



RESEARCH ARTICLE

Variation in pre-therapy levels of selected *Mycobacterium tuberculosis* transcripts in sputum and their relationship with 2-month culture conversion [version 1; peer review: awaiting peer review]

Neesha Rockwood ^{1,2}, Rachel P.J. Lai ³, Ronnett Seldon⁴, Douglas B. Young^{3,5}, Robert J. Wilkinson ¹⁻³

¹Department of Medicine, Imperial College London, London, W2 1PG, UK

²Wellcome Center for Infectious Diseases Research in Africa, Institute of Infectious Disease and Molecular Medicine and Department of Medicine, University of Cape Town, Cape Town, South Africa

³Francis Crick Institute, London, NW1 2AT, UK

⁴Molecular Mycobacteriology Research Unit, Institute of Infectious Disease and Molecular Medicine and Department of Pathology, University of Cape Town, Cape Town, South Africa

⁵MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, SW7 2AZ, UK

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Abstract

Background: The abundance of transcripts arising from *Mycobacterium tuberculosis* (MTB) in sputum pre-chemotherapy may enhance our understanding of factors influencing treatment response. We hypothesized that differences in the prevalence of pre-existing slowly metabolizing MTB in sputum may be partially responsible for differences in the rate of sputum clearance during treatment.

Methods: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to characterize a selected limited transcription profile of MTB in sputum pre-chemotherapy and assess inter-individual variation. The difference in cycle threshold (Ct) per gene, normalized to 16S, between exponential/stationary phase culture and sputum was calculated and stratified by 2-month culture converter status.

Results: HIV-1 uninfected patients with rifampicin-susceptible tuberculosis provided sputum pre-chemotherapy; 11 patients were negative for MTB culture after two months of therapy and 8 remained culture-positive. Increased *icl1* and *prpD* and *rpsN2:rpsN1* in sputum relative to culture suggested cholesterol utilization and a low-zinc environment respectively. Increased *hspX* and decreased *atpA* and *nuoG* relative to exponential culture suggested a slowly metabolizing subpopulation of MTB. While the *hspX*^{hi}*atpA*^{lo}*nuoG*^{lo} signal varied, we did not observe statistically significant enrichment of this phenotype in the non-converter population nor an association with MTB-lineage.

Conclusion: Differential abundance of selected informative transcripts suggested a metabolically less-active subpopulation with a prevalence that varied between individual untreated patients.

Open Peer Review

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Keywords

Mycobacterium tuberculosis, quantitative reverse transcriptase polymerase chain reaction, gene expression, sputum, persistent infection, treatment response



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Corresponding author: Neesha Rockwood (neesha.rockwood@doctors.org.uk)

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Introduction

Tuberculosis (TB) causes over a million deaths annually¹. One attribute believed to contribute to its success as a pathogen is its ability to persist as phenotypically-adapted subpopulations under antimicrobial, environmental or immune stress.

A well-defined feature of *Mycobacterium tuberculosis* (MTB) phenotypic adaptation involves up-regulation of the DosR regulon in response to reduced oxygen availability, or exposure to nitric oxide or carbon monoxide^{2,3}. This regulon consists of 48 genes under the control of transcription factor DosR, including *hspX* coding for alpha-crystallin stress protein. Previous studies have reported abundant DosR-regulated transcripts in sputum pre-chemotherapy⁴⁻⁶. The DosR regulon includes a small RNA(sRNA) transcript MTS1388. This was reported to accumulate in infected tissues and we anticipated that its enhanced stability might make it a particularly sensitive target for reverse transcription-polymerase chain reaction (RT-PCR)⁷. It was of interest to examine the possible influence of MTB genotype on transcriptional profiles observed in sputum. It has been reported that a mutation in strains from the Beijing family introduces a new transcriptional start site that results in constitutive expression of the *dosR* gene (and hence the associated regulon) independent of stress induction⁸.

MTB phenotypes with reduced replication and metabolism, particularly in the context of hypoxia, are characterized by a low level of ATP and a switch from proton-pumping NADH dehydrogenase-1 to non-proton-pumping NADH dehydrogenase-2^{9,10}. We therefore included *atpA*, *nuoG* and *ndh* in our panel of target genes. In addition, we included genes encoding the major sigma factors *sigA* and *sigB*, together with *icl1* and *prpD* as markers of the methylcitrate cycle that has been shown to be induced as a consequence of cholesterol catabolism during infection¹¹. A second abundant sRNA, MTS2823, has been linked to the methylcitrate cycle¹² and was also included in the set of target genes.

Finally, we included two paralogous ribosomal protein genes, *rpsN1* and *rpsN2*, on the basis of previous studies of the MTB sputum phenotype (RPL, unpublished data). MTB has four ribosomal proteins that can be expressed as alternative zinc-binding or zinc-independent forms dependent on the availability of zinc in the growth medium¹³; *rpsN1* encodes a zinc-binding S14 protein and *rpsN2* the corresponding zinc-independent form.

We hypothesized that differences in the prevalence of pre-existing drug-tolerant MTB in sputum may be partially responsible for differences in the rate of sputum clearance during treatment. To test this, we used qRT-PCR to measure the abundance of the selected transcripts in sputum samples from a panel of patients infected with different MTB lineages and with differing responses to subsequent antitubercular chemotherapy. To determine inter-individual variation in m/sRNA expression patterns, our protocol lacked an amplification step after the random primer based cDNA synthesis, prior to quantification by qRT-PCR¹⁴.

Methods

Setting

Recruitment was at Site B Ubuntu Clinic, a primary care integrated HIV/tuberculosis clinic, in Khayelitsha township, Western cape, South Africa as part of a prospective study (University of Cape Town Human Research Ethics Committee approval 568/2012) assessing frequency and determinants of acquired drug resistance. The study was carried out from March 2013-July 2014, with clinical follow-up until November 2015. A subset of the patients was invited to provide sputum for qRT-PCR in this nested study. All participants gave written consent.

Participants

HIV-uninfected patients with GenXpert® MTB/Rif-confirmed rifampicin-susceptible pulmonary TB, were recruited pre commencement of quadruple therapy with rifampicin/isoniazid/ethambutol/pyrazinamide. Patients were excluded if they were under 18, had received treatment for tuberculosis in the previous 6 months, had a positive pregnancy test, were unable to give informed consent, declined testing for HIV or were unable to expectorate sputum.

Sample size considerations

This was an exploratory study and there was no formal sample size calculation. A sample size of 20, was selected to compare variability of expression of selected transcripts, stratified by culture converter status and MTB strain.

Sample collection

Full details of clinical variables collected and follow up procedures have been described elsewhere¹⁵. In this sub-study, patients produced spontaneous sputum samples TRIzol®LS (10296028, Invitrogen, Carlsbad, USA) in 3:1 volume was immediately added to spontaneously expectorated sputum, vortexed until the mixture was homogenous. Samples were transported 30 minutes on ice from the clinical site to the laboratory to be frozen at -20°C.

RNA extraction from sputum

1 ml aliquots thawed and were ribolyzed at 6.5 m/sec for 45 seconds and again after 5 minutes with a FastPrep-24 ribolyzer (MPBio, UK). 200µl of 100% chloroform (CHCl₃) was added, the mixture vortexed and centrifuged at 3000 rpm for 10 minutes. The liquid content was transferred to a new tube, 600µl CHCl₃/ isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm for 1 minute. The top aqueous layer was transferred to a new tube and mixed well with an equal volume of 100% ethanol. The RNA ethanol mixture was then extracted using the RNA Clean and Concentrator kit (R1013, Zymo, Irvine, USA).

Axenic culture of reference *M. tuberculosis*

M. tuberculosis H37Rv (Systems strain) was available in-house and cultured in 7H9 media (M0178, Sigma-Aldrich, UK) containing 0.2% v/v glycerol, 0.05% v/v Tween 80 and 10% ADC in roller bottle. Exponential phase culture was harvested when OD 600 measured between 0.7-1 (Biochrom WPA

CO8000 Cell Density Meter) and stationary phase culture was harvested 4 weeks after the OD 600 reached a plateau of 1.0. 10 mL of culture was pelleted by centrifugation and washed once with phosphate buffered saline (PBS) before addition of 2ml Trizol@LS.

cDNA synthesis, primer design and qPCR

First strand cDNA synthesis was performed from 1µg RNA from each sputum using Superscript VILO system (11754-050, Invitrogen Carlsbad, USA) and random hexamers (N8080127, Invitrogen). This process was also carried out for H37Rv exponential and stationary phase cultures. Serial dilutions were carried out on H37Rv cDNA until the average cycle threshold (Ct) for 16S was comparable to values obtained from sputa to allow optimal comparability.

Primers and probes complementary to 8 mRNAs (atpA, SigA, SigB, nuoG, ndh, icl1, prpD, rpsN1, rpsN2, HspX), 2 sRNAs (MTS2823 and MTS1338) and 16S ribosomal RNA were designed using [Primer Quest Tool](#) (Integrated DNA Technologies, UK, see [Table 1](#)). Primers and probes with potential to form hairpin structure were excluded and potential cross-reactivity was checked by the [BLASTN](#) tool. qPCR was carried out on customized TaqMan array plates pre-printed with primers and probes described above. Reaction was run in triplicate on a ABI Prism 7000 (Applied Bioscience, UK). Conditions were 95°C/10 minutes, followed by 40 cycles of 95°C/15 seconds and 60°C/60 seconds. Separate sputum samples taken

simultaneously at baseline, were grown in MGIT liquid cultures using the BD BACTEC MGIT 960 system at a routine diagnostic laboratory, National Health Laboratory Service (Groote Schuur Hospital). Isolates were collected and genotyped by spoligotyping. Spoligotyping was done according to the internationally standardized method. 500µl aliquots of MGIT culture were heat killed at 80°C for 1 hour and 5µl of lysate used for PCR. Chromosomal DNA of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 were used as positive controls and water as a negative control. Primers DRa and DRb were used to amplify spacers in the direct repeat (DR) region. Biotin labeled PCR products were then hybridized to immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers was visualized on film as black squares after incubation with streptavidin-peroxidase and electrochemical luminescence system detection¹⁶. Hybridisation patterns obtained were used to assign the isolates to genotype families using the [SPOTCLUST](#) SpolDB3 database¹⁷.

Data normalization

To normalize for overall bacterial load, all cycle thresholds (Ct) of the genes of interest (GOI) were divided by Ct of the house-keeping gene 16S (Ct_{GOI} / Ct_{16S} , not ; denoted as $SPUT_{GOI}$). For individual GOIs (atpA, SigA, SigB, nuoG, ndh, icl1, prpD, rpsN2, HspX, MST2823, and MTS1338), the difference between exponential phase culture (Ct_{GOI} / Ct_{16S} , denoted as EXP_{GOI}) and $SPUT_{GOI}$ was calculated for each patient, along with the median for all patients (ΔGOI_{EXP}).

Table 1. Primer and probe sequences.

Gene	Name	Probe (dye-sequence)	Forward primer	Reverse primer
atpA	SP_Rv1308	FAM-CTGGATGTACGAGGGCAAGCATGT	AGTCCGCCGGTTTCAAT	CTTAGTCAGGTCGTCGAAGATG
sigA	SP_Rv2703	FAM-ACGCAAGGACGCCGAACCTCA	GTGATTTCTGCTGGGATGAAGA	TACCTTGCCGATCTGTTTGAG
sigB	SP_Rv2710	FAM-TATCTGAACGGCATCGGCAAGACG	AGCGATCTGGATGCTCAAAG	TTGGCCAGTTCGACTTCAC
prpD	SP_Rv1130	FAM-TTGCACGAGCACAAGATCGACCAT	CTATGAGATCCACATCGACCTAAC	GCGTGGTAGATGGTCTCTTG
icl1	SP_Rv0467	FAM-TCTGCTCGGCTCGC	CAGCACATCCGCACTTTGAC	ATCACCACCGTGGGAACATC
nuoG	SP_Rv3151	FAM-CCTTGTCGCACATCGGGCAATC	GTGATGGAAGTCTGCTGAT	CCGTTAGACATTGCCTGGTT
ndh	SP_Rv1854c	FAM-AAGATCGGTCCCGTTGAGTTCAGC	ATTCCAGTACTTCGACAAGGG	CAAATCAGCCAGGCGATAAAG
rpsN1	SP_Rv0717	FAM-TCTACCGCAAGTTCGGGCTGTG	CGAAGAAGGCACTGGTCAA	ATCTCGCGCAGGCAAAT
rpsN2	SP_Rv2056c	FAM-CCACCGTCGCCCCGT	AAGAAGTCCAAGATCGTCAAGAATC	GGGATCGGATGATGTCTTTGAG
MTS2823	SP_MTB000078	FAM-CAGATGGCATCGACGG	GGTCTGCGTAATTGCGAACA	CTGTAGCCCCACCCAAAGG
hspX	SP_Rv2031c	FAM-CGGCTGGAAGACGAG	CCTTCGACACCCGGTTGAT	CGTAGCGCCCTCTTTCA
MTS1338	SP_MTB000077	FAM-CAGTATTGGTGATACCGGG	CCGCCTGCTGTGTTTGGT	GGGCAGATCACCGGGTTT
16S	SP_MTB000019	FAM-TGAAATCTCACGGCTTAA	GGTGGTTTGTGCGGTTGTTT	GCCCGCACGCTCACA

For individual GOIs, (the difference between stationary phase culture (Ct_{GOI}/Ct_{16S} , denoted as $STAT_{GOI}$) and $SPUT_{GOI}$ was calculated for each patient, along with the median for all patients (ΔGOI_{STAT}).

The difference in abundance of the mRNA/sRNA transcripts, in sputum compared with the reference H37Rv cultures, was summarized as an overall 'mean deviation' from exponential/stationary culture. The overall 'mean deviation from exponential phase culture' (MDEx) was calculated as the mean of all values of $[EXP_{GOI} - SPUT_{GOI}]$ inclusive of 11 GOIs and inclusive of all patients ($N=19$).

$$MDEx = \frac{\sum_j (\sum_{i=1}^N (EXP_{GOI} - (SPUT_{iGOI})))}{j \cdot N}$$

where j represents each GOI and N = total number of patients

The 'mean deviation from stationary phase culture' (MDStat) was calculated as the mean of $[STAT_{GOI} - SPUT_{GOI}]$ inclusive of all GOIs and inclusive of all patients.

$$MDStat = \frac{\sum_j (\sum_{i=1}^N (STAT_{GOI} - (SPUT_{iGOI})))}{j \cdot N}$$

where j represents each GOI and N =total number of patients

The Wilcoxon rank-sum test was used compare statistical significance between groups. Correlation between continuous variables

was assessed by the Spearman correlation co-efficient. All analyses were performed using [GraphPad Prism](#) version 8 (La Jolla CA).

Results

In total, 20 participants provided sputum for qRT-PCR, of whom 19 were included in the study (see underlying data¹⁸). One was excluded due to high Ct values for all m/sRNAs analysed, indicative of insufficient MTB RNA. Eight out of 19 participants were non-converters who remained MTB culture-positive at 2 months, and 11 were converters who became culture-negative by 2 months. All patients were culture-negative after 5 months of therapy. Seven patients were infected by a strain from the MTB lineage 2 (Beijing), one with a Lineage 3 strain, and 11 with strains belonging to Lineage 4 ([Table 2](#)).

The bacterial load in sputum was assessed by time to positivity (TTP) in MGIT culture and by the Ct for 16S ribosomal RNA. Median TTP at baseline for non-converters who remained culture-positive at 2 months was 7.5 days (IQR 6-9) and was 7 days (IQR 6-11.5) for converters who were culture-negative at 2 months. 16S Ct values were significantly correlated with TTP at baseline ($R=0.70$, $p<0.0001$).

[Figure 1A](#) illustrates primary Ct data for *rpsN1* and *rpsN2* transcripts. In exponential cultures of MTB H37Rv, the Ct for *rpsN1* was lower than *rpsN2* – i.e. expression of the *rpsN2* transcript was repressed, consistent with growth in zinc-replete 7H9 medium¹³ – while the reverse was observed in stationary phase. Ct values were much higher in sputum, reflecting the lower number of bacteria, with the ratio of *rpsN1* to *rpsN2* suggestive of growth in a low-zinc environment.

Table 2. Characteristics of study participants.

	All participants (n=19)	Non-converters (n=8)	Converters (n=11)
% Male	79	100	64
Median age (IQR)	34 (27–45)	39 (28–50)	29 (26–39)
%Smear grading			
Neg/scanty	11	0	18
1+	21	12.5	27
2+	37	37.5	37
3+	31	50	18
Median time to culture positivity at baseline (IQR)	7 (6–10)	7.5 (6–9)	7 (6–11.5)
% Extensive radiological disease*	95	100	91
% Cavities on CXR*	63	50	73
%SPOTCLUST Classification			
Beijing (lineage 2)	37	50	27
CAS (lineage 3)	5	12.5	0
LAM8 (lineage 4)	5	12.5	0
X3 (lineage 4)	16	12.5	18
T1 (lineage 4)	21	12.5	27
LAM3 (lineage 4)	11	0	18
T4 (lineage 4)	5	0	10

* The presence of cavitation >1 cm maximum diameter was noted. Extensive disease on chest radiograph was noted as either involvement of >1 lung or involvement of ≥ 1 out of 3 (upper, mid or lower) zones per lung. CXR – Chest X-ray

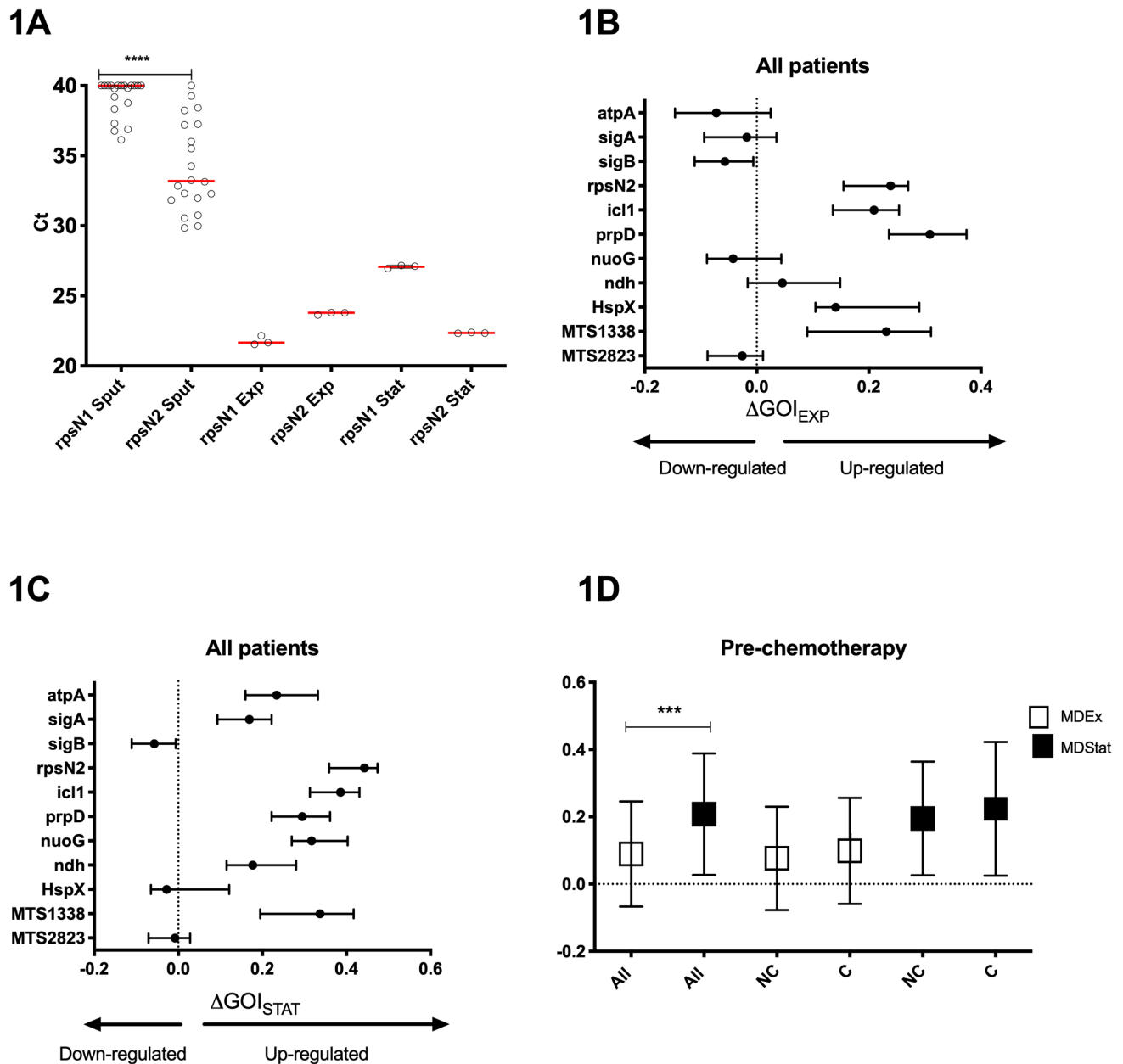


Figure 1. Comparison of cycle threshold (Ct) of genes of interest in sputum and reference exponential and stationary phase H37Rv cultures. A) rpsN1 and rpsN2. Where readings were below the level of detection, they were allocated Ct value of 40. Ct data for rpsN1 and rpsN2 expression in H37Rv in exponential and stationary phase cultures (1 mcg MTB RNA) is also shown. **B)** Differences in sputum compared with exponential phase cultures normalized to 16S. ΔGOI_{EXP} Difference in expression of gene of interest in exponential phase culture compared with gene of interest in sputum at baseline (median $\pm$ IQR). A positive number indicates increased relative to exponential phase culture and negative number indicates decreased relative to exponential phase culture. **C)** Differences in sputum compared with stationary phase cultures normalized to 16S. ΔGOI_{STAT} Difference in expression of gene of interest in stationary phase culture compared with gene of interest in sputum at baseline (median $\pm$ IQR). A positive number indicates increased relative to stationary phase culture and negative number indicates decreased relative to stationary phase culture. **D)** Overall mean deviation ($\pm$ SD) from H37Rv growth status. The further away from 'zero' line, the greater the difference in patterns of abundance. At both time points, sputum was closer in pattern of abundance to exponential phase H37Rv cultures. MDEx Mean deviation from Exponential phase; MDStat Mean deviation from Stationary phase; NC non-converter; C converter.

Figure 1B and 1C illustrate 16S-normalized transcript abundance in sputum compared to exponential and stationary phase culture. Median levels of *atpA*, *sigA* and *nuoG* in sputum were slightly lower than exponential culture but significantly higher ($p < 0.01$) than stationary phase. Conversely, *hspX* in sputum was higher than in exponential culture but lower than stationary phase. The 16S-normalized abundance of *rpsN2*, *icl1* and *prpD* transcripts in sputum was significantly higher ($p < 0.01$) than under either phase of laboratory culture. The mean deviation of the total set of transcripts in sputum from H37Rv growth status at baseline (MDEx and MDStat) showed that the overall sputum profile resembled exponential culture more closely than stationary phase (Figure 1D).

To further define a potential reduced-metabolism phenotype in sputum, we searched for a *hspX^{hi}atpA^{lo}nuoG^{lo}* signal and its variability between individuals. A strong correlation was observed between 16S-normalized abundance of *atpA* and *nuoG* ($R = 0.92$, $p < 0.001$). There was a non-significant trend towards reduced expression of these markers of oxidative phosphorylation in non-converters. 6/8 (75%) had below median levels for both *nuoG* and *atpA*. This was compared with 4/11 (36%, $p = 0.1$) and 5/11 (45%, $p = 0.2$) of converters, (Figure 2A). There was a non-significant trend of increased expression of the DosR regulated transcript (*hspX*) above the median of the entire group, in non-converters 5/8 (63%) compared with converters 4/11 (36%, $p = 0.2$) (Figure 2B).

The 16S-normalized transcript abundance of *hspX* showed no significant difference ($p = 0.8$) between the 7 Beijing and 12 non-Beijing samples included in the study (Figure 2C), consistent with the conclusion that the DosR phenotype detected in sputum reflects an induced response to hypoxia, nitric oxide or carbon monoxide.

Discussion

This study adds to literature describing the sputum phenotype of *M. tuberculosis* by demonstrating the potential application of qRT-PCR to analyse differences in well-characterized patient cohorts. By avoiding the pre-amplification step characteristic of previous protocols, the present study avoids potential technical issues involving non-linear amplification and preferential enrichment of abundant transcripts.

Based on the selected set of target genes, the overall transcription profile detected for MTB in sputum more closely resembled H37Rv in exponential culture than in stationary phase culture. It has previously been proposed that MTB assumes a non-replicating phenotype in sputum⁵, but comparison of our own with published data^{6,19} indicates that the sputum profile falls between replicating and non-replicating states, and that differing interpretations may partially reflect inter-laboratory variation in reference cultures. The extent to which MTB continues to replicate within the respiratory tract could have an important influence on disease transmission.

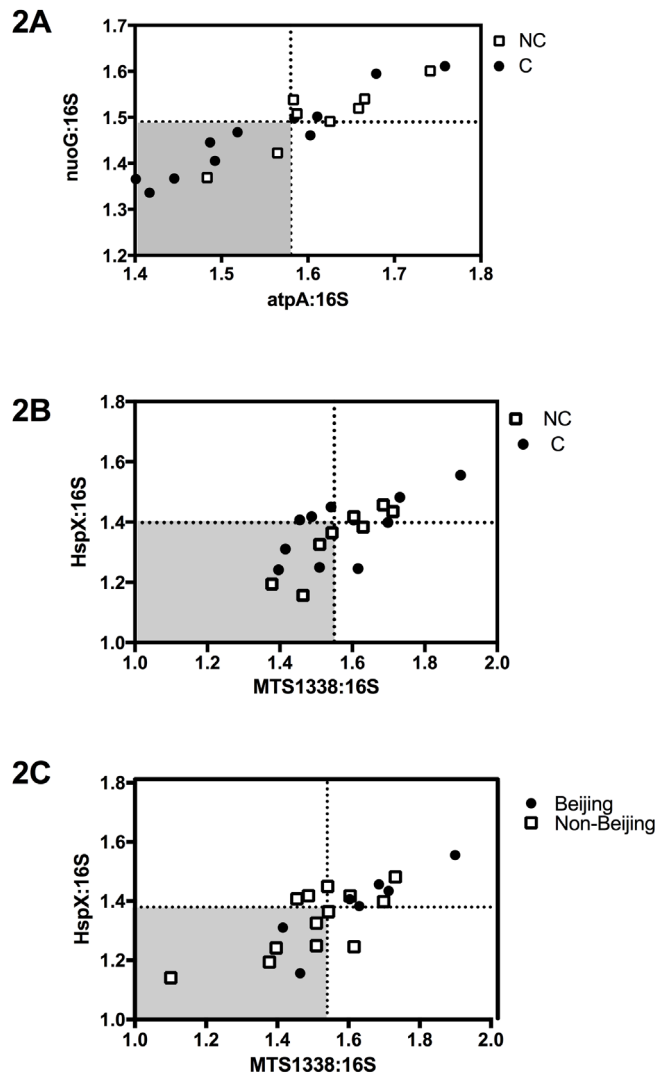


Figure 2. Pre-chemotherapy expression of transcripts influenced by oxygen gradients in sputum. Lower values for (Ct_{GOI}/Ct_{16S}) represent higher levels of expression. The median abundance as shown by the dotted lines, were used to distinguish the highest expressors (shaded grey). **A)** *nuoG*, *atpA* stratified by converter status **B)** *HspX*, *MTS1338* stratified by converter status **C)** *HspX*, *MTS1338* stratified by Beijing/non-Beijing status.

Consistent with previous descriptions of the transcriptional phenotype of MTB during infection⁴⁻⁶, *icl1* and *prpD* transcripts were significantly higher in sputum than in laboratory culture. The products of these two genes participate in the methylcitrate cycle that is required to process potentially toxic propionate generated during metabolism of cholesterol and odd branch length fatty acids. This suggests that MTB in sputum resembles intracellular MTB in utilizing cholesterol as a carbon source²⁰. In contrast, abundant representation of *rpsN2*

transcript in sputum is characteristic of growth in a low-zinc environment, which is inverse to the zinc intoxication associated with MTB in activated macrophages²¹.

Increased abundance of *hspX* and decreased abundance of *atpA* and *nuoG* transcripts relative to exponential culture is consistent with the presence of a metabolically less-active MTB phenotype in sputum. While a trend towards enrichment of this sub-population in patients who required longer treatment to achieve culture conversion was observed, it did not reach statistical significance. By knowing the lineage of the infecting strain in each case, we were able to exclude the possibility that genotypic variation – for example, constitutive *dosR* expression in Beijing strains – was responsible for the observed variable representation of the low-metabolism phenotype.

Findings were limited by the relatively small sample size and suggest the need for expanded studies focusing on more phenotypically informative transcripts. As the sputum RNA was not pre-amplified, certain genes of interest had very high Ct values which should be interpreted with care. An important limitation relates to the use of 16S rRNA to normalize samples with respect to bacterial load. The relative stabilities of rRNA and mRNA in the respiratory tract are unknown and preferential enrichment for one or the other in non-replicating or dead bacteria could skew the data. A further limitation is comparison of transcripts from sputum to exponential and stationary phase cultures of H37Rv instead of isolates cultured from the original sputa.

In conclusion, direct qRT-PCR interrogation of sputum samples using a limited panel of MTB transcripts reveals an overall transcriptional phenotype that bears closer resemblance exponentially-growing bacteria than to stationary phase culture. Differential abundance of selected informative transcripts suggested a metabolically less-active subpopulation with a prevalence that varied between individual untreated patients. We were unable to demonstrate a statistically significant correlation between this subpopulation and subsequent response to treatment.

Data availability

Underlying data

Figshare: Selected Pre-therapy MTB transcripts. <https://doi.org/10.6084/m9.figshare.8295779.v1>¹⁸

This project contains the following underlying data:

- selected pre-therapy MTB transcripts.xlsx (Demographic and pre-therapy *Mycobacterium tuberculosis* transcript level data for participants)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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