in helping clinicians discriminate between states of health, contamination, colonization, and infection. Although in principle our metagenomic approach is capable of identifying nonbacterial pathogens, we have not yet established whether our current approach can detect fungal, viral, or protozoal pathogens in the lungs. Additional methodological work is needed to optimize the detection of microbial signal in host-DNA–rich respiratory specimens. Finally, further work is needed to streamline the bioinformatic analysis of metagenomic sequencing data before this approach can be scaled for testing in a clinical context.

Pneumonia is a 21st-century problem, yet its diagnosis still relies on 19th-century tools. Our results demonstrate the feasibility and promise of introducing real-time metagenomics to our diagnostic arsenal. Clinical study is warranted, as the revolution in molecular microbiology has at last reached the bedside. ■

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Triplicate Sputum Cultures for Efficacy Evaluation of Novel Antituberculosis Regimens

To the Editor:

Sputum culture conversion from positive to negative remains the standard endpoint in recent studies of antituberculosis drugs (1–3). In most protocols, a single sputum sample is collected for culture at each patient visit. With ongoing efficacious treatment, however, sputum production and quality decreases (4), while the frequency of "contaminated" results increases. Such results are equivocal, do not prove the absence of viable mycobacteria, and increase the cost of the trial. Various measures at different steps of the sputum collection and culture process can reduce, but not completely eliminate, the problem of contaminated cultures (5–8).

TMC207-C208 (NCT00449644) was a 120-week, randomized, double-blind, phase 2 study that examined addition of bedaquiline or placebo to the first 24 weeks of a five-drug treatment regimen in 160 adults with multidrug-resistant tuberculosis (2). For the first time, we used a simple strategy to minimize the occurrence of microbiologically uninformative visits by collecting triplicate sputa at 30-minute intervals during each visit, separate routine processing of each sputum sample using only mycobacteria growth indicator

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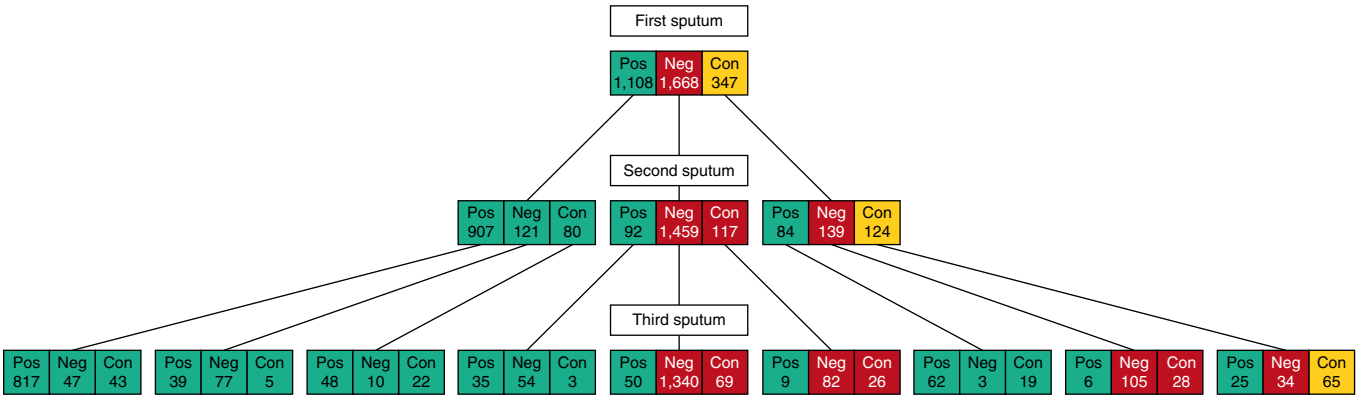

Figure 1. Distribution of single sputum results over 27 possible permutations in triplicate and the aggregate result after addition of the second and third sputa. The text and numbers in the boxes indicate the occurrence of single sputum outcomes in an analysis limited to patients who had three sputum results. Single sputum results are combined to aggregate duplicate and triplicate scores following the principle that an aggregate score with at least one positive single sputum is positive, an aggregate score with no positive but at least one negative single sputum is negative, and an aggregate score of contaminated is awarded only if all available sputa are contaminated. The colored blocks indicate the aggregate result after each additional sputum, with an overall positive result shown as *green*, an overall negative result shown as *red*, and an overall contaminated result shown as *yellow*. The figure shows clearly that the chance of scoring a positive (*green*) or negative (*red*) aggregate increases with each sputum, whereas the chance of scoring a contaminated aggregate (*yellow*) decreases. Con = contaminated; Neg = negative; Pos = positive.

tube (MGIT) liquid culture (Becton Dickinson, Sparks, MD), and scoring of triplicate results for each visit (2). Single sputa were categorized as positive, negative, contaminated, or missing. All instrument-positive cultures were reported as positive if blood agar was negative and contaminated if blood agar was positive, regardless of the presence or absence of acid-fast bacilli as identified by Ziehl-Neelsen staining. Cultures were declared negative if no growth occurred 6 weeks after incubation. Duplicate and triplicate sputa were scored as positive aggregates if one or more sputum sample was positive, negative aggregates if one or more sputum sample was negative and none were positive, or contaminated if none were valid. All samples produced were processed unless there was evidence of leakage or bloody admixture. If a visit produced fewer than three samples, the aggregate result of available sputa was used.

We examined the concordance of results between the first sputum and aggregate scores from triplicate sputa, and the effect of triplicate versus single sputum sampling on the number of evaluable patients in a hypothetical TMC207-C208 trial scenario comparing sputum conversion from positive at baseline to positive/negative at Weeks 8, 16, or 24. Partial results of this analysis have been previously reported (9).

Over the course of 120 weeks, 160 participants submitted 9,685 evaluable sputa with 536 missing samples, resulting in 3,077 complete triplicates (88.8%), 244 duplicates (7%), and 146 single sputa (4.2%). Overall, of the 9,685 single sputum samples, 34.6% (n = 3,410) scored positive, 55.1% (n = 5,437) were negative, and 10.3% (n = 1,018) were contaminated. There were comparable rates of positive (range, 34.4–34.9%), negative (54.3–56.0%), or contaminated scores (9.1–11.3%) for single sputum samples in triplicates, showing that single sputum results were not affected by 30-minute intervals between collections.

Rates for first sputa and aggregate triplicate scores (n = 3,467 samples), respectively, were 34.4% (n = 1,194) and 42.3% (n = 1,467) for positive, 54.3% (n = 1,882) and 55.1% (n = 1,911) for negative, and 11.3% (n = 391) and 2.6% (n = 89) for contaminated. Use of aggregate scores versus the first sputum sample increased the proportion of visits with informative culture results mainly by

Table 1. Distribution of Single Sputum Results in Triplicates Scored Positive or Negative

Triplicates Scored Positive	**First Sputum (*N* = *1,467*)**	**Second Sputum (*N* = *1,441*)**	**Third Sputum (*N* = *1,352*)**
Positive, n (%)	**1,194 (81.4)**	1,142 (79.3)	1,074 (79.4)
Negative, n (%)*	**154 (10.5)**	178 (12.4)	186 (13.8)
Contaminated, n (%)*	**119 (8.1)**	121 (8.4)	92 (6.8)
Triplicates Scored Negative	**First Sputum (*N* = *1,911*)**	**Second Sputum (*N* = *1,807*)**	**Third Sputum (*N* = *1,660*)**
Negative, n (%)	**1,728 (90.4)**	1,654 (91.5)	1,537 (92.6)
Contaminated, n (%)*	**183 (9.6)**	153 (8.5)	123 (7.4)

Definition of abbreviations: N = total number of sputum samples; n = number of sputum samples scoring positive, negative, or contaminated.
The first sputum results are bold for comparison with the aggregate triplicate score.
*These rows indicate false-negative results compared with the triplicate result.

scoring negative first samples as positive triplicates (+7.9%) and contaminated first samples as negative triplicates (+8.7%). The chance of scoring a positive or negative aggregate increased with each sputum in a triplicate, whereas the chance of scoring a contaminated aggregate decreased (Figure 1).

Compared with 1,467 positive aggregate scores, 18.6% of the first sputa were determined to be false-negative because they failed to detect viable bacteria at this visit (Table 1). Of 1,911 negative triplicates, 9.6% of the first sputa were contaminated (Table 1) and failed to demonstrate the absence of viable mycobacteria.

False-negative results based on first sputa increased over time for positive aggregate scores, peaking at the critical phase of the study when patients were nearing culture conversion and levels of viable mycobacteria were low, and declining again thereafter (data not shown). The use of the aggregate scores thus identified patients who still had an active infection and could help prevent overestimation of treatment efficacy.

In the hypothetical trial scenario, 7 (5%), 16 (13%), 10 (9%), and 18 (18%) more patients with contaminated first sputa had informative positive/negative aggregate scores at baseline and Weeks 8, 16, and 24, respectively. The extra work involved for the additional evaluable patients is performing three sputum collections and MGIT cultures instead of a single one at each visit. The consequent "cost" in extra sputum cultures was 275 (199%), 247 (183%), 227 (183%), and 218 (185%) sputum cultures, respectively, equating to an average of 19 more samples collected and analyzed per extra evaluable patient gained. It is more than likely that the cost of one trial patient is several times the cost of the additional MGIT cultures required.

A limitation of this analysis is the lack of an early morning sputum for comparison, although data supporting this approach are spurious and only available from a diagnostic setting (5). No statistical significance testing was performed; however, we consider a decrease from 11.3 to 2.6% in the proportion of uninformative visits of considerable clinical importance. Finally, we did not examine grades of positivity within triplicates but consider it beyond the scope of this work.

In conclusion, collection of triplicate instead of single sputa and deriving an aggregate score increases the sensitivity for efficacy determination in studies of antituberculosis regimens by increasing the proportion of positive and negative visits and reducing the proportion of uninformative visits. This increased sensitivity of aggregate scores makes culture conversion harder to achieve, as single sputum sampling may disadvantage a more efficacious treatment. As such, results of comparative studies such as TMC207-C208 are more robust if performed with triplicate samples. Triplicate sputum sampling is relatively little extra work and increases the proportion of evaluable patients, thereby reducing the size, duration, and cost of studies, which is an important consideration in planning *Mycobacterium tuberculosis* clinical trials in resource-limited settings. ■

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Diagnosis of Western Red Cedar Asthma Using a Blood-based Gene Expression Biomarker Panel

To the Editor:

Western red cedar asthma (WRCA) is the most common form of occupational asthma in the Pacific Northwest region of North America (1). It has been shown that WRCA is caused by sensitivity to plicatic acid (PA), which is a low-molecular-weight molecule found in the dust from western red cedar (*Thuja plicata*) (2). To date, no molecular biomarkers have been reported that can diagnose WRCA (3). At this time, the diagnosis involves multiple bronchial challenges, which are time-consuming, expensive, and logistically challenging. Peripheral whole blood is a useful and easily obtainable resource for studying WRCA, and transcriptional changes in blood have been observed after methacholine inhalation challenge in WRCA individuals (4). In the study reported here, we have developed a blood-based biomarker panel that can classify, at baseline (before PA challenge), PA-positive subjects from PA-negative subjects.

Methods

On written informed consent, 24 male subjects (17 subjects in the discovery set and 7 subjects in the independent validation set) were recruited and underwent a methacholine challenge (Day 1), followed by a PA challenge (Day 2), using standardized protocols (5). On both days, FEV_1 was monitored at regular intervals until 6 hours after the commencement of inhalational challenge. On the basis of the FEV_1 data collected during the Day 2 PA challenge, eight subjects in the discovery set were classified as PA-negative (defined as neither a 20% drop in FEV_1 within the first 2 hours nor a 15% drop in FEV_1 at 2–6 h), whereas the rest of the subjects in the discovery set were classified as PA-positive (all these subjects had a drop in FEV_1 $\geqslant$20% during the first 2 h). All 7 subjects in the validation set were classified as PA-positive. Peripheral whole-blood samples were collected on Day 2 before the PA challenge.

The blood samples were collected using PAXgene Blood RNA Tubes, and total RNA was extracted using the PAXgene Blood miRNA Kit (PreAnalytiX, Hombrechtikon, Switzerland). One hundred nanograms of the purified RNA was used in a custom NanoString nCounter Elements assay (NanoString Technologies, Seattle, WA) to simultaneously quantify the expression of 166 transcripts, which are mostly involved in immune processes such as the T helper type 2 (Th2) cell pathway (6). The data were normalized and analyzed using the statistical computing environment, R (version 3.2.4), and the biomarker panel was identified using various classification methods such as random forests and elastic net. The performance of the panel was evaluated using an area under the receiver operating characteristic curve (AUC) obtained from leave-one-out cross-validation and further tested with samples from the validation set. The differential expression of the panel transcripts was identified using the linear models for microarray data R package.

This study was reviewed and approved by the University of British Columbia Clinical Research Ethics Board, and written informed consent was obtained from all the study participants.

Results and Discussion

Table 1 shows the clinical and demographic characteristics of the subjects in both discovery and validation sets. In the discovery set, there was no significant difference in age, body mass index, and baseline FEV_1 (before methacholine challenge and before PA challenge) between the PA-positive group and the PA-negative group, using Mann-Whitney *U* tests. There was no significant difference in percentage change in FEV_1 at any point between the two groups after methacholine challenge (Day 1). During the course of PA challenge (Day 2), the PA-positive subjects experienced significant drops in FEV_1 compared with the PA-negative subjects at all points after challenge ($P < 0.001$).

Having demonstrated that there was no difference in the clinical and demographic characteristics between the PA-positive and the PA-negative groups (excepting after PA challenge) in the discovery set, we undertook a biomarker discovery approach to identify a classifier of PA-positive subjects at baseline. Using a candidate-directed approach of random forests, a biomarker panel consisting of 2 transcripts, *MAP2K2* and *MAPKAPK2*, demonstrated the highest AUC performance of 0.847 (95% confidence interval, 0.631–1.000) (Figure 1). The phenotypic labels (PA-positive or PA-negative) were then reshuffled, and the AUC was recalculated 200 times using leave-one-out cross-validation. The mean and standard deviation AUC of this random classifier was 0.600 ± 0.095 (Figure 1). To validate this panel, seven additional independent PA-positive subjects (validation set) were tested. Using the threshold (Youden index) that was previously established using the discovery samples, 6 (86%) of these new subjects were correctly classified as PA-positive.

We further compared the expression of the 2 panel transcripts between PA-positive and PA-negative groups at baseline (before challenge) in the discovery set. Both *MAP2K2* and *MAPKAPK2* showed differential expression between PA-positive and PA-negative subjects (Benjamini-Hochberg false discovery rate = 0.027).

Kinases such as MAPKAPK2 and MAP2K2 in the mitogen-activated protein kinase (MAPK) signaling pathway play an important role in regulating inflammatory responses. The MAPK signaling pathway divides into three major groups: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase, and p38 MAPK. MAPKAPK2 is a downstream molecule of p38 MAPK, whereas MAP2K2 belongs to the ERK group. The p38 MAPK group is involved in many aspects of asthma such as cytokine release, mast cell migration, and neutrophil recruitment (7). It is a key player in activating transcription factor GATA-binding protein (GATA) 3, which further regulates Th2 cell differentiation and Th2 cytokine expression (8). It also contributes to eosinophil differentiation (9) and inhibition of eosinophil apoptosis (10), which may be associated with the elevated levels of these cells observed in WRCA (2). The

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