(e.g., intracranial hemorrhage). In our patient, repeated failure of thrombus extraction using traditional bronchoscopy tools, coupled with urgency to improve lung expansion and wean ECMO support, prompted a trial of cryoextraction, which was ultimately successful.

This example of flexible bronchoscopic cryoextraction in a critically ill, anticoagulated neonate on ECMO provides proof of concept for children of almost any age and size, although risks must be weighed before such an undertaking. This should only be considered in facilities with the experienced, multidisciplinary team necessary to support cryoextraction in high-risk situations. The ongoing development of advanced tools able to deploy via small-working-channel bronchoscopes will empower novel therapeutic and diagnostic approaches in pediatric bronchoscopy. ■

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Resistance-Conferring Mycobacterial Mutations and Quantification of Early Bactericidal Activity

To the Editor:

We write in reference to our recent article (1), which describes an early bactericidal activity (EBA) study investigating the optimal dose of isoniazid (INH) for patients with INH-resistant tuberculosis mediated by *inhA* mutations. Here, we reexamine the measures of sputum bacterial load used with regard to the presence of drug resistance–conferring mutations.

The parent study, AIDS Clinical Trials Group A5312, is an ongoing phase 2A, open-label trial in which individuals with smear-positive pulmonary tuberculosis with INH resistance mediated by *inhA* mutation were randomized to receive INH at 5, 10, or 15 mg/kg daily for 7 days. Control subjects with no mycobacterial INH resistance received INH at the standard dose of 5 mg/kg daily. Overnight sputum samples were collected daily to assess the fall in $\log_{10}$ colony-forming units (CFUs) and the increase in time to positivity (TTP) for mycobacterial culture. The study's main outcome, which remains unchallenged by this *post hoc* analysis, is

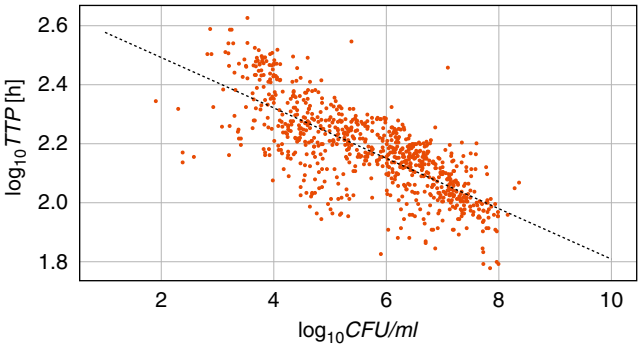

Figure 1. Daily $\log_{10}$ colony-forming units ($\log_{10}$CFUs) and $\log_{10}$ time to positivity ($\log_{10}$TTP) for mycobacteria over 7 days for all 59 participants. The relationship between TTP and CFUs is determined using an ordinary least-squares regression model: $\text{TTP} = b_0 + b_1 \times \text{CFUs}$. The resulting equation is $\log_{10}\text{TTP} = 2.663 - 0.0854 \times \log_{10}\text{CFU}$; $r^2 = 0.555$; $P < 0.0001$ for b_0 and b_1.

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that INH given at doses of 10–15 and 5 mg/kg to participants with and without *inhA*-mediated mycobacterial INH resistance, respectively, provide similar 7-day EBA.

Our interest was sparked by the observation that baseline sputum bacillary loads, measured by CFU counts on solid culture medium, were lower in control subjects (median, 5.41 $\log_{10}$CFU) than in participants with mycobacterial *inhA* mutations (median, 5.65–7.04 $\log_{10}$CFU). However, the baseline TTP for mycobacteria in liquid cultures was shorter overall in control subjects (median, 97 h) than in participants with mycobacterial *inhA* mutations (median, 124–142 h). This is noteworthy because lower CFU counts would be expected to translate into a higher TTP rather than into the lower TTP observed.

To test whether microbial fitness cost conferred by mycobacterial mutations could explain this incongruence, we reclassified the baseline isolates of participants for the presence of bacterial mutations. Of 59 participants enrolled, 43 participants (73%) had mycobacteria with *inhA* mutations at least. Thirty-one participants (52%) had mycobacteria with additional mutations conferring rifampicin resistance, three participants (5%) had mycobacteria with mutations conferring fluoroquinolone resistance, and one participant (2%) had mycobacteria with mutations conferring aminoglycoside resistance. Of the 16 control subjects, only 13 subjects had mycobacteria with no detectable resistance, whereas three had mycobacteria with rifampicin monoresistance and were thus reclassified into the mutation group. This resulted in 13 participants (22%) without mycobacterial mutations contributing 168 TTP and CFU results and in 46 participants (88%) with at least one resistance-conferring mycobacterial mutation contributing 643 data points.

Figure 1 shows all available CFUs and TTPs plotted against each other. A linear ordinary least-squares regression model with $\log_{10}$TTP as a dependent variable, $\log_{10}$CFU as an independent variable, and an intercept term fitted the data well ($r^2 = 0.555$; $P < 0.0001$ for slope and intercept). The introduction of a mutation variable separated the mutated and nonmutated samples in two almost parallel lines (Figure 2) and improved the correlation ($r^2 = 0.631$). The difference in intercepts was significant ($P = 0.002$), but there was no significant difference in the steepness of the slopes ($P = 0.852$). This means that the presence of bacterial mutations significantly prolongs the expected TTP for a given $\log_{10}$CFU by about 50 hours, independent of the CFU count.

Microbial fitness cost due to mutations is a well-known concept (2). Bacterial resistance-conferring mutations are generally costly, but compensatory mutations can alleviate some of the fitness costs of resistance (3). An increased generation time has been described in tuberculosis bacilli with a *rpoB* mutation under stress (4). Time to culture positivity for bacteria is vulnerable to changes in metabolic activity because its readout is dependent on consumption of nutrients, which in turn depends on both the activity and number of bacteria present. Counting of CFUs, however, only depends on numbers and is thus not as affected. Colonies growing more slowly will be smaller but will still contribute to enumeration.

This finding will not be of much consequence if only differences of the same endpoint marker between time points are measured, such as in EBA studies. The parallel lines in Figure 2 illustrate that a dropping mycobacterial load will change at the same rate if mutated or nonmutated bacteria are measured. A potential pitfall comes in when comparison to an absolute value is made, such as in studies that measure the rate of or time to conversion of culture results from positive to negative.

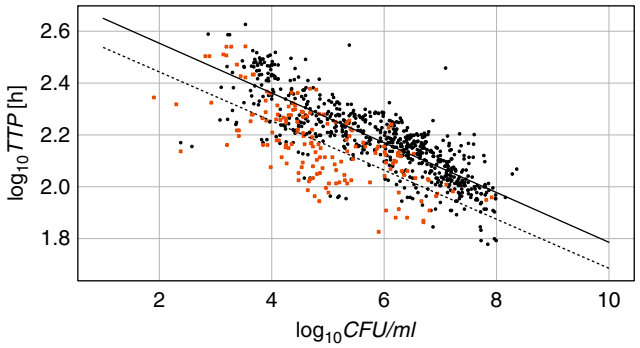

Figure 2. Daily $\log_{10}$ colony-forming units ($\log_{10}$CFU) and $\log_{10}$ time to positivity ($\log_{10}$TTP) for mycobacteria over 7 days for all 59 participants with mutated (black dots) and nonmutated (red crosses) bacteria. The model includes a mutation variable (0, dashed line, or 1, full line): $\text{TTP} = b_0 + b_1 \times \text{CFUs} + b_2 \times \text{mutations} + b_3 \times \text{CFUs} \times \text{mutations}$. This formula resulted in $\log_{10}\text{TTP} = 2.6324 - 0.0947 \times \log_{10}\text{CFUs} + 0.1133 \times \text{mutations} - 0.0013 \times \log_{10}\text{CFUs} \times \text{mutations}$; $r^2 = 0.631$; $P < 0.0001$ for b_0, b_1; $P = 0.002$ for b_2; $P = 0.852$ for b_3.

Consider a clinical study with an all-novel regimen composed of agents with new mechanisms of action that are not susceptible to mutations conferring resistance to conventional agents. This new regimen would be tested in a patient population with disease caused by bacteria resistant to conventional agents. The control group would be on the conventional regimen and would consist only of subjects with mutation-free bacterial infection. The endpoint of having conversion from positive to negative liquid culture results would require the TTP for mycobacteria to increase beyond 42 days for negative results to be declared. According to our findings, if both treatments are equally efficacious, culture conversion is likely to occur sooner in the all-novel treatment group because not only the treatment but also the mutation-associated fitness cost is slowing down the cultures. Mutation-free control cultures would grow faster, and conversion to negative results using the reference treatment would be harder. Faster culture conversion by the all-novel regimen would lead to the conclusion that it is superior to the conventional treatment, even in patients who may be described as "hard to treat."

Our observations are biologically plausible and based on data collected in a prospective clinical trial, but this *post hoc* analysis has inherent weaknesses. Mutations were detected by line probes only, thus not allowing quantification of proportions of mutated bacilli and varying degrees of fitness cost or compensatory effects. Larger cross-sectional studies including whole-genome sequencing to detect a broader diversity of strains, mutations, and subpopulations is needed to investigate this further.

Until it is clear how mutation-related fitness cost influences culture-based endpoints, it seems prudent to not use liquid culture results as the only or even the primary endpoint in studies involving participant groups with disease caused by bacteria with different degrees of resistance. Comparisons between groups with disease due to mutated and nonmutated bacilli in clinical trials should be made with caution. Solutions to the problem might come from nucleotide-based alternatives, such as bacterial sputum DNA (5), transrenal DNA

(6), or RNA (7, 8); from measurements of bacterial components such as sputum lipoarabinomannan (9); or from genetic epistasis analysis (10). Future clinical trials should test these readouts against each other to better understand their significance. ■

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Cystic Fibrosis Lung Function Decline after Within-Host Evolution Increases Virulence of Infecting *Pseudomonas aeruginosa*

To the Editor:

People with cystic fibrosis (CF) and *Pseudomonas aeruginosa* (*Pa*) lung infections often experience unexplained clinical deterioration, and intensive efforts have been made to understand causes of these declines. We present a patient with CF who suffered marked reduction in lung function, link the decline to the emergence of point mutations in infecting *Pa*, and discuss the implications of the findings for within-host evolution of pathogens, antibiotic treatment, and personalized medicine.

The male patient with *F508del/F508del CFTR* genotype became infected with *Pa* at 0.5 years of age. Genome sequencing determined that the same *Pa* strain was present until his death, as is common in CF (1). At approximately 15.6 years of age, the patient developed reduced lung function (Figure 1, top). He received multiple antibiotic courses; however, his lung function decline continued. Evaluation including repeated cultures to search for new bacterial, mycobacterial, fungal, and viral pathogens; allergic bronchopulmonary aspergillosis; and lung imaging identified no etiology other than long-standing

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Data availability: The genome sequencing data are uploaded to the National Center for Biotechnology Information and are now available. The accession number is Bioproject PRJNA669532.

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