

Rationale underlying the measurement of fractional exhaled nitric oxide in systemic sclerosis patients

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ABSTRACT

Systemic sclerosis (SSc) is an autoimmune disease characterised by tissue fibrosis leading to vascular injury. Nitric oxide (NO) has been implicated in the pathogenesis of autoimmune diseases. A deficiency in basal NO production by the constitutive endothelial isoform of nitric oxide synthase may promote vasoconstriction and vascular wall thickening.

In January 2017, we searched the PubMed/Medline, Cochrane Library and Embase/Medline databases for studies analysing physio-pathological correlations with lung fractional exhaled NO (FeNO) production. This review describes the rationale underlying possible applications of FeNO measurements in the management of SSc.

Measuring NO levels at multiple expiratory flow rates makes it possible to distinguish airway NO production and distal airway/alveolar NO concentration (ANOC), and there is increasing evidence indicating that it may be useful in many non-respiratory conditions. FeNO levels are increased in SSc patients with fibrosing lung disease, whereas those with pulmonary hypertension have relatively low FeNO levels, thus suggesting that NO plays an important role in regulating pulmonary vascular resistance in SSc. However, a number of studies have shown increased ANOC in SSc patients without increased FeNO levels. The relationship between lung diffusing capacity for carbon monoxide and ANOC may be related to increased alveolar membrane thickness impeding NO diffusion or alveolar inflammation in SSc lung disease.

The findings concerning the usefulness of FeNO measurements in SSc patients are discordant, but the available papers suggest that ANOC is a more accurate

indicator of progressive lung dysfunction and an increase in ANOC could assess the extent of interstitial lung disease non-invasively.

Introduction

Systemic sclerosis (SSc) is characterised by cellular and humoral autoimmunity, tissue and skin fibrosis, vascular injury, and heart, lung, kidney and gastrointestinal tract involvement. Functional and structural vascular injury frequently appear years before the other manifestations, whereas the fibrosis is due to an excessive accumulation of collagen and components of the extra-cellular matrix. In the recent times it has become increasingly evident as genetic factors and inflammatory trigger may also play a pathogenetic role by affecting host susceptibility or modifying the clinical presentation of the disease and organ damage (1).

Patients with SSc often report dyspnea upon exertion, fatigue and reduced exercise tolerance, which are frequently due to the involvement of the musculoskeletal system, lungs, heart, chest wall, and pulmonary vasculature. They are also at particular risk of developing pulmonary arterial hypertension (PAH) or interstitial lung disease (ILD) with pulmonary hypertension (PH), which may lead to right ventricular failure and early death (2). ILD and PH are leading causes of disease-related morbidity and mortality (3), and now account for respectively 33% and 28% of deaths (3); the proportion of deaths due to ILD and PAH has increased, whereas deaths due to renal crises have significantly decreased (from 42% to 6%) (3). A European Scleroderma Trials and Research (EUSTAR) group analysis of a cohort of 3,656 SSc patients found the presence of ILD in 53% of the cases

with diffuse cutaneous SSc and 35% of those with limited cutaneous SSc (4). The cumulative post-diagnosis survival rate of SSc patients is 84.1% after five years and 74.9% after ten years (5) and, although the reported 5-year survival rate of SSc-ILD patients is similar to that of patients without ILD, the 10-year rate is significantly lower (29-69%) (6).

Autopsy studies have found that up to 100% of patients show parenchymal involvement (5); high-resolution computed tomography has shown that as many as 90% show interstitial abnormalities (7); and the results of lung function tests are altered in 40-75% (8). The pathogenesis of SSc is unclear but it is thought that immune activation and vascular endothelial cell injury are central features (9). Interstitial and alveolar inflammation is well documented in patients with SSc-ILD, which is pathologically similar to idiopathic pulmonary fibrosis (IPF) (10), and inflammatory mechanisms may also play a role in the obliterative vascular wall cell proliferation of PH associated with SSc, which resembles that seen in primary pulmonary hypertension (PPH) (11). Immuno-inflammatory processes are accompanied by cytokine-mediated up-regulation of the inducible isoform of nitric oxide synthase (iNOS), which leads to the production of large quantities of nitric oxide (NO) (12) that may have strong pro-inflammatory and cytotoxic properties (13). NO has been implicated in the pathogenesis

of autoimmune diseases (14) including SSc (15-18), as well as in that of IPF (19), whereas a deficiency in basal NO production by the constitutive endothelial isoform of NOS (eNOS) may promote vasoconstriction and vascular wall thickening, and be involved in the pathogenesis of PPH (20).

Measuring NO in exhaled air has been increasingly used to assess the potential role of endogenous lung NO in different diseases (21), and the growing availability of NO analysers and official guidelines has made the measurement of fractional exhaled NO (FeNO) a useful means of managing various pulmonary and extra-pulmonary diseases (Table I).

In January 2017, we searched the PubMed/Medline, Cochrane library and Embase/Medline databases for studies analysing the physio-pathological correlations of lung FeNO production, and we here describe the rationale underlying FeNO measurements and their possible applications in the management of SSc.

Exhaled nitric oxide measurements

Upon different stimuli, all of the iso-enzymes of NOS convert L-arginine to L-citrulline and generate NO (22). Constitutive NOS include neuronal NOS (NOS1 or nNOS), endothelial NOS (NOS3 or eNOS) and mitochondrial NOS (mtNOS) (22), whereas inducible NOS (NOS2 or iNOS) is only expressed in response to inflammatory and infectious triggers and produces

large quantities of NO regardless of calcium ion influx (23). Pulmonary NO is mainly produced by the airway epithelium and the alveoli, and its transfer to the bronchial lumen is driven by a concentration gradient, thus making FeNO a flow-dependent measure (24). The gold standard for assessing FeNO levels is the on-line method, which involves the continuous sampling of expiration by a NO analyser, and the real-time display of the NO profile versus time or exhaled volume, together with airway flow rate and/or pressure (24). Patients are asked to use a mouthpiece to inhale NO sampled air (pre-assessed in order to calibrate the analyser) up to total lung capacity, and then to exhale without holding their breath, which would otherwise increase the NO diffusion time from the airway wall to air. For the same reason, low exhalation flow rates increase FeNO values and high rates decrease them. It is common practice to display the pressure or expiratory flow rate to the subject undergoing the test, who is asked to maintain a positive mouthpiece pressure of 5-20 cmH₂O in order to avoid the nasal backflow of NO (thus avoiding the need for a nose clip). A correct exhalation should last for >6 seconds (an exhaled volume of at least 0.3 L at 50 mL/sec) and provides a single-breath NO profile (exhaled NO vs. time) that consists of a washout phase followed by a plateau of at least three seconds. Repeated and reproducible exhalations should be made in order to obtain at

Table I. Respiratory and non-respiratory conditions in which FeNO measurements may play a management role.

Increased FeNO levels	Variable changes in FeNO levels	Decreased FeNO levels
Asthma	Bronchiectasis	Cystic fibrosis
Late asthmatic response	Chronic obstructive pulmonary disease	Primary ciliary dyskinesia
Allergic rhinitis	Fibrosing alveolitis	Pulmonary hypertension
Viral infections	Sarcoidosis	HIV infection
Hepato-pulmonary syndrome	Systemic sclerosis	Acute respiratory distress syndrome
Liver cirrhosis		
Acute/chronic rejection of lung transplant		
Bronchiolitis obliterans		
Hodgkin's lymphoma		
Sleep apnea syndrome		
Crohn's disease and other Inflammatory bowel diseases		
Obesity		

Table II. Factors that may interfere with FeNO measurements.

FACTORS INCREASING FeNO LEVELS	FACTORS DECREASING FeNO LEVELS
Drugs: <ul style="list-style-type: none"> • Angiotensin-converting-enzyme inhibitors • Papaverine • Sodium nitroprusside 	Drug intake: <ul style="list-style-type: none"> • Oxymetazoline • NOS inhibitors
Food and beverages: <ul style="list-style-type: none"> • Nitrite/nitrate-enriched food • Arginine ingestion 	Food and beverages: <ul style="list-style-type: none"> • Curcumin and resveratrol
Environmental factors: <ul style="list-style-type: none"> • NO in air • Calibration of FeNO analyser • Occupational exposure to ozone, chlorine dioxide, formaldehyde 	Environmental factors: <ul style="list-style-type: none"> • Calibration of FeNO analyser
Physiological and pathological conditions and tests: <ul style="list-style-type: none"> • Airway infections • Systemic inflammatory conditions • Chronic cough 	Physiological and pathological conditions and tests: <ul style="list-style-type: none"> • Tobacco use • Alcohol ingestion • Chronic hypoxia • Spirometry • Sputum induction • Hypothermia • Hyperventilation • Physical exercise • Menstrual cycle • Childhood • Female gender

Sickle cell anaemia, circadian rhythms and pregnancy are currently under consideration.
FeNO: fraction exhaled nitric oxide; NO: nitric oxide; NOS nitric oxide synthase.

least two values within 10% of each other, and exhaled NO is then calculated as the mean of the two values. At least 30 seconds of tidal breathing off-circuit should elapse between any two manoeuvres (24).

From a practical standpoint, the FeNO value obtained at a flow rate of 50 mL/sec is a reliable surrogate of the production of NO in the respiratory airways, but there is no simple surrogate for determining the distal airway/alveolar NO concentration (ANOC). In order to distinguish airway NO production from ANOC, it is necessary to measure FeNO at 50 mL/sec, followed by multiple (usually three) flow rates ranging from 100 mL/sec to 400 mL/sec (24). There are many physiological and pathological conditions that can interfere with FeNO measurements (Table II). According to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines, non-disease related factors influencing FeNO values are a very young age (children) or female sex (during menses or pregnancy), respiratory manoeuvres (performing spirometry reduces FeNO levels),

airway calibre, some foods and beverages, smoking (reduces FeNO values chronically and acutely), infections, hypoxia and exercise (both reduce FeNO levels), and medications directly related to NO synthesis or airway calibre (Table II) (24).

Exhaled nitric oxide as a marker of inflammatory disease

FeNO in pulmonary disease

Athopic asthma is characterised by an inflammatory infiltrate in the airways with predominance of mast cells and eosinophils and FeNO measurement are highly correlated with eosinophilic airway inflammation (25).

It is 25 years since it was first reported that FeNO levels are increased in bronchial asthma (26); FeNO can provide added value in the early diagnosis of active pulmonary inflammatory processes (27, 28). It has already been clinically accepted as an indirect means of monitoring airway inflammation and the response to inhaled corticosteroids, and thus facilitates the management of asthma and the prevention of recurrences (29).

There is contradictory evidence regarding the exact function of NO in others lungs disease; in some NO is a pro-inflammatory mediator with immunomodulatory effects (30). This appears to predispose an airway hyper-responsiveness (30-31); but on the other hand, under physiological condition NO acts as a weak mediator of smooth muscle relaxation and protects against the airway hyperresponsiveness (32).

A reduction in FeNO levels in smokers was first observed by Schilling *et al.* (33); this effect was found after both acute and chronic smoking exposure (34). Passive smoking has also been found to reduce levels of FeNO in healthy subjects (35). Smoking cessation is accompanied by an increase in FeNO levels (36). The possible mechanism by which FeNO levels are a potential negative feed-back mechanism of the NO from the cigarette smoke, which could lead to down regulation iNOS in the lung (37-38), an inadequate supply of cofactors necessary for NO production, such as tetrahydrobiopterin (39), and an increase in the breakdown of NO (40-41).

FeNO levels are inconsistent in chronic obstructive pulmonary disease (COPD) patients, this may be due to the confounding effect of smoking or it may reflect the heterogeneity of underlying airway inflammation (25). If the FeNO diagnostic label in COPD patients is not accurate, the response to anti-inflammatory treatment can be predicted with FeNO (25).

Respiratory infections of presumed viral origin were early recognised to be associated with an increased in FeNO levels (42). This increase in FeNO is primarily related to rhinovirus infections (43), whereas both infections with respiratory syncytial virus (44) and influenza virus (45) seems to be associated with reductions rather than increases in FeNO levels. The increase of FeNO levels in rhinovirus infections may involve up-regulation of interferons causing increased iNOS expression in the bronchial epithelium for the activation of signal activator of transcription (STAT)-1 (42).

Bacterial pneumonias are not generally related to increased FeNO levels (46)

and neither is the exposure to endotoxin and other bacterial components (47).

FeNO in non pulmonary disease

It has also been suggested that FeNO can act as a biomarker of a number of non-pulmonary diseases (Table I). There are various theories as to why FeNO levels are increased in such cases, including leukocyte priming and subsequent homing in embryonally related tissues (*e.g.* between the lung and bowel) (48, 49) and the blood transport of pro-inflammatory cytokine spill-over from injured tissue to the lung (50), and an increasing body of evidence indicates the usefulness of measuring FeNO levels in everyday clinical practice. Decreased FeNO levels may suggest a failure in compensatory circulatory mechanisms such as that found in cases of congestive heart failure (CHF) (51) and pulmonary hypertension (PH), and a low ANOC is considered a marker of hypoxia and endothelial dysfunction as hypoxia induces vasoconstriction (23), and the same is true in the case of obstructive sleep apnea syndrome (OSAS) (52), in which low ANOC is probably partially due to hypoxia, even though in these patients overall FeNO values are increased, not decreased, probably because the majority is produced by the upper airways inflammation (53). Conversely, increased FeNO levels might reflect a hyperdynamic circulatory syndrome as in the case of hepato-pulmonary syndrome (54), and/or be the consequence of a high systemic level of inflammatory cytokines as in Hodgkin's lymphoma (55), Crohn's disease and other inflammatory bowel disease (IBDs) (48, 49) (Fig. 1). Metabolism disorders such as obesity that lead to a pro-inflammatory condition may also increase FeNO, as is demonstrated by the positive correlation between the body mass index and FeNO levels (56).

FeNO in other rheumatology disease

Elevated levels of FeNO in patients with Sjögren's syndrome were demonstrated (57). The elevated FeNO levels in these patients may derive from epithelium, or from macrophages activated by cytokines released from lymphocytes. The third possibility is that NO is

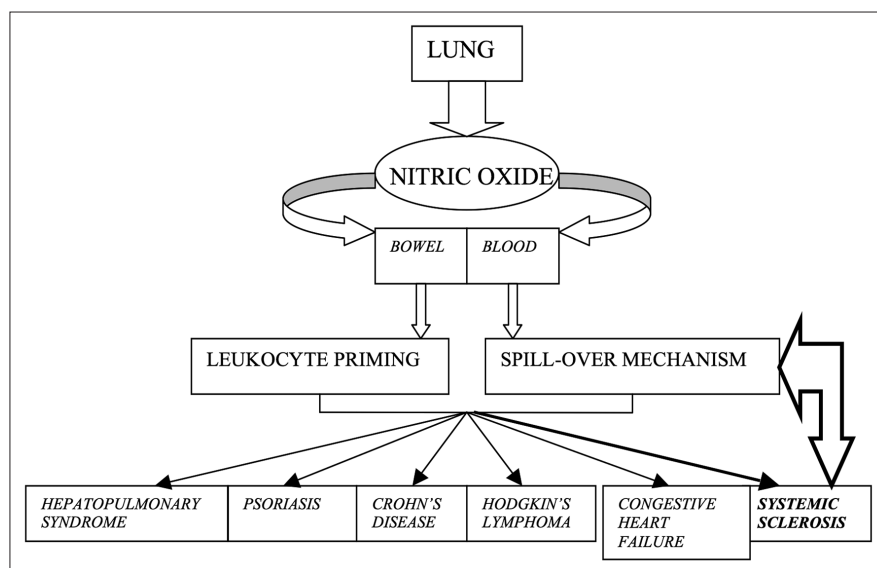


Fig. 1. Schematic representation of FeNO as a biomarker of some non-pulmonary diseases.

produced by inflammatory cells in the epithelium.

In rheumatoid arthritis ANOC and tissue concentration of NO in the airway wall were lower compared with the healthy subjects (58). A possible explanation for this phenomenon is an increased oxidative stress. Oxidative stress is believed to play an important role in the pathogenesis of autoimmunity by enhancing inflammation and affecting the immunological tolerance (59). Increased oxidative stress in rheumatoid arthritis has been found by measuring oxidative stress related molecules in biological fluid (60) and is known that the oxidative stress reduce NO with the formation of potent oxidising reactive nitrogen species (61), and increased metabolites of NO have detected in the blood and synovial fluid of rheumatoid arthritis patients (62).

FeNO in patients with systemic lupus erythematosus (SLE) is significantly increased and correlated with the disease activity (63-64). NO increase may depend on respiratory tract inflammation, or on circulating cytokines produced elsewhere (65). Increased expression of iNOS in response to inflammatory stimuli present in SLE may lead to increased tissue damage, altered enzyme activity, and increased expression of neoepitopes in self antigens.

There is compelling evidence that pharmacologic inhibition of iNOS leads to reduce disease activity and damage in

murine models of lupus. In humans, observational studies, suggest that overexpression of iNOS led to glomerular and vascular pathology (65).

SSc and nitric oxide production

The microvascular bed of SSc patients is the target of an immuno-inflammatory injury that dysregulates vascular tone control and leads to the progressive disorganisation of vascular architecture and subsequent vascular obliteration, thus reducing blood flow to the organs involved (66); furthermore, at cellular level, endothelial dysfunction is characterised by inflammatory and vasospastic potential (67). This dysfunction is reflected in the clinical sign of Raynaud's phenomenon, which may be due to one or more of a variety of proposed pathological mechanisms (68). The endothelial hypothesis suggests that it may be caused a reduction in the production of endothelium-derived vasodilatory mediators (prostacyclin, NO) and an increase in endothelial vasoconstrictive signals (endothelin) (69-71) but, although it is unanimously reported that endothelin levels are high, there is confusion concerning the exact status of NO production in SSc because both increased and decreased circulating total nitrate levels have been observed (Table III) (15, 16, 71-73).

Early studies indicated that FeNO levels are increased in SSc patients (74),

Table III. Summary of trends in FeNO and ANOC measurements among SSc patients vs. controls in the papers considered.

Author	FeNO vs. controls	ANOC vs. controls
Fajac <i>et al.</i> (47)	↑ SSc ↓ SSc PAH	-
Paredi <i>et al.</i> (48)	↑ SSc ILD	-
Rolla <i>et al.</i> (49)	↑ SSc ↑ SSc ILD ↓ SSc PAH	-
Girgis <i>et al.</i> (45)	↓ SSc	↑ SSc ↓ SSc PAH
Malinovschi <i>et al.</i> (28)	↓ SSc ↓ SSc PAH	↑ SSc ↑ SSc PAH
Guillan-del Castillo <i>et al.</i> (53)	↓ SSc ILD	-
Tiev, Hua-Huy, Kettaneh <i>et al.</i> (61)	↑ SSc ↓ SSc ILD	↑ SSc
Tiev, Hua-Huy, Riviere <i>et al.</i> (60)	-	↑ SSc ↑ SSc-ILD
Tiev, Cabane <i>et al.</i> (56)	↓ SSc (non-significant)	↑ SSc ↑ ↑ SSc-ILD
Tiev, Coste <i>et al.</i> (57)	-	↑ SSc ↑ ↑ SSc-ILD
Kozij <i>et al.</i> (54)	↓ SSc ILD	↑ ↑ SSc-PAH
Tiev, Le-Dong <i>et al.</i> (59)	↓ SSc (non-significant)	↑ SSc ↑ ↑ SSc-ILD ↑ ↑ SSc-PAH
Moodley Juben <i>et al.</i> (50)	↑ SSc = SSc ILD	-
Malerba <i>et al.</i> (51)	↑ SSc = SSc ILD = SSc PAH ↓ SSc ILD + PAH	-
Kharitonov <i>et al.</i> (52)	↓ SSc = SSc PAH	-
Wuttgeet <i>et al.</i> (58)	-	↑ SSc ↑ SSc ILD

FeNO: fraction exhaled nitric oxide; ANOC: airway/alveolar nitric oxide concentration; SSc: systemic sclerosis; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease.

particularly in those with fibrosing lung disease (75, 76), but later studies found lower FeNO levels in SSc patients with ILD than in those without: Moodley *et al.* (77) did not report any differences between SSc patients with ILD and healthy subjects, whereas Malerba *et al.* (78) found that SSc patients without ILD had higher FeNO levels than healthy subjects.

Patients with SSc and PAH have relatively low FeNO levels, which suggests that NO plays an important role in regulating pulmonary vascular resistance in SSc (76, 79). Some of the earlier studies indicating increased FeNO

levels used higher exhalation flow rates and therefore probably sampled the peripheral airways to a larger extent (76, 77), whereas others showing that SSc patients with lower FeNO levels have worse outcomes during follow-up, which may indicate the presence of aggressive ILD (80). The feasibility of measuring FeNO in patients with SSc lung disease and its discriminative ability have recently been demonstrated, with the authors concluding that FeNO is a potential biomarker distinguishing SSc patients with pulmonary arterial hypertension and those with interstitial lung disease (81).

A number of studies have found increased ANOC in SSc patients with no detectable increase in FeNO levels (82-85). ANOC specifically increases in patients with early SSc and reflects early inflammatory lung involvement (85). Other studies have found a higher ANOC in SSc patients than in healthy controls (74, 83-86) that is related to the extent of ILD; it is a more accurate marker than serum NO levels (83) or alveolitis (87), and has been shown to be a valuable means of predicting the ILD (84) and pulmonary deterioration. High ANOC levels have been associated with declining lung function or death, with a specificity of 90% (88).

The negative relationship between pulmonary carbon monoxide diffusion capacity (DLCO) and ANOC may be related to increased thickness of alveolar membrane impeding NO diffusion (DLNO) or alveolar inflammation in SSc lung disease. Girgis *et al.* found that the DLNO/DLCO ratio of 4.3 observed in patients with chronic obstructive pulmonary disease (COPD) was applicable to SSc patients, and showed that ANOC in SSc patients was significantly increased in comparison with healthy controls (83). However, the DLNO/DLCO ratio recently reported in SSc patients with ILD (89) was slightly higher than that in patients with COPD, thus suggesting that the increased NO concentration in the alveolar space was unlikely to be due to NO diffusion across the alveolar membrane. On the other hand, it has been reported that increased FeNO is related to alveolitis as documented by bronchoalveolar lavage (BAL) cell counts (75). Taken together, these data are consistent with the hypothesis that alveolar inflammation is probably the main factor increasing ANOC in SSc patients. Moreover ANOC is directly related to the presence of ILD on chest HRCT scans (84), probably because of still unknown biological mechanisms linking active alveolitis to cell proliferation and lung fibrosis in patients with SSc (90).

Conclusions

As we have seen, FeNO is a parameter subject to multiple interferences, as it

is conditioned by both voluptuous habits and acute and chronic diseases.

For example, both active and passive cigarette smoking can reduce it while obesity increases it. Acute virosis of upper respiratory tract may increase FeNO, while its behaviour in chronic pathologies is not unequivocal. FeNO assessment in COPD is contradictory; CHF and PH are characterised by low FeNO, the latter also reducing ANOC; FeNO is increased in Hodgkin's lymphoma, Crohn's disease and other IBDs, while in OSA FeNO is increased and ANOC is decreased.

FeNO can easily fluctuate over time in patients with SSC because of changes in their voluptuous habits, chronic comorbidity and viral infections of upper and lower airways, especially in patients treated with immunosuppressants.

It is therefore currently impossible to indicate FeNO "tout court" as a reliable marker of the evolution of the SSC.

On the other hand, ANOC seems to be a more accurate marker of progressive lung dysfunction and could be used for non-invasive assessment of the extent of ILD in conditions such as SSC, given the direct correlation between ANOC and ILD valued through chest HRCT and the negative correlation between ANOC and DLCO.

In conclusion, using ANOC as a marker of ILD in the follow-up of SSC patients should be encouraged because it is certainly less invasive than HRCT and easier to perform than DLCO, as the latter may be unreliable in patients with poor lung volumes, such as many patients affected by both SSC and ILD.

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