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Journal of Breath Research

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To cite this article before publication: Paolo Cameli *et al* 2019 *J. Breath Res.* in press https://doi.org/10.1088/1752-7163/ab0233

EVALUATION OF MULTIPLE-FLOWS EXHALED NITRIC OXIDE IN IDIOPATHIC AND NON-IDIOPATHIC INTERSTITIAL LUNG DISEASE

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

Declarations of interest: none

[1]

ABSTRACT

BACKGROUND: Fractional exhaled nitric oxide (FeNO) is a non-invasive and reproducible marker of nitrosative stress and lung inflammation. More recently, FeNO has been proposed as a marker of severity of idiopathic pulmonary fibrosis (IPF) and systemic sclerosis associated ILD.

AIM AND OBJECTIVES: to evaluate the role of FeNO in the diagnostic pathway of ILDs.

METHODS: According to ERS guidelines for exhaled biomarkers in lung diseases, FeNO at multiple flow-rates (50-100-150 and 350 ml/s) and alveolar concentration of NO (CaNO) were collected in 60 healthy controls and 134 patients affected by ILD: 50 with IPF, 19 with fibrotic non-specific interstitial pneumonia (NSIP), 19 with chronic hypersensitivitis pneumonia (cHP) and 46 with connective tissue disease related ILD (CTD-ILD). ROC curves were performed to investigate the potential role of eNO parameters in discriminating between idiopathic and non-idiopathic ILDs.

RESULTS: All ILD groups reported higher levels of FeNO 150-350 ml/s and CaNO than controls. Among ILDs, CTD-ILD showed more elevated FeNO 350 ml/s and CaNO levels than other ILD. In particular, CaNO reported the best diagnostic accuracy to discriminate CTD-ILD from idiopathic ILDs.

CONCLUSIONS: Patients affected by ILD reported increased FeNO 150-350 ml/s and CaNO in respect with healthy controls, indicating a potential role of nitrosative stress in lung fibrosis. The significant difference of CaNO levels between idiopathic ILDs and CTD-ILD is interesting and may suggest that NO is also implicated in lung inflammation associated with rheumatological disease. Further evidence is necessary to establish if CaNO is worthy of attention in the differential diagnosis of ILDs.

Keywords: Exhaled nitric oxide; interstitial lung disease; biomarkers; connective tissue disease

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

[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).

INTRODUCTION

Connective tissue diseases associated with Interstitial lung disease (CTD-ILD) is a heterogeneous group of pathologies characterized by the presence of different patterns of interstitial pulmonary involvement associated to a specific connective tissue disorder. The onset of pulmonary disease during the clinical course of CTDs is associated with a significant morbidity and it can impair quality of life and life expectancy. Specific CTDs are often related with particular radiological and histopatological ILD patterns, such as systemic sclerosis (SSC) with non specific interstitial pneumonia (NSIP) [1], Sjogren syndrome (SS) with NSIP or lymphocytic interstitial pneumonia [2] and rheumathoid arthritis (RA) with usual interstitial pneumonia (UIP) [3]. Lung involvement in CTDs is undoubtedly harmful and, in patients affected by SSc, is the main cause of death in endstage disease [4].

The pathogenesis of CTD-ILD is not well understood yet, although it has been reported that immune dysregolation, autoimmune processes, cell senescence, oxidative stress and epithelial dysfunction participate to bronchoalveolar epithelial depauperation, followed by an aberrant proliferation of fibroblasts and epithelial-mesenchymal transition (EMT), that eventually leads to pulmonary fibrosis [5, 6, 7].

Even though the exclusion of CTD in the diagnostic pathway of ILD patients is mandatory, recent researches underlined how idiopathic interstitial pneumonias (IIPs), such as IPF or NSIP, share not only common pathogenethic pathways with CTD-ILD, but also similar outcomes, especially CTD with a pattern of UIP [8].

After the introduction of new specific antifibrotic drugs for IPF patients, new tools to discriminate IIP from CTD-ILD are necessary. Nitric oxide (NO) is a intracellular mediator involved in vasodilation, inflammation and nitrosative-oxidative stress; experimental researches reported an aberrant NO production associated to abnormal lung fibrogenesis [9, 10]. Recent clinical studies have suggested a potential role of fractional exhaled nitric oxide (FeNO) as severity biomarker in patients with ILDs [11].

Tiev et al. suggested that eNO parameters, in particular alveolar concentration of NO (CANO), could be useful in patients with SSC to detect the presence of ILD [12], to predict functional deterioration [13] and

response to cyclophosphamide [14]. They didn't mention a potential role of this parameter to differentiate IIP from CTD-ILDs.

The present study aimed to compare FeNO parameters among patients with IIPs, non-idiopathic ILD (chronic hypersensitivity penumonia, cHP) and CTD-ILDs in order to evaluate the different behaviour in each specific diffuse lung disease and the potential prognostic role of this indicator.

MATHERIALS AND METHODS

1.1 Study population and study design

134 patients with ILDs were recruited at Siena Regional Referral Centre for Sarcoidosis and Interstitial Lung Diseases. ILD population was composed by 50 patients with IPF (30 males, mean age 65 ± 9.1 years), 19 with NSIP (9 males, 66 ± 10 years), 19 with cHP (11 males, 59.2 ± 13 years) and 46 with CTD-ILD (17 males, 62.4 ± 11 years). Among CTD-ILD group, 15 patients had SS, 14 had RA and 17 SSC. Diagnosis was performed according to international guidelines [15,16,17]. No histological sampling (such as transbronchial biopsy, criobiopsy or surgical lung biopsy) has been performed for diagnostic purpose in this population. Medical history was collected in order to investigate professional exposure, smoking status and therapeutic options. All patients were able to perform pulmonary function tests (PFTs), including single-breath diffusing capacity for carbon monoxide. High resolution computed tomography (HRCT) of the chest was performed in all patients for diagnostic purposes to evaluate the specific radiological patterns. All included patients were clinically stable, free of respiratory infections and/or acute exacerbations for at least 8 weeks. Patients with atopy, asthma, cancer or in current therapy with biological agents were excluded in all cases.

60 healthy volunteers (32 males, mean age 61 ± 5 years) were enrolled in the study as control group. History of asthma, recent respiratory infections, inhalant allergies as well as therapy with phosphodiesterase-5 inhibitors were considered as exclusion criteria. They did not receive any pharmacological therapy.

All patients and controls gave their informed consent to the study. The study was conducted according to Declaration of Helsinki principles.

1.2 Study protocol

Exhaled nitric oxide measurements was performed in the morning, in sitting position in a comfortable environment: all participants had been fasting for at least 8 hours before the detection avoiding foods containing nitrates (lettuce, spinach, cabbage, sausages) and high fat foods for at least 12 hours. All participants had a mouthwash with water just before the test. Participants underwent PFTs the same morning after FeNO evaluation (with a distance of almost 2 hours).

1.3 Pulmonary function tests

The following lung function measurements were recorded according to ATS/ERS standards [18, 19], 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 capacity carbon monoxide lung transfer factor/alveolar volume (TLCO/VA). TLCO measurement could not be collected in 10 patients unable to perform properly single-breath manoeuvres. PFTs were performed after at least 2 hours from exhaled NO measurements.

1.4 Exhaled nitric oxide measurements

NO measurements were performed using a chemo-luminescence analyser (model Hypair FeNO medisoft Cardioline Exp'air, 2010) according to the ATS recommendations for online measurement of FeNO in adults [20]. The analyser was sensitive to NO from 1 to 500 ppb with a resolution of 1 ppb. All measurements were undertaken at ambient NO level of < 10 ppb. Exhaled NO was measured during slow exhalation from total lung capacity against a positive pressure kept constantly between 5-20 cm H2O. The exhalation flow rate was kept constant through the use of 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 adequate measurements were performed and if a difference of more than 10% between these measurements was observed, a third assay was done. The flow-independent NO parameters, CANO and maximum airway flux of NO (J'awNO), were calculated using the linear model endorsed by ERS recent technical standard [21]: CANO and J'awNO, respectively, correspond to Y-intercept and the slope of the linear relation between flow rate and FeNO•flow product. For each patient a linear relationship was evaluated between the three points (100, 150 and 350 ml/s) of NO flux against the flow.

1.5 Data collection and reproducibility

Each measurement was considered acceptable with a confidence rate > 95% and a flow stability > 90%. A single investigator made FeNO measurements, guaranteeing inter- and intra-observer agreement.

1.6 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons between the IIP groups, CTD-ILD group and controls were performed by Kruskall-Wallis test, with Dunn's multiple comparison test to compare single groups. Correlations between CANO and PFT values parameters were made by Spearman's test. We used non parametric tests because variables didn't show a normal distribution. ROC curves were performed in order to evaluate the diagnostic accuracy of FeNO parameters for CTD-ILD. A p-value < 0.05 was considered statistically significant. Statistical analyses and figures were performed using Graph Pad Prism 5.

RESULTS

1.1 Population of the study

Demographic, functional, therapeutic, radiological features and eNO parameters were reported in Table 1. Populations of patients and controls were comparable: no significant differences were observed regarding age, smoking status and body mass index (BMI).

As expected, CTD-ILD patients were predominantly female, while the majority of IPF patients were male. The cohorts of healthy controls, NSIP and cHP patients were equally sex-matched. Chi-square test didn't reveal any statistical significant difference between the groups of patients and controls. Concerning PFTs, globally the cohort of patients reported a mild restrictive functional impairment associated to a moderate reduction of DLCO with no significant differences for any specific group, except for IPF patients that showed significantly lower values of TLC in respect to CTD-ILD patients.

1.2 eNO parameters and clinical-radiological features

All ILD patients presented significantly higher FeNO150 and CANO levels than healthy controls ($p<0.0001$ for both), while FeNO 350 values were significantly increased only in IPF, CTD-ILD and NSIP patients than controls ($p<0.0001$). No statistical significant differences were found among FeNO50, 100 and J'awNO levels of each subgroup. Concerning ILD population, CTD-ILD patients reported significantly higher values of FeNO 350 and CANO than the other subgroups (Fig.1).

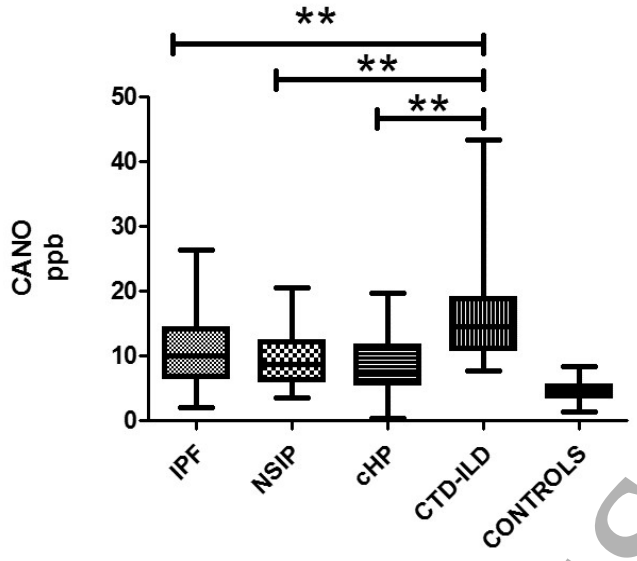

Fig.1 Comparison of CANO levels in IPF, NSIP, cHP, CTD-ILD patients and healthy controls. CANO: alveolar concentration of nitric oxide; ppb: pars per billion. **: $p<0.001$. (1.5 column fitting image)

No significant differences were reported among naive patients and patients in therapy with Pirfenidone, Nintedanib and immune-suppressants.

Concerning radiological data, there were no significant differences of eNO parameters among different CT patterns (typical UIP, probable UIP, indeterminate UIP and alternative to UIP) [22] in ILD population as well as in IPF and CTD-ILD subgroups.

1.3 Correlation with functional parameters

In the overall ILD population, we reported a significant inverse correlation between CANO and FVC (r=-0.3864, $p<0.0001$) and DLCO (r=-0.4129, $p<0.0001$). Regarding CTD-ILD, IPF and NSIP subgroups, CANO correlated inversely with FVC (r=-0.3877, p=0.0457; r=-0.4429; p=0.0026; r=-0.7521; p=0.0030, respectively) and DLCO percentages (r=-0.4589, p=0.0025; r=-0.5171; p=0.0005; r=-0.6521; p=0.0030, respectively); no other significant correlations were found.

1.4 Discriminating idiopathic IIPs and CTD-ILDs

ROC curves were performed in order to identify eNO parameters with adequate accuracy in detecting the presence of CTD. CTD-ILD patients showed higher FeNO 150 and 350 and CANO values than the other ILDs patients and were chosen for this purpose. CANO reported the best performance (area under the curve (AUC) = 0.795, p<0.0001) in respect to FeNO 350 (AUC=0.751, p=0.0001) and FeNO 150 (AUC=0.559, p=0.267) (Fig. 2); a CANO cut-off value of 13.09 ppb showed a sensibility of 60% and a specificity of 80% in discriminating CTD-ILD from ILD.

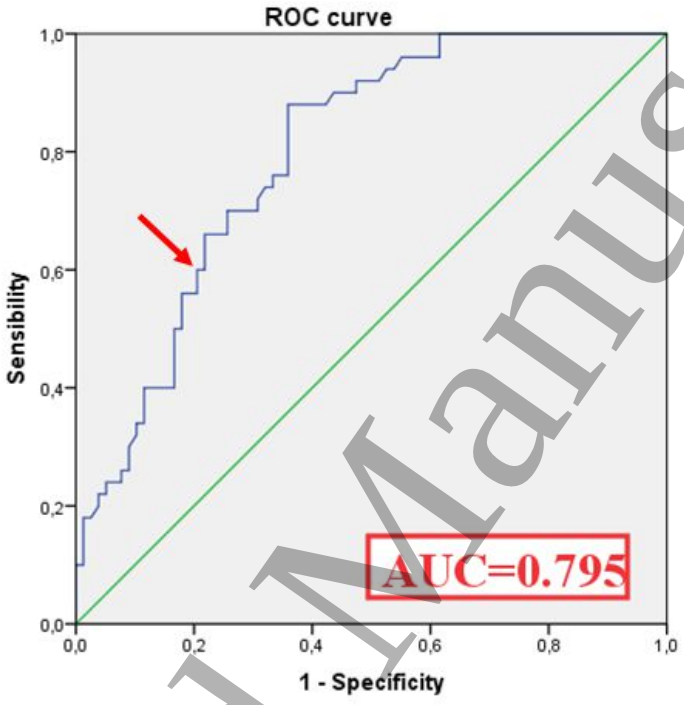

Fig.2 ROC curve for CANO for the detection of CTD in the ILD population. AUC: area under the curve; CANO: alveolar concentration of nitric oxide. (1.5 column fitting image)

DISCUSSION

In this study, the features of eNO parameters in idiopathic (IPF, NSIP) and non-idiopathic ILDs (cHP and CTD-ILD) were analysed; in particular, our aim was to evaluate the potential utility of this non-invasive, reproducible biomarker in the diagnostic algorithm of lung diseases and to evaluate its potential as severity

marker of pulmonary interstitial diseases. Our study suggested the importance to perform multiple-flow measurements of FeNO in patients with diffuse lung diseases as ILD patients reported higher values of peripheral exhaled NO than controls, in line with previous findings [11]. Among eNO parameters, FeNO 350 and CANO, detected according to recent ERS statement, resulted the most promising biomarkers with the highest statistical differences.

CTD-ILD patients showed the highest values of peripheral lung NO and CANO than other ILDs; this finding could be explained by a more pronounced inflammatory process occurring in these rheumatic diseases stimulating an aberrant overexpression of inducible NO synthetase in lung tissue, as reported [23]. Moreover, DLCO percentages were not significantly different among ILD subgroups, suggesting that increased CANO levels in CTD-ILD patients were not related to a worse diffusion lung capacity. Our results confirmed previous findings by Tiev et al, who reported significantly increased CANO levels in SSC-ILD patients in respect to IPF patients and healthy controls: our study enlarged these assumptions to other CTD-ILDs (like RA-ILD and SS-ILD), contributing to the comprehension of NO pulmonary dynamics in these rare diseases. Unfortunately, bronchoalveolar lavage (BAL) cellular analysis

Interestingly, CANO significantly correlated with FVC and DLCO, that are the most important prognostic lung function parameters in ILDs. In previous studies, our group of research hypothesized that CANO could be a surrogate marker of NO diffusion through alveolar capillary membrane: its progressive impairment due to thickening of the membrane reduces washout of NO in alveolar spaces, inducing a NO retention proportional to disease severity. The inflammatory burden in CTD-ILD may alter this relation between CANO and alveolar diffusion of NO, impairing its potential role as severity biomarker. However, these assumptions need to be further validated and the next step of our study will be to detect specific physiological parameters, such as diffusion capacity of lung for NO (DLNO) in these populations of patients.

Concerning radiological data, no significant differences of eNO parameters were found among different CT pattern of ILDs: to our knowledge, this is the first study investigating this task, regardless the publication of the recent Fleischner society guidelines for IPF [24]. In 2007, Tiev reported a significant correlation between CANO and quantitative radiological indexes of lung fibrosis, like ground-glass and reticular score: however, the study included only patients with SSC-ILD and no specific CT patterns were reported [25]. According to

our results, eNO parameters fail to distinguish different CT patterns in a qualitative way, in agreement with our previous study, where IPF and idiopathic NSIP didn't show significant differences [11].

Another relevant aim of our study was to determine the potential diagnostic role of NO parameters in the diagnostic pathway of ILDs: both CANO and FeNO 350 showed a good accuracy in discriminating the presence of CTD in patients with ILDs. International guidelines considered the exclusion of autoimmune diseases as a primary requirement to perform a diagnosis of IPF: this task is not always simple as many ILD patients have clinical or serological features of autoimmunity without reaching a definitive diagnosis of CTD or reports lung fibrosis as first manifestation of CTD. Therefore, the need for new tools for detecting the presence of CTD in ILDs is compelling. eNO measurement may contribute to this topic with numerous advantages including the non-invasivity, the high reproducibility and very low cost.

In conclusion, a potential role of eNO measurement in the evaluation of ILD diagnosis and in the management of ILD patients may be suggested. Among eNO parameters, FeNO 350 and CANO, detected according to recent ERS statement, resulted the most promising biomarkers over-expressed in ILD patients than controls. CTD-ILD patients showed the highest values of peripheral lung NO and CANO than other ILDs, as a possible expression of more pronounced inflammatory processes occurring in these rheumatic diseases.

	ILD population	CTD-ILD	IPF	NSIP	cHP	Controls	p-value
N°	134	46	50	19	19	60	
Male (%)	67 (50)	17 (37)	30 (60)	9 (47)	11 (58)	32 (53)	0.2112 Chi square: 5.843, df 4
Age (yrs)	63.5 ± 10.8	62.4 ± 11	65 ± 9.1	66 ± 10	59.2 ± 13	61 ± 5	0.2087 K=5.874
Smoking history (pack/year)	15 ± 23.8	12.5 ± 14.7	17.7 ± 24.9	19.2 ± 36.5	4.7 ± 7.8	5.2 ± 7.2	0.0987 K=9.271
Current smoker (%)	10 (7)	3 (6)	5 (10)	1 (5)	1 (5)	7 (11)	0.3956 Chi-square: 8.398, df 8
Former smoker (%)	64 (47)	21 (45)	29 (58)	8 (42)	6 (31)	18 (30)	0.3956 Chi-square: 8.398, df 8
Never smoker (%)	60 (45)	22 (48)	16 (32)	10 (52)	12 (63)	35 (58)	0.3956 Chi-square: 8.398, df 8
NO parameters							
FeNO 50 (ppb)	20.4 ± 8.4	19.8 ± 8.3	20.1 ± 8.1	19.9 ± 6.1	20.5 ± 10.8	15.8 ± 4.1	0.1400 K=6.923
FeNO 100 (ppb)	17.9 ± 7.4	18.8 ± 8	17 ± 6.9	16.8 ± 4.9	16.7 ± 6.5	13.1 ± 2.8	0.0954 K=9.054

FeNO 150 (ppb)	15.6 ± 6.9	16.3 ± 6.5	15.3 ± 7.1	15 ± 4.3	15 ± 4.2	10.8 ± 2.9	<0.0001† K=24.26
FeNO 350 (ppb)	10.7 ± 2.8	12.3 ± 2.7	10.2 ± 2	9.6±2.3	9.4 ± 3.8	7.2 ± 1.9	<0.0001‡ K=57.59
J'aw NO (nl/min)	38.7 ± 31.9	31.4 ± 30.8	40.1±32.6	41.3±29.2	49.4±37.5	35.3±22.3	0.1209 K=7.300
CANO (ppb)	12.3 ± 6.61	15.6 ± 7.2	10.9 ±5.4	10.1 ± 5.1	9 ± 5.1	4.5 ± 1.6	<0.0001¶ K=73.18
PFTs						n.a.	
FVC % predicted value (l)	78.6 ± 23 (2.4 ± 0.9)	87.5 ± 21.4 (2.5 ± 0.7)	75.9 ± 22.2 (2.4 ± 0.9)	71 ± 23.4 (2.1 ± 0.8)	76.1 ± 23.3 (2.4 ± 1.1)		0.0831 K=6.671
FEV1 % predicted value (l)	77.9 ± 23 (1.8 ± 1.5)	85 ± 19 (1.9 ± 0.9)	77.1 ± 22.3 (1.9 ± 1.2)	73 ± 23.4 (1.8 ± 1.1)	70.2 ± 27.5 (1.8 ± 1.3)		0.0884 K=6.553
FEV1/FVC	80 ± 7.8	78.7 ± 6.2	79.7 ± 7.6	81.9 ± 9.4	81.8 ± 8.7		0.2881 K=3.764
TLC % predicted value (l)	81.9 ± 17.7 (4.5 ± 1.4)	90.6 ± 18.2 (4.7 ± 1.3)	77.6 ± 15.2 (4.4 ± 1.3)	75.5 ± 13.7 (4.3 ± 1.6)	85.3 ± 20.2 (4.9 ± 1.5)		0.0155* K=10.40
DLCO % predicted value	48.3 ± 18.7	53 ± 16.8	43.8 ± 17.2	45.3 ± 19.4	54.8 ± 21.5		0.0829 K=6.677
KCO % predicted value	72.5 ± 22.1	75.1 ± 22.7	68.1 ± 20.6	73 ± 21.2	80.6 ± 22		0.2647 K=3.970
HRCT scan pattern							
Typical UIP (%)	54 (40)	16 (35)	38 (76)	0	0		
Probable UIP (%)	17 (13)	7 (15)	8 (16)	0	2 (10)		

Indeterminate UIP (%)	23 (17)	14 (30)	4 (8)	0	5 (26)
Not UIP (%)	40 (30)	9 (19)	0	19 (100)	12 (63)
Therapy ongoing	70	36	18	10	6
Steroid (prednisone use mg/die)	51	25 (12.4 ± 9.3)	9 (11.1 ± 4)	11 (12.5 ± 5.9)	6 (14.5 ± 8.1)
Pirfenidone	6	0	6	0	0
Nintedanib	3	0	3	0	0
Methotrexate (mg/sett)	4	4 (12.5 ± 1.7)	0	0	0
Azathioprine (mg/die)	10	2 (100 ± 0)	5 (90 ± 20)	3 (100 ± 0)	0
Mycophenolate mophetile (g/die)	5	5 (1.7 ± 0.4)	0	0	0
Cyclophosphamide	2	1	0	1	0

Table 1. Demographic, functional, therapeutic data, radiological features and eNO parameters of the study population. †: difference in rank sum among IPF, NSIP, cHP, CTD-ILD and healthy controls: 38.2 ($p<0.001$); 47.43 ($p<0.001$); 44.9 ($p<0.05$); 51.28 ($p<0.0001$), respectively. ‡: difference in rank sum among IPF, NSIP, cHP, CTD-ILD and healthy controls: 50.74 ($p<0.0001$); 38.46 (not significant); 31.35 (not significant); 81.73 ($p<0.0001$), respectively; difference in rank sum among CTD-ILD patients and IPF, NSIP and cHP patients: 30.99 ($p<0.05$); 43.28 ($p<0.001$); 50.38 ($p<0.0001$), respectively. ¶: difference in rank sum among IPF, NSIP, cHP, CTD-ILD and healthy controls: 58.98 ($p<0.0001$); 52.72 ($p<0.0001$); 43.69 ($p<0.05$); 90.90 ($p<0.0001$), respectively; difference in rank sum among CTD-ILD patients and IPF, NSIP and cHP patients: 31.91 ($p<0.001$); 38.18 ($p<0.001$); 47.2 ($p<0.001$), respectively. *: difference in rank sum between IPF and CTD-ILD patients:-20.02; $p<0.05$. eNO: exhaled nitric oxide; FeNO: fractional exhaled nitric oxide; J'awNO: maximum airway flux of NO; CANO: alveolar concentration of nitric oxide; HRCT: high resolution computed tomography; K=Kruskal Wallis statistic. Df; degree of freedom.

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