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Letter to the Editor

The evaluation of anti-tuberculosis drug effects on phenotypes of *Mycobacterium tuberculosis* not detected by culture methods

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The only accepted surrogate biomarker of tuberculosis (TB) treatment success is two months sputum culture conversion to negativity [1,2]. Yet, the standard TB treatment comprising rifampicin (RIF) as sterilising drug has to be extended for six months [3] and attempts to shorten this duration have resulted in increased TB recurrence [4,5]. The clinical observation that TB treatment must be continued beyond the point of bacillary elimination from sputum to achieve durable cure has been linked to residual proportions of persisters, i.e. slow-growing or dormant phenotypes of *Mycobacterium tuberculosis* tolerant to antibiotics. Auramine O (acid-fast) and Nile Red (lipophilic) staining of smears made from sputum samples collected from TB patients revealed that the mycobacterial population in sputum is not as homogenous as previously thought [6,7]. In fact, *M tuberculosis* from *in vitro* cultures under stress show a mixture of acid fast (green phenotype), partially lipophilic (cream phenotype) and totally lipophilic (red phenotype) mycobacteria corresponding to fast-growing, slow-growing and dormant cells [8]. Slow-growing and dormant phenotypes represent persisters that cannot grow on solid medium [9], but there is evidence that slow-growing persister phenotypes might grow in liquid media [6].

We hypothesized that liquid culture might reflect treatment effects on some intermediate phenotypes better than solid culture. We investigated this theory on sputum samples collected daily from 15 newly diagnosed, smear positive TB patients who received daily RIF as monotherapy (10 mg/kg). Using confocal microscopy on Nile Red and Auramine O stained sputum smears, we determined the proportions of fast-growing (green), slow-growing (cream) and dormant phenotypes (red). We also determined colony forming unit (CFU) counts on solid agar and time to positivity (TTP) in liquid medium. In total, 171 CFU, 206 TTP and 203 phenotype counts were available. Missing values were evoked by contaminated or missing solid or liquid cultures and unreliable phenotype counts due to high levels of background staining. For details on experiments and data analyses see online methodology.

Before treatment start, small proportions of persisters were predominated by a much larger proportion of fast-growing bacteria. As expected, there was a rapid fall in $\log_{10}$(CFU) counts and increase in TTP [10]. To look at how each of the three phenotypes participated in these changes we modelled each value of $\log_{10}$(CFU) and TTP for the percentage of each phenotype (green, cream or red) present at that time point over treatment days 1–14, adjusting for baseline $\log_{10}$(CFU) or TTP. The figures show the change per day in $\log_{10}$(CFU) and TTP for phenotype specific percentages of 0, 25, 50, 75 and 100. Strong mycobacterial killing, represented by significantly steep $\log_{10}$(CFU) or TTP slopes, is seen when fast-growing cells dominate (depicted green, Fig. 1A) and when dormant cells are absent (depicted red, Fig. 1B). Interestingly, for slow-growing cells (depicted cream, Fig. 1C) the pattern of change was similar but only TTP and not $\log_{10}$(CFU) showed a statistically significant difference in day slopes with increasing percentages (Fig. 1C). This means that killing by RIF is only detected by $\log_{10}$(CFU) when fast-growing cells predominate and dormant cells are absent. Drug activity on intermediate cells cannot be predicted by $\log_{10}$(CFU). TTP, in contrast, reflects killing activity on all three phenotypes.

These results support previous studies showing that liquid culture is better able to recover persisting mycobacterial subpopulations from sputum and can remain positive longer than solid culture if sputum is collected repeatedly under treatment [6]. CFU and TTP measures depend on actively replicating or metabolizing mycobacteria. Dormant cells have been shown to be able to regain activity after stimulation by resuscitation promoting factors, which were not available for this study [11].

In summary, RIF treatment successfully reduced the number of viable bacteria in sputum, but this was limited to the elimination of fast-growing and, to a lesser degree, slow-growing bacterial phenotypes, the killing of which was detected by liquid but not by solid culture. Treatment effects on dormant bacteria were not measurable. This is not really surprising, given that all currently available anti-TB drugs were discovered by means of phenotypical methods. In the absence of an assay that can reliably detect drug effects on persisters in both preclinical and clinical experiments the characterisation of drug activity will remain erroneous as drug effects on persisting mycobacteria are not detected. Our inability to investigate drug effects on the phenotypes believed to be the main cause for TB reoccurrence after treatment completion is a barrier to TB eradication. The development of new anti-TB drugs able to eliminate persisting mycobacteria and thus shorten

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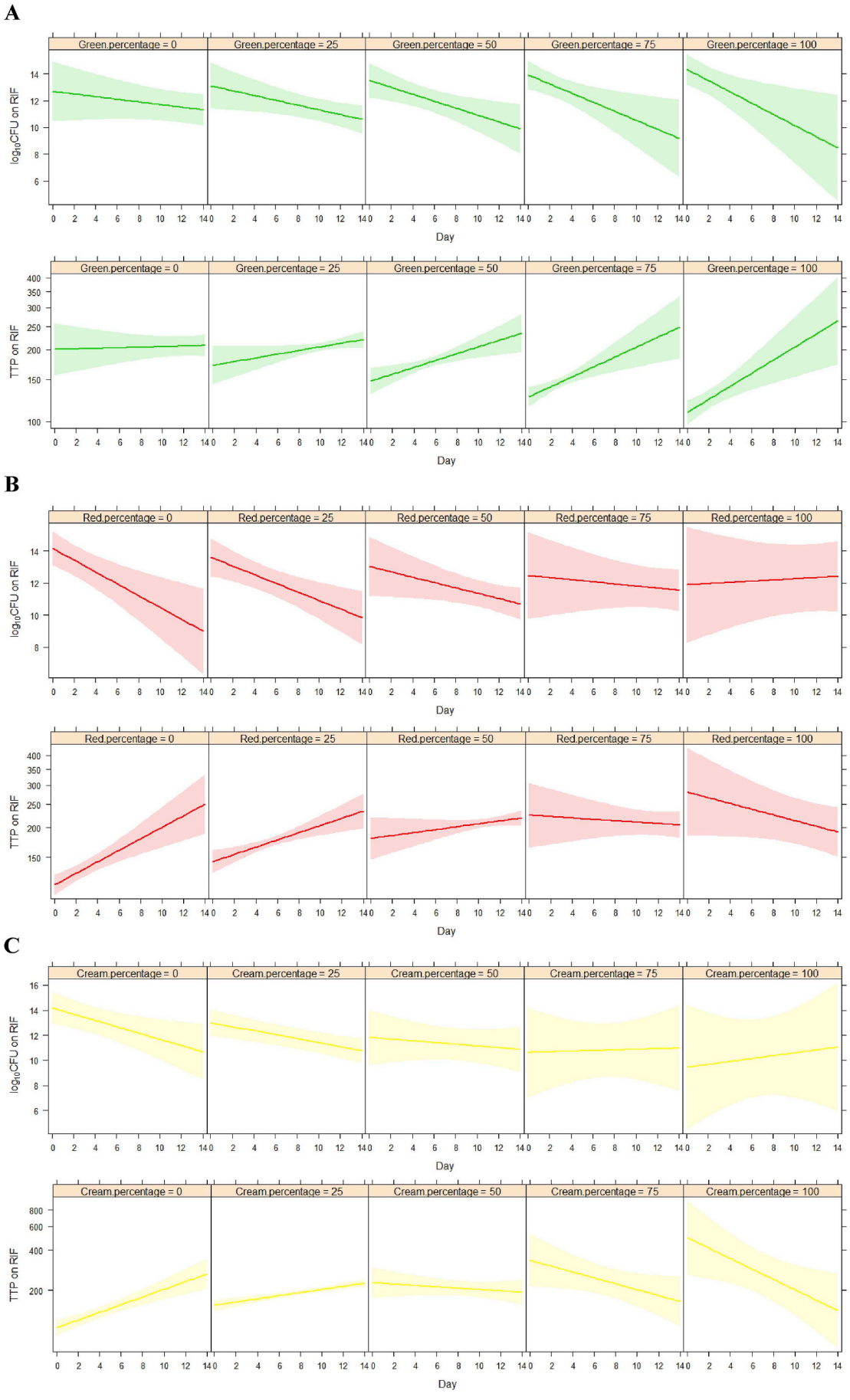

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Fig. 1. The graphs show estimates of bactericidal activity with 95% CIs along 14 days of RIF treatment in plots of exactly 0%, 25%, 50%, 75% and 100% proportion of each cell phenotype. Each row of graphs is from the interaction term in a linear mixed-effects model of $\log_{10}$(CFU) or TTP for the statistical interaction between phenotype percentage and day, adjusting for the baseline values. A *P*-value for the interaction in a row of graphs smaller than 0.05 indicates that the estimated bacterial activities (slopes) differ significantly from one proportion to the next. Fast-growing cells are depicted in green, intermediate cells in cream, and dormant cells in red. **A:** The estimates of bactericidal activity measured with $\log_{10}$(CFU) and TTP increase with increasing proportion of fast-growing cells from flat slopes for 0% green cells to steep slopes for 100% green cells. $P = 0.0486$ for $\log_{10}$(CFU) and $P = 0.0020$ for TTP. **B:** The estimates of bactericidal activity measured with $\log_{10}$(CFU) and TTP decrease with increasing proportion of dormant cells from flat slopes for 100% red cells to steep slopes for 0% red cells. $P = 0.0247$ for $\log_{10}$(CFU) and $P = 0.0004$ for TTP. **C:** An increase in slow-growing cells also increases bactericidal activity but this is significant only for estimates of TTP ($P = 0.0016$) and not for estimates of $\log_{10}$(CFU) ($P = 0.3298$) that shows less steep slopes and much larger variation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatment will critically depend on methods for quantification of drug activities on all phenotypes becoming available.

Conflicts of interest

The authors declare no conflicts of interests.

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References

[1] Perrin FMR, Lipman MCI, McHugh TD, Gillespie SH. Biomarkers of treatment response in clinical trials of novel antituberculosis agents. Lancet Infect Dis 2007;7:481–90.
[2] Mitchison DA. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. Am Rev Respir Dis 1993;147:1062–3.
[3] World Health Organization. Treatment of tuberculosis: guidelines for national programmes. WHO; 2010http://www.who.int/tb/features_archive/new_treatment_guidelines_may2010/en/, Accessed date: 24 February 2015.
[4] Merle CS, Fielding K, Sow OB, Gninafon M, Lo MB, Mthiyane T, et al. A four-month gatifloxacin-containing regimen for treating tuberculosis. N Engl J Med 2014;371:1588–98.
[5] Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. N Engl J Med 2014;371:1577–87.
[6] Dhillon J, Fourie PB, Mitchison DA. Persister populations of Mycobacterium tuberculosis in sputum that grow in liquid but not on solid culture media. J Antimicrob Chemother 2014;69:437–40.
[7] Garton NJ, Christensen H, Minnikin DE, Adegbola RA, Barer MR. Intracellular lipophilic inclusions of mycobacteria in vitro and in sputum. Microbiology (Rea, Engl) 2002;148:2951–8.
[8] Deb C, Lee C-M, Dubey VS, Daniel J, Abomoelak B, Sirakova TD, et al. A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. PLoS One 2009;4:e6077.
[9] Garton NJ, Waddell SJ, Sherratt AL, Lee S-M, Smith RJ, Senner C, et al. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. PLoS Med 2008;5:e75.
[10] Kayigire XA, Friedrich SO, van der Merwe L, Donald PR, Diacon AH. Simultaneous staining of sputum smears for acid-fast and lipid-containing Myobacterium tuberculosis can enhance the clinical evaluation of antituberculosis treatments. Tuberculosis (Edinb) 2015;95:770–9.
[11] Mukamolova GV, Turapov O, Malkin J, Woltmann G, Barer MR. Resuscitation-promoting factors reveal an occult population of tubercle Bacilli in sputum. Am J Respir Crit Care Med 2010;181:174–80.

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