Host and pathogen factors that influence variability of *Mycobacterium tuberculosis* lipid body content in sputum from patients with tuberculosis: an observational study

Baye G Tarekegn, Leopold D Tientcheu*, Jonathan Decker*, Andrew J Bell, Galina V Mukamolova, Beate Kampmann, Gashaw Messele, Tadeye Abeje, Abraham Aseffa, Hazel M Dockrell, Pranabashis Haldar, Michael R Barer, Natalie J Garton*

Summary

Background High proportions of *Mycobacterium tuberculosis* cells in sputum containing triacylglycerol-rich lipid bodies have been shown to be associated with treatment failure or relapse following antituberculous chemotherapy. Although lipid body determination is a potential biomarker for supporting clinical trial and treatment decisions, factors influencing variability in sputum frequencies of lipid body-positive (%LB$^+$) *M tuberculosis* in patients are unknown. We aimed to test our hypothesis that exposure to host-generated NO and *M tuberculosis* strains are factors associated with differences in sputum %LB$^+$.

Methods In this observational study, we determined %LB$^+$ frequencies before treatment by microscopy in patients with smear-positive tuberculosis from two separate prospective observational study settings (Gondar, Ethiopia, recruited between May 1, 2010, and April 30, 2011, and Fajara, The Gambia, who provided sputum samples before treatment between May 5, 2010, and Dec 22, 2011). In Ethiopia, fractional exhaled nitric oxide (FeNO) was measured as a biomarker of host NO, and *M tuberculosis* strain differences were determined by spoligotyping. Treatment response was assessed by percentage weight change after 7 months. In The Gambia, treatment responses were assessed as change in BMI and radiographic burden of disease after 6 months. Sputum *M tuberculosis* isolates were studied in vitro for their %LB$^+$ and triacylglycerol synthase 1 (*tgs1*) mRNA responses to NO exposure. Propidium iodide staining was used as a measure of NO strain toxicity. Correlation between in vitro %LB$^+$ frequencies following NO exposure and those of the same strain in sputum was examined with linear regression and Dunnett's multiple comparison test.

Findings In Ethiopia, 73 patients who were smear positive for pulmonary tuberculosis were recruited (43 [59%] were male and 30 [41%] were female). Of these, the %LB$^+$ in the sputum of 59 patients showed linear correlation with $\log_{10}$ FeNO (r^2=0·28; p<0·0001) and an association with strain spoligotype was suggested. Seven *M tuberculosis* strains from The Gambia showed different dose-responses to NO in vitro, demonstrated by changing lipid body content, *tgs1* transcription, and bacterial toxicity. In sputum %LB$^+$ frequencies correlated with in vitro %LB$^+$ responses to NO of the corresponding isolate. In a subset of 34 patients across both cohorts, higher sputum %LB$^+$ frequencies before treatment were associated with weaker responses to treatment than lower sputum %LB$^+$ frequencies.

Interpretation *M tuberculosis* strain and exposure to host-generated NO are associated with sputum %LB$^+$. Our results support the use of *M tuberculosis* strain-dependent sputum %LB$^+$ as a predictive biomarker of treatment response.

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*Contributed equally

Department of Respiratory Sciences, University of Leicester, Leicester, UK (B G Tarekegn PhD, J Decker MSc, A J Bell PhD, Prof G V Mukamolova PhD, P Haldar MD, Prof M R Barer PhD, N J Garton PhD); **Leicester Tuberculosis Research Group, University of Leicester, Leicester, UK** (Prof G V Mukamolova, P Haldar, Prof M R Barer, N J Garton); **National Institute for Health and Care Research Leicester Biomedical Research Centre, Leicester, UK** (Prof G V Mukamolova, P Haldar, Prof M R Barer, N J Garton); **Department of Medical Microbiology** (B G Tarekegn) **and Department of Surgery** (G Messele MD), **University of Gondar, Gondar, Ethiopia; Medical Research Council Unit, The Gambia at London School of Hygiene & Tropical Medicine, Vaccines and Immunity Theme, Fajara, The Gambia** (L D Tientcheu PhD, Prof B Kampmann PhD); **Institut für Internationale Gesundheit and Centre for Global Health, Charité - Universitätsmedizin Berlin, Berlin, Germany** (Prof B Kampmann); **Armauer Hansen Research Institute, Addis Ababa, Ethiopia** (T Abeje MD, A Aseffa MD); **Department of Infection Biology, London School of Hygiene & Tropical Medicine, London, UK** (L D Tientcheu, Prof H M Dockrell PhD); **Department of Clinical Microbiology, University Hospitals of Leicester NHS Trust, Leicester, UK** (Prof M R Barer)

Introduction

Clinical laboratory analyses have made key contributions to patient and public health management of tuberculosis. In the past two decades, analytical developments have advanced the diagnosis,[1] treatment,[2,3] and epidemiology[4] of tuberculosis. However, a key challenge remains the assessment and understanding of the persister *Mycobacterium tuberculosis* population, which is widely believed to contribute to slow treatment response and relapse risk.[5–8] Assessment of this bacterial subpopulation, at commencement of and during therapy, has the potential to inform prognosis and personalise management.

Our previous work[9,10] demonstrated the occurrence of *M tuberculosis* triacylglycerol lipid bodies in sputum samples and their association with the persister state. Expression of *tgs1* (which encodes the most active triacylglycerol synthase enzyme) in *M tuberculosis* is regulated by the dormancy-associated and NO-responsive transcriptional regulator DosR.[11] Lipid body-positive and triacylglycerol-rich mycobacterial cells have been associated with bacterial dormancy elicited by multiple stimuli and with tolerance to

Correspondence to:
Dr Natalie J Garton, Department of Respiratory Sciences, University of Leicester, Leicester LE1 9HN, UK
njg17@le.ac.uk

Research in context

Evidence before this study

The prolonged treatment required for tuberculosis is considered to reflect the burden of antibiotic-tolerant *Mycobacterium tuberculosis* bacilli, known as persisters. Patient-specific frequencies of lipid body-positive (%LB$^+$) *M tuberculosis* determined by sputum microscopy have emerged as a candidate assessment of this population and are associated with treatment outcomes. Lipid bodies comprise intrabacillary accumulations of triacylglycerol associated with expression of triacylglycerol synthase 1 (*tgs1*). *Tgs1* expression increases on exposure to NO, a key stimulus of the dormancy-associated DosR regulon in *M tuberculosis*, providing potential linkage between lipid bodies and bacterial growth states that are associated with persisters. Using the terms "*Mycobacterium tuberculosis*", "lipid bodies", "triacylglycerol synthase", and "nitric oxide", we searched PubMed for peer-reviewed studies published between database inception and July 1, 2023. We identified no studies relating *M tuberculosis tgs1* expression and lipid body response to nitric oxide exposure in sputum. Our aim here was to elucidate host and bacterial factors underpinning different sputum %LB$^+$ and their linkage to treatment responses.

Added value of this study

We combined data from two independently conceived clinical studies (in Ethiopia and The Gambia) to assess the basis for %LB$^+$ variation between patients. In Ethiopian patients we correlated lipid body frequencies with patient exhaled NO levels, and spoligotyping indicated that this feature was *M tuberculosis* strain and genotype dependent. In both locations, assessments further supported the view that higher %LB$^+$ associates with weaker treatment responses. In vitro analysis of seven Gambian *M tuberculosis* sputum isolates revealed strain-specific NO dose-responses with %LB$^+$ and *tgs1* transcript increases at lower exposures and lethality at higher exposures. A correlation was observed between isolate-specific in vitro %LB$^+$ and that which was determined in the cognate sputum sample.

Implications of all the available evidence

Our results support use of sputum %LB$^+$ as a predictive biomarker of treatment response. Calibration of this biomarker is *M tuberculosis* strain dependent and could be developed to inform clinical prognosis.

antimycobacterial agents.[10,12–15] In the sputum of patients with tuberculosis, lipid body-positive *M tuberculosis* are abundant and transcriptional and growth characteristics (ie, subpopulations that cannot be cultivated by traditional techniques) consistent with slow replication and dormancy are present.[10,16] High lipid body-positive *M tuberculosis* frequencies (%LB$^+$) in sputum are patient specific and are associated with treatment failure or relapse.[17]

We combine results from separately conceived studies in Ethiopia and The Gambia, investigating the clinical significance of %LB$^+$ frequencies in sputum. In vitro experiments elucidating bacterial interactions with NO link these studies and address the hypothesis that sputum %LB$^+$ frequencies reflect host-specific and *M tuberculosis* strain-specific properties with implications for the outcome of chemotherapy. In Ethiopia we studied the relationship between fractional exhaled NO (FeNO) and sputum %LB$^+$, in The Gambia we studied %LB$^+$ alone, and at both sites we investigated treatment responses. Our in vitro studies enabled us to propose a model linking bacterial responses to NO and our clinical findings.

Methods

Study design and participants

In this observational study, we included patients with tuberculosis who were part of two separate prospective observational studies in Gondar, Ethiopia[18] and Fajara, The Gambia.[19] Both cohorts had sputum containing acid-fast bacilli and received 6 months of standard directly observed treatment.

In the Ethiopian cohort, to investigate the relationship between %LB$^+$ and FeNO, 73 patients who were smear positive for pulmonary tuberculosis were recruited between May 1, 2010, and April 30, 2011, independent of HIV status.[18] The inclusion criteria were provision of informed written consent to participate, acid-fast bacilli-positive sputum sample (grades 2+–4+), and aged 18 years or older. Sputum %LB$^+$ rates before treatment were determined, FeNO measurement taken, blood samples were collected for full blood count and HIV-status, and stool samples were collected for intestinal parasite determination. Individuals providing FeNO measurements that did not exceed a background threshold of 5 parts per billion (ppb) or were more than 3 SD from the cohort mean were excluded from analyses. The Ethiopian study was approved by the University of Gondar Ethics Committee.

In The Gambia, 29 patients without HIV participating in a study of immunological responses to tuberculosis[19] provided sputum samples before treatment between May 5, 2010, and Dec 22, 2011, for %LB$^+$ analysis in the present study. The inclusion criteria for the Gambian study and the present study were provision of informed written consent to participate, no previous history of tuberculosis disease, and aged 15 years or older. The Gambian study was approved by the Gambian Government Medical Research Council Joint Ethics Committee in The Gambia and the London School of Hygiene & Tropical Medicine Ethics Committee in the UK.

Formaldehyde-fixed sputum smears from the Ethiopian study were frozen and shipped to the University of Leicester, UK, for %LB$^+$ determination from June to September, 2011. Decontaminated sputum samples collected in The Gambia for %LB$^+$ determination were frozen and shipped to the

University of Leicester, UK, for analysis from Oct 25, 2013, to Nov 25, 2013. Additional details of sample collection and a flow diagram indicating the organisation of the two studies are in the appendix (pp 2–5, 8).

Procedures

FeNO concentration was measured within 24 h of delivery of the sputum samples, using a NIOX MINO airway inflammation monitor (Aerocrine AB, Solna, Sweden) according to the manufacturer's instructions.

Sputum samples were decontaminated with sodium hydroxide and N-acetyl cysteine, prepared as smears and fixed with formaldehyde. Smears were then stained with auramine and either Nile Red or LipidTox Red for %LB$^+$ assessment.

Weight change (as percentage change from baseline) was determined at 7 months after treatment initiation in the Ethiopian cohort. BMI and chest x-ray were measured in the Gambian cohort.[19] In this study, the relationships between %LB$^+$ and changes between baseline and post-treatment BMI and chest x-ray were assessed.

In addition to *M tuberculosis* H37Rv, Gambian sputum isolates with the following designations and spoligotype were used for assessing responses to NO in vitro: H1 (Haarlem 1), L10 (Latin or central American), L2 (Latin American), L9 (Latin American), Bj (Beijing), T3 (T), and U (Uganda). Details of initial *M tuberculosis* H37Rv NO studies are in the appendix (p 3).

M tuberculosis H37Rv and the sputum isolates from The Gambia were cultured on Middlebook 7H11 medium until just subconfluent as a fine lawn (appendix p 13) and then they were harvested for induction with an NO donor (spermine.NONOate) or a control compound (spermine.4HCl). After 4 h incubation at 37°C, cells were harvested for determination of *tgs1* and 16SrRNA expression with qRT-PCR, %LB$^+$ with Nile Red labelling, and assessment of propidium iodide staining indicating membrane damage. For comparison between samples, *tgs1* transcript levels were normalised with levels of 16SrRNA transcripts. Assessment of NO toxicity in treated samples was made by examining the relationship between the propidium iodide-positivity (as a proportion of control treated samples) and %LB$^+$ with linear regression analysis. Additional details of procedures are in the appendix (pp 2–5).

Statistical analysis

The primary outcome of this study was evaluation of the association between %LB$^+$ and FeNO. Secondary outcomes were the effect of strain on this association and evaluation of association of %LB$^+$ and treatment response. Statistical analyses were performed using Stata 12, SPSS, and GraphPad Prism v9. We used a multivariate generalised regression model, including both continuous and categorical variables recorded at baseline, to explore factors associated with %LB$^+$ *M tuberculosis* in sputum. Non-parametric continuous variables normalised by log-transformation were appropriate before statistical analysis, as indicated (table 1). ANCOVA was performed in SPSS to compare the linear relationship of %LB$^+$ acid-fast bacilli and FeNO, according to HIV status.

We used one-way ANOVA to compare the %LB$^+$ of the different spoligotype groups. Comparison between more than two groups was done using a Kruskal–Wallis test with Dunnett's correction for multiple comparisons with adjusted p values provided as directed by GraphPad Prism. Univariate associations between stated continuous variables were performed using linear regression. For all analyses statistical significance was considered as p of or less than 0·05.

	β coefficient	Standard error	p value
Gender, reference group is female	0·319	3·512	0·93
Age, years	0·083	0·106	0·43
Parasite infection, reference group is infection present	–1·176	3·329	0·72
HIV status, reference group is HIV positive	–12·620	3·322	<0·0010
FeNO*	33·748	7·275	<0·0010
Blood eosinophils*	–9·220	3·972	0·020

FeNO=fractional exhaled nitric oxide. *These variables were log transformed to give normal distributions.

Table 1: **Factors associated with lipid body frequencies of acid-fast bacilli in sputum in Ethiopian cohort (n=73)**

Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

Results

In Ethiopia, 73 patients were recruited, of whom 43 (59%) were male and 30 (41%) were female, and the mean age was 32 years (SD 16; appendix p 6). FeNO results from 14 patients were excluded from the overall analyses: 13 patients' results did not exceed background (5 ppb) and the FeNO for one patient was more than 3 SD away from the mean, which was probably driven by factors other than those assessed here. The 59 FeNO values included for analysis ranged from 6 to 49 ppb. Because parasitic infection was found to be frequent (32 [44%] of patients; appendix p 7) we included this in our analyses.

In The Gambia, 29 HIV-negative patients were recruited as previously described.[19] Nine (31%) of the 29 patients were female and 20 (69%) were male, and the median age was 27 years (range 15–64).

In a multivariate general linear model, HIV status, FeNO, and blood eosinophils were statistically significant independent predictors of %LB$^+$, with FeNO showing the strongest association (table 1). The univariate linear correlation (r^2) of FeNO with %LB$^+$ was 0·28 (p<0·0001; figure 1A). In contrast with FeNO, blood eosinophil count was negatively correlated with %LB$^+$ (β coefficient –9·22; table 1), possibly reflecting the association between blood

See online for appendix

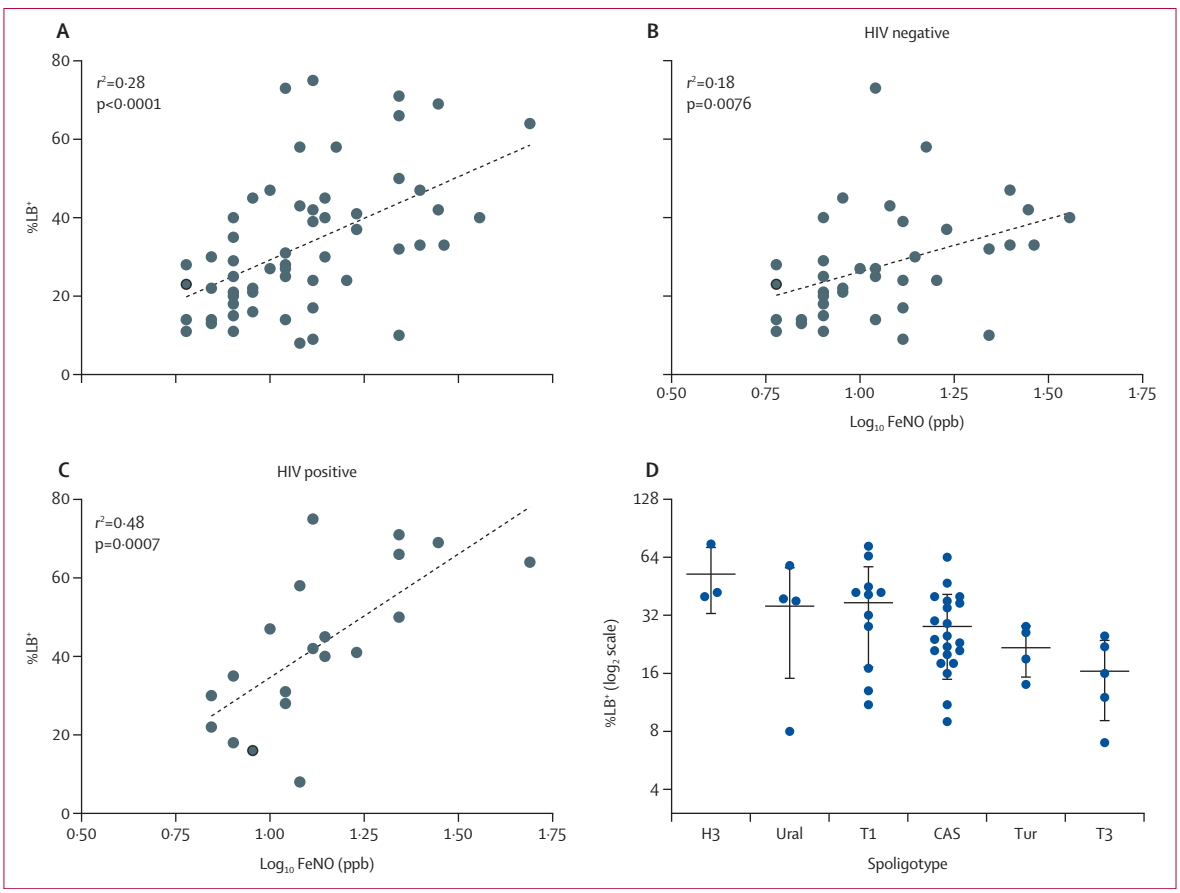

Figure 1: **The correlation of pretreatment *Mycobacterium tuberculosis* %LB$^+$ in sputum with FeNO in the whole patient dataset (A), HIV-negative patients (B), and HIV-positive patients (C), and %LB$^+$ in sputum by *M tuberculosis* spoligotype (D)**

The result from each sample is displayed as a single point. In panel D the midline of the bars represents the mean and the whiskers represent the SD. %LB$^+$=frequencies of lipid body-positive *M tuberculosis*. FeNO=fractional exhaled nitric oxide. ppb=parts per billion. H3=Haarlem 3. T1=T family 1. CAS=Central Asian Strain. Tur=Turkey. T3=T family 3.

eosinophils and parasitic infection in this cohort. HIV-positive status was associated with a significantly higher mean %LB$^+$ than HIV-negative status (38·8 [SD 18·4] *vs* 26·8 [SD 14·0]; mean difference 12·0 [95% CI 4·0–19·9]; p<0·0050). Using ANCOVA, HIV status was found to be a significant determinant of the linear relationship between %LB$^+$ and FeNO (figure 1B, C), with a significantly steeper gradient in HIV-positive patients (p=0·043).

We explored the possibility that %LB$^+$ frequencies might associate with spoligotypes available for Ethiopian isolates (figure 1D). There was a significant difference in the mean %LB$^+$ across different spoligotypes (p=0·033), suggesting that spoligotype might be an important determinant of %LB$^+$. No pairwise comparisons reached statistical significance. Exploration of spoligotype-specific relationships between FeNO and %LB$^+$ suggested different relationships within the different spoligotype groups (appendix p 9). A significant positive correlation between FeNO and %LB$^+$ was observed within the Central Asian Strain (CAS) group (p=0·0060); however, meaningful statistical inferences were precluded in other groups by low numbers.

In Ethiopian patients at 7 months, there was a significant negative correlation between %LB$^+$ and percentage weight gain (r^2=0·45 p=0·049; figure 2A). Although the number of patients was small, stratification by HIV status revealed a closer significant negative correlation in the HIV-negative group (r^2=0·98, p=0·013; n=4) compared with the HIV-positive group that showed non-significant correlation (r^2=0·42, p=0·24; n=5; figure 2B, C). Baseline FeNO showed a negative correlation with weight change but was not statistically significant (r^2=0·52, p=0·11; appendix p 10).

In The Gambian cohort there was a significant positive association between baseline sputum %LB$^+$ and extent of chest x-ray disease after 6 months of chemotherapy (n=24; r^2=0·23; p=0·013; figure 2D). There was also a non-significant negative correlation between BMI gain and %LB$^+$ (n=25; r^2=0·102; p=0·12; figure 2E).

As a point of reference, we first studied the *M tuberculosis* type strain, H37Rv. NO exposure elicited dose-dependent decreases in growth (^{3}H-uracil uptake) together with increases in *tgs1* expression, triacylglycerol, and lipid body content, and increased tolerance to isoniazid

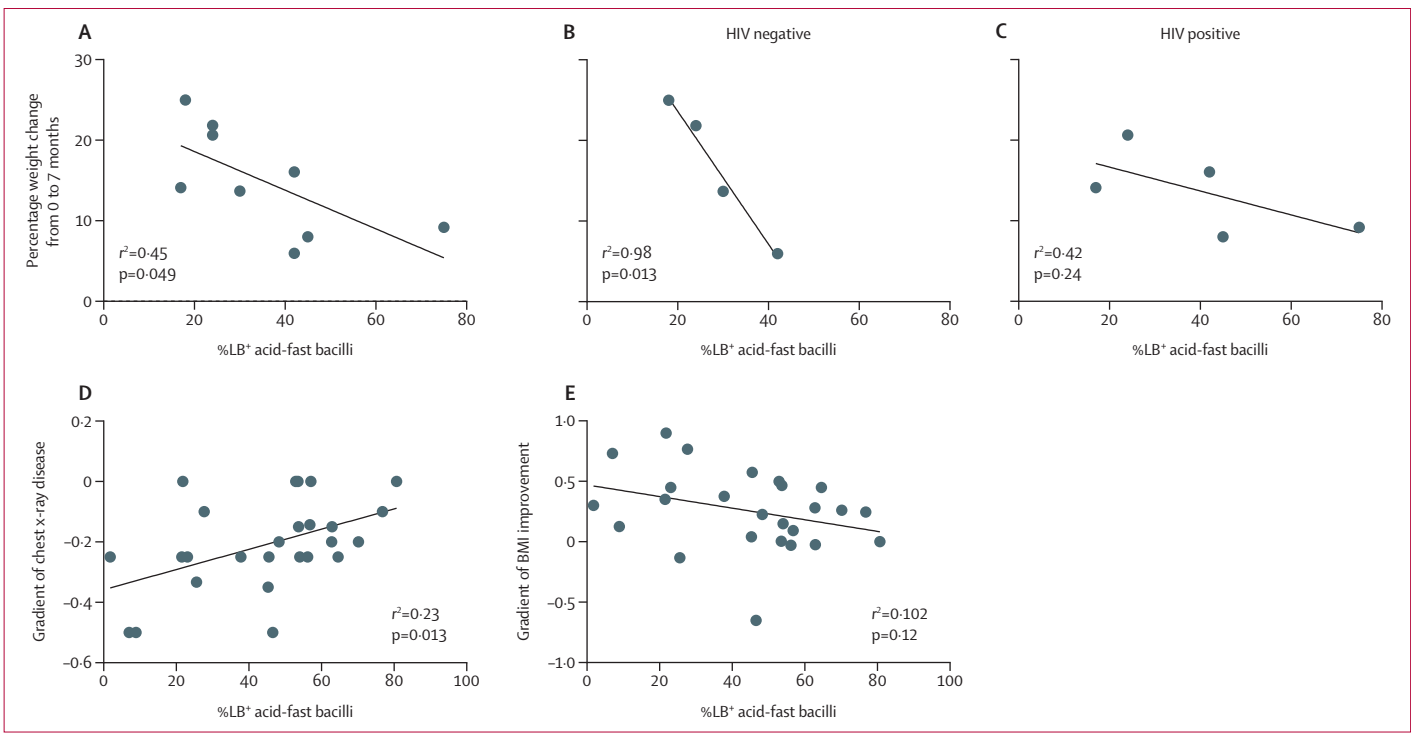

Figure 2: **The correlation of sputum %LB⁺ at diagnosis with treatment responses**
The percentage weight gain of Ethiopian patients at 7 months after initiation of therapy in all patients (A), HIV negative patients (n=5; B), and HIV-positive patients (n=4; C). (D) Chest x-ray improvement of Gambian patients (n=24), whereby a negative score denotes a reduction in disease. (E) BMI improvement whereby a positive score indicates an increase in BMI of Gambian patients (n=25). %LB⁺=frequencies of lipid body-positive *Mycobacterium tuberculosis*.

(appendix pp 11–12). These features reinforce our basic premise that growth state, lipid content, and antibiotic tolerance are inter-related, and the possibility that strain-related variations in these responses might substantially impact on treatment responses.

Seven *M tuberculosis* isolates from the Gambian cohort and H37Rv were available for comparison. Having established experimental conditions enabling comparison of multiple strains on the same day (appendix p 13), the effects of exposure to NO (50–500 μM) for 4 h on %LB⁺ were determined (figure 3A). Each strain showed distinct absolute values; the predominant pattern was of an initial increase in %LB⁺ (50–100 μM NO) and a decline at higher doses. Notably, Beijing, with the highest baseline value, showed no significant increase. To test the possibility that the declines reflected toxic effects of NO, we assessed propidium iodide staining (reflecting cell membrane damage) of the bacilli after 4 h NO exposure. A striking negative correlation was observed between increase in propidium iodide-positivity and associated declines in observed %LB⁺ (r^2=0·96, p<0·0001; figure 3B), supporting the view that the observed declines in lipid body content reflected *M tuberculosis* cell injury. Strain H1 showed the greatest susceptibility to NO with an increase in propidium iodide labelling of over 30% following this exposure.

We also compared the effects of NO on *tgs1* transcription across our strains. In contrast to the effect on %LB⁺, all the strains increased *tgs1* transcription in response to 250 μM and 500 μM NO (figure 3C, D). There were clear differences in both baseline expression and responses to NO. Distinct patterns were shown by the Beijing strain, with high baseline *tgs1* expression, but only a three-fold increase with NO, and by H37Rv, with very low baseline activity, but with an increase of over 200-fold with NO induction; the remaining strains all had similar maximal expression (145×10^{-5} [SD 15×10^{-5}] *tgs1* copies per 16SrRNA transcript).

To address our primary hypothesis, that sputum *M tuberculosis* %LB+ reflects exposure to differing NO levels in vivo and secondarily, that this should be related to bacterial strain, we examined correlations between the in vitro %LB⁺ following NO exposure and those determined in sputum. At baseline (without exposure to NO) and NO concentrations of 50 μM and 100 μM, strong (r^2 >0·9) and significant (p<0·0020) correlations with the sputum %LB⁺ were revealed by linear regression (table 2). The lower r^2 values for 250 μM and 500 μM exposures reflect increasing lethal effects. Gradients close to unity indicated that strains are ranked the same by the %LB⁺ in sputum and in vitro. Paired analyses clearly indicated that the 250 μM NO exposure gave %LB⁺ values close to those determined in sputum, with 50 μM a close second (Dunnett's multiple comparisons test; mean difference of 5·429 [adjusted p=0·96] for 250 μM NO exposure and 3·700 [adjusted p=0·83] for 50 μM NO exposure; where p=1 indicates

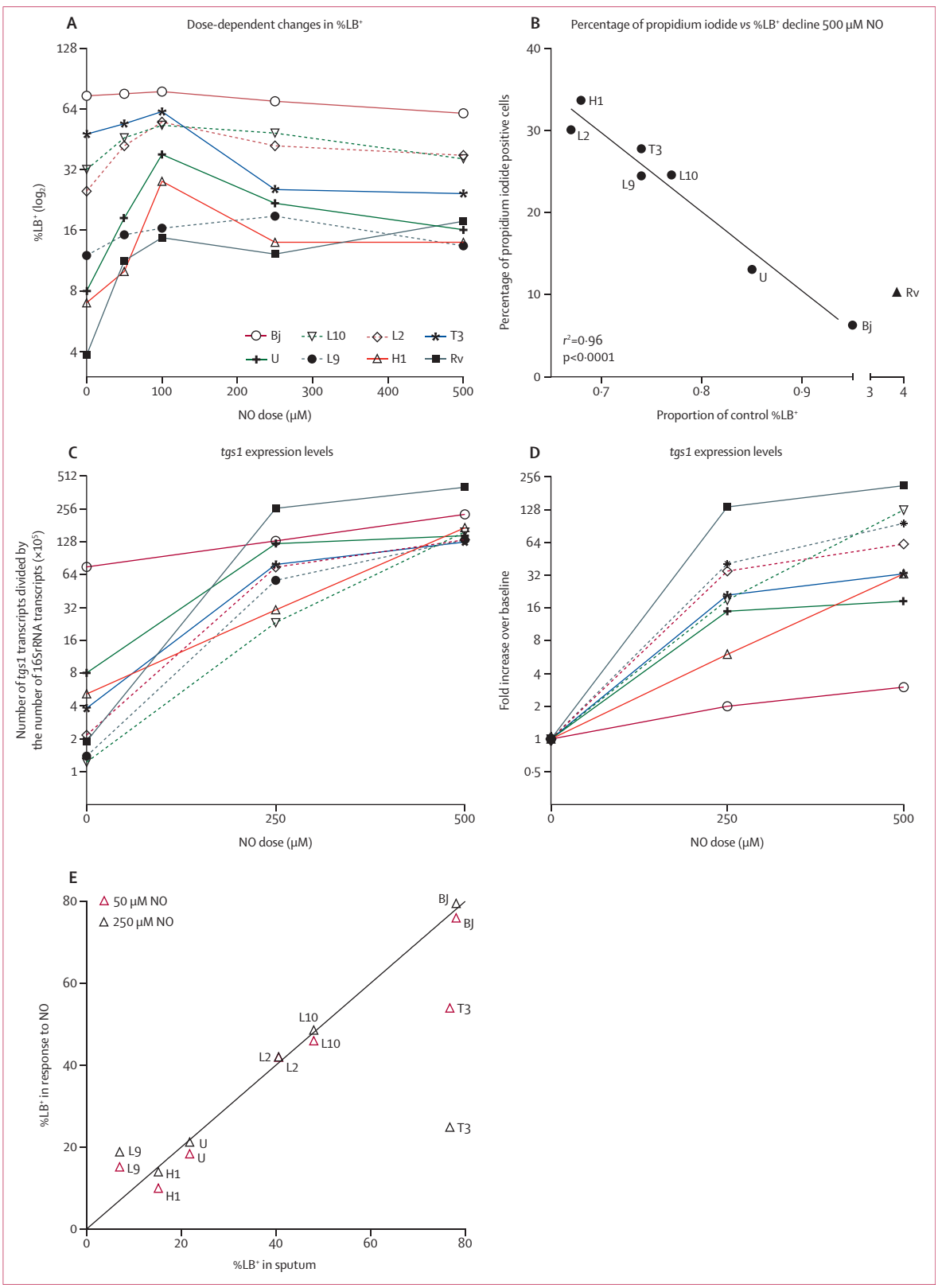

Figure 3: **NO exposure in vitro**
(A) %LB$^+$ assessed 4 h after exposure to varying concentrations of NO. (B) Percentage of propidium iodide-stained cells after 4 h 500 μM NO exposure compared with the corresponding %LB$^+$ as proportion of control %LB$^+$ for each individual strain. All assessments were made in duplicate. (C) *tgs1* transcript responses (quantitative RT-PCR) normalised to 16SrRNA transcript numbers assessed following 4 h NO exposure. (D) *tgs1* transcript fold increases over baseline levels. (E) Strain-related association between %LB$^+$ in sputum and in vitro in response to 50 μM and 250 μM NO exposure of the seven *Mycobacterium tuberculosis* strains from The Gambia (table 2). %LB$^+$=frequencies of lipid body-positive *Mycobacterium tuberculosis*. Bj=Beijing. H1=Haarlem 1. L2=Latin American. L9=Latin American. L10=Latin or central American. Rv= H37Rv. T3=T family 3. U=Uganda.

identity); these two results are shown in figure 3E compared with a reference line of identity. Strain T3 is clearly an outlier with its in vitro %LB$^+$ responses well below identity with sputum, suggesting that it might have greater sensitivity to NO than the other isolates.

Discussion

We have used results from patients with tuberculosis in two African countries and the in vitro responses of related *M tuberculosis* isolates to improve our understanding of the factors determining %LB$^+$ frequencies observed in sputum samples. Clinical and laboratory data indicate that %LB$^+$ frequencies are linked to treatment responses in tuberculosis.[10,17] Our results show that %LB$^+$ frequencies in sputum were associated with patient FeNO and bacterial strain. Bacterial responses to NO in vitro were strain variable. We found further evidence that high %LB$^+$ frequencies in sputum correlate with poor responses to treatment. Finally, we showed that %LB$^+$ of different *M tuberculosis* strains induced with NO in vitro strongly correlated with the %LB$^+$ of the same strain in sputum. We propose that in vivo exposure to NO contributes to this correlation.

In Ethiopian samples, sputum %LB$^+$ and FeNO analysis revealed clear correlation. Multivariate analysis showed that FeNO, blood eosinophils, and HIV status were all independent determinants of sputum %LB$^+$ (table 1), with FeNO and blood eosinophils showing significant linear associations, and with FeNO the strongest association. Eosinophilia is well known to associate with FeNO, possibly reflecting the high rate of enteric helminth parasitism in this population.[20–22] Although %LB$^+$ was significantly associated with HIV status, there was also evidence that *M tuberculosis* produced more lipid bodies relative to the concurrent FeNO level in HIV-positive patients (figure 1C).

Because 28% of the variation seen in patients' sputum %LB$^+$ was associated with their measured FeNO, we considered other contributing factors. Examination of spoligotype and associated FeNO provided suggestion that strain differences might be important, although group numbers were low (appendix p 9). Review of the ranges of sputum %LB$^+$ for the separate spoligotypes suggested strain-associated differences (figure 1D) within the Ethiopian isolates. It is also possible that local hypoxia could have contributed to the observed %LB$^+$ values via DosR activation,[23] but this factor was not assessable.

Treatment responses were monitored in a subset of patients by weight gain in Ethiopia, and by chest x-ray score and BMI in The Gambia (figure 2). Both studies provide modest support for the hypothesis that high pretreatment sputum %LB$^+$ frequencies associate with less favourable treatment responses, as found previously.[17] Interestingly, although only four individuals were available for inclusion, weight gain showed a particularly strong association with HIV-negative individuals in Ethiopia.

The associations between %LB$^+$, FeNO, bacterial strain, HIV status, and treatment responses raise questions concerning the underlying mechanisms linking these observations. Our central hypothesis was that bacterial strain differences in response to NO exposure might reveal distinct dose–response relationships and that this could account for the variation in sputum %LB$^+$, beyond that associated with FeNO.

	NO exposure				
	0 μM	50 μM	100 μM	250 μM	500 μM
Linear regression					
r^2	0·93	0·90	0·90	0·49	0·56
p value	0·0005	0·0011	0·0011	0·081	0·054
Equation	Y=0·69X – 2·32	Y=0·8X + 4·57	Y=0·7X + 18·4	Y=0·56X + 12·48	Y=0·44X + 11·15
Dunnett's multiple comparisons test					
Mean difference	15·010	3·700	–6·129	5·429	11·830
Adjusted p value*	0·038	0·83	0·56	0·96	0·51

*Adjusted as directed by GraphPad package.

Table 2: **Association between the frequency of lipid body-positive *Mycobacterium tuberculosis* in vitro following NO exposure with those observed in the sputum from which they were isolated**

Seven Gambian *M tuberculosis* clinical isolates and the type strain, H37Rv, showed a range of baseline %LB$^+$, and differing NO responses with respect to %LB$^+$, cell membrane integrity, and *tgs1* transcription. Culture as a fine, subconfluent lawn that enabled us to compare multiple strains on the same day resulted in minimal H37Rv %LB$^+$ and the highest value for the Beijing strain as expected (figure 3A). Constitutive expression of the DosR regulon (and hence *tgs1*) has been reported in Beijing strains during growth in liquid culture, and results in increased triacylglycerol content compared with H37Rv.[24] In our study, the Beijing strain had the highest baseline *tgs1* expression of all tested strains, nearly ten-fold greater than the strain with the next-highest expression, the Uganda strain, and 40-fold higher than that of H37Rv (figure 3C). To our knowledge, differences in *tgs1* expression and triacylglycerol LB%$^+$ content for other strains during growth have not been reported previously. Differences in *tgs1* expression and %LB$^+$ during growth might reflect differing regulation of DosR, as seen with the Beijing strain,[25] or activity of additional triacylglycerol synthases,[23] or both, and remain to be investigated. The %LB$^+$ of most strains showed biphasic NO responses, increasing over baseline with lower NO exposure and decreasing at higher levels (figure 3A). With constitutive *tgs1* expression[24] and high baseline %LB$^+$, the absence of a NO response in the Beijing strain was unsurprising. We confirmed that NO toxicity occurred at higher doses with the propidium iodide exclusion data (figure 3B), but this does not show a relationship with strain baseline %LB$^+$. There was a dissociation between the transcriptional responses, resulting in increased %LB$^+$, and cell toxicity elicited by NO. Variable susceptibility to NO of different *M tuberculosis* strains has previously been reported,[26] but how this might relate to increased *tgs1* expression and lipid body content is not known.

We examined the degree to which our individual strain %LB$^+$ correlated with the cognate sputum %LB$^+$. Most striking was the consistent rank order of the %LB$^+$ between

sputum and in vitro determinations, irrespective of the in vitro conditions applied. This finding raises the possibility that %LB$^+$ determinations applied to patients' *M tuberculosis* isolates directly from laboratory growth might be as strongly related to treatment outcome as determinations on sputum. Given the biphasic patterns of in vitro responses to NO noted earlier, we suggest that any sputum %LB$^+$ might reflect either stimulation, or toxicity associated with host NO production. Thus, we propose a general pattern of *M tuberculosis* dose-responses to NO with initial induction of *tgs1* and associated triacylglycerol synthesis increasing %LB$^+$, moving on to cell damage with increasing dose, all in a strain-specific manner.

The overall biphasic responses to NO shown in our study could have substantial clinical implications. Although the NO concentrations experienced by *M tuberculosis* in the lung are unknown, we suggest that levels equivalent to our lower doses will elicit lipid body production and DosR-mediated responses that could produce antibiotic tolerance.[10,23] At higher NO exposure, bactericidal effects dominate, and bacterial elimination will be favoured. Thus, where patients produce high, sustained FeNO concentrations as observed by Ralph and colleagues,[27] treatment is enhanced by host NO production, as evidenced in their study by increased culture conversion at 2 months. This pattern will be modulated by the NO response characteristics of the infecting *M tuberculosis* strain. Based on our results, we would expect Beijing strains to be relatively unaffected by host NO, and other strains would be susceptible. It is also interesting to note that H37Rv, the laboratory strain that is widely used to explore *M tuberculosis* biology, is an outlier in terms of its NO response.

Our study was limited by the small number of patients, suboptimal clinical metadata, inadequate representation of spoligotype groups, and technical challenges in the laboratory work. Although Ethiopian treatment response numbers were very low, the pattern observed was consistent with that seen in The Gambia, and with treatment outcomes in the earlier Malawi study.[17] Fully powered studies mapping %LB$^+$ to treatment outcome are currently in progress. Small numbers of isolates in Ethiopian spoligotype groups (with the exception of CAS) limited the conclusion that different spoligotype-specific relationships between FeNO and %LB$^+$ exist.

Preparation of multiple clinical isolates with differing in vitro growth characteristics in comparable physiological states for parallel exposures to multiple concentrations of NO presents a formidable technical challenge. The solid medium lawn growth approach developed here (appendix p 13) allowed us to make the comparisons presented, but the experiments were limited by the small number replicates and the range of conditions studied. Nonetheless, we have observed consistent patterns across multiple experiments between in vitro growth and sputum samples analysed at an earlier date.

We conclude that mycobacterial %LB$^+$ in tuberculosis patients' sputum reflects both the bacterial genotype and the host NO response specific to that infection. We suggest that analyses on clinical samples, such as %LB$^+$, where the results reflect both host and pathogen and can be linked to treatment responses, might inform personalised patient management in the future. In tuberculosis this insight could assist in the earlier recognition of effective treatments in clinical trials, and in routine practice, identification of patients who could safely receive shorter treatment might be enabled.

Contributors

MRB, BGT, LDT, and NJG conceived and designed the study. BGT, LDT, JD, GVM, TA, and NJG conducted the experiments. GM was involved in patient recruitment and clinical screening. AJB developed the software used for lipid body analysis. MRB, PH, BGT, LDT, JD, and NJG analysed and interpreted the data. MRB and BK acquired funding that supported the study. MRB, BK, HMD, and AA provided supervision. MRB and NJG verified the underlying data. MRB and NJG drafted the manuscript. All authors reviewed, revised, and gave final approval to the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit the manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

The data described in the results of this study are presented within the paper and appendix. The raw data generated and analysed here are available from the corresponding author following publication.

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