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# Pulmonary nitric oxide bioavailability is impaired in pulmonary tuberculosis and predicts mycobacterial clearance with treatment

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## ABSTRACT

**Background:** Nitric oxide (NO), produced by macrophages infected with *Mycobacterium tuberculosis*, is measurable in exhaled breath in pulmonary tuberculosis (PTB). We aimed to investigate concentration of fractional exhaled NO ( $FE_{NO}$ ) at PTB diagnosis, change during treatment, and relationship with 2-month sputum culture-conversion.

**Methods:** In Papua, we measured baseline  $FE_{NO}$  in PTB patients then serially over 6 months, and once in healthy controls. Treatment outcomes were 2-month sputum culture and time to sputum microscopy-conversion.

**Results:** Among 200 PTB patients and 88 controls,  $FE_{NO}$  was lower in PTB patients at diagnosis (geometric mean  $FE_{NO}$  12.7ppB, 95%CI 11.6-13.8) than controls (16.6ppB, 95%CI 14.2-19.5,  $p=0.002$ ), fell further after treatment initiation (nadir at one week), then recovered by 6 months ( $p=0.03$ ). More-severe TB was characterized by lower  $FE_{NO}$ :  $FE_{NO}$  was directly proportional to weight ( $p<0.001$ ) and forced vital capacity ( $p=0.001$ ), and inversely proportional to radiological score ( $p=0.03$ ). People whose  $FE_{NO}$  increased or remained unchanged by 2 months were over twice as likely to achieve sputum culture-conversion than those whose  $FE_{NO}$  decreased (OR 2.72, 95%CI 1.05-7.12,  $p=0.04$ ).

**Conclusion:** Among PTB patients, 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

## INTRODUCTION

Investigation of tuberculosis (TB) pathophysiology provides the basis for research into TB adjunctive therapies and biomarkers. These fields are considered to be priority research areas as improved TB treatments are sought [1, 2]. Nitric oxide (NO) has a fundamental physiological role in innate immunity in TB. NO is derived chiefly from the amino acid L-arginine. Its production in macrophages expressing nitric oxide synthase 2 (*NOS2*) provides a key component of the human immune response to *Mycobacterium tuberculosis* (Table 1) [3-11]. *M. tuberculosis* – and other bacterial genera - have specific immune-evasion strategies including nitric oxide detoxification systems, to mitigate NO-mediated microorganism toxicity [12, 13], and variable susceptibility to NO [14]. Resistance of *M. tuberculosis* to reactive nitrogen species correlates with virulence in guinea pig models [15], and with resistance to isoniazid [16], the latter possibly attributable to upregulation of *ahpC* (encoding an alkyl hydroperoxide reductase thought to be involved in protection against oxidative and nitrosative stress), to restore virulence due to loss of *katG* [17].

NO is measurable in exhaled breath using validated chemiluminescence analysers [18, 19]. Determinants of fractional exhaled NO ( $F_{E_{NO}}$ ) are shown in the Panel. Analysis of breath components provides a non-invasive means of directly sampling the site of pathology in pulmonary TB (PTB). Differences in exhaled breath characteristics in people with TB compared with controls include significantly lower pH [20], and elevated levels of volatile metabolites [21]. Investigations of  $F_{E_{NO}}$  in PTB to date have been limited by small sample sizes, post-hoc analyses and/or different methodologies for  $F_{E_{NO}}$  measurement, and have provided conflicting results, including reports of both increased [22] or decreased [23]  $F_{E_{NO}}$  in TB compared with controls.

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

Given the immunological rationale and inconclusive findings to date, we aimed to investigate  $F_{E_{NO}}$  in PTB and healthy controls in a high TB-burden tropical setting. Our objectives were to determine the accuracy and precision of  $F_{E_{NO}}$

measurement in the field; compare  $FE_{NO}$  in pulmonary TB and in healthy volunteers; investigate relationships between  $FE_{NO}$  and PTB severity, and determine the relationship between change in  $FE_{NO}$  and microbiological response (2-month sputum culture conversion and time to sputum microscopy clearance).

## **METHODS**

This study was performed as part of a clinical trial of adjunctive L-arginine and vitamin D in TB in Timika, Papua Province, Indonesia ([clinicaltrials.gov/NCT00677339](https://clinicaltrials.gov/NCT00677339)). In this study, neither intervention affected  $FE_{NO}$  (unpublished data). The Timika population comprises people of Papuan (Melanesian) and Non-Papuan (Asian) ethnicity. Eligible study participants with PTB, enrolled at the TB clinic were  $\geq 15$  years, sputum smear-positive for acid fast bacilli, and provided written informed consent. Standard TB treatment comprised rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, then rifampicin and isoniazid for 4 months. Local healthy controls were  $\geq 18$  years, gave written informed consent, and had no co-morbidities. Data from additional healthy controls (age, sex, ethnicity, weight and  $FE_{NO}$  only) from the same location whose results have been reported elsewhere [24], were also made available for this analysis. Enrolments of TB patients and controls occurred across all months; environmental conditions in Timika, comprising tropical temperatures (25-30°C daily maxima), 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<sup>®</sup> (Aerocrine, Sweden). This device is well-validated [18] and employs single-use, disposable, filtered mouthpieces without infection control risks.  $FE_{NO}$  measurements complied with 2005 American Thoracic Society Guidelines [25].  $FE_{NO}$  was measured on one occasion in healthy controls, and serially in TB patients at baseline, 1 and 2 weeks, then 1, 2 and 6 months of follow-up. 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<sup>®</sup> and a 'gold standard' non-portable NiOX FLEX<sup>®</sup> (not located at the TB clinic), calibrated fortnightly with 200ppB NO.

Chest radiographs, pulmonary function, a 6-minute walk test (6MWT) and locally adapted St George's Respiratory Questionnaire (SGRQ, in Indonesian) were performed at baseline then at 2 and 6 months. Chest x-rays were scored according to a previously-reported method [26]. Pulmonary function (forced vital capacity, FVC and forced expiratory volume in 1 minute, FEV<sub>1</sub>) was measured outdoors (for infection control purposes) using a handheld spirometer (MicroLoop<sup>®</sup>, MicroMedical, UK), with individual-use filtered one-way mouthpieces (Sure-Gard<sup>®</sup>) suitable for use in smear-positive PTB. Percentage of predicted FEV<sub>1</sub> was calculated from local normal reference ranges [27]. 6MWT was assessed according to standard procedures [28].

Sputum microscopy was undertaken at the Timika laboratory weekly for 2 months then at months 5 and 6. Baseline and 2-month sputum cultures were processed at the University of Indonesia, Jakarta, using BACTEC<sup>®</sup> Mycobacterium Growth Indicator Tube (MGIT) 960 system. Sputum smear conversion was defined as  $\geq 2$  consecutive negative smears without a subsequent positive. Sputum culture conversion at 2 months is a standard early measure of TB treatment response because it predicts 6-month treatment outcome and 2-year relapse [2, 29].

## Statistical methods

Analyses were undertaken using Stata 12.1. Scatter plots and Pearson's correlation coefficient were used to evaluate differences in FE<sub>NO</sub> within and between analysers. FE<sub>NO</sub> data were log-normal; geometric means were compared between TB patients and healthy controls using Student's 2-sample T-test, and between different time points using paired T-tests. Associations between FE<sub>NO</sub> 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<sub>NO</sub> and weight was displayed graphically using values predicted from the regression model of FE<sub>NO</sub> against weight. Incremental changes in FE<sub>NO</sub> ( $\Delta$ FE<sub>NO</sub>) were calculated as  $(\log \text{FE}_{\text{NO}} [\text{week8}]) - (\log \text{FE}_{\text{NO}} [\text{week0}])$ . The association between  $\Delta$ FE<sub>NO</sub> and culture or smear

conversion at week 8 was tested using logistic regression, and receiver-operator characteristic (ROC) scores given as area under the curve (AUC), compared using chi-squared tests.

## RESULTS

Two hundred participants with PTB and 40 healthy controls were enrolled in the study June 2008-February 2010, and 48 additional controls in 2005 (Figure 1). PTB patients had lower weight, pulmonary function measures, 6MWT and worse SGQR scores than controls, comprised more non-Papuans, and were more likely to have quit smoking (Table 2).

### **FE<sub>NO</sub> measurement**

The average difference between paired FE<sub>NO</sub> measures within 15 minutes by an individual using a single NiOX MINO<sup>®</sup> analyser/sensor was -0.4 ppb (95% limits of agreement -6.2 to 5.5), and on two different analysers/sensors was 0.8 ppb (95% limits of agreement -4.3 to 5.8) (Figure 2A-B). FE<sub>NO</sub> values obtained from the NiOX MINO<sup>®</sup> portable analysers were highly correlated with those from the NiOX FLEX<sup>®</sup> 'gold standard' analyser (Pearson's R 0.95), but consistently lower (Figure 2C). Rather than applying a correction factor, we have used raw FE<sub>NO</sub> results given that NiOX MINO<sup>®</sup> devices are now commonly used by ourselves and others in field research [23, 30].

### **TB versus healthy controls**

FE<sub>NO</sub> was significantly lower in TB patients (geometric mean FE<sub>NO</sub> 12.7ppB, 95% CI 11.6-13.8) than controls (16.6ppB, 95% CI 14.2-19.5), p=0.002 (Figure 3). The relationship between weight and FE<sub>NO</sub> seen in PTB patients at baseline was different from that observed in the healthy controls (p=0.02 for test of interaction): weight was directly proportional to FE<sub>NO</sub> in PTB patients, but not among healthy volunteers (Figure 4). The FE<sub>NO</sub> difference between patients and controls were unchanged when controlling for co-variables, or when restricting analyses to only contemporaneously-enrolled controls.

## Determinants of baseline $FE_{NO}$

Associations between clinical measures and  $FE_{NO}$  in controls and PTB patients at baseline are shown in Table 3; regression coefficients were calculated for continuous variables according to groups as shown. Among PTB patients, measures signifying greater TB severity such as lower weight (or lower BMI), worse pulmonary function measures, lower haemoglobin and higher x-ray score, were each associated with lower  $FE_{NO}$ . However, these factors only accounted for a small proportion of the overall variation in  $FE_{NO}$ ; e.g. for each 10kg 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+ and HIV- patients (geometric mean 11.2ppB, 95% CI 7.8-16.2) versus HIV- TB patients (13.3ppB, 95% CI 11.8-15.0) ( $p=0.3$ ), 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 15.1ppB, 95% CI 11.9-19.3) and non-smokers (18.3ppB, 95% CI 14.7-22.9) was not statistically significantly different ( $p=0.2$ ).

## $FE_{NO}$ during follow-up

After starting TB treatment, the low baseline  $FE_{NO}$  fell further to 10.7ppB (95% CI 9.4-12.1) with a nadir at one week ( $p=0.0006$  compared with baseline  $FE_{NO}$ ), then gradually recovered by treatment completion to 15.1ppB (95% CI 13.3-17.2) ( $p=0.03$ ), creating a j-shaped curve (Figure 5).  $FE_{NO}$  at TB treatment completion was not statistically significantly different from that obtained from healthy controls (16.6ppB, 95% CI 14.2-19.5). The longitudinal trend seen in  $FE_{NO}$  in the TB patients was not observed in the quality control measures performed in healthy staff members.

## Association with microbiological outcomes

The proportion of study participants whose 2-month sputum was culture negative for *M. tuberculosis* was 78.9% (97/123). Change in  $FE_{NO}$  from baseline to 2 months ( $\Delta FE_{NO}$ ) was significantly associated with sputum culture conversion by 2 months. Specifically, people whose  $FE_{NO}$  remained unchanged or increased were approximately twice as likely to have achieved sputum culture conversion to negative by 2 months, than those whose  $FE_{NO}$

decreased (OR 2.72, 95% CI 1.05-7.12,  $p=0.04$ ). The mean absolute change in 2-month  $FE_{NO}$  was +1.6ppB in those achieving culture negativity, and -4.7ppB in those remaining culture positive ( $p=0.02$ ).

The diagnostic utility of  $\Delta FE_{NO}$  compared with sputum smear conversion time for predicting 2-month culture conversion is shown in Figure 6. The ROC score for  $\Delta FE_{NO}$  (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 areas under the curve was not statistically significant ( $p=0.1$ ,  $\chi^2$  test).

Additional to the association with culture conversion, people in whom  $FE_{NO}$  stayed the same or increased had a slightly shorter time to sputum smear conversion than those whose  $FE_{NO}$  fell (OR 1.06, 95% CI 1.00-1.12),  $p=0.06$ . Changes achieved in  $FE_{NO}$  were unrelated to baseline weight ( $p=0.9$ ), weight at week 8 ( $p=0.8$ ) or change in weight between weeks 0 and 8 ( $p=0.3$ ) using regression analyses. Including these weight variables in multivariable models had a negligible effect on the relationship between  $\Delta FE_{NO}$  and culture or microscopy conversion.

## DISCUSSION

In this largest study of  $FE_{NO}$  in pulmonary TB to date, we provide the first human *in vivo* evidence that pulmonary NO bioavailability is significantly associated with microbiological outcomes. Additionally, we show that  $FE_{NO}$  is low in TB patients compared with controls, lower still in worse 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. As NO kills *M. tuberculosis* bacilli, it is biologically plausible that low pulmonary NO, due to impaired production or increased NO clearance, would be associated with more severe disease and worse outcomes, as we have shown.

$FE_{NO}$  thus shows promise as a biomarker of TB treatment response; however, further work is required given the importance of  $FE_{NO}$  determinants other than TB [19, 31] (Panel), the wide overlap with values obtained from healthy controls, and the modest increments seen in  $\Delta FE_{NO}$ . The diagnostic utility of  $\Delta FE_{NO}$  in predicting culture conversion is limited (Figure 6), but interestingly, is not statistically significantly worse than sputum conversion time, a widely-used measure of response to TB treatment.

NO kills *M. tuberculosis* by direct bacterial cell damage and induction of apoptosis of TB-harboring macrophages [4]. NO is capable of killing TB bacilli *in vitro* with a molar potency comparable to that of antibiotics [32]. At low concentrations, NO also plays a role in driving *M. tuberculosis* into a non-replicating persistor state, resistant to antibiotics [33], through up-regulation of a dormancy regulon [34]. Thus high NO concentrations are mycobactericidal, but sub-toxic 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. In TB-infected NOS2<sup>-/-</sup> knockout mice and mice treated with NOS2 inhibitors, larger and more destructive lung lesions are seen [3, 4]. NO also inhibits production of TNF- $\alpha$ , a mediator of caseating necrosis of lung tissue and weight loss in TB. Although pulmonary pathology in TB in murine models differs from that in humans, results from our study and others [22, 23, 30] suggest a disease-protective role for NO in people, not just mice. Of relevance, lung function (FVC) was independently associated with FE<sub>NO</sub> in our TB patients in a multivariable model.

An early study of pulmonary NO in TB found that *NOS2* gene expression in participants' alveolar macrophages correlated significantly with their exhaled NO [22]. FE<sub>NO</sub> was significantly *higher* in 19 people with newly diagnosed PTB compared with 14 control subjects, but the FE<sub>NO</sub> measurement technique comprised 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). FE<sub>NO</sub> was inversely associated with TB severity, as we have also shown, and normalised by 3 months of treatment [22]. Idh and colleagues a decade later reported lower FE<sub>NO</sub> in PTB patients than controls using a chemiluminescent analyser and an exhalation flow rate of  $50 \pm 5$  mL/s [23] as used here. In further work by the same group, the median baseline FE<sub>NO</sub> was approximately 15 ppb in PTB patients overall, which did not significantly change during 8 weeks of follow up, and also was unaffected by supplementation with an arginine-rich food (peanuts) [30]. In this study, post-hoc subgroup analyses did detect increased cure rates in the HIV+ patients given arginine-rich food, but in the comparative arm of 32 HIV+ patients randomised 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+/TB

patients was associated with a decreased cure rate. Including our data, the cumulative evidence suggests that a poorer pulmonary NO bioavailability in active TB is detrimental to the host.

Adjunctive therapy with inhaled NO has been tested in eight TB patients and 10 controls [35]. Inhaled NO (80ppB, for 72 hours, from day 2 after starting TB treatment), was well-tolerated but did not impact significantly on microbiological outcomes. Mean times to sputum culture conversion were 35.5 days in the NO group and 37.2 in controls. Cystic fibrosis (CF) is another lung disease in which  $FE_{NO}$  is low [36] and *NOS2* expression in bronchial epithelial cells is impaired [37]. Pulmonary NO deficiency may be an important factor in CF patients' susceptibility to pulmonary bacterial colonization [19]. A clinical trial of inhaled NO in 13 CF patients also demonstrated safety, but failed to show benefits [38]. Inhaled L-arginine offers a potentially more long-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 1.3g 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 [36].

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 (since arginase degrades L-arginine to ornithine without producing NO), *NOS2* expression and *NOS2* function, and the presence of NO-quenching molecules. L-arginine, the main NO source in humans, is a conditionally-essential amino acid. Thus hypoargininaemia can develop when catabolism exceeds supply, and has been demonstrated in TB [39] and other infections (see review[3]). Micro- and macro-nutrient deficiencies are well-recognised in TB; it is plausible that a vicious cycle could develop between increasing TB severity, escalating nutritional deficiencies, decreased NO formation and impaired NO-dependent macrophage anti-TB effects. Arginase over-production is also recognised in TB [39, 40]. Intracellular pathogens including *M. tuberculosis* can promote macrophage arginase production, presumably as a pathogen-induced immune-evasion strategy [41]. Additionally, circulating or local asymmetric dimethylarginine, an endogenous NOS inhibitor, might play a role in TB, as in other infections (e.g. sepsis, malaria), in limiting NO bioavailability and thereby being associated with worse clinical outcomes [42, 43].

We found that body weight had an important association with  $FE_{NO}$  in TB patients, but not among healthy controls (Figure 4). Weight distributions in the healthy and TB study populations were clearly different (Table 2). Weight is a well-recognised measure of TB severity and treatment response [44]. Other severity markers, including x-ray score [26], pulmonary function and haemoglobin also predicted  $FE_{NO}$  in TB patients in this study. There are multi-factorial explanations for anaemia in this environment (e.g. malaria, iron deficiency), but chronic disease is a likely contributor in these TB patients. We hypothesise that the association between weight and  $FE_{NO}$  in TB occurs because decreased NO bioavailability reflects impaired *M. tuberculosis* immune responses and hence more severe TB disease.

Among controls, those who smoked had only slightly lower  $FE_{NO}$  values than non-smokers, with the difference being non-significant. Smoking is usually associated with low  $FE_{NO}$  [19], but the identification of consistent associations with  $FE_{NO}$  in healthy populations requires much larger sample size than used here [31].

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 TB [22] supports the validity of  $FE_{NO}$  as a measure of macrophage NO production in this disease.  $FE_{NO}$  measures total lung NO production, therefore the real extent of NO impairment at sites of TB 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 other factors as being responsible for both impaired  $FE_{NO}$  and poorer clinical outcomes. Differences in  $FE_{NO}$  concentration between analyser types were noted. The hand-held devices systematically under-estimated  $FE_{NO}$  compared with the 'gold standard', which has not been reported previously. This difference was small in the usual  $FE_{NO}$  range. Notably, our median  $FE_{NO}$  amongst controls (16.6 ppB) is the same as that considered 'normal' (16 ppB) [19]. Discrepancies between devices may have been due to ambient conditions in the TB clinic (high temperature, often  $\geq 30^{\circ}C$ , and humidity  $\geq 75\%$ ). We made concerted efforts to shield the NiOX MINO<sup>®</sup> from these conditions (e.g. storage in a  $19^{\circ}$  refrigerator between patients, and in an air-conditioning overnight). Downward drift in NiOX MINO<sup>®</sup> performance over time has been recognised [45], but we did not observe this, making longitudinal comparisons legitimate.  $FE_{NO}$  researchers in tropical locations should be cognisant of susceptibility of NiOX devices to ambient conditions, and the possibility of systematic under-estimation of  $FE_{NO}$  using NiOX MINO<sup>®</sup> compared with the 'gold standard'.

We found a marked fall in  $FE_{NO}$  in the first week after commencing TB treatment, before eventual recovery. This could be attributable to longitudinal changes in the predominant macrophage phenotype; specifically, M2 responses (characterized by a high arginase1/low NOS2 phenotype i.e. low NO) might predominate in advanced TB 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 ( $TNF\alpha/IFN\gamma/NOS2$  phenotype i.e. high NO) during recovery. This hypothesis is supported by a model proposed by Lugo-Villarino et al describing: 1) predominance of M1 responses soon after infection with *M. tuberculosis*, in which appropriate *NOS2* upregulation occurs, and  $TNF\alpha/IFN\gamma$  predominate; 2) subsequent transition to M2-predominant phenotype during the course of untreated illness, characterised by poorly microbicidal responses and progressive disease; and 3) restoration of M1 predominance after successful treatment [46]. Concentrations of both M2 cytokines (IL10 and IL13) and M1 cytokines (IL-12p40, macrophage inflammatory proteins-1 $\alpha$  and 1 $\beta$ ,  $TNF\alpha$ ) have been shown to increase at week 1 or other time points shortly after TB treatment initiation, before returning to pre-treatment levels [47]. This would support our hypothesis for the longitudinal  $FE_{NO}$  trend, assuming the M2 responses initially outweigh M1 [47].

Our findings support further research into adjunctive treatments for PTB to increase pulmonary NO production. Systemic administration of oral L-arginine (1 to 6g) has thus far been disappointing in this regard [30, 48](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, both to improve *M. tuberculosis* killing, and to prevent the low intracellular NO environments which promote the development of the non-replicating antibiotic-resistant state in *M. tuberculosis*. Overall the clinical data presented here support the *in vitro* evidence on the importance of NO in human antimycobacterial immune responses, describe the utility of  $FE_{NO}$  as a biomarker of the immune response to TB and response to treatment, and provide the basis for future studies of adjunctive therapy to augment pulmonary NO production.

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## **Footnote**

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### **Conflicts of interest**

We have no conflicts of interest to declare.

### **Conference presentations relevant to this publication**

Preliminary results from this study were presented at the Australasian Society for Infectious Diseases Annual Scientific Meeting (ASID Clinical Research Network Workshop), Fremantle, March 2012.

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## Tables

**Table 1: Evidence for the role of nitric oxide (NO) in tuberculosis immunology**

Study Type	Results	Selected references
<i>M. tuberculosis</i> in vitro studies	Mycobacteria are susceptible to NO and other reactive nitrogen species <i>in vitro</i>	[15]
	<i>M. tuberculosis</i> isolates differ in their susceptibility to reactive nitrogen intermediates: - less-susceptible isolates are more virulent in guinea pigs - less-susceptible isolates from human cases are less susceptible to anti-tuberculosis drugs	[15, 16]
	<i>M. tuberculosis</i> immune-evasion strategies include the induction of arginase expression (thereby decreasing NO availability) in macrophages	[41]
Mouse macrophage studies	Arginine-derived reactive nitrogen intermediates in mouse macrophages effectively kill <i>M. tuberculosis</i>	[4]
	<i>M. tuberculosis</i> lacking genetic resistance to reactive nitrogen intermediates cannot grow in mouse macrophages, in contrast with wild-type <i>M. tuberculosis</i>	[32]
Human macrophage studies	Alveolar macrophages from healthy humans infected <i>ex vivo</i> with <i>M. tuberculosis</i> produce NO, and NO production correlates with intracellular growth inhibition of <i>M. tuberculosis</i>	[11]
	Blood mononuclear cells from healthy donors infected <i>ex vivo</i> with <i>M. tuberculosis</i> , and from people with pre-existing TB infection, produce NO.	[8]
	Pulmonary macrophages kill mycobacteria only if they express NOS2; killing is prevented with a NOS inhibitor	[10]
<i>In vivo</i> mouse studies	NOS2 is expressed at sites of disease in immunocompetent mice, but is deficient in immunocompromised mice with progressive TB disease	See review[49]
	<i>M. tuberculosis</i> infection is poorly contained in mice treated with NOS inhibitors	[4]
	Fulminant <i>M. tuberculosis</i> disease develops in NOS2 knockout mice (NOS2 <sup>-/-</sup> ) in contrast with controls	[49]
<i>In vivo</i> human studies	In lung resection studies, NOS2 and nitrotyrosine (a tissue marker of NO metabolism) are expressed in macrophages within granulomata and areas of TB pneumonitis.	[7]
	NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls	[9]
	NOS2 is expressed in macrophages from lungs of patients with tuberculosis	[22]

**Table 2: Baseline characteristics of study participants**

	<b>TB patients</b>	<b>Healthy controls (contemporaneous)</b>	<b>Healthy controls (previous)</b>	<b>p value (TB versus all available controls)</b>
<b>Number of study participants</b>	200	40	48	-
<b>Age in years: median (range)</b>	28 (15-73)	29 (18-65)	27 (18-42)	0.2
<b>Papuan: no. (%)</b>	89 (45%)	20 (50%)	44 (92%)	<0.0001
<b>Female: no. (%)</b>	69 (35%)	9 (23%)	17 (35%)	0.4
<b>HIV positive: no./no.tested (%)</b>	19/145 (13%)	-	-	-
<b>Papuan</b>	15/71 (21%)			
<b>Non-Papuan</b>	4/74 (5%)			
<b>Weight (kg): mean (range)</b>	48.5 (26.3-74.0)	62.5 (41.6-84.0)	59.8 (45.0-85.0)	<0.0001
<b>Body mass index (kg/m<sup>2</sup>): mean (range)</b>	19.2 (12.0-32.5)	24.3 (17.1-33.6)	-	<0.0001
<b>Height (m): mean (range)</b>	1.58 (1.35-1.79)	1.61 (1.48-1.73)	-	0.08
<b>Smoking status: no. (%)</b>				
<b>Current</b>	56 (28%)	18 (45%)	23 (48%)	<0.0001
<b>Ex-smoker</b>	53 (27%)	1 (3%)	3 (6%)	
<b>Never smoked</b>	91 (46%)	21 (53%)	22 (46%)	
<b>Fractional exhaled nitric oxide (ppB): geometric mean (95% CI)</b>	12.7 (11.6-13.8)	16.2 (12.2-21.5)	17.0 (14.1-20.5)	0.002
<b>FVC (L): mean (range)</b>				
<b>Female</b>	1.62 (0.63-2.96)	1.91 (1.20-2.99)	-	<0.0001
<b>Male</b>	2.36 (0.84-4.45)	3.37 (1.57-4.22)		
<b>FEV<sub>1</sub> (L): mean (range)</b>				
<b>Female</b>	1.43 (0.63-2.47)	1.71 (1.18-2.52)	-	<0.0001
<b>Male</b>	2.03 (0.59-4.00)	3.20 (2.16-5.30)		
<b>Percent predicted FEV<sub>1</sub> (%): mean (range)</b>				
<b>Female</b>	62.8 (31.0-99.0)	81.0 (52.6-108.5)		<0.0001
<b>Male</b>	63.6 (16.5-108.5)	95.3 (67.8-148.4)		
<b>St George's Respiratory Questionnaire total score (units): median (range)</b>	40.7 (5.2-91.9)	0 (0-9.23)	-	<0.0001
<b>6-minute walk test (m): median (range)</b>				
<b>Female</b>	375 (20-490)	460 (360-528)	-	<0.0001
<b>Male</b>	425 (40-612)	511 (370-640)		
<b>Chest radiograph</b>				
<b>Cavity size: no. (%)</b>				
<b>0</b>	69 (44%)	-	-	-
<b>≤4cm</b>	53 (34%)			
<b>&gt;4cm</b>	35 (22%)			
<b>% lung affected: median (IQR)</b>	40 (23-62)			
<b>X-ray score: median (IQR)</b>	68 (34-94)			
<b>Sputum acid fast bacilli density: no.(%)</b>				
<b>0 or Scanty</b>	55 (28%)	-	-	-
<b>1+</b>	57 (29%)			
<b>2+</b>	50 (25%)			
<b>3+</b>	38 (19%)			
<b>Sputum culture at diagnosis: no.(%)</b>				
<b>No growth, contaminated or no     result unavailable</b>	36 (18%)	-	-	-
<b><i>M. tuberculosis</i> identified</b>	164 (82%)			
<b><i>M. tuberculosis</i> susceptibility: no.(%) *</b>				
<b>Fully susceptible</b>	126/149 (85%)	-	-	-
<b>Monoresistance or other (not MDR)</b>	23/149 (15%)			
<b>INH &amp; RIF resistant (MDR-TB)</b>	2 /149 (1%)			

\* Reasons for missing culture results at baseline (and 2 month follow-up) included specimen contamination, specimen loss during transit, power-outage in the laboratory, or participant loss to follow-up prior to 2 months.

**Table 3: Associations between F<sub>E</sub>NO and clinical variables in univariate analyses**

Independent variable	Dependent variable	Tuberculosis patients		Healthy controls	
		Geometric mean ratio (95% CI) per increase in continuous variable	p value	Geometric mean ratio (95% CI) per increase in continuous variable	Significance (p value)
Log FE <sub>NO</sub>	Female (vs male)	0.82 (0.75-0.91)	<b>0.05</b>	1.22 (0.86-1.73)	0.3
	Age (years)	1.06 (0.97-1.15) per 10 years	0.2	0.98 (0.79-1.21) per 10 years	0.8
	Weight (kg)	1.24 (1.15-1.28) per 10 kg	<b>&lt;0.001</b>	0.97 (0.81-1.15) per 10 kg	0.7
	Height (cm)*	1.13 (1.07-1.20) per 10 cm	<b>0.03</b>	1.06 (0.67-1.69) per 10 cm	0.8
	Current smoker	1.01 (0.81-1.26)	0.9	0.83 (0.60 - 1.14)	0.2
	Ex-smoker	1.11 (0.89-1.39)	0.4	N/A (too few values)	
	Non-Smoker (reference group)	1		1	
	Papuan (vs Non-Papuan)	1.00 (0.83-1.20)	1.0	1.39 (0.98-1.99)	0.07
	HIV+ (vs HIV-)	0.85 (0.60-1.18)	0.3	-	-
	FVC (L)*	1.24 (1.10-1.39) per 1L	<b>0.001</b>	0.86 (0.56-1.33) per 1L	0.5
	FEV1 (L)*	1.23 (1.07-1.40) per 1L	<b>0.003</b>	1.02 (0.66-1.57) per 1L	0.9
	6 minute walk test (m)*	1.15 (0.96-1.38) per 200 m	0.1	0.56 (0.23-1.39) per 1L 200 m	0.2
	St George's Respiratory Questionnaire (units)*	0.78 (0.60-1.03) per 50 units	0.08	0.43 (0.00-84.6) per 1L 50 units	0.7
	White cell count (x10 <sup>9</sup> /L)*	0.70 (0.53-0.94) per 10 x10 <sup>9</sup> /L	<b>0.02</b>	1.04 (0.35-3.10) per 1L 10 x10 <sup>9</sup> /L	0.9
	Haemoglobin (g/dL)*	1.74 (1.16-2.62) per 10 g/dL	<b>0.007</b>	0.76 (0.31-1.87) per 1L 10 g/dL	0.5
	Xray score (units)	0.86 (0.76-0.98) per 50 units	<b>0.03</b>	-	-
	Sputum acid fast bacillus density (grade)	1.00 (0.91-1.09) per 1 grade	0.9	-	-

\*Only available in 40 healthy controls. Other measures available in all 88 controls.

**Panel: Determinants of exhaled nitric oxide concentration**

**Overall determinants of  $F_{E_{NO}}$  [19, 31]**

- Availability of substrate L-arginine
- Expression of NOS2 by alveolar leucocytes [22]
- Expression of enzymes promoting alternative L-arginine degradation pathways (e.g. arginases) by alveolar leucocytes and endothelial cells
- Presence of NOS2 inhibitors such as asymmetric dimethylarginine
- Pulmonary blood flow [50]

**Factors associated with increased  $F_{E_{NO}}$  [19, 31]**

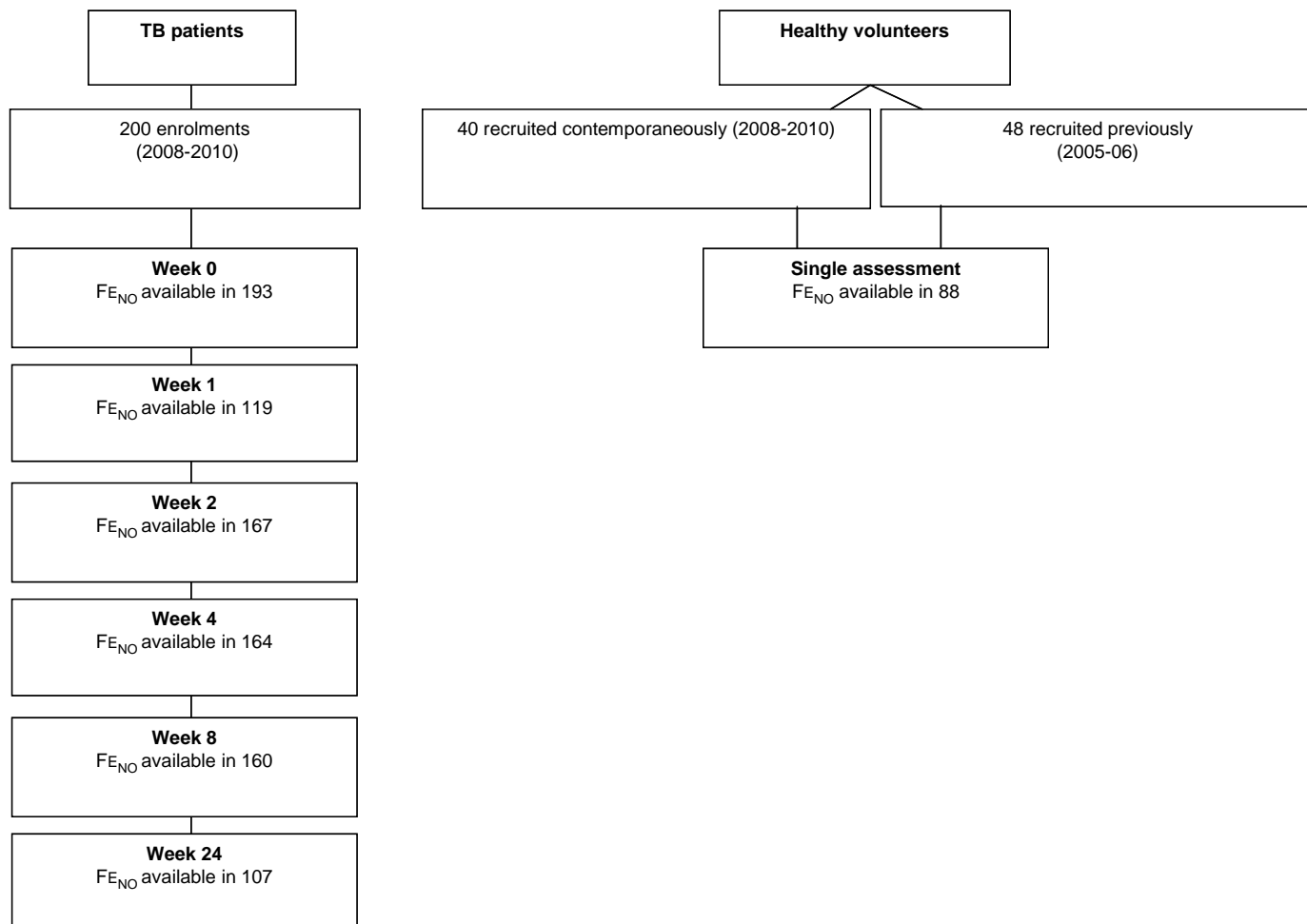
- Older age
- Male sex
- Increasing height
- Nitrate-rich diet
- Disease states: Asthma exacerbation, viral respiratory tract infection, bronchiectasis

**Factors associated with decreased  $F_{E_{NO}}$  [19, 31]**

- Smoking
- Alcohol consumption [51]
- High altitude [52]
- Disease states: chronic cystic fibrosis, pulmonary hypertension

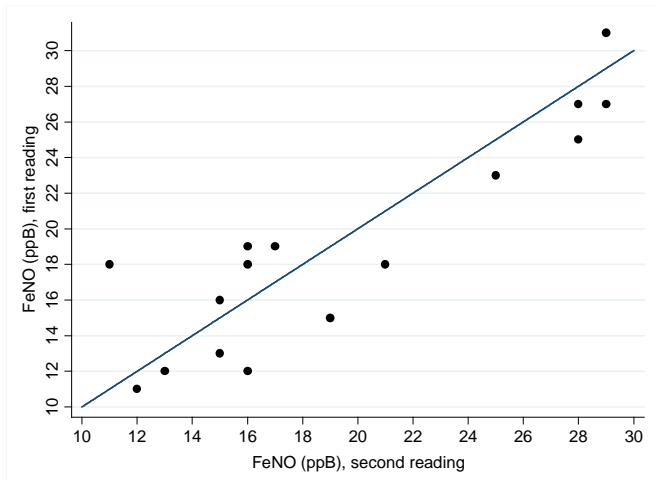
## Figures

Figure 1: Study diagram



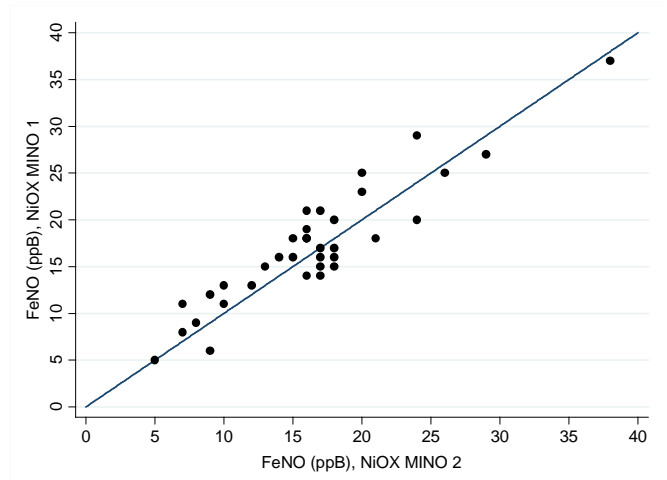
**Figure 2A: Repeated  $FE_{NO}$  measures using one NiOX MINO<sup>®</sup> analyser**

Dots show individual data points, line represents  $y=x$



**Figure 2B: Paired  $FE_{NO}$  measures using two NiOX MINO<sup>®</sup> analysers**

Dots show individual data points, line represents  $y=x$



**Figure 2C: Paired  $FE_{NO}$  measures using NiOX MINO<sup>®</sup> and NiOX FLEX<sup>®</sup> analysers**

Dots show individual data points, line represents  $y=x$

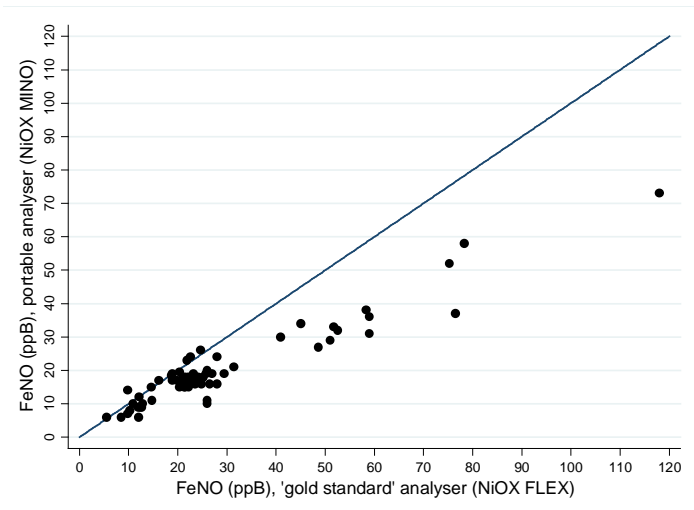
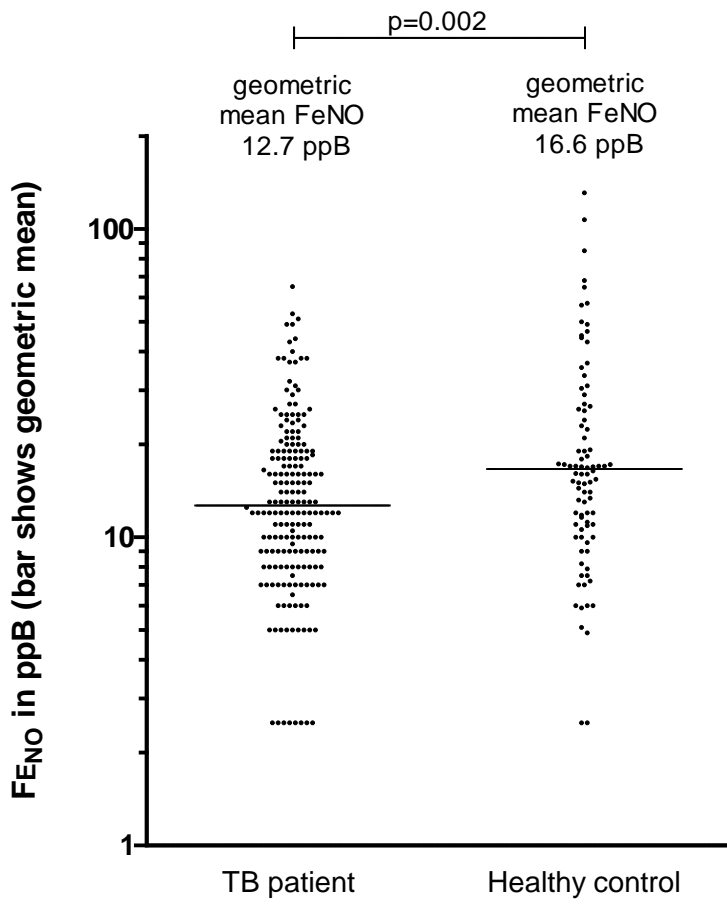
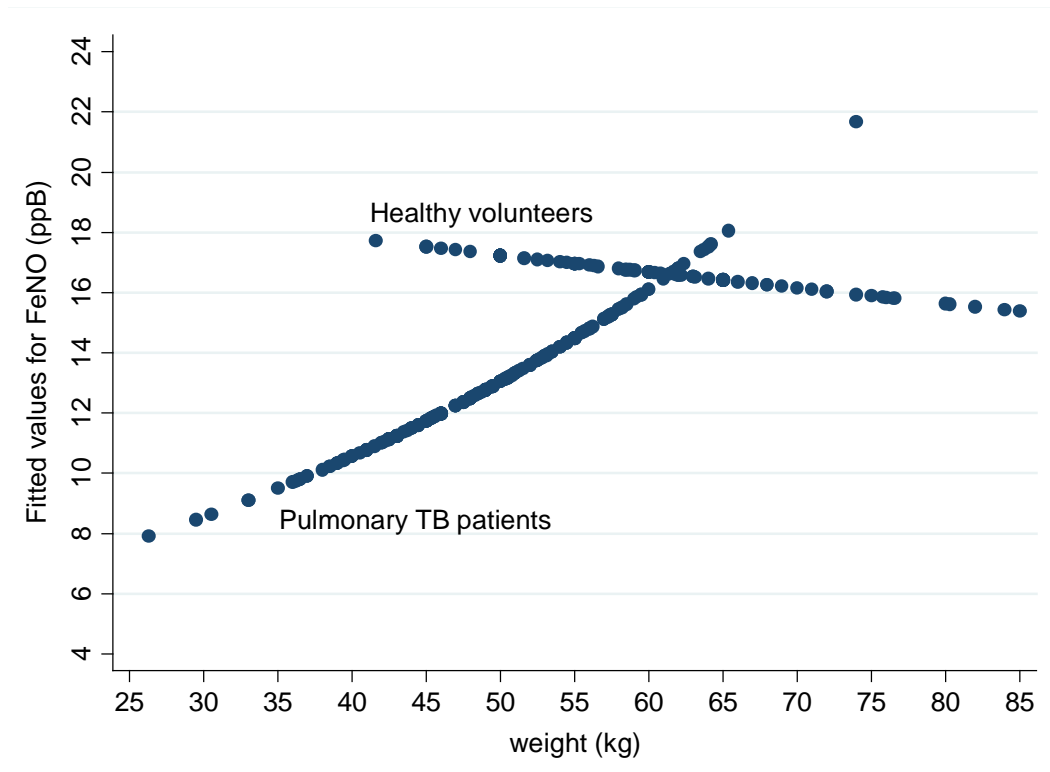


Figure 3: FE<sub>NO</sub> in healthy controls compared with pulmonary TB patients at diagnosis

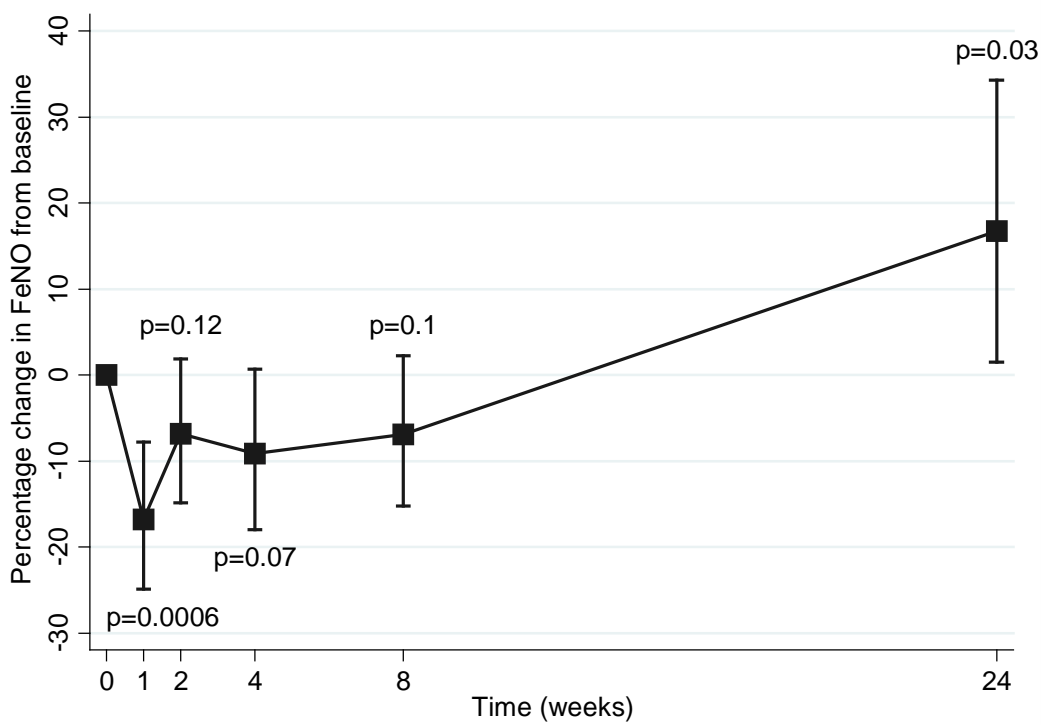


**Figure 4: Fitted values of  $FE_{NO}$  by weight, in pulmonary tuberculosis patients and healthy controls**



**Figure 5: Change in  $FE_{NO}$  in TB patients between diagnosis and treatment completion**

Means (squares) and 95% confidence intervals (whiskers) are shown. P values relate to differences in  $FE_{NO}$  compared with baseline.



**Figure 6: Receiver-operator characteristics for change in  $FE_{NO}$  compared with sputum microscopy conversion time\* as predictors of 2-month sputum culture status (where 1=culture negative, 0=culture positive).**

\*given as inverse to generate comparable ROC curves since higher values for sputum smear clearance time are associated with positive sputum culture, but higher values of  $\Delta FE_{NO}$  are associated with negative sputum culture.

