

9. Diacon A, van Brakel E, Lounis N, Meyvisch P, Van Baelen B, de Marez T, Jenkins E, Dannemann B. Triplicate versus single sputum samples improves sensitivity for efficacy determination in studies of anti-tuberculosis (TB) regimens [abstract]. *Int J Tuberc Lung Dis* 2015;19 (Suppl 2):S284. Abstract OA-405-05.

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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, FEV₁ was monitored at regular intervals until 6 hours after the commencement of inhalational challenge. On the basis of the FEV₁ 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 FEV₁ within the first 2 hours nor a 15% drop in FEV₁ 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 FEV₁ ≥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 FEV₁ (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 FEV₁ 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 FEV₁ 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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Table 1. Clinical and Demographic Characteristics of the Subjects in the Discovery and Validation Sets

	Discovery Set			Validation Set	
	PA Positive (n = 9)	PA Negative (n = 8)	P Value*	PA Positive (n = 7)	P Value†
Age, yr	54.0 (43.0 to 56.0)	38.0 (31.5 to 47.5)	0.152	50.0 (44.0 to 60.0)	0.122
BMI	28.70 (25.10 to 30.40)	27.65 (24.85 to 33.02)	0.962	29.30 (27.25 to 30.95)	0.971
Predicted FEV ₁ , L	3.91 (3.61 to 3.97)	4.36 (3.73 to 4.56)	0.290	4.06 (3.10 to 4.48)	0.509
Predicted FEV ₁ /FVC	0.81 (0.79 to 0.84)	0.82 (0.79 to 0.83)	0.736	0.79 (0.79 to 1.06)	0.928
Baseline FEV ₁ , L					
Before methacholine challenge	2.93 (2.31 to 3.63)	3.49 (2.79 to 3.65)	0.481	3.20 (2.58 to 3.41)	0.618
Before PA challenge	2.68 (2.32 to 3.47)	3.43 (2.77 to 3.59)	0.481	3.07 (2.18 to 3.25)	0.516
Percentage change in FEV ₁ from baseline (after methacholine challenge)					
1 h	−4.45 (−8.71 to 6.70)	−8.63 (−10.93 to 6.70)	0.423	−8.38 (−11.98 to −3.19)	0.466
2 h	−4.02 (−10.82 to 4.46)	−7.71 (−15.11 to 1.26)	0.321	−4.10 (−11.88 to 1.48)	0.560
4 h	−0.47 (−3.77 to 7.14)	−2.60 (−6.68 to 4.45)	0.815	−1.54 (−12.02 to 2.86)	0.715
6 h	−2.12 (−8.56 to 6.25)	0.27 (−1.79 to 3.65)	0.606	−4.40 (−5.23 to 2.05)	0.828
Percentage change in FEV ₁ from baseline (after PA challenge)					
1 h	−32.45 (−35.74 to −24.00)	9.24 (4.11 to 15.64)	<0.001	−15.94 (−21.70 to −8.01)	<0.001
2 h	−29.20 (−34.60 to −23.21)	1.88 (−0.65 to 12.13)	<0.001	−12.50 (−15.34 to −5.95)	<0.001
4 h	−24.64 (−31.64 to −19.38)	7.43 (1.61 to 14.47)	<0.001	−11.22 (−13.59 to −7.67)	<0.001
6 h	−28.60 (−42.74 to −23.27)	3.88 (0.27 to 10.96)	<0.001	−12.96 (−19.32 to −12.35)	<0.001
Current smoker, n	0	1		0	

Definition of abbreviations: BMI = body mass index; PA = plicatic acid.

Statistics are shown as median (interquartile range) unless otherwise indicated.

*Mann-Whitney *U* test comparing PA-positive subjects and PA-negative subjects in the discovery set.

†Kruskal-Wallis *H* test comparing PA-positive subjects and PA-negative subjects in the discovery set, and PA-positive subjects in the validation set.

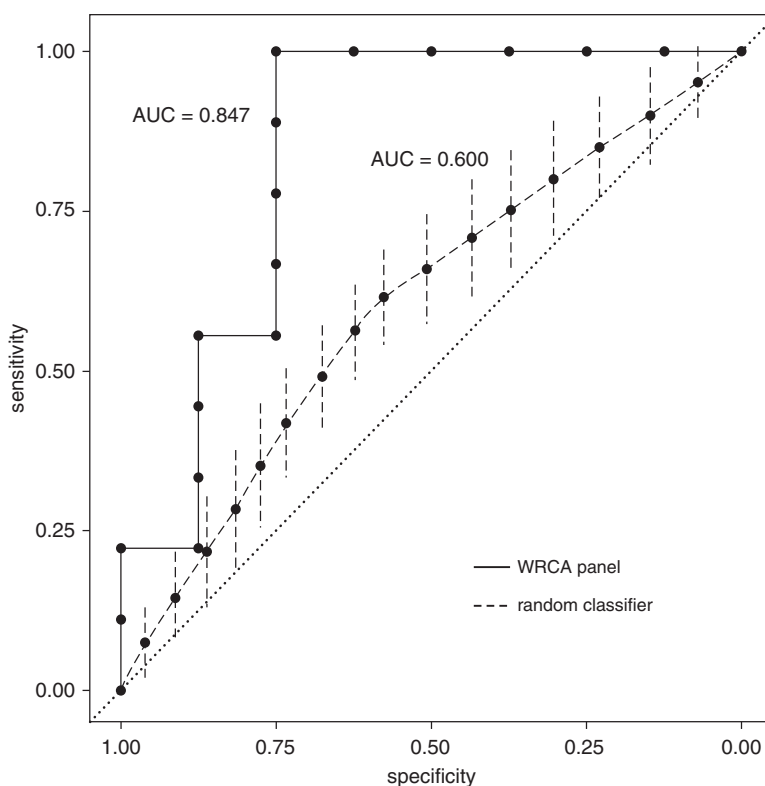


Figure 1. Area under the curve (AUC) performance of the reported western red cedar asthma (WRCA) biomarker panel (solid line) and the AUC performance after the reshuffling of phenotypic labels (dashed line) in the discovery set. Error bars indicate SDs of sensitivities.

ERK group is involved in Th2 cell differentiation as well (7). It also regulates airway smooth muscle cell proliferation (11), which may further contribute to remodeling and narrowing of the airway.

Limitations of this study include small sample size (for both discovery and validation) and the functional significance of blood reflecting changes in lung function. To ensure the results were not confounded by other variables, we carefully matched the clinical and demographic characteristics of the two groups. In conclusion, we have demonstrated that peripheral whole blood may have utility as a resource to help diagnose WRCA and that the transcriptional signatures may help to further elucidate the disease mechanisms of WRCA. Further validation of the biomarker panel with larger cohorts and additional PA-negative subjects is required to confirm its robustness. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Chen Xi Yang, B.Sc.
Amrit Singh, Ph.D.
Young Woong Kim, M.Sc.
University of British Columbia
Vancouver, British Columbia, Canada
and
Prevention of Organ Failure Centre of Excellence
Vancouver, British Columbia, Canada

Edward M. Conway, M.D., Ph.D.
Christopher Carlsen, M.D., M.P.H.
University of British Columbia
Vancouver, British Columbia, Canada

Scott J. Tebbutt, Ph.D.
University of British Columbia
Vancouver, British Columbia, Canada
and
Prevention of Organ Failure Centre of Excellence
Vancouver, British Columbia, Canada

ORCID IDs: 0000-0002-8033-4769 (C.X.Y.); 0000-0002-7475-1646 (A.S.); 0000-0002-3470-6728 (Y.W.K.); 0000-0003-0081-0305 (E.M.C.); 0000-0003-2455-6575 (C.C.); 0000-0002-7908-1581 (S.J.T.).

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Clinical Metagenomics for the Diagnosis of Hospital-acquired Infections: Promises and Hurdles

To the Editor:

We read with interest the paper by Pendleton and colleagues (1) [this issue, pp. 1610–1612] about the rapid identification of respiratory bacterial pathogens directly from a mini-bronchoalveolar lavage sample using the MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). Within hours of the samples being taken, they obtained for the first sample a single long read corresponding to *Pseudomonas aeruginosa*, and for the second, six reads assigned to *Staphylococcus aureus*, which were later proven to originate from the pathogens found by conventional culture.

We indeed share the enthusiasm of the authors about the application of next-generation sequencing methods to clinical samples (which we refer to as clinical metagenomics [CMg]), but we believe it is important to stress the many hurdles that need to be addressed before clinical metagenomics can be used routinely (2, 3).

Currently, the main challenge of CMg lies in the preanalytic stage: clinical samples (especially in case of bacterial infections) can contain a substantial concentration of leukocytes. Importantly, the human genome is approximately 1,000 times longer than an average bacterial genome. Hence, when not applying a DNA extraction method aimed at reducing the load of human DNA, the vast majority of sequenced reads may be of human origin. This could explain the very low number of bacterial reads reported by Pendleton and colleagues (1).

Another major challenge of CMg is to differentiate between sequence reads that originate from the “real” microbiota and those that originate from reagent contaminants. Although *S. aureus* is not a common contaminant, culture-free profiling of microbial communities showed that sequence reads assigned to *Pseudomonas* spp. may also be of exogenous origin (4). This argues for the systematic use of negative extraction controls in real-time sequencing, as already recommended for 16S ribosomal DNA profiling and whole metagenome shotgun sequencing of microbial communities (5).

Furthermore, in the context of the microbiological diagnosis of hospital-acquired pneumonia, the distinction between colonization and infection is based on quantification thresholds

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