

Title: Severity of scleroderma lung disease is related to alveolar concentration of nitric oxide

Short title: Exhaled C_{ANO} and scleroderma lung disease

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Abstract

Alveolar concentration of exhaled NO (C_{ANO}) is increased in systemic sclerosis (SSc) patients, but whether this increase is related to the severity of interstitial lung disease (ILD) in SSc has not yet been investigated.

Fifty eight SSc patients prospectively underwent pulmonary function tests (PFTs), echocardiogram, fibrosis scoring on pulmonary CT scan. Patients were subdivided into 2 groups according to the existence (or not) of ILD. Measurements of C_{ANO} were assessed in all SSc patients and compared with those obtained in 19 healthy volunteers. Relationships were sought between C_{ANO} , PFTs and CT scan fibrosis scores.

In overall, C_{ANO} was significantly increased in SSc patients (median, range: 6.2 ppb, 3.8-9.9) as compared with controls (2.0 ppb, 1.2-3.0; $p<0.001$). Among SSc patients, C_{ANO} was significantly higher in patients with ILD ($n=33$; 7.5 ppb, 5.2-11.9) as compared with patients without ILD ($n=25$; 4.9 ppb, 3.1-7.0 ppb; $p<0.01$). C_{ANO} was inversely related to total lung capacity ($r= -0.34$; $p<0.01$) and DLCO ($r=-0.37$; $p<0.01$) and was directly related to CT scan fibrosis scores ($r= 0.36$; $p<0.01$).

Increased C_{ANO} could, at least in part, either reflect or contribute to the severity of lung disease and could be noninvasively used to assess the extent of ILD in SSc.

(Words count: 200)

Keywords: exhaled nitric oxide, interstitial lung disease, nitric oxide, systemic sclerosis.

Systemic sclerosis (SSc) is a multi-system disorder of unknown aetiology. It is characterized by fibrosing skin and organs such as lung, heart, gastro-intestinal tract and kidneys [1]. Interstitial lung disease (ILD) occurs in 35 to 40% of patients with SSc. Multiple factors such as oxidative stress and chronic inflammation may be responsible for lung injury leading to pulmonary fibrosis, resulting in restrictive lung function and reduced gas lung transfer. The extent of ILD could be evaluated by pulmonary CT scan, showing ground glass attenuation or reticular fibrosis aspect that are related to the presence of usual interstitial pneumonia or nonspecific interstitial pneumonia in pathologic pulmonary biopsy specimens [2, 3]. These CT scan signs, particularly ground glass attenuation, could reflect the area of active pulmonary lesion in SSc [4]. Although the pathogenetic aspects of scleroderma lung disease remains unclear and the reversibility of ILD under treatment was uncommon in SSc. Currently, assessment of lung involvement or prognosis in SSc is often difficult and unreliable, direct evidence of alveolitis or detailed histological diagnosis are only provided by invasive means such as bronchoscopy and broncho-alveolar lavage (BAL) cells count [5] or transbronchial biopsy, which are difficult to perform on a regular basis. Hence, there is a need to develop non invasive tools to assess severity of lung impairment in SSc.

Partitioning NO in the exhaled air [6, 7], into alveolar concentration (C_{ANO}) and conducting airway flux (J'_{awNO}), can differentiate the anatomic origin of the inflammation, i.e. distinguishing alveoli from airways inflammatory disorders, thus providing an useful tool to evaluate the local NO production in respiratory disease. C_{ANO} is increased in patients with SSc and ILD [8]. However, it has not yet been investigated to see whether this increase actually reflects the importance of lung

functional impairment and the extent of lung morphological involvement in SSc. Therefore, the aim of this study is to investigate the relationship between exhaled NO levels and the detailed lung function testing and high resolution CT fibrosis scoring in SSc patients. We hypothesize that the degree of lung involvement as assessed by exhaled NO could reflect the extent of ILD in SSc.

Methods

Subjects

Between November 2004 and December 2005, 58 patients (52 women, 6 men, median of age, 1st and 3rd quartile: 53.5 years, 45-60) who met the American College of Rheumatology criteria of SSc [9], were included in the prospective study conducted in two academic referral centres of SSc in France. Clinical data, including modified Rodnan skin score, were collected from all SSc patients (Table 1). Patients were subdivided into 2 groups according to the existence (or not) of ILD documented by pulmonary CT scan. Thirty three SSc patients (55.8 years; 47.3-60.8) had ILD and 25 patients (52.4 years; 44.7-59.0) had not pulmonary involvement (Table 2). Patients with a recent respiratory tract infection (<3 months) and patients who take immunosuppressive treatment (cyclophosphamide, mycophenolate mofetil), corticosteroids higher than 10 mg/day, or bosentan, were excluded from this study. Among the 58 patients with SSc enrolled, 11 patients with SSc took low dose corticosteroid (5 – 10 mg /day), and 10 patients were current smokers. None of the SSc patient had either a history of atopy or took NO donors. Carbon monoxide transfer factor (DLCO) could not be performed in 2 SSc patients. Exhaled NO was measured in all SSc patients and in 19 healthy non-smokers controls (9 women, 10 men, 42.7 years, 28.1-50.4). This study was approved by the local ethics committee and all subjects provided informed consent.

Pulmonary CT scanning

All patients underwent a high-resolution computed tomogram (HRCT) of lung in the week prior the measurement of exhaled NO. Interstitial lung disease was considered present if the HRCT of the chest demonstrated compatible changes in reticular or air

space opacities. The extent of the individual CT scan patterns was estimated in each lobe into 5 levels for ground glass score and fibrosis score [10] as follows: grade 0= no abnormality, grade 1= less than 5% of the lobe, grade 2:6-25% of the lobe, grade 3: 26-50% of the lobe, grade 4=51-75% of the lobe, and grade 5=76-100% of the lobe. Ground glass attenuation was defined as a hazy increase of lung parenchymal attenuation and fibrosis score included lobular septal thickening and subpleural honeycomb change.

Echocardiogram

All patients underwent an echocardiogram (Vivid® 7 G.E medical systems, Norway) in the week prior to measurement of the partitioned of exhaled NO. Pulmonary hypertension was defined by a right ventricular systolic pressure of > 40 mmHg on echocardiogram.

Lung function measurement

Pulmonary function tests (PFT) (forced vital capacity, forced expiratory volume in one second), DLCO (MasterScreen® Body, VIASYS Healthcare GmbH, Hoechst, Germany) and blood gas measurement were performed at the same visit than exhaled NO. PFT parameters were expressed as percentage of predicted normal [11].

Fractional exhaled NO measurement

NO was measured using chemoluminescent NO analyzer (EndoNO 8000®, SERES, Aix-en-Provence, France), according to validated method for the online measurement of the exhaled NO concentration (FE_{NO}) in adults [12]. After full inspiration from room

air with ambient NO levels less than 20 part per billion (ppb), the subject exhaled against positive pressure that was constantly kept between 5 cmH₂O (lower limit) and 20 cmH₂O (upper limit) to generate exhalation flow rates (V'_E) of 50, 100, 150 and 200 ml/s ($F_{E_{NO50-200}}$). For each V'_E , the elimination rate of NO (V'_{NO}) was calculated ($V'_{NO} = V'_E \cdot F_{E_{NO}}$) [6, 7]. $F_{E_{NO}}$ is inversely related to V'_E , whereas V'_{NO} varies directly as a function of V'_E . At the flow rate > 50 ml/s, the latter relationship is linear and can be expressed as $V'_{NO} = V'_E \cdot F_{E_{NO}} = C_{A_{NO}} \cdot V'_E + J'aw_{NO}$. [6, 7] For each patients and controls, the R^2 values of the relationship between $F_{E_{NO}}$ and V'_E were calculated.

Statistical Analysis

All results were expressed as medians and 1st - 3rd quartile. Comparisons between 2 SSc groups (with and without ILD) and control group were performed by Kruskal-Wallis test ($\alpha=0.05$). The difference was significant if α is below 0.05. Comparison between the subgroup of patients with SSc associated with ILD and that without ILD and the comparison between the subgroup of patient with pulmonary hypertension and the healthy control group were performed by Mann-Whitney U test ($\alpha=0.05$). Correlations between $C_{A_{NO}}$ and TLC, ground glass score, fibrosing score, systolic pulmonary arterial pressure (sPAP) were made by Spearman test ($\alpha=0.05$). Sigmastat[®] (version 3.1 for windows, Systat software Inc) was used to perform the statistical analysis.

Results

Demographic characteristics and pulmonary function tests' results are given in table 1. The median C_{ANO} was significantly increased in whole group of SSc patients ($n=58$; 6.2 ppb, 3.8-9.9) as compared with healthy controls ($n=19$; 2.0 ppb, 1.2-3.0; $p<0.001$) (Table 1, Fig 1). The median R^2 values of the relationship F_{ENO} versus V'_{exh} were 0.95 for patients and 0.96 for controls. The median level of $J'aw_{NO}$ of patients with SSc ($n=58$; 6.3 nl/min, -9.8-31.3) was lower than that of healthy controls ($n=19$; 35.5 nl/min, 25.8-54.9) (Table 1). F_{ENO50} values measured in SSc patients (10.1, 5.9-17.8) did not significantly differ from those obtained from healthy controls (13.1, 11.6-19.6) (Table 1).

Among the 58 patients with SSc, HRCT images revealed heterogeneous areas of both ground glass and honeycombing in 33 patients and were consistent with ILD associated with SSc. Total lung capacity (TLC) was significantly reduced, that was consistent with restrictive lung disease, in the SSc group with ILD (82%, 68-95) as compared with the SSc group without ILD (105%, 90-107; $p<0.001$). DLCO/VA values were not different between the two groups of SSc patients (Table 2). Reticular score and ground glass score were significantly higher in SSc patients with ILD (7, 4-9 and 8, 6-12) as compared with SSc patients without ILD ($p<0.001$). C_{ANO} was significantly higher in SSc patients with ILD ($n=33$; 7.5 ppb, 5.2-11.9) as compared with SSc patients without ILD ($n=25$; 4.9 ppb, 3.1-7.0; $p<0.01$). Conversely, $J'aw_{NO}$ from SSc patient with ILD was lower than that from patients without ILD (Table 2).

In patients with SSc, C_{ANO} was inversely related to TLC ($r=-0.34$; $p<0.01$) and DLCO ($r=-0.37$, $p<0.01$). Furthermore, C_{ANO} was directly related to lung fibrosis radiological

indexes, *i.e.* ground glass attenuation score ($r=0.36$; $p<0.01$) and reticular score ($r=0.33$; $p<0.05$).

No correlation was found between sPAP estimated by echocardiogram and C_{ANO} . Furthermore, the median level of C_{ANO} from SSc patients with pulmonary hypertension (sPAP ≥ 40 mmHg), ($n=8$; 6.9 ppb; 5.9-8.9) was comparable to that from SSc patients whose sPAP was under 40 mmHg ($n=50$; 6.0 ppb; 3.4-9.0). Note that C_{ANO} from SSc patients with pulmonary hypertension was significantly higher than that from healthy controls ($n=19$; 2.0 ppb; 1.2-3.0; $p<0.01$). The treatment by low dose of corticosteroid, under 10 mg/day, did not significantly affect the C_{ANO} in treated SSc patients as compared with untreated patients.

Discussion

In this study, we have found that alveolar concentration of exhaled NO is significantly increased in SSc patients as compared with healthy controls, a result consistent with previous findings using the two compartment-model method partitioning exhaled NO into alveolar concentration and conducting airway flux [8]. The observation that exhaled NO is increased in SSc patients is also consistent with previous studies measuring mixed NO concentration in the exhaled air of scleroderma patients with (or without) interstitial lung disease [13, 14]. We have also demonstrated that C_{ANO} is inversely related to lung volumes, and directly related to the extent of ILD, as assessed by the ground glass score and the reticular score on pulmonary CT scan in patients with SSc. Moreover, we have found that the levels of C_{ANO} are different among SSc patients, being significantly higher in patients with ILD as compared with patients without ILD. The low r -values, however, suggest that increased C_{ANO} only accounts for part of the mechanisms causing lung impairment in SSc patients. The question as to whether how the relative importance of NO varies amongst patients and during the time course of the disease still remains to be addressed.

The inverse correlation between C_{ANO} and DLCO may reflect greater oxidative stress and/or more severe inflammation causing an increase in alveolar production of NO that results in impaired pulmonary gas transfer. Conversely, reduced gas diffusion (as reflected by low DLCO) may in turn account for the accumulation of NO, hence its high concentration in the alveolar compartment. However, DLCO/VA values did not significantly differ between these two groups of patients (with vs without ILD). It is unlikely that diffusion abnormalities could account for increased C_{ANO} .

No relationship was found between C_{ANO} and systolic pulmonary arterial pressure. Our results are consistent with those from previous study showing increased exhaled NO in a limited number of SSc patients with pulmonary arterial hypertension [8]. By contrast, measuring F_{ENO} in mixed expired air, Kharitonov *et al.* have found decreased exhaled NO in SSc patients with pulmonary hypertension [15]. It is, however, difficult to compare these results with data from our study due to different methodological approaches to measure exhaled NO.

Many factors might account for increased pulmonary vascular tone, including overproduction of vasoconstrictors and growth factors [16], inflammatory cytokines [17], or reduced synthesis of pulmonary vasodilators [17, 18]. Our results are consistent with the hypothesis suggesting that pulmonary hypertension in SSc is due, at least in part, to inflammatory processes, rather than reduced endothelial NOS activity, as seen in idiopathic pulmonary hypertension [19].

Pulmonary inflammation and lung damage leading to subsequent fibrosis can be assessed by measuring exhaled NO. We showed that C_{ANO} was correlated positively with ground glass attenuation score and with reticular score, further supporting the association between severity of ILD and increased alveolar production of NO.

Together with the increase in C_{ANO} , we have found that J'_{awNO} values are significantly lower in SSc patients as compared with those from control subjects. Such difference has not yet been described. We hypothesize that the low J'_{awNO} could reflect reduced NO production in the airways of SSc patients as a result from the inhibitory effects that high alveolar concentration of NO might exert on bronchial epithelial NO

synthase activity [20]. Alternatively, as C_{ANO} and J'_{awNO} are derived from the slope and intercept of the linear relationship between V'_E and V'_{NO} [6, 7], it is conceivable that the higher the slope, the lower the intercept, based on the two compartment-model of pulmonary nitric oxide exchange dynamics [6, 7].

The limitation of our results relates to the fact that alveolitis was not evidenced by another method, such as bronchoscopy and broncho-alveolar cells count. These investigations are invasive. Moreover, they could yield false negative results, for example with BAL cells counts, particularly when alveolitis involves the lower lung lobe [21].

We conclude that alveolar NO production is significantly increased in patients with SSc, probably as a result of a lung fibrosis development. We submit that C_{ANO} could be used as a surrogate marker for scleroderma lung disease. Furthermore, increased C_{ANO} may reflect the extent of lung restriction. Our data highlight the potential interest of this none-invasive method in the diagnosis and the monitoring of ILD in patients with SSc.

(Words count: 2161)

ACKNOWLEDGEMENTS

The authors would like to thank the staff of technicians and nurses of the 'Service de Physiologie-Explorations Fonctionnelles', Cochin Hospital, for their technical help. This study was supported by the 'Association des Sclérodermiques de France'.

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Table 1: Alveolar concentration and conducting airway flux of nitric oxide from the systemic sclerosis patients and healthy volunteers

	SSc (n=58)	Ctrl (n=19)	<i>p</i>
Age, yrs	53.5 (45 - 60)	42.7 (28.1- 50.4)	0.01
Smoker	10	0	
Duration of disease, yrs	5.5 (3.0 - 10.4)		
Modified Rodnan skin score	8 (4 -13)		
$J'aw_{NO}$, nl/min	6.3 (-9.8 - 31.3)	35.5 (25.8-54.9)	<0,001
CA_{NO} , ppb	6,2 (3.8 - 9.9)	2.0 (1.2-3.0)	<0.001
R^2	0,95 (0.92 - 0.98)	0.96 (0.95-0.98)	NS
FE_{NO50} , ppb	10.1 (5.9-17.8)	13.1 (11.6-19.6)	NS
TLC, % pred	92 (77 - 105)		
FVC, % pred	90 (75 - 104)		
FEV_1 , % pred	88 (74 - 101)		
PaO_2 , mmHg	85 (80 - 98)		
DLCO*, % pred	61 (46 - 71)		
DLCO/VA*, % pred	74 (64 – 85)		
sPAP, mmHg	31 (26 – 36)		
sPAP \geq 40 mmHg	8		

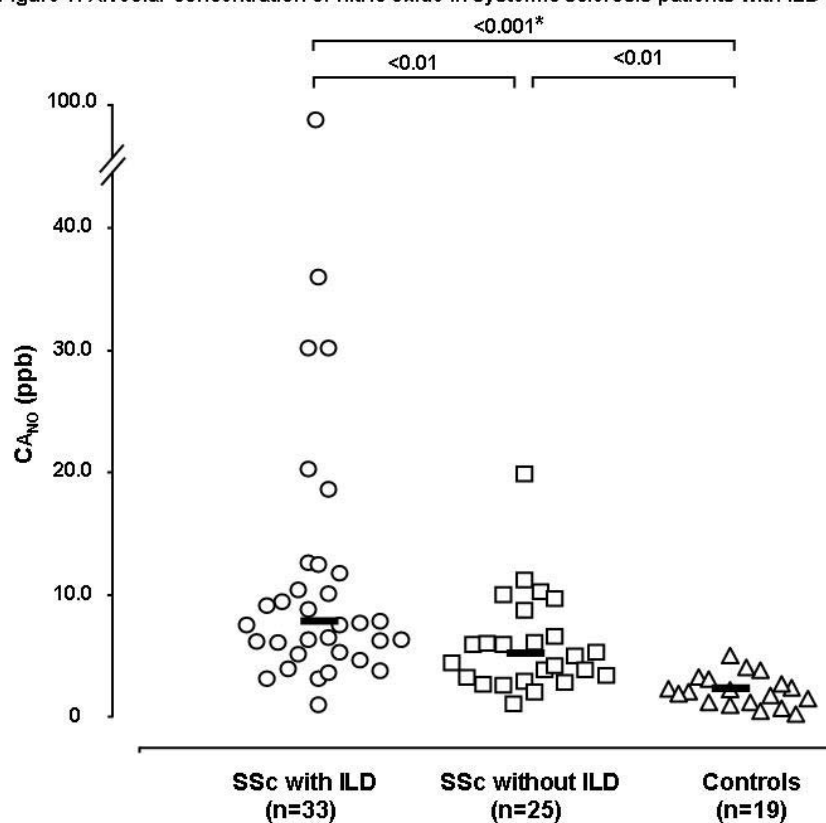
SSc: systemic sclerosis, Ctrl: controls (healthy volunteers), yrs: years, NO: nitric oxide, $J'aw_{NO}$: conducting airway flux of NO, CA_{NO} : alveolar concentration of NO, *p* value was significant if <0.05, NS: the difference was not statistically significant, R^2 : relationship between V'_{NO} and V'_{exh} , FE_{NO} : fractional exhaled NO concentration at 50 ml/s constant flow rate, % pred: % of predicted value, TLC: total lung capacity, FVC: forced vital capacity, FEV_1 : forced expiratory volume in one second, PaO_2 : arterial oxygen tension, DLCO: transfer lung carbon monoxide factor, VA: volume alveolar, sPAP: Systolic pulmonary artery pressure all data were presented as medians and 1st and 3rd quartile, * Maxima of each score is 25.

Table 2: Exhaled nitric oxide levels from systemic sclerosis patients with and without interstitial lung disease

	SSc (n=58)		<i>p</i>
	SSc with ILD (n=33)	SSc without ILD (n=25)	
Age, yrs	55.8 (47.3 – 60.8)	52.4 (44.7 - 59.0)	NS
Smoker	4	6	NS
$J'aw_{NO}$, nl/min	4.1 (-18.9 -13.6)	18.0 (-0.2 - 39.6)	<0.05
CA_{NO} , ppb	7.5 (5.2 -11.9)	4.9 (3.1 - 7.0)	<0.01
R^2	0.96 (0.92 - 0.98)	0.95 (0.93 – 0.98)	NS
FE_{NO50} , ppb	9.3 (5.8-17.1)	11.1 (6.1-19.0)	NS
TLC,% pred	82 (68 – 95)	105 (90 - 107)	<0.001
FVC, % pred	81 (70 – 97)	97 (83 - 115)	<0.01
FEV_1 , % pred	83 (74 – 100)	92 (83 - 105)	NS
PaO_2 ,mmHg	84 (79 – 98)	86 (82 - 97)	NS
DLCO,% pred	53 (42 – 66)	65 (59 - 82)	<0.01
DLCO/VA ,% pred	73 (58 – 82.7)	76 (71 - 85)	NS
Ground glass score*	8 (6-12)	0 (0 - 0)	<0.001
Reticular score*	7 (4 - 9)	0 (0 - 0)	< 0.001

SSc: systemic sclerosis, ILD: interstitial lung disease. NO: nitric oxide, yrs: years, $J'aw_{NO}$: conducting airway flux of NO, CA_{NO} : alveolar concentration of NO, R^2 : relationship between V'_{NO} and V'_{exh} , FE_{NO} : fractional exhaled NO concentration at 50 ml/s constant flow rate, *p* value was significant if <0.05, NS: the difference was not statistically significant, FEV_1 : forced expiratory volume in one second, FVC: forced vital capacity, PaO_2 : arterial oxygen tension, TLC: total lung capacity, DLCO: transfer lung carbon monoxide factor, VA: volume alveolar, all data were presented as medians and 1st and 3rd quartile, * Maxima of each score is 25.

Figure 1: Alveolar concentration of nitric oxide in systemic sclerosis patients with ILD or without ILD.



SSc: systemic sclerosis, ILD: interstitial lung disease, NO: nitric oxide, CA_{NO} : alveolar concentration of nitric oxide, * Comparisons between the 3 groups by Kruskal-Wallis test.