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Sputum is a surrogate for bronchoalveolar lavage for monitoring *Mycobacterium tuberculosis* transcriptional profiles in TB patients☆

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SUMMARY

Pathogen-targeted transcriptional profiling in human sputum may elucidate the physiologic state of *Mycobacterium tuberculosis* (*M. tuberculosis*) during infection and treatment. However, whether *M. tuberculosis* transcription in sputum recapitulates transcription in the lung is uncertain. We therefore compared *M. tuberculosis* transcription in human sputum and bronchoalveolar lavage (BAL) samples from 11 HIV-negative South African patients with pulmonary tuberculosis. We additionally compared these clinical samples with *in vitro* log phase aerobic growth and hypoxic non-replicating persistence (NRP-2). Of 2179 *M. tuberculosis* transcripts assayed in sputum and BAL via multiplex RT-PCR, 194 (8.9%) had a p-value <0.05, but none were significant after correction for multiple testing. Categorical enrichment analysis indicated that expression of the hypoxia-responsive DosR regulon was higher in BAL than in sputum. *M. tuberculosis* transcription in BAL and sputum was distinct from both aerobic growth and NRP-2, with a range of 396–1020 transcripts significantly differentially expressed after multiple testing correction. Collectively, our results indicate that *M. tuberculosis* transcription in sputum approximates *M. tuberculosis* transcription in the lung. Minor differences between *M. tuberculosis* transcription in BAL and sputum suggested lower oxygen concentrations or higher nitric oxide concentrations in BAL. *M. tuberculosis*-targeted transcriptional profiling of sputa may be a powerful tool for understanding *M. tuberculosis* pathogenesis and monitoring treatment responses *in vivo*.

1. Introduction

Understanding the physiologic state and adaptations of *Mycobacterium tuberculosis* (*M. tuberculosis*) in human patients with tuberculosis (TB) is important because the effectiveness of antibiotics depends on the physiologic state of the bacterium [1–3]. It is therefore essential to develop robust methods for *in vivo* monitoring and evaluation of pathogen physiology, since the physiologic state of *M. tuberculosis* in human tissues likely differs from the state of *M. tuberculosis* in *in vitro* and murine models of tuberculosis [4–7].

The physiologic state of *M. tuberculosis* can be deduced in human clinical samples using pathogen-targeted transcriptional profiling [5,6]. A promising practical application of this technique is monitoring how drug treatment alters the *M. tuberculosis*

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transcriptome. Drug treatment of human patients induces drug-tolerant "persister" phenotypes that require prolonged treatment [5,8,9]. Monitoring *M. tuberculosis* transcriptional responses during treatment may provide novel pharmacodynamic measures that will enhance evaluation of new drug regimens and help identify patients who are responding poorly to treatment.

In order to use *M. tuberculosis* gene expression profiling as a treatment monitoring tool, it is important to determine whether *M. tuberculosis* gene expression in sputum recapitulates bacterial adaptations in the lung. *M. tuberculosis* rapidly adapts its physiologic state to environmental conditions [4,10] and therefore could have a different transcriptional pattern in sputum than in the lung. Bronchoalevolar lavage (BAL) is an invasive technique for obtaining alveolar and small airways specimens that more directly samples the sites of disease within the lung than does sputum [11]. Since serial collection of BAL for treatment monitoring is infeasible on a large scale, we sought to determine how similar *M. tuberculosis* in sputum is to *M. tuberculosis* in BAL.

We therefore compared the gene expression patterns of *M. tuberculosis* in contemporaneously-collected sputa and BAL fluid. We also examined and compared these results with *in vitro* samples representing two extremes along the continuum of oxygen availability: the aerobic log-phase growth model and the gradual oxygen deprivation model, known as Non-Replicating Persistence 2 (NRP-2) [12], to examine whether either model reflected the *in vivo* state. Since transcriptional profiling has potential practical application for treatment monitoring, we contextualized transcriptional differences between sputum and BAL by comparison with transcriptional changes induced in human sputum by drug treatment. Finally, to determine if differences in *M. tuberculosis* expression between sputum and BAL might reflect different host immune responses, we assayed selected human genes associated with granuloma formation and inflammatory response in the same sputa and BAL.

2. Methods

2.1. Enrollment and collection of human specimens

HIV-uninfected adults with sputum acid-fast bacillus positive pulmonary TB were enrolled prior to antibiotic treatment at Stellenbosch University, South Africa. Spontaneously expectorated sputum was collected 15–30 min post bronchoscopy in approximately 10 mL of guanidine thiocyanate (GTC) solution (5 M guanidinium thiocyanate, 0.5% w/v sodium N-lauryl sarcosine, 25 mM trisodium citrate, 0.1 M 2-mercaptoethanol, 0.5% w/v Tween 80 [pH 7.0]) per 5 mL sputum, according to published methods [5]. During bronchoscopy, the right middle lobe bronchus was lavaged with two 60 mL aliquots of saline. 15 mL of returned aspirate was immediately aliquoted into 30 mL GTC solution. Samples were needle-sheared, centrifuged at 9000 × g for 3 min, and stored in Trizol at −80 °C. The Human Research Ethical Committee of the Faculty of Medicine and Health Sciences at Stellenbosch University approved the study. All patients provided written informed consent.

2.2. In vitro experiments

M. tuberculosis H37Rv for log phase aerobic growth was grown in 100 mL 7H9 at 37 °C with four replicates. At mid-log phase growth, 1 mL was transferred to a 250 mL vented flask containing 30 mL 7H9 media (0.05% Tween 80, 0.2% glycerol, 10% ADC supplement) and incubated at 37 °C. At mid-log phase growth 20 mL were transferred into 20 mL of 4M GTC with 10uL B-mercaptoethanol per mL GTC, centrifuged, resuspended in trizol, cells lysed, and RNA extracted using chloroform.

M. tuberculosis for the NRP-2 model was grown in 100 mL Dubos Tween albumin (DTA) at 37 °C with 6 replicates. At mid-log phase growth, 1 mL was transferred into 250 mL of DTA in a 250 mL vented cap flask, incubated, and grown until an OD_{600} of 0.40. Twenty mL were then transferred into a falcon tube, and centrifuged at 800 rpm for 3 min to sediment clumped colonies. Then, 170 uL of this culture were transferred into 17 mL of DTA in a test tube along with a magnetic stir bar. Test tubes were sealed with parafilm and placed on a Wheaton BioStir4 plate with stirring set at 120 rpm. After 14 days, the solution was transferred into 17 mL of 4M GTC with 10uL B-mercaptoethanol per mL GTC, centrifuged, and resuspended in Trizol. The cells were then lysed and RNA extracted.

2.3. RNA extraction and transcriptional profiling

Total RNA was extracted using a phenol/chloroform protocol described in the Supplementary Materials. We assayed expression of 2179 selected *M. tuberculosis* genes (54% of the genome) via multiplex quantitative RT-PCR (TaqMan) with a LightCycler 480 (Roche, Indianapolis, Indiana) using methods previously described in detail [5]. A panel of 262 human genes was also assayed due to their association with inflammatory cytokines and immune response. Details of TaqMan primers are available at http://genes.stanford.edu/oli/genes.php?organism=h.

2.4. Data pre-processing

Data from both the *in vitro* experiments and clinical samples were batch corrected using a median approach. This median approach involves taking the median value of genes resampled across batches and normalizing the subsequent batches to the first batch. Since BAL and sputa were paired samples, the *M. tuberculosis* transcriptional data from clinical samples was normalized using a previously-described minimum variance method [13]. A separate normalization was then performed on the combined *in vitro* and clinical data. As this data was not paired, a median was first taken for each gene, and then the same minimum variance method was used to normalize each sample to this median.

2.5. Analysis

Differential expression was tested via paired t-tests (between BAL and sputum from the same patients) and unpaired, unequal variance t-tests (between clinical samples and *in vitro* samples) with Benjamini-Hochberg (BH) multiple testing correction (Supplemental Data 1). Categorical enrichment in TB-specific functional categories [5] (Supplemental Data 2) and Gene Ontology (GO) [14,15] were evaluated with a modified Fisher's exact test [14,16]. We used different gene lists to calculate enrichment for clinical and *in vitro* data. Since no genes were differentially expressed between BAL and sputum after correction for multiple comparisons, we assessed trends in categorical enrichment using genes with a nominal p-value of <0.05. The resulting categorical enrichment p-values were then corrected using BH multiple testing correction. For comparisons using *in vitro* data, categorical enrichment was quantified using genes with a BH-corrected p-value of <0.05. These categorical enrichments were not further corrected for multiple comparisons. Because of the potential use of *M. tuberculosis* transcriptional profiles for treatment monitoring, we additionally compared differences between sputum and BAL with transcriptional changes induced by drug treatment. For this analysis we used our previously-published data on *M. tuberculosis* expression in the sputum of patients treated for TB [5], a project that used the same platform and approximately the same number of samples.

Host expression profiles were first normalized by minimizing the variance of medians of the housekeeping genes: EEF1A1, GAPD, PPIA, RPL13A. They were then normalized for differential expression by minimizing the variance of the housekeeping genes of the paired sputum and BAL samples.

3. Results

3.1. Greater M. tuberculosis mRNA abundance in sputum than in BAL

mRNA abundance (quantified as median CT of the 100 most highly expressed *M. tuberculosis* transcripts assayed) was lower in BAL than in sputum (Table S1). Even after adjusting for the dilution caused by saline used for alveolar lavage (See supplement), *M. tuberculosis* mRNA was 8.4-fold more concentrated in sputum than in BAL. Measurement of *M. tuberculosis* mRNA was highly repeatable (Figure S1).

3.2. Increased expression of DosR in BAL relative to sputum

A paired comparison of sputum with BAL identified 194 genes (8.9% of genes assayed) with p-value <0.05, suggesting marginally higher differences between sputum and BAL than expected by chance alone. However, after adjustment for multiple comparisons, no genes were significantly differentially expressed.

Although no genes passed multiple comparison adjustment, we explored trends in gene categories using a nominal p-value threshold of 0.05 on the genes with multiple testing correction performed on the categorical enrichments p-values. Enrichment analysis of functional gene categories in genes with p-value <0.05 showed that expression of genes in the DosR regulon was higher in BAL than in sputum (adjusted p-value = 0.0012) (Figure 1a). Of the 48 DosR regulon genes, 45 had higher mean expression in BAL. Bronchoalveolar lavage also had lower expression of ribosome proteins (adjusted p-value of 0.036).

3.3. Transcription in clinical samples differs markedly from in vitro models

As expected, the transcriptional profile of NRP-2 was markedly different from transcription in log-phase aerobic growth (Figure 2a). Consistent with previous studies [17], hypoxic stress in NRP-2 was associated with profound up-regulation of the DosR regulon, oxidative stress responses, and anaerobic respiration and down-regulation of genes related to growth and metabolism.

Both *in vitro* models were also distinct from the clinical samples. A Principal Components Analysis plot (Figure 3) shows clusters of aerobic and NRP-2 samples that are distinct from one another and also distinct from the clinical samples. By contrast, sputum and BAL samples partially overlap. This pattern is corroborated by differential expression testing using an unpaired t-test. A range of 396–1020 genes (18%–47% of genes assayed) was differentially expressed between clinical and *in vitro* samples after multiple comparison correction (Table 1a and Table 1b). Transcription in sputum and BAL was highly correlated (Pearson = 0.91, Table S2), while correlation of these clinical samples to *in vitro* samples was lower (ranging from 0.74 between BAL and NRP-2 to 0.86 between sputum and aerobic growth).

Categorical evaluation of transcriptional differences using genes significantly different between clinical and *in vitro* specimens (p-value <0.05 after Benjamini-Hochberg multiple comparison testing) revealed that BAL and sputum had similar enrichment in functional processes relative to aerobic growth (Figure 2b–c). Relative to aerobic growth, sputum and BAL had significant up-regulation of the DosR regulon and significant down-regulation of ribosomal genes and primary metabolism genes (TCA cycle, ATPases, NADH, and acyl/acetyl CoAs). However, the magnitude of these differences was not as profound as observed in aerobic respiration compared to NRP-2. For example, while DosR is upregulated in clinical samples relative to aerobic growth, clinical samples have significantly lower DosR expression than NRP-2 (Figure 2d–e). In addition to lower DosR expression, sputum and BAL had lower expression of oxidative stress genes than NRP-2. Finally, Figure 2f indicates that the magnitude of changes in expression between sputum and BAL is diminutive relative to comparisons with *in vitro* data.

3.4. Drug effects outweigh differences between sputum and BAL

We compared the relative magnitude of transcriptional differences between sample type (BAL versus sputum) with transcriptional changes induced by drug treatment. In previously-reported (5) *M. tuberculosis* sputum transcriptome data from Ugandans, the

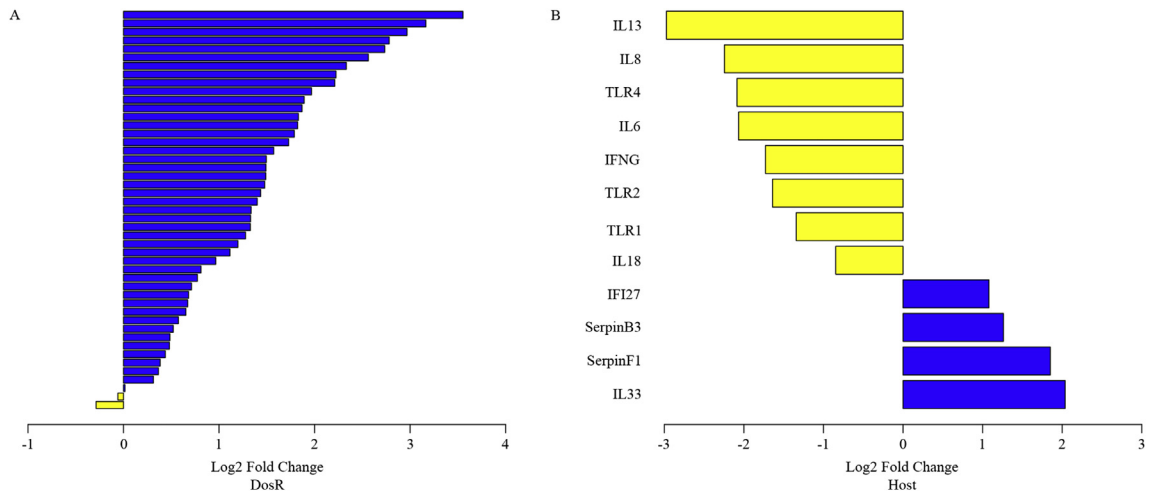

Figure 1. Expression of key *M. tuberculosis* and host genes in BAL relative to sputum (log_2 scale). Upregulation shown in blue, downregulation is shown in yellow. A) Mean expression of 48 *M. tuberculosis* DosR regulon genes in BAL relative to sputum demonstrates up-regulation of DosR in BAL. B) Mean expression of select human immune genes in BAL relative to sputum is consistent with decreased inflammation in BAL.

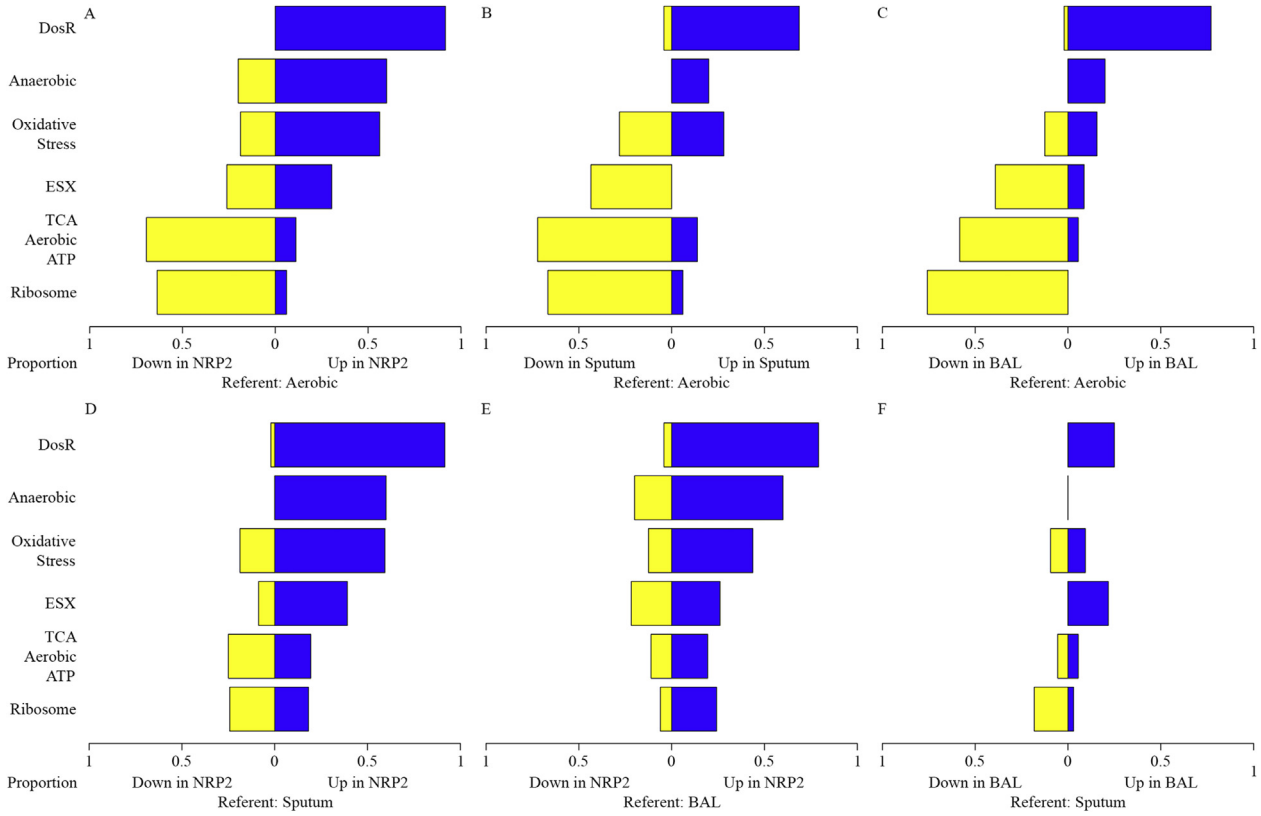

Figure 2. Differential expression of categories related to primary metabolic function, oxygen availability, and virulence between clinical and *in vitro* samples. The proportion of genes in each category that were significantly differentially expressed is illustrated. Genes that did not display statistically significant change are not shown. Categories are ordered from most up-regulated to most down-regulated in NRP2 relative to aerobic growth. Comparison of categorical expression in A) aerobic growth versus NRP-2, B) aerobic growth versus sputum, C) aerobic growth versus BAL, D) sputum versus NRP-2, E) BAL versus NRP-2, and F) sputum versus BAL.

number of genes differentially expressed (p-value < 0.05) before and after two days of drug treatment was 785, more than 3-fold higher than in the BAL-sputum comparisons (454 genes remained significant after multiple comparisons correction). After seven days of treatment, the number of genes with p-value < 0.05 increased to 1017 (714 remained significant after correction for multiple comparisons). In contrast to the sputum-BAL comparison, drug treatment did not alter DosR expression, and expression of ESAT genes decreased significantly [5].

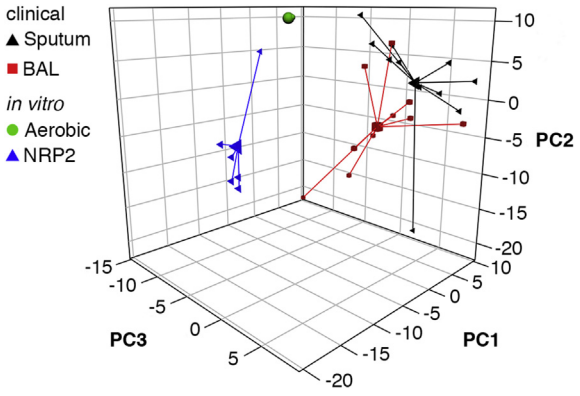

Figure 3. Principal components plot of the *M. tuberculosis* transcriptome in sputum, BAL, the *in vitro* aerobic-growth model, and the hypoxic non-replicating persister (NRP-2) model. Transcriptional differences between sputum and BAL are minor relative to *in vitro* samples, demonstrating distinct *M. tuberculosis* phenotypes in clinical samples.

3.5. Decreased expression of key human immune-related genes in BAL relative to sputum

To determine if the immune milieu of sputum and BAL differ, we assayed 262 human genes. The number of genes with a nominal p-value <0.05 was 73 (28%) in sputum versus BAL comparison with expression of select genes shown in Figure 1b. Relative to sputum, BAL had decreased expression of inflammatory cytokines including interferon-γ, IL-6, IL-8, IL-13, IL-18 and toll-like receptors 1, 2, and 4.

4. Discussion

Our analysis identified only minor differences between *M. tuberculosis* transcription in BAL and sputum. BAL had higher expression of genes of the hypoxia-responsive DosR regulon

Table 1
Genes that are significantly (a) up- and (b) down-regulated (p < 0.05 after multiple testing correction) in the column versus the row.

	Sputum	BAL	Aerated	NRP2
a. Upregulated genes				
Sputum		–	–	–
BAL	0		–	–
Aerated	511	277		–
NRP2	520	367	588	
b. Downregulated genes				
Sputum		–	–	–
BAL	0		–	–
Aerated	302	119		–
NRP2	500	208	897	

suggesting a lower oxygen tension in BAL than in sputum. However, the differences in *M. tuberculosis* transcription between BAL and sputum were minor relative to the massive transcriptional reprogramming in human sputum samples following drug exposure, suggesting sputum can be a surrogate for BAL in monitoring treatment response. Comparison with *in vitro* data suggests that *M. tuberculosis* transcriptional patterns in BAL and sputum are distinct from standard *in vitro* models and may represent a midpoint on a spectrum of oxygen availability between aerobic growth and NRP-2. These findings suggest that sputum is an acceptable surrogate sample for monitoring the bacterial physiologic adaptations of *M. tuberculosis* in the lower airway of humans with pulmonary tuberculosis.

Studying pathogen phenotypes in human patients is important because, as this study reconfirmed, the physiologic state of *M. tuberculosis* in humans differs from that observed *in vitro* and in murine models [4–7]. Systematic evaluation of transcriptional differences between sputum and invasive sampling like bronchoscopy is essential because *M. tuberculosis* rapidly adapts to environmental perturbations and different sample types could represent different environments. TB lesions evolve rapidly [18], displaying a spectrum of cell composition, necrosis and fibrosis, a range of nutrient availability, pH and oxygen tension and variable proportions of intra- and extra-cellular bacilli [3,4]. Spontaneously-expectorated sputum is thought to originate primarily from the air-liquid interface of necrotic granulomas that drain to airways [3]. We hypothesize that bronchoscopic lavage may force saline into less-well-aerated lesions that would not otherwise be open to airways, resulting in relatively greater sampling of hypoxic regions.

Several *M. tuberculosis* transcriptional profiling studies have been performed using surgical lung specimens [4,7], but lung tissue has important disadvantages relative to sputum or BAL. Practically and ethically, lung tissue can be obtained only from patients failing drug treatment or as management of massive hemoptysis [4,7]. Since drug exposure greatly alters the *M. tuberculosis* transcriptome [5], post-treatment lung specimens provide little information about treatment naïve *M. tuberculosis* or transcriptional changes that are induced by drug exposure. On a practical note, longitudinal sputum sampling is a much more feasible approach, whereas repeating BAL or surgical lung biopsy would be logistically impractical if not impossible.

In contrast to *M. tuberculosis* gene expression, which is relatively similar in BAL and sputum, host profiling revealed differences in expression of human immune genes between the two sample types. Specifically, sputum had a proinflammatory transcriptome relative to BAL.

This report has several limitations. First, this study used BAL with the intention of obtaining a more direct sample of diseased regions of the lung than sputum provides. However, in the process of sampling alveolar spaces and diseased lung segments, BAL also washes medium to small airways, resulting in a mixed sample that may include airway *M. tuberculosis* destined to appear in sputum. It is unclear whether any single tissue type could capture the diversity and heterogeneity of lesions present in human TB [3]. By sampling a range of microenvironments, we believe BAL provides an acceptable aggregate measure of the mean expression pattern of the *M. tuberculosis* population in the accessible lower airway of patients with pulmonary tuberculosis. Second, our *in vitro* comparisons used *M. tuberculosis* H37Rv, a laboratory strain. Inherent between-strain differences could confound our comparisons between clinical and *in vitro* specimens. Third, comparing BAL-sputum transcriptional profiles during drug treatment would have been optimal, but repeating bronchoscopy was not feasible. Since the pre-treatment transcriptional profiles were similar between sputum and BAL, we think it is likely that transcriptional profiles during treatment will be similar between sputum and BAL. Finally, the relatively small number of participants limited our statistical power to detect differences between BAL and sputum. However, it is likely that we identified differences with meaningful effect sizes.

4.1. Conclusion

Monitoring *M. tuberculosis* gene expression profiles in clinical specimens provides a more accurate representation of the physiologic state of *M. tuberculosis* in human disease than samples obtained in experimental models. Our analysis showed that transcriptional patterns in BAL and sputum were generally similar, albeit with higher expression of DosR regulon genes in BAL. However, the differences in *M. tuberculosis* expression between sputum and BAL were substantially smaller than changes in expression in sputum that occur during drug treatment. These results suggest that transcriptional profiling of *M. tuberculosis* in sputa can be used to monitor the physiological state of *M. tuberculosis* in the lower airway of patients with pulmonary tuberculosis, and that resulting information may enable novel insights into drug effects and lead to the identification of pharmacodynamic markers.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2016.07.004.

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