MAJOR ARTICLE

Impaired Pulmonary Nitric Oxide Bioavailability in Pulmonary Tuberculosis: Association With Disease Severity and Delayed Mycobacterial Clearance With Treatment

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Background. Nitric oxide (NO), a key macrophage antimycobacterial mediator that ameliorates immunopathology, is measurable in exhaled breath in individuals with pulmonary tuberculosis. We investigated relationships between fractional exhale NO (FE_{NO}) and initial pulmonary tuberculosis severity, change during treatment, and relationship with conversion of sputum culture to negative at 2 months.

Methods. In Papua, we measured FE_{NO} in patients with pulmonary tuberculosis at baseline and serially over 6 months and once in healthy controls. Treatment outcomes were conversion of sputum culture results at 2 months and time to conversion of sputum microscopy results.

Results. Among 200 patients with pulmonary tuberculosis and 88 controls, FE_{NO} was lower for patients with pulmonary tuberculosis at diagnosis (geometric mean FE_{NO}, 12.7 parts per billion [ppb]; 95% confidence interval [CI], 11.6–13.8) than for controls (geometric mean FE_{NO}, 16.6 ppb; 95% CI, 14.2–19.5; $P = .002$), fell further after treatment initiation (nadir at 1 week), and then recovered by 6 months ($P = .03$). Lower FE_{NO} was associated with more-severe tuberculosis disease, with FE_{NO} directly proportional to weight ($P < .001$) and forced vital-capacity ($P = .001$) and inversely proportional to radiological score ($P = .03$). People whose FE_{NO} increased or remained unchanged by 2 months were 2.7-fold more likely to achieve conversion of sputum culture than those whose FE_{NO} decreased (odds ratio, 2.72; 95% CI, 1.05–7.12; $P = .04$).

Conclusions. Among patients with pulmonary tuberculosis, impaired pulmonary NO bioavailability is associated with more-severe disease and delayed mycobacterial clearance. Measures to increase pulmonary NO warrant investigation as adjunctive tuberculosis treatments.

Keywords. tuberculosis; exhaled nitric oxide; L-arginine; M2 macrophages; biomarker.

Investigation of tuberculosis pathophysiology provides the basis for research into tuberculosis adjunctive therapies and biomarkers. These fields are considered to be priority research areas as improved tuberculosis treatments are sought [1, 2]. Nitric oxide (NO) has a fundamental physiological role in innate immunity in tuberculosis. Derived chiefly from the amino acid L-arginine, its production in macrophages expressing

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the gene encoding nitric oxide synthase 2 (*NOS2*) provides a key component of the human immune response to *Mycobacterium tuberculosis*, both in mycobacterial killing and in ameliorating immunopathology (Table 1) [3–12]. *M. tuberculosis*, along with organisms in other bacterial genera, have specific immune-evasion strategies, including NO detoxification systems, to mitigate NO-mediated microorganism toxicity [13, 14], with variable susceptibility to NO [15]. Resistance of *M. tuberculosis* to reactive nitrogen species correlates with virulence in guinea pig models [16] and with resistance to isoniazid [17].

NO is measureable in exhaled breath, using validated chemiluminescence analyzers [21, 23]. Determinants of fractional exhaled NO (FE_{NO}) are shown in the Supplementary Panel. Analysis of breath components provides a noninvasive means of directly sampling the site of pathology in pulmonary tuberculosis. Differences in exhaled breath characteristics among people with tuberculosis, compared with controls, include significantly lower pH [24] and elevated levels of volatile metabolites [25]. Investigations of FE_{NO} in pulmonary tuberculosis to date have been limited by small sample sizes, post hoc analyses, and/or different methods for FE_{NO} measurement and have provided conflicting results, including reports of both increased [21] or decreased [26] FE_{NO} in patients with tuberculosis, compared with controls.

FE_{NO} has potential as a tuberculosis biomarker. Ideal biomarkers should be able to predict clinical and microbiological responses, identify active tuberculosis, and normalize with therapy [2]. Novel biomarkers capable of predicting early treatment response are sought for use as clinical trial outcome measures, because of the impracticality of relying on traditional, later tuberculosis treatment end points [2].

Given the immunological rationale and inconclusive findings to date, we aimed to investigate FE_{NO} in patients with pulmonary tuberculosis and healthy controls in a tropical setting where the tuberculosis burden is high. Our objectives were to determine the accuracy and precision of FE_{NO} measurement in the field, compare FE_{NO} in patients with pulmonary tuberculosis and healthy volunteers, investigate relationships between FE_{NO} and pulmonary tuberculosis severity, and determine the relationship between change in FE_{NO} and microbiological response (ie, sputum culture conversion at 2 months and time to sputum microscopy clearance).

METHODS

This study was performed as part of a clinical trial of adjunctive treatment with L-arginine and vitamin D for tuberculosis in Timika, Papua Province, Indonesia (clinicaltrials.gov identifier NCT00677339). In this study, neither intervention affected FE_{NO} [27]. The Timika population comprises people of Papuan (Melanesian) and non-Papuan (Asian) ethnicity. Eligible study participants had pulmonary tuberculosis, were enrolled at the

Table 1. Evidence for the Role of Nitric Oxide (NO) in Tuberculosis Immunology

Study Type, Results	Selected Reference(s)
M. tuberculosis in vitro studies	
Mycobacteria are susceptible to NO and other reactive nitrogen species in vitro	[16]
M. tuberculosis isolates differ in their susceptibility to reactive nitrogen intermediates: less susceptible isolates are more virulent in guinea pigs, and less susceptible isolates from human cases are less susceptible to antituberculosis drugs	[16, 17]
M. tuberculosis immune-evasion strategies include the induction of arginase expression (thereby decreasing NO availability) in macrophages	[18]
Mouse macrophage studies	
Arginine-derived reactive nitrogen intermediates in mouse macrophages effectively kill *M. tuberculosis*	[4]
M. tuberculosis lacking genetic resistance to reactive nitrogen intermediates cannot grow in mouse macrophages, in contrast with wild-type *M. tuberculosis*	[19]
Human macrophage studies	
Alveolar macrophages from healthy humans infected ex vivo with *M. tuberculosis* produce NO, and NO production correlates with intracellular growth inhibition of *M. tuberculosis*	[10]
Blood mononuclear cells from healthy donors infected ex vivo with *M. tuberculosis* and from people with preexisting tuberculosis produce NO	[7]
Pulmonary macrophages kill mycobacteria only if they express NOS2; killing is prevented with a NOS inhibitor	[9]
In vivo mouse studies	
NOS2 is expressed at sites of disease in immunocompetent mice but is deficient in immunocompromised mice with progressive tuberculosis	[20]
M. tuberculosis infection is poorly contained in mice treated with NOS inhibitors	[4]
Fulminant *M. tuberculosis* infection develops in *NOS2* knockout mice ($NOS2^{-/-}$) in contrast with controls	[20]
NO ameliorates inflammatory tissue damage in pulmonary tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β	[11]
In vivo human studies	
In lung resection studies, NOS2 and nitrotyrosine (a tissue marker of NO metabolism) are expressed in macrophages within granulomata and areas of tuberculosis pneumonitis	[6]
NOS2 expression is increased in peripheral blood monocytes from people with tuberculosis, compared with healthy controls	[8]
NOS2 is expressed in macrophages from lungs of patients with tuberculosis	[21]

Abbreviations: IL-1β, interleukin 1β; *M. tuberculosis, Mycobacterium tuberculosis;* NOS2, nitric oxide synthase.

tuberculosis clinic, were aged ≥15 years, were sputum smear–positive for acid fast bacilli, and provided written informed consent, with demographic details and human immunodeficiency virus (HIV) seroprevalence reported previously [28]. Standard tuberculosis treatment comprised rifampicin, isoniazid, pyrazinamide, and ethambutol for 2 months and then rifampicin and isoniazid for 4 months. Local healthy controls were aged ≥18 years, gave written informed consent, and had no comorbidities. Data (ie, age, sex, ethnicity, weight, and FE_{NO} only) from additional healthy controls from the same location whose results have been reported elsewhere [29] were also made available for this analysis. Enrollments of patients with tuberculosis and controls occurred across all months; environmental conditions in Timika, comprising tropical temperatures (25°C–30°C daily maxima) and high humidity (65%–90%), show minimal seasonality. The study was approved by the Human Research Ethics Committees of Menzies School of Health Research, Darwin, Australia, and the National Institute for Health Research and Development, Jakarta, Indonesia.

Procedures

FE_{NO} was measured using a portable NiOX MINO device (Aerocrine, Sweden). This device is well validated [22] and employs single-use, disposable, filtered mouthpieces without infection control risks. FE_{NO} measurements complied with 2005 American Thoracic Society guidelines [30]. FE_{NO} was measured on 1 occasion in healthy controls and serially, at baseline and 1 week, 2 weeks, 1 month, 2 months, and 6 months after baseline, in patients with tuberculosis. Quality control measures comparing results from biological controls (staff) or foil bags filled with varying NO concentrations were compared on a weekly basis throughout the 20-month study period between the NiOX MINO and a gold standard nonportable NiOX FLEX (not located at the tuberculosis clinic), calibrated fortnightly with 200 parts per billion (ppb) NO.

Chest radiographs, pulmonary function tests, a 6-minute walk test, and a locally adapted St George's Respiratory Questionnaire (SGRQ; in Indonesian) were performed at baseline and at 2 and 6 months. Chest Timika Tuberculosis X-ray were scored according to a previously reported method [31]. Pulmonary function (forced vital capacity [FVC] and forced expiratory volume in 1 minute [FEV_1]) was measured outdoors (for infection control purposes), using a handheld spirometer (MicroLoop), with individual-use, filtered, 1-way mouthpieces (Sure-Gard) suitable for use in smear-positive pulmonary tuberculosis. Percentage of predicted FEV_1 was calculated from local normal reference ranges [32]. A 6-minute walk test was assessed according to standard procedures [33].

Sputum microscopy was undertaken at the Timika laboratory weekly for 2 months and then at months 5 and 6. Baseline and month 2 sputum cultures were processed at the World Health Organization (WHO)–accredited laboratory at University of Indonesia, Jakarta, using the Bactec Mycobacterium Growth Indicator Tube 960 system. Conversion of sputum smear findings was defined as ≥2 consecutive *M. tuberculosis*–negative smears without a subsequent *M. tuberculosis*–positive smear. Conversion of sputum culture to negative at 2 months is a standard early measure of tuberculosis treatment response [2].

Statistical Methods

Analyses were undertaken using Stata, 12.1. Scatterplots and the Pearson correlation coefficient were used to evaluate differences in FE_{NO} within and between analyzers. FE_{NO} data were log normal; geometric means were compared between patients with tuberculosis and healthy controls, using the Student 2-sample *t* test, and between different time points using paired *t* tests. Associations between FE_{NO} and other variables were tested using univariate and multivariate regression models. Regression coefficients were exponentiated and interpreted as geometric mean ratios. Distribution of residuals was checked for normality. The relationship between FE_{NO} and weight was displayed graphically using values predicted from the regression model of FE_{NO} against weight. Incremental changes in FE_{NO} (ΔFE_{NO}) were calculated as [log FE_{NO} at week 8]−[log FE_{NO} at week 0]. The association between ΔFE_{NO} and culture or smear conversion at week 8 was tested using logistic regression, and receiver-operator characteristic (ROC) scores, given as areas under the curve (AUCs), were compared using χ^2 tests.

RESULTS

Two hundred participants with pulmonary tuberculosis and 40 healthy controls were enrolled during June 2008–February 2010, and 48 additional controls were enrolled in 2005 (Figure 1). Patients with pulmonary tuberculosis had lower weight, pulmonary function measures, 6-minute walk test results and worse SGQR scores than controls. Greater percentages of patients with pulmonary tuberculosis were non-Papuans and ex-smokers (Table 2).

FE_{NO} Measurement

The average difference between paired FE_{NO} measurements obtained within 15 minutes by an individual using a single NiOX MINO analyzer/sensor was −0.4 ppb (95% limits of agreement, −6.2 to 5.5) and by an individual using 2 different analyzers/sensors was 0.8 ppb (95% limits of agreement, −4.3 to 5.8; Figure 2*A* and *B*). FE_{NO} values obtained from the NiOX MINO portable analyzers were highly correlated with those from the NiOX FLEX gold standard analyzer (Pearson R, 0.95) but consistently lower (Figure 2*C*). Rather than applying a correction factor, we used raw FE_{NO} results because NiOX MINO devices are now commonly used by ourselves and others in field research [26, 34].

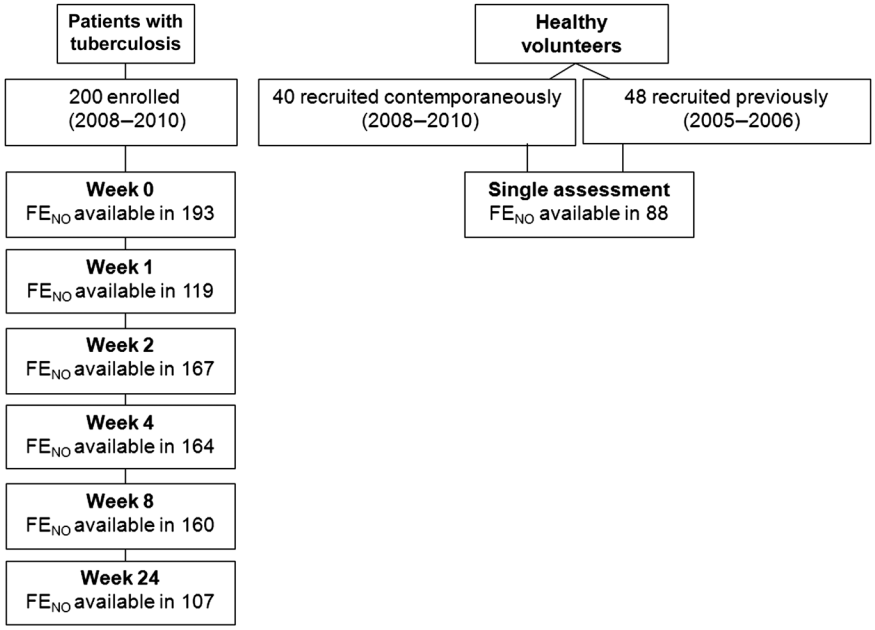

Figure 1. Number of patients with tuberculosis and healthy volunteers enrolled in the study and availability of fractional exhaled nitric oxide (FE_{NO}) data.

Patients With Tuberculosis Versus Healthy Controls

The FE_{NO} was significantly lower in patients with tuberculosis (geometric mean FE_{NO}, 12.7 ppb; 95% confidence interval [CI], 11.6–13.8) than in controls (geometric mean FE_{NO}, 16.6 ppb; 95% CI, 14.2–19.5; $P = .002$; Figure 3). The relationship between weight and FE_{NO} seen in patients with pulmonary tuberculosis at baseline was different from that observed in the controls ($P = .02$ for test of interaction): weight was directly proportional to FE_{NO} in patients with pulmonary tuberculosis, but this finding was not observed among healthy volunteers (Figure 4*A*). The difference in FE_{NO} between patients and controls was unchanged when controlling for covariables or when restricting analyses to only contemporaneously enrolled controls.

Determinants of Baseline FE_{NO}

Associations between clinical measures and FE_{NO} in controls and patients with pulmonary tuberculosis at baseline are shown in Table 3; regression coefficients were calculated for continuous variables according to groups as shown. Among patients with pulmonary tuberculosis, measures signifying greater tuberculosis severity, such as lower weight (or lower body mass index [BMI; calculated as the weight in kilograms divided by the height in meters squared), worse pulmonary function measures, lower hemoglobin level, and higher radiograph score, were each associated with lower FE_{NO}. However, these factors only accounted for a small proportion of the overall variation in FE_{NO} (eg, for each 10-kg weight increment, FE_{NO} increased by a factor of 1.25; Table 3). When controlling for weight (or BMI), the only remaining significant association with FE_{NO}, apart from weight/BMI, was FVC. FE_{NO} was not significantly different between HIV-positive patients with tuberculosis (geometric mean FE_{NO}, 11.2 ppb; 95% CI, 7.8–16.2) and HIV-negative patients with tuberculosis (geometric mean FE_{NO}, 13.3 ppb; 95% CI, 11.8–15.0; $P = .3$), but ascertainment of HIV status was incomplete (Table 2). Among healthy controls, no predictors of FE_{NO} were identified. The difference in FE_{NO} between smokers (geometric mean FE_{NO}, 15.1 ppb; 95% CI, 11.9–19.3) and nonsmokers (geometric mean FE_{NO}, 18.3 ppb; 95% CI, 14.7–22.9) was not statistically significantly different ($P = .2$).

FE_{NO} During Follow-up

After starting tuberculosis treatment, the low baseline FE_{NO} fell further to 10.7 ppb (95% CI, 9.4–12.1), with a nadir at 1 week ($P = .0006$, compared with baseline FE_{NO}), and then gradually recovered by treatment completion, to 15.1 ppb (95% CI, 13.3–17.2; $P = .03$), creating a J-shaped curve (Figure 4*B*). The FE_{NO} at tuberculosis treatment completion was not statistically significantly different from that obtained from healthy controls (16.6 ppb; 95% CI, 14.2–19.5). The longitudinal trend in FE_{NO} among patients with tuberculosis was not observed in the quality control measures involving healthy staff members.

Association With Microbiological Outcomes

The proportion of study participants whose 2-month sputum specimen was culture negative for *M. tuberculosis* was 78.9% (97/123) [27]. Change in FE_{NO} from baseline to 2 months (ΔFE_{NO}) was significantly associated with sputum culture conversion by 2 months. Specifically, people whose FE_{NO} remained

Table 2. Baseline Characteristics of Study Participants

Characteristic	Patients With Tuberculosis (n = 200)	Healthy Controls		P[a]
		Contemporaneous (n = 40)	Previous (n = 40)	
Age, y, median (range)	28 (15–73)	29 (18–65)	27 (18–42)	.2
Papuan	89 (45)	20 (50)	44 (92)	<.0001
Female sex	69 (35)	9 (23)	17 (35)	.4
HIV positive				
Overall	19/145 (13)	. . .	. . .	
Papuan	15/71 (21)	. . .	. . .	
Non-Papuan	4/74 (5)	. . .	. . .	
Weight, kg, mean (range)	48.5 (26.3–74.0)	62.5 (41.6–84.0)	59.8 (45.0–85.0)	<.0001
Body mass index,[b] mean (range)	19.2 (12.0–32.5)	24.3 (17.1–33.6)	. . .	<.0001
Height, m, mean (range)	1.58 (1.35–1.79)	1.61 (1.48–1.73)	. . .	.08
Smoking status				
Current	56 (28)	18 (45)	23 (48)	<.0001
Ex-smoker	53 (27)	1 (3)	3 (6)	
Never smoked	91 (46)	21 (53)	22 (46)	
FE_{NO}, ppb, geometric mean (95% CI)	12.7 (11.6–13.8)	16.2 (12.2–21.5)	17.0 (14.1–20.5)	.002
FVC, L, mean (range)				
Female sex	1.62 (0.63–2.96)	1.91 (1.20–2.99)	. . .	<.0001
Male sex	2.36 (0.84–4.45)	3.37 (1.57–4.22)	. . .	
FEV_1, L, mean (range)				
Female sex	1.43 (0.63–2.47)	1.71 (1.18–2.52)	. . .	<.0001
Male sex	2.03 (0.59–4.00)	3.20 (2.16–5.30)	. . .	
Percentage of predicted FEV_1, mean (range)				
Female sex	62.8 (31.0–99.0)	81.0 (52.6–108.5)	. . .	<.0001
Male sex	63.6 (16.5–108.5)	95.3 (67.8–148.4)	. . .	
SGRQ score, U, median (range)	40.7 (5.2–91.9)	0 (0–9.23)	. . .	<.0001
6-min walk test, m, median (range)				
Female	375 (20–490)	460 (360–528)	. . .	<.0001
Male	425 (40–612)	511 (370–640)	. . .	
Chest radiograph finding				
Cavity size in cm				
0	69 (44)	. . .	. . .	
≤4	53 (34)	. . .	. . .	
>4	35 (22)	. . .	. . .	
Percentage of lung affected, median (IQR)	40 (23–62)	. . .	. . .	
Radiography score, median (IQR)	68 (34–94)	. . .	. . .	
Sputum acid fast bacilli density				
0 or scanty	55 (28)	. . .	. . .	
1+	57 (29)	. . .	. . .	
2+	50 (25)	. . .	. . .	
3+	38 (19)	. . .	. . .	
Sputum culture result at diagnosis				
No growth, contaminated, or no result unavailable	36 (18)	. . .	. . .	
M. tuberculosis identified	164 (82)	. . .	. . .	

Table 2 continued.

Characteristic	Patients With Tuberculosis (n = 200)	Healthy Controls		P^a
		Contemporaneous (n = 40)	Previous (n = 40)	
M. tuberculosis susceptibility, proportion tested (%)[c]				
Fully susceptible	126/149 (85)	. . .	. . .	. . .
Monoresistance or other (not MDR)	23/149 (15)	. . .	. . .	
INH and RIF resistant (MDR)	2 /149 (1)	. . .	. . .	

Data are no. of subjects with the characteristic/no. tested (%) or no. (%) of subjects, unless otherwise indicated.

Abbreviations: CI, confidence interval; FE_{NO}, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 minute; FVC, forced vital capacity; HIV, human immunodeficiency virus; INH, isoniazid; IQR, interquartile range; MDR, multidrug resistant; *M. tuberculosis, Mycobacterium tuberculosis;* ppb, parts per billion; RIF, rifampin; SGRQ, St George's Respiratory Questionnaire.

[a] Patients with tuberculosis versus all available controls.

[b] Calculated as the weight in kilograms divided by the height in meters squared.

[c] Reasons for missing culture results at baseline and the 2-month follow-up visit included specimen contamination, specimen loss during transit, power outage in the laboratory, or participant loss to follow-up <2 months after baseline.

unchanged or increased were approximately twice as likely to have achieved conversion to *M. tuberculosis*–negative sputum cultures by 2 months, compared with individuals whose FE_{NO} decreased (odds ratio, 2.72; 95% CI, 1.05–7.12; $P = .04$). The mean absolute change in 2-month FE_{NO} was +1.6 ppb in those achieving culture negativity and –4.7 ppb in those remaining culture positive ($P = .02$).

The diagnostic usefulness of ΔFE_{NO} compared with sputum smear conversion time for predicting 2-month culture conversion is shown in Supplementary Figure 1. The ROC score for ΔFE_{NO} (AUC, 0.64) was lower than desirable for a reliable diagnostic test and lower than that for sputum smear conversion (AUC, 0.77), but the difference in these AUCs was not statistically significant ($P = .1$, by the χ^2 test).

In addition to the association with culture conversion, people in whom the FE_{NO} stayed the same or increased had a slightly shorter time to sputum smear conversion than those whose FE_{NO} decreased (OR, 1.06; 95% CI, 1.00–1.12; $P = .06$). Changes achieved in FE_{NO} were unrelated to baseline weight ($P = .9$), weight at week 8 ($P = .8$), or change in weight between weeks 0 and 8 ($P = .3$), according to regression analyses. Inclusion of these weight variables in multivariable models had a negligible effect on the relationship between ΔFE_{NO} and culture or microscopy conversion.

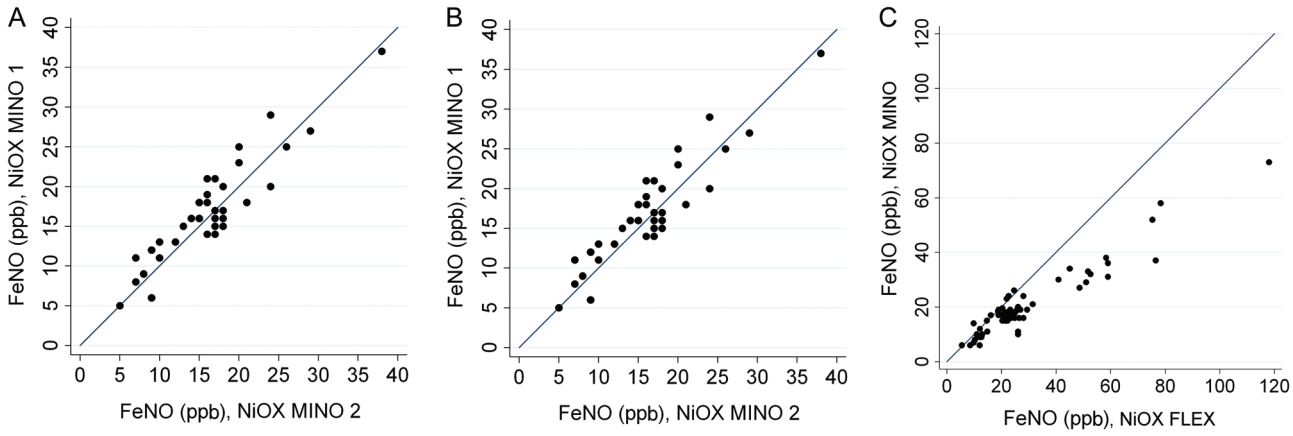

Figure 2. *A*, Repeated fractional exhaled nitric oxide (FE_{NO}) measurements using 1 NiOX MINO analyzer. Dots show individual data points, and the line represents y = x. *B*, Paired FE_{NO} measurements using 2 NiOX MINO analyzers. Dots show individual data points, and the line represents y = x. *C*, Paired FE_{NO} measurements using NiOX MINO (portable analyzer) and NiOX FLEX (gold standard analyzer). Dots show individual data points, and the line represents y = x. Abbreviation: ppb, parts per billion.

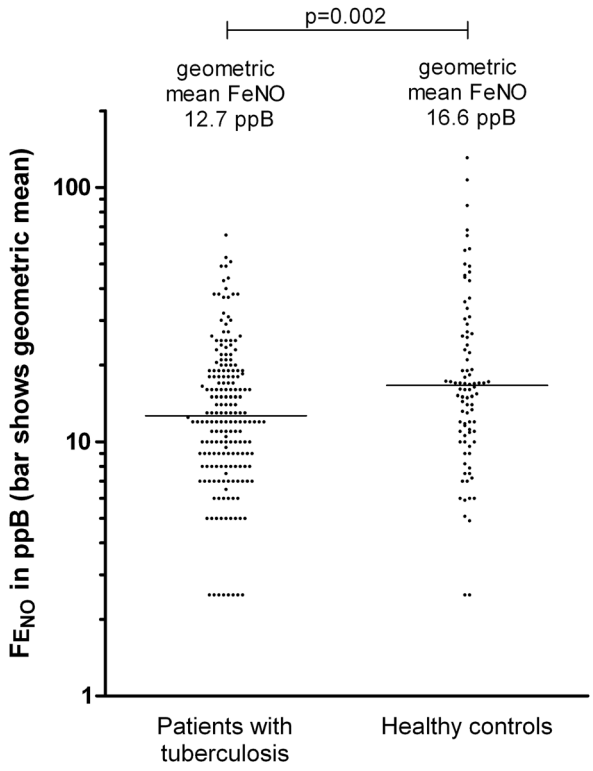

Figure 3. Fractional exhaled nitric oxide (FE_{NO}) in healthy controls and patients with pulmonary tuberculosis at diagnosis. Horizontal lines indicate geometric mean values. Abbreviation: ppb, parts per billion.

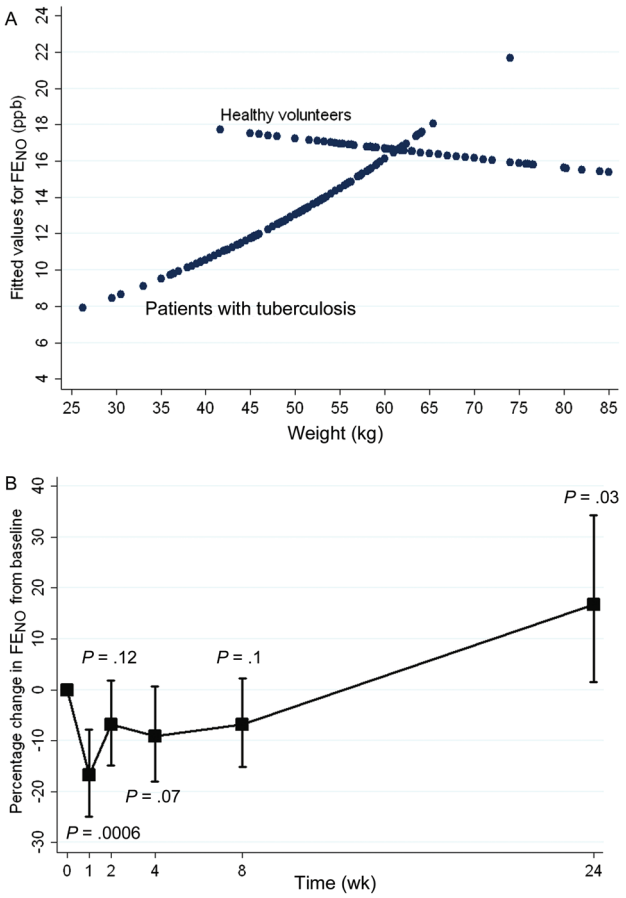

Figure 4. *A*, Fitted values for fractional exhaled nitric oxide (FE_{NO}), by weight, in patients with pulmonary tuberculosis and healthy controls. *B*, Change in FE_{NO} between diagnosis and treatment completion in patients with tuberculosis. Mean values (squares) and 95% confidence intervals (whiskers) are shown. *P* values relate to differences in FE_{NO}, compared with baseline. Abbreviation: ppb, parts per billion.

DISCUSSION

In this study, the largest investigation to date of the FE_{NO} in patients with pulmonary tuberculosis, we provide the first human in vivo evidence that pulmonary NO bioavailability is significantly associated with both microbiological outcomes and reduced disease severity. Additionally, we show that FE_{NO} is low in patients with tuberculosis, compared with controls, lower still in patients with more-severe disease, and recovers over time. This provides clinical support for in vitro data implicating NO as an important component of the human antimycobacterial immune response. Because NO kills *M. tuberculosis* bacilli and ameliorates immunopathology [3, 4, 11], it is biologically plausible that a low pulmonary NO level, due to impaired production or increased NO clearance, would be associated with more severe disease and worse outcomes, as we have shown.

FE_{NO} shows promise as a biomarker of tuberculosis treatment response; however, further work is required, given the importance of FE_{NO} determinants other than tuberculosis [23, 35] (Supplementary Panel), the wide overlap with values obtained from healthy controls, and the modest increments seen in ΔFE_{NO}. The diagnostic usefulness of ΔFE_{NO} in predicting culture conversion is limited (Supplementary Figure 1), but interestingly is not statistically significantly worse than sputum conversion time, a widely used measure of response to tuberculosis treatment.

NO kills *M. tuberculosis* by direct bacterial cell damage and induction of apoptosis of *M. tuberculosis*–harboring macrophages [4]. NO is capable of killing tuberculosis bacilli in vitro with a molar potency comparable to that of antibiotics [19]. At low concentrations, NO also plays a role in driving *M. tuberculosis* into a nonreplicating persister state, resistant to antibiotics [36], through upregulation of a dormancy regulon [37]. Thus, high NO concentrations are mycobactericidal, but subtoxic NO levels contribute to intracellular *M. tuberculosis* persistence. Impaired NO production as seen in this study may thus be doubly disadvantageous to the host.

As we have reviewed elsewhere [3], animal studies demonstrate that impaired NO production is associated with greater degrees of lung damage [4]. NO ameliorates inflammatory tissue damage in pulmonary tuberculosis by inhibiting NLR3

Table 3. Univariate Analyses of Associations Between Fractional Exhaled Nitric Oxide (FE_{NO}) and Clinical Variables

Variable	Patients With Tuberculosis		Healthy Controls	
	GMR (95% CI)	*P*	GMR (95% CI)	*P*
Female sex (vs male sex)	0.82 (.75–.91)	.05	1.22 (.86–1.73)	.3
Age (per 10 y)	1.06 (.97–1.15)	.2	0.98 (.79–1.21)	.8
Weight (per 10 kg)	1.24 (1.15–1.28)	<.001	0.97 (.81–1.15)	.7
Height (per 10 cm)[a]	1.13 (1.07–1.20)	.03	1.06 (.67–1.69)	.8
Smoking status				
Current smoker	1.01 (.81–1.26)	.9	0.83 (.60–1.14)	
Ex-smoker	1.11 (.89–1.39)	.4	NA[b]	.2
Nonsmoker	1 (reference)		1 (reference)	
Papuan (vs non-Papuan)	1.00 (.83–1.20)	1.0	1.39 (.98–1.99)	.07
HIV positive (vs HIV negative)	0.85 (.60–1.18)	.3	. . .	. . .
FVC (per 1 L)[a]	1.24 (1.10–1.39)	.001	0.86 (.56–1.33)	.5
FEV_1 (per 1 L)[a]	1.23 (1.07–1.40)	.003	1.02 (.66–1.57)	.9
6-minute walk test (per 200 m)[a]	1.15 (.96–1.38)	.1	0.56 (.23–1.39)	.2
SGRQ score (per 50 U)[a]	0.78 (.60–1.03)	.08	0.43 (.00–84.6)	.7
WBC count (per 10×10^9/L)[a]	0.70 (.53–.94)	.02	1.04 (.35–3.10)	.9
Hemoglobin level (per 10 g/dL)[a]	1.74 (1.16–2.62)	.007	0.76 (.31–1.87)	.5
Timika Tuberculosis X-ray score (per 50 U)	0.86 (.76–.98)	.03	. . .	. . .
Sputum acid fast bacillus density (per 1 grade)	1.00 (.91–1.09)	.9	. . .	. . .

Data are factor increases in FE_{NO}. For example, among patients with tuberculosis, for each 10-y age increment, the FE_{NO} increases by a factor of 1.06.

Abbreviations: CI, confidence interval; FEV_1, forced expiratory volume in 1 minute; FVC, forced vital capacity; GMR, geometric mean ratio; HIV, human immunodeficiency virus; NA, not available; SGRQ, St George's Respiratory Questionnaire; WBC, white blood cell.

[a] Data are only available for 40 healthy controls. Other values are available are available for all 88 controls.

[b] Too few values were available for comparison.

inflammasome-dependent processing of interleukin [11] and production of tumor necrosis factor α (TNF-α), a mediator of caseating necrosis of lung tissue and weight loss in tuberculosis. Although the pulmonary pathology of tuberculosis in murine models differs from that in humans, results from our study and others [21, 26, 34] suggest a disease-protective role for NO in people, not just mice. Importantly, lung function (FVC) was independently associated with FE_{NO} in our patients with tuberculosis in a multivariable model.

An early study of pulmonary NO in tuberculosis found that alveolar macrophage *NOS2* gene expression correlated significantly with exhaled NO level [21]. FE_{NO} was significantly higher in 19 people with newly diagnosed pulmonary tuberculosis, compared with 14 control subjects, but the FE_{NO} measurement technique involved a now-obsolete method (exhalation at an uncontrolled flow rate of around 200 mL/s through an open tube, with a probe sampling from the stream of expired breath). The FE_{NO} was inversely associated with tuberculosis severity, as we have also shown, and normalized by 3 months of treatment [21]. A decade later, Idh and colleagues reported lower FE_{NO} in patients with pulmonary tuberculosis, compared with controls, using a chemiluminescent analyzer and an exhalation flow rate of 50 ± 5 mL/seconds [26], as used here. In further work by the same group, the median baseline FE_{NO} was approximately 15 ppb in patients with pulmonary tuberculosis overall, which did not significantly change during 8 weeks of follow-up, and also was unaffected by supplementation with an arginine-rich food (peanuts) [34]. In this study, post hoc subgroup analyses detected increased cure rates in the HIV-positive patients given arginine-rich food, but in the comparative arm of 32 HIV-positive patients randomly assigned to the non–arginine-rich food, the cure rate was anomalously low, at 53%. Notably, they reported that low baseline eNO (<10 ppb) in HIV-positive patients with tuberculosis was associated with a decreased cure rate. Including our data, the cumulative evidence suggests that poorer pulmonary NO bioavailability during active tuberculosis is detrimental to the host.

Adjunctive therapy with inhaled NO has been tested in 8 patients with tuberculosis and 10 controls [38]. Inhaled NO (80 ppb for 72 hours, from day 2 after starting tuberculosis treatment) was well tolerated but did not have a significant impact on microbiological outcomes. Mean times to sputum culture conversion were 35.5 days in the NO group and 37.2 days in controls. Cystic fibrosis (CF) is another lung disease associated with a low FE_{NO} [39] and impaired *NOS2* expression in bronchial epithelial cells [40]. Pulmonary NO deficiency may be an important factor in CF patients' susceptibility to pulmonary bacterial colonization [23]. A clinical trial of inhaled NO in 13 CF patients also demonstrated safety but failed to show benefits [41]. Inhaled L-arginine offers a potentially longer-acting solution to the challenge of enhancing pulmonary NO production, compared with inhaled NO itself, given the short half-life of NO and requirement for prolonged administration regimens. A clinical trial of inhaled L-arginine (1.3 g) versus placebo (normal saline) in CF showed that FE_{NO} increased significantly for several hours, and a significant improvement in FEV_1 was sustained for over 24 hours after inhalation of L-arginine, but not placebo [39].

It is likely that a combination of factors results in impaired ability to produce NO, since NO bioavailability depends on substrate (L-arginine) availability, arginase production, *NOS2* expression, and NOS2 function, as well as the presence of NO-quenching molecules. L-arginine, the main NO source in humans, is a conditionally essential amino acid. Thus, hypoargininemia

can develop when catabolism exceeds supply and has been demonstrated in tuberculosis [42] and other infectious diseases [3]. Micro- and macronutrient deficiencies are well-recognized in tuberculosis; it is plausible that a vicious cycle could develop between increasing tuberculosis severity, escalating nutritional deficiencies, decreased NO formation, and impaired NO-dependent macrophage antituberculosis effects. Arginase overproduction is also recognized in tuberculosis [12, 42]. Intracellular pathogens including *M. tuberculosis* can promote macrophage arginase production as a pathogen-induced immune-evasion strategy [18]. Additionally, circulating or local asymmetric dimethylarginine, an endogenous NOS inhibitor, might play a role in tuberculosis, as in other infections (eg, sepsis and malaria), in limiting NO bioavailability and thereby being associated with worse clinical outcomes [43, 44].

Body weight had an important association with FE_{NO} in patients with tuberculosis but not in healthy controls. Weight distributions in the healthy and tuberculosis study populations were clearly different. Weight is a well-recognized measure of tuberculosis severity and treatment response [45]. Other severity markers, including radiography score [31], pulmonary function, and hemoglobin level also predicted FE_{NO} in patients with tuberculosis in this study. There are multifactorial explanations for anemia in this environment (eg, malaria and iron deficiency), but chronic disease is a likely contributor here. We hypothesize that the association between weight and FE_{NO} in tuberculosis occurs because decreased NO bioavailability allows unchecked immunopathology and more-severe disease.

A limitation of this study is that FE_{NO} may imprecisely reflect intracellular alveolar macrophage NO concentrations; however, the correlation shown previously between alveolar macrophage *NOS2* expression and FE_{NO} in tuberculosis [21] supports the validity of FE_{NO} as a measure of macrophage NO production in this disease. FE_{NO} measures the total lung NO production; therefore, the real extent of NO impairment at sites of tuberculosis pathology may be underestimated. Our study was designed to investigate association rather than causality. Therefore, since determinants of FE_{NO} are varied and incompletely understood, we cannot exclude the possibility that other factors contribute to both impaired FE_{NO} and poorer clinical outcomes. Differences in FE_{NO} concentration between analyzer types were noted. The handheld devices systematically underestimated FE_{NO}, compared with the gold standard, which has not been reported previously. This difference was small in the usual FE_{NO} range. Notably, our median FE_{NO} among controls (16.6 ppb) is the same as that considered normal (16 ppb) [23]. Discrepancies between devices may have been due to ambient conditions in the tuberculosis clinic (high temperatures, often ≥30° C, and humidity levels of ≥75%). We made concerted efforts to shield the NiOX MINO from these conditions (eg, storage in a 19°C refrigerator between patients and in an air-conditioner overnight). Downward drift in NiOX MINO performance over time has been recognized [46], but we did not observe this, making longitudinal comparisons legitimate. FE_{NO} researchers in tropical locations should be cognizant of the susceptibility of NiOX devices to ambient conditions and of the possibility of systematic underestimation of FE_{NO} when using NiOX MINO, compared with the gold standard.

We found a marked fall in the FE_{NO} in the first week after commencing tuberculosis treatment, before eventual recovery. This could be attributable to longitudinal changes in the predominant macrophage phenotype. Specifically, M2 responses (characterized by a high arginase 1 level and a low NOS2 level [ie, a low NO level]) might predominate in advanced tuberculosis at the time of treatment commencement, become exaggerated during the initial inflammatory cascade occurring in response to treatment initiation, then become gradually superseded by M1 responses (characterized by a TNF-α/interferon γ [IFN-γ]/NOS2 phenotype [ie, a high NO level]) during recovery. This hypothesis is supported by a model proposed by Lugo-Villarino et al, describing (1) the predominance of M1 responses soon after infection with *M. tuberculosis,* in which appropriate *NOS2* upregulation occurs and the TNF-α/IFN-γ phenotype predominates; (2) subsequent transition to an M2-predominant phenotype during the course of untreated illness, characterized by poorly microbicidal responses and progressive disease; and (3) restoration of M1 predominance after successful treatment [47]. Concentrations of both M2 cytokines (interleukin 10 and interleukin 13) and M1 cytokines (interleukin 12p40, macrophage inflammatory protein-1α and -1β, and TNF-α) have been shown to increase at week 1 or other time points shortly after tuberculosis treatment initiation, before returning to pretreatment levels [48]. This would support our hypothesis for the longitudinal FE_{NO} trend, assuming that M2 responses initially outweigh M1 responses [48].

Our findings support further research into adjunctive treatments for pulmonary tuberculosis to increase pulmonary NO production. Systemic administration of oral L-arginine (1–6 g) has thus far been disappointing in this regard [27, 34, 49] (Ralph et al, unpublished data), but inhaled L-arginine or alternative NO-donors require investigation. Adjunctive treatments to support NO production might be particularly valuable during the early period of treatment response, to improve *M. tuberculosis* killing, to reduce inflammatory pulmonary tissue damage, and to prevent the low intracellular NO environments that promote the development of the nonreplicating, antibiotic-resistant state among *M. tuberculosis* strains. Overall, the clinical data presented here support the in vitro evidence of the importance of NO in human antimycobacterial immune responses, describe the usefulness of FE_{NO} as a biomarker of the immune response to tuberculosis and response to treatment, and provide the basis for future studies of adjunctive therapy to augment pulmonary NO production.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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