratory Disease and Lung Transplantation Unit.

Declarations of interest: none

[1] Abbreviations: nitric oxide (NO), exhaled nitric oxide (eNO), fraction of exhaled NO (FeNO), alveolar concentration of NO (CaNO), maximum conducting airway wall flux (J'awNO), pulmonary function tests (PFT).

ABSTRACT

BACKGROUND: Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrotic lung disease leading to respiratory failure and death in 2-5 years from diagnosis. To date, clinical course of disease and prognosis cannot be predicted with an acceptable accuracy. Recently, alveolar concentration of nitric oxide (CaNO) has been proposed as a marker of severity of IPF, but its prognostic value in this setting is unknown.

AIM OF THE STUDY: To evaluate the reliability of CaNO as a prognostic biomarker in patients with IPF.

METHODS: In the Siena Referral Centre for Interstitial Lung Diseases, multiple-flows exhaled nitric oxide analysis was performed to measure CaNO in a cohort of 88 patients with IPF and in 60 healthy controls. In this population, we evaluate functional disease progression and survival according to the follow-up of our Centre. Clinical, functional and radiological data were collected at baseline to investigate correlations with CaNO levels.

RESULTS: IPF patients showed significantly higher levels of CaNO than healthy controls ($p < 0.0001$); CaNO was significantly correlated with many pulmonary functional parameters. Survival analysis showed that all patients with CaNO $\geq$ 6 ppb reported a significantly worse outcome. Disease progression, expressed as FVC time to decline to 10% (TTD10), occurred significantly earlier in patients with CaNO $\geq$ 9 ppb.

CONCLUSION: We confirm that CaNO was significantly higher in IPF patients than in healthy controls and its correlation with functional parameters. Moreover, CaNO $\geq$ 6 and $\geq$ 9 ppb were significantly correlated with mortality and disease progression, respectively. These data suggest that CaNO, a non-invasive and reproducible biomarker, may predict disease progression and survival outcome in patients with IPF.

Keywords: Exhaled nitric oxide; idiopathic pulmonary fibrosis; biomarkers; alveolar nitric oxide

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of interstitial lung disease (ILD) and is characterized by chronic and progressive deterioration of lung volumes, leading to chronic respiratory failure and finally death. The clinical course of IPF is heterogeneous: some patients experience much faster progression than others, sometimes developing acute exacerbations. Certain biomarkers, such as Krebs Von Den Lungen-6 protein [1], periostin [2], CA-19.9 and CA.125 [3], have been proposed as having prognostic value in IPF but none has shown sufficient reliability to be included in routine clinical care.

Exhaled breath analysis, including fractional exhaled nitric oxide (FeNO), could provide novel biomarkers for IPF. In the lungs, nitric oxide (NO) is a intracellular mediator that can be produced constitutively, by endothelial and epithelial cells [4], or through inducible NO syntase (iNOS), expressed by a large variety of resident cells, including alveolar macrophages and fibroblasts [5,6]. An excessive production of NO through iNOS correlated with the burden of pulmonary nitrosative-oxidative stress and has been related to aberrant fibrogenesis processes in murine model of ILD [7]. FeNO can be considered a reliable and reproducible biomarker of nitrosative stress in lungs; moreover, a multiple-flows evaluation can estimate both bronchial and alveolar NO parameters, enabling the study of NO dynamics also in diffuse lung disease in a non-invasive way [8]. Our previous studies show that alveolar concentration of nitric oxide (CaNO) was significantly elevated in patients with IPF and correlated with severity of disease [9,10]. Moreover, CaNO distinguished connective tissue disease associated with ILD (CTD-ILD) from idiopathic interstitial pneumonias (IIP) [11]. The prognostic value of CaNO in IPF is unknown: in a small, non-control-matched study, Kotecha et al. reported a non significant link between higher CaNO and sooner progression and death [12].

Here we investigated the prognostic value of CaNO for mortality and disease progression in a cohort of newly diagnosed IPF patients.

MATERIALS AND METHODS

1.1 Study population and design of the study

We retrospectively recruited 88 patients with IPF (68 males, 65 ± 9.8 years old), seen at Siena Regional Referral Centre for Interstitial Lung Diseases between November 2011 and January 2016. IPF was diagnosed according to 2011 ATS/ERS guidelines [13]. Medical history was recorded in order to evaluate comorbidities and smoking status. None of the patients included in the study were on steroid therapy, antifibrotic treatment for IPF or oxygen therapy. Patients with history of atopy, concomitant asthma, cancer or autoimmune disorders were excluded from the study. All patients performed basal pulmonary function tests (PFTs), including single breath diffusing capacity for carbon monoxide, within 3 months of exhaled NO analysis. Gender-Age-Physiology (GAP) score and Composite Physiological Index (CPI) were calculated, as previously reported [14,15].

Clinical and functional follow-up were performed every 6 months or according to individual clinical needs. Every PFT performed was recorded to evaluate functional progression, expressed as >10% relative decline in FVC% predicted (time to decline of FVC > 10%: TTD10).

As control group, we enrolled 60 healthy volunteers (40 males, 63.1 ± 10.6 years old). History of asthma, recent respiratory infections, inhalant allergies and phosphodiesterase-5 inhibitor therapy were exclusion criteria. Controls were not on any pharmacological therapy.

All patients and controls gave their informed consent to the study. The study was approved by Siena Local Ethics Committee C.E. A. V. S. E. (code number 180712). The study was conducted according to the principles of the Declaration of Helsinki.

1.2 Pulmonary function tests

The following lung function measurements were recorded according to ATS/ERS standards [16,17] using a Jaeger Body Plethysmograph with corrections for temperature and barometric pressure: forced expiratory volume in the first second (FEV1), forced vital capacity (FVC), FEV1/FVC, total lung capacity (TLC), residual volume (RV), carbon monoxide lung transfer factor (TLCO) and

carbon monoxide lung transfer factor/alveolar volume (TLCO/VA). TLCO measurement was impossible in seven patients who could not properly perform single-breath manoeuvres. PFTs were performed at least 2 hours after exhaled NO measurements.

1.3 Extended exhaled NO analysis

Nitric oxide measurements were performed with an electrochemical analyser (model Hypair FeNO Medisoft Cardioline Exp'air, 2010) according to ATS recommendations for online measurement of FeNO in adults [18]. The analyser was sensitive from 1 to 500 ppb NO with a resolution of 1 ppb. All measurements were made at an ambient NO concentration of < 10 ppb. Exhaled NO was measured during slow exhalation from total lung capacity against a positive pressure in the range 5-20 cm H2O. Exhalation flow rate was kept constant by a biofeedback visual display. FeNO was measured at flow-rates of 50, 100, 150 and 350 ml/s. For each flow rate, at least two technically satisfactory measurements were performed and in the case of a difference of more than 10% between these measurements, a third measurement was taken. The flow-independent NO parameters, CaNO and maximum airway flux of NO (J'awNO), were calculated by the device software using the linear model endorsed by the recent ERS technical standard [8]: CaNO and J'awNO were the Y-intercept and the slope of the linear relationship between flow rate and FeNO•flow product, respectively. For each patient, the linear relationship was evaluated between the three points (100, 150 and 350 ml/s) of NO flux versus flow.

1.4 Data collection and reproducibility

Each measurement was considered acceptable with a confidence rate > 95% and a flow stability > 90%. All FeNO measurements were performed by a single investigator.

1.5 Statistical analysis

Comparisons of eNO levels between patients and controls were performed by the t-test. Correlations between variables were performed with the Pearson test. Unadjusted survival estimates were obtained using Kaplan-Meier curves for the outcomes TTD-10 and all-cause mortality. Cox regression analysis was used to calculate hazard ratios (HR) for mortality and

functional disease progression according to baseline CaNO. Comparisons between Kaplan-Meier curves were obtained using the log-rank test statistic. A p-value < 0.05 was considered statistically significant. Data is expressed as mean ± standard deviation (SD). Statistical analysis and figures were conducted with Stata version 15.1 (Stata corp, College Station, TX), Microsoft Excel and Graphpad Prism 5.0 for Windows.

RESULTS

1.1 Study population

Demographic features, smoking status and functional data and exhaled biomarker assessments of IPF patients and controls are reported in Table 1. No significant differences were found concerning age, sex or smoking status. Regarding PFTs, on average IPF patients showed mild restrictive impairment associated with a moderate reduction in TLCO.

1.2 Extended exhaled nitric oxide analysis

Patients showed significantly higher FeNO at expiratory flows of 100, 150 and 350 ml/s and CaNO than healthy controls ($t = 3.291$, $p = 0.0013$; $t = 2.941$, $p = 0.0040$; $t = 4.035$, $p < 0.0001$; $t = 5.947$, $p < 0.0001$, respectively) (Fig.1), while there were no significant differences in FeNO at expiratory flow 50 ml/s and J'awNO ($t = 1.913$, $p = 0.0582$, $t = 1.256$, $p = 0.1235$). No differences in eNO were reported in relation to age, sex, smoking status or biometric data.

Regarding functional and eNO parameters, CaNO was inversely correlated with TLCO% ($r = -0.2682$, $p = 0.0155$) (Fig. 2); there was also a similar but not significant trend ($r = -0.2045$; $p = 0.0604$) between CaNO and FVC%. The same was true between CaNO and CPI ($r = 0.2120$, $p = 0.0575$). No other significant correlations were found between eNO levels and functional parameters.

1.3 Outcome analysis

Results of PFTs beyond baseline were available for 81 patients (92%). On 1st August 2018 (1086.8 ± 630 days of follow-up), 37 (42%) IPF patients were alive, eight (9%) had undergone lung transplant and 43 (48%) had died. Median survival was 941 days. Among eNO parameters, Kaplan-Meier analysis showed better survival in patients with CaNO < 6 ppb (HR 0.4684; p=0.0230;), irrespective of age, sex or smoking status (Fig. 3). Baseline DLCO was also inversely correlated with mortality (DLCO>50%: HR 0.22, p<0.001); when we combine DLCO<50% and CaNO > 6 ppb in the same model, both remain significant (HR 0.19, p<0.001; HR: 2.7, p=0033, respectively). Concerning disease progression, CaNO $\geq$ 9 ppb was associated with a significantly

shorter TTD10 (HR 2.1; p 0.0021), while baseline DLCO% was not significantly associated with TTD10 (HR 0.57, p=0.08); CaNO > 9 ppb remain significant also if combined with DLCO% in the same model (HR 2.2, p=0.022; HR:0.55, p=0.07, respectively).

DISCUSSION

The aim of this study was to evaluate the prognostic value of CaNO for mortality and disease progression in a cohort of IPF patients. Our results confirm that CaNO, a reliable and non-invasive biomarker, was significantly elevated in IPF and related to disease severity, especially with regard to impairment of alveolar diffusion. These findings are in line with previous studies [1,19], and furtherly confirmed in this study based on a wider cohort. The correlation between CaNO and TLCO% may result both by an impairment of alveolar diffusion and by the increase of NO production at alveolar levels, as demonstrated by Pullamssetti [20]. The reliability of CaNO were confirmed by a recent metanalysis in which CaNO emerged as the most promising of various breath biomarkers for clinical management of IPF [21].

To our knowledge, this is the second study to investigate the prognostic value of this biondicator in IPF. Kotecha et al. reported a significant difference in baseline CaNO between IPF patients with stable and progressive disease, as well as a non significant decreasing trend in survival and time to progression and higher CaNO [1]. Our results confirmed and strengthened these assumptions, showing that CaNO may be a reliable predictive marker of disease progression and mortality. The difference between our study and Kotecha's could depend on our larger cohort (88 vs 27 patients, respectively) and also on the different multiple-flows eNO analysis techniques (trumpet-shape method by Condorelli [22] vs Tsoukias and George method [23], respectively). This discrepance may be furtherly investigated in a future prospective study, to clarify the predictive prognostic value in IPF. Another study investigated the potential role of FeNO 50 in predicting mortality or development of pulmonary hypertension in ILD patients, with negative results [24]. Our study further confirmed the limited prognostic value of FeNO 50 in IPF. These results seem reasonable

because exhaled samples for FeNO 50 originate in the proximal airways, which are not involved in pathogenesis of IPF [25], while CaNO allows us to estimate eNO burden in the alveolar district.

The significantly higher CaNO and its potential prognostic value suggest that NO can be useful not only for insights into the pathogenesis of lung fibrosis but also for predicting disease progression. Tiev et al. proposed CaNO as a marker of pulmonary functional deterioration, leading to respiratory failure or death, in a cohort of patients with ILD associated with systemic sclerosis [26]. The authors suggested that increased CaNO was related to more severe alveolar inflammation and played a leading role in the progression of pulmonary fibrosis. This assumption cannot apply to IPF, where inflammation appears to play a secondary role [27], as demonstrated by the lack of efficacy of steroid and immunosuppressive treatment [28]. The relation between CaNO, progression of disease and mortality may indicate that nitrosative stress, overexpressed in pulmonary fibrosis [29,30], plays an important role not only in inducing but also in protracting lung fibrogenesis.

The study has some limitations. First of all, retrospective studes are by nature associated with recall and selection bias, even though all patients were followed in our Center, ensuring the homogeneity of the collection data. Therefore, our results need to be confirmed in a larger, longitudinal and prospective study. Unfortunately, we did not perform sequential measurements of eNO parameters to evaluate their trend during follow-up. Repeated measures could presumably be useful to compare CaNO in progressive and non-progressive IPF and to investigate any effects of antifibrotic treatment on eNO.

The present study confirms that IPF patients have significantly elevated CaNO. Higher levels of this biomarker were associated with worse survival and sooner disease progression. These results may suggest a potential utility of CaNO as a non-invasive cost-effective biomarker useful in the clinical management of IPF for early identification of progressive disease.

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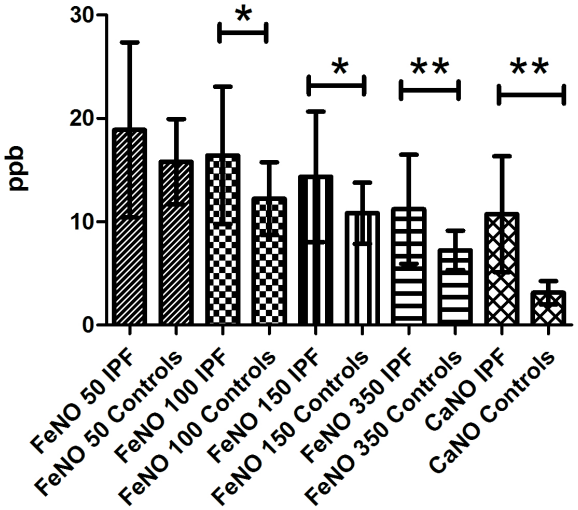
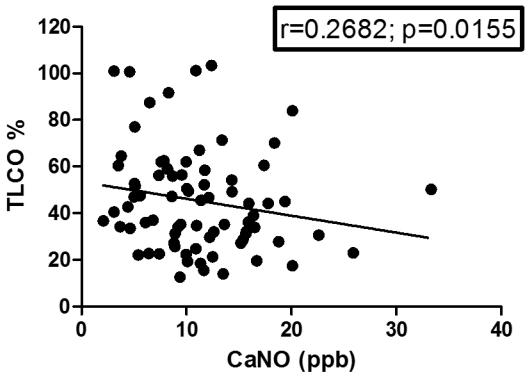
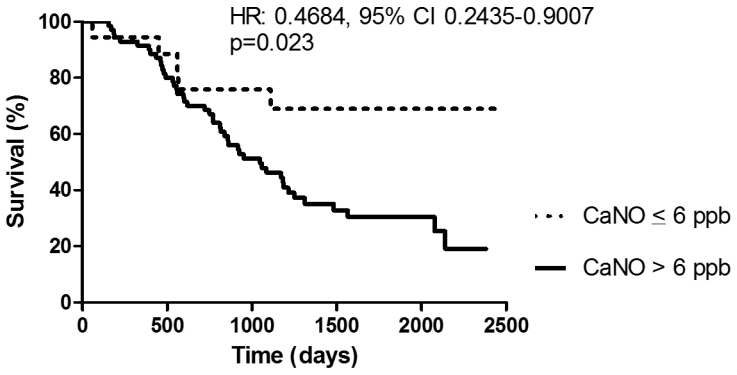
Parameters	IPF	Controls	p-value
N°	88	60	
Male sex (%)	68 (77)	40 (66)	0.1878
Age (years)	65 ± 9.8	63.1 ± 10.6	0.1255
Current smokers (%)	2 (2)	4 (7)	0.4048
Former smokers (%)	57 (65)	38 (63)	0.4048
Never smokers (%)	29 (33)	18 (30)	0.4048
Pack-year	20.9 ± 22.4	15 ± 13.1	0.2245
PFTs		n.a.	
FVC %	77.6 ± 24		
FVC ml	2590 ± 926.4		
FEV1 %	79.9 ± 23.6		
FEV1 ml	2084 ± 743.7		
FEV1/FVC	80.8 ± 7.3		
RV %	90.6 ± 28.1		
RV ml	2125 ± 650.6		
TLC %	79.8 ± 20.5		
TLC ml	4845 ± 1408		
TLCO % (81 pz)	45.4 ± 21.8		
TLCO/VA % (81 pz)	72.2 ±20.6		
GAP score			

0-3 (%)	37 (42)		
4-5 (%)	35 (39)		
6-8 (%)	16 (18)		
CPI (81 pz)	47.4 ± 18.4		
Exhaled biomarkers	88	60	
FeNO 50 (ppb)	18.8 ± 8.4	15.8 ± 4.1	0.0582
FeNO 100 (ppb)	16.4 ± 6.6	12.2 ± 3.5	0.0013
FeNO 150 (ppb)	14.3 ± 6.3	10.8 ± 2.9	0.0040
FeNO 350 (ppb)	11.2 ± 5.2	7.2 ± 1.9	<0.0001
J'awNO (nl/min)	32.5 ± 26.1	40.3 ± 32.3	0.1259
CaNO (ppb)	10.7 ± 0.6	3.1 ± 1.2	<0.000001

Table 1. Demographic features, smoking status, functional assessment and eNO parameters in IPF patients and healthy controls. All data are expressed as mean ± standard deviation, unless otherwise indicated. eNO: exhaled nitric oxide; FeNO: fractional exhaled nitric oxide; J'awNO: maximum airway flux of NO; CaNO: alveolar concentration of nitric oxide, ppb: pars per billion.







HIGHLIGHTS

IPF patients showed significantly higher alveolar concentration of nitric oxide.

Alveolar nitric oxide is inversely related to lung functional impairment.

Higher alveolar nitric oxide levels are associated with worse survival

Alveolar nitric oxide conentration may predict disease progression.