Volatile sputum biomarkers can monitor the response to treatment of *nontuberculous mycobacteria* disease: a pilot study

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Abstract— ***Nontuberculous mycobacteria*** **disease can cause severe comorbidity and high mortality. Tracking treatment response and determining treatment endpoint remains a major challenge in the clinical management of NTM disease. The current approach for monitoring treatment response requires multiple cultures and radiographic results, which is time-consuming and relatively insensitive. Here we report nine biomarkers selected by comparing paired pre-treatment (n=6) and post-treatment (n=6) sputum samples. The suggested biomarkers can distinguish the pre-treatment group from the post-treatment group. The results demonstrate that detecting volatile sputum biomarkers is a potential supplementary tool for monitoring the response to the treatment of NTM disease.**

Keywords— **NTM, volatile biomarker, GC×GC-ToFMS**

I. Introduction

Nontuberculous mycobacteria (NTM) are ubiquitous bacteria. Among over 190 known NTM species, some are opportunistic pathogens that can cause severe infections in the lung and other organs. The incidence of pulmonary disease caused by NTM infections is increasing globally, including in North America, Europe, and Asia [1]. In Canada specifically, a study on the Ontario population reported that the NTM disease rate doubled from 1998 to 2010 [2].

NTM infections cause airway inflammation and progressively result in severe lung damage. The most encountered pathogenic NTM species are *M. avium* complex (MAC) and *M. abscessus* complex (MABSC). The slowly growing MACs are the most prevalent, while the MABSC strains are less common but have, on average, poorer treatment outcomes. NTM disease, and more importantly, comorbidities, lead to a high 5-year mortality rate of about 40% [3,4].

People with compromised immune systems, including underlying lung diseases, are susceptible to NTM infection. For example, people with cystic fibrosis (pwCF) are at a significantly higher risk of acquiring NTM infections than general populations. The NTM detection rate in the general population is approximately 1 in 20,000, whereas in pwCF, the rate is 20% [5]. In Canada, it is estimated that 5% of pwCF have an NTM infection over a lifetime [6]. Besides the lengthy NTM diagnosis process, which can take 4-6 months, another major challenge in the clinical management of NTM is evaluating the response to treatment [7]. The treatment response assessment regime currently includes microbiologic culture, radiography, and symptomatic and quality-of-life assessment. Determining treatment efficacy, i.e., NTM eradication, requires at least three consecutive negative mycobacterial cultures from respiratory samples collected at least four weeks apart. And determining treatment failure is suggested to have at least two positive cultures of the causative species from respiratory samples [8]. Alternative biomarkers, such as blood-based humoral [9] and NTM-driven immune responses [10], have been reported, but the approaches are invasive and need further development. Thus, developing a faster, culture-independent tool to identify NTM disease and track treatment is necessary.

The use of airway volatile molecules from patients' breath has been reported as a potential approach to distinguish people with NTM disease from those who are NTM-free. In this pilot study, we report the possible use of volatile molecules from patients' sputum samples to differentiate pre- and post-treatment samples.

II. Material and Methods

A. Study Design

Six pwCFs (subjects A to F) diagnosed with NTM lung disease were enrolled in a clinical study in National Jewish Health. Three of those six subjects were diagnosed with *M. avium* complex (MAC), and three were *M. abscessus* complex (MABSC) (Table 1). The six subjects started antibiotic treatment at the beginning of the study, and paired sputum samples were collected from pre-treatment and during treatment or post-treatment. After a period of treatment, subjects A, D, and E became NTM clear, and the remaining four patients were still infected with NTM and continued on treatment.

Table 1. Clinical information of the enrolled subjects.

ID	Age	Sex	CF Mutations	Species	*Tx duration	Tx outcomes
A	54	M	F508del	MABSC	399	Cleared
B	21	M	2184insA/ H199Y	MABSC	224	Not cleared
C	26	M	F508del/406-1G->A	MABSC	28	Cleared
D	26	F	F508del/N1303K	MAC	823	Cleared
E	23	F	F508del/621+1G->T	MAC	386	Not cleared
F	36	F	F508del	MAC	114	Not cleared

F and M stand for females and males. Tx stands for treatment. Tx duration means the duration between two sampling times in days. For those patients who did not get NTM cleared, the treatment continued afterward.

B. Experimental Method

Sputa were collected and homogenized with Dulbecco's phosphate-buffered saline (volume = 3 × weight of sputum) and 10% sputolysin solution (volume = 4 × weight of sputum). Thereafter, 0.5 mL of the sputum supernatant was transferred to a 20 mL headspace vial. The sputum headspace was extracted by a 2 cm length solid phase microextraction (SPME) fiber which was coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) df 50/30 µm from Supelco (Bellefonte, PA, USA). The sample was incubated at 43 °C for 15 minutes and extracted for 30 minutes. The fiber was then desorbed at 270 °C for 3 minutes into the 2-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-ToF MS) for analysis. The primary column was a Rxi-624 Sil MS with mid polarity, and the second column was Stabilwax with high polarity. The generated chromatograms were processed and aligned by ChromaTOF (version 4.72, LECO, MI, USA).

C. Data Processing

A signal-to-noise (S/N) cutoff of 50:1 was used for peak finding, and the NIST 11 library was used for peak identification. A match score over 800 was required for the putative peak identification. For the alignment of peaks, a maximum retention time deviation for the first and second dimensions was set at 3 s and 0.1 s, with an inter chromatogram spectral match threshold set at 750. Data analysis was performed using R version 4.1.1. Contaminants and artifacts were removed before further data analysis [11]. Probabilistic Quotient Normalization (PQN) was then applied to correct the dilution effect during the sampling process. A frequency of observation (FOO) cutoff of 100% in either the pre-treatment or post-treatment groups was implemented. The remaining features were $\log_{10}$ transformed, mean-centered, and auto-scaled. The Boruta feature selection method was then applied for feature selection [12]. The principal component analysis (PCA) score plot and hierarchical heatmap were employed as visualization tools. (Figure 1)

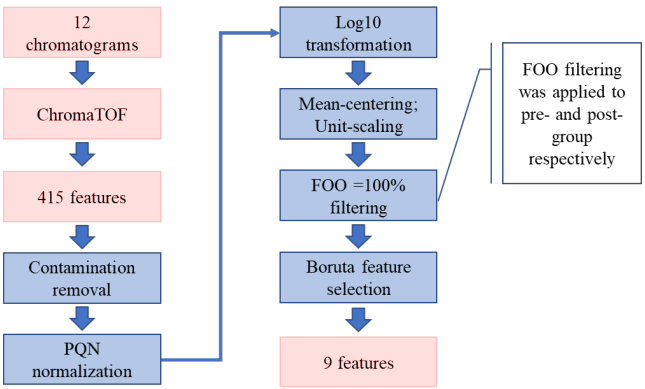

Fig. 1 Schematic of data processing workflow. PCA and heatmap were used for visualization after selecting 9 features.

III. Results and Discussion

A. The sputum VOCs can monitor the response to the treatment

In this pilot study, we hypothesized that a set of volatile molecules in the headspace of sputum samples could be used for discriminating between the pre- and post-treatment groups. Across the 12 samples, 415 unique features were found. From these, nine features that can best differentiate the two groups were selected using the Boruta feature selection algorithm (Table 2 and visualized in Figure 2).

Among the nine potential biomarkers we reported, five molecules were given putative names, out of which four molecules are hydrocarbons, and one is a ketone. The remaining four analytes did not meet the library match score of 800, thus, were not given a putative name. Eight out of nine features have a higher relative abundance in the pre-treatment group, and one feature shows the reverse trend.

The first and second principal components (PC) capture 46% and 22% of the total variance of the nine selected features, respectively. The hierarchical heatmap illustrates the complete clustering of the two groups and the relative abundances of the selected biomarkers (Fig. 2b). The relative abundance differences between pre-treatment and post-

Table 2. The set of Boruta-selected features discriminated between the pre-and post-treatment samples.

ID	Putative Name	Chemical Formula	Chemical Class	Before Tx vs. After/During Tx	1st RT	2nd RT	Kovats Retention Index for 1st RT
1	p-acetylacetophenone	$C_{10}H_{10}O_2$	Ketone	↓*	1717	1.0	1146
2	Analyte 220 (A)	Unknown	Hydrocarbon	↑	1473	0.5	1026
3	3,5-dimethyl-Octane	$C_{10}H_{22}$	Hydrocarbon	↑*	1438	2.0	1009
4	2,4-Dimethyl-1-heptene	C_9H_{18}	Hydrocarbon	↑*	1070	0.6	846
5	Analyte 312 (B)	Unknown	Hydrocarbon	↑*	1959	1.6	1275
6	3,3-dimethyl-Hexane	C_8H_{18}	Hydrocarbon	↑*	2019	1.1	1360
7	Analyte 207 (C)	Unknown	Ketone	↑*	1441	0.8	1011
8	Analyte 334 (D)	Unknown	Unknown	↑*	2093	0.7	1354
9	4,4,5-trimethyl-2-Hexene	C_9H_{18}	Hydrocarbon	↑*	1020	0.6	826

* p-value < 0.05. Wilcoxon test method was used. Tx stands for treatment. 1st and 2nd RT represent the retention time on the primary and secondary columns, respectively

treatment groups of all features except Analyte A are statistically significant.

Discussion

This is the first study investigating how volatile molecules change through antibiotic treatment in patients with NTM disease. The observed clustering patterns in the PCA space and heatmap provided pilot project-level evidence supporting our hypothesis that the volatile molecules in the sputum samples' headspace have the potential to monitor the response to the treatment of NTM disease. The nine biomarkers consisted of ketones and branched hydrocarbons (including alkanes and alkenes). Among the nine molecules we reported, eight demonstrate higher relative abundance in the pre-treatment group.

We hypothesize that the alkanes among the biomarker might originate from lipid peroxidation. Lipid peroxidation is a consequence of oxidative stress, a process linked to pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) [13], lung cancer [14], and tuberculosis (TB) [15]. A previous TB breath research study [16] reported biomarkers in the same chemical classes as we reported, showing higher abundances in the TB disease group. The author linked those biomarkers to oxidative stress. We observed that most biomarkers became less abundant after treatment. Thus, we hypothesize that antibiotic treatment could lead to a decreased level of oxidative stress, and the products of lipid peroxidation decrease as a consequence. This study shows no direct evidence between oxidative stress and NTM treatment. However, other studies demonstrated that the serum lipid peroxidation product, malondialdehyde, was more abundant in TB patients [15] and decreased in abundance after two months of chemotherapy [17].

The possible biochemical pathways of other biomarkers, including ketones and alkenes, can only be speculated. They may be the metabolism of microorganisms [18].

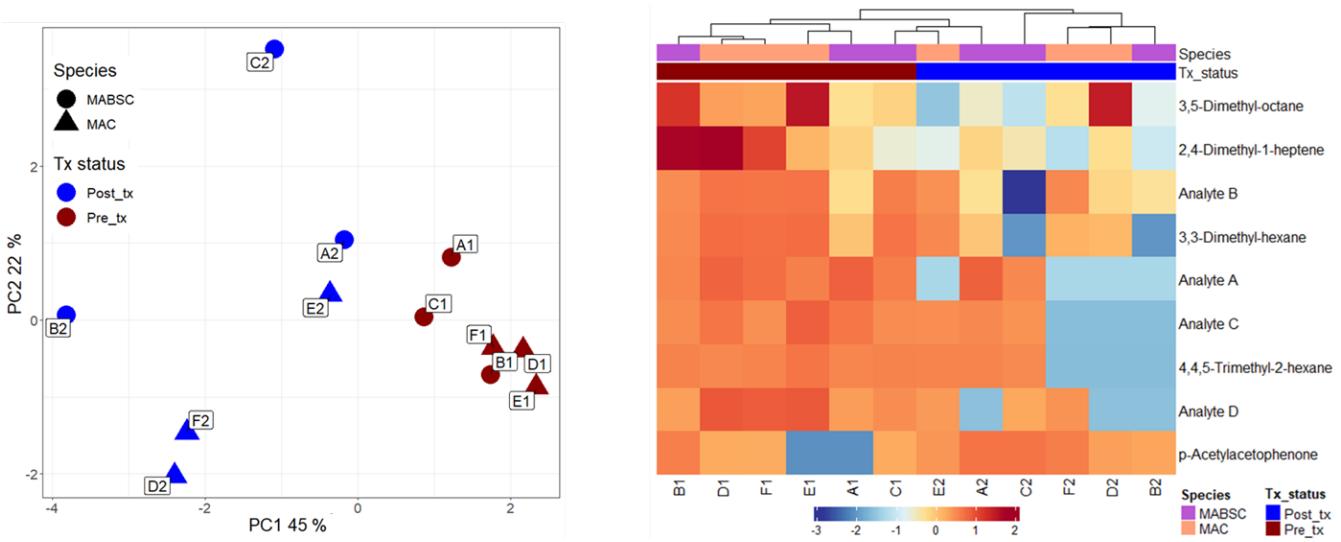

Fig. 2 a) The principal component analysis (PCA) plot generated using the nine selected features. b) the hierarchical heatmap of the nine features. Clustering distance method = Euclidean. A – F represent the subject A to F. Tx stands for treatment. Subject A, D and E were cleared from NTM after treatment.

In addition to separating the pre- and post-treatment groups, we also observed that the MAC and MABSC groups tend to sub-cluster. Based on this observation, we could conjecture that a subset of these biomarkers could drive separation between the two groups.

There are several limitations in this pilot study. Most importantly, the sample size is tiny, and the time between samples collected for each paired patient sample varies largely, i.e., from 28 to 823 days. In addition, the collection of only two samples per subject did not allow us to track the potential dynamic variation of the biomarkers. Future studies should include a larger cohort of NTM patients, and a longitudinal sampling regime over the treatment period. Moreover, we are still unsure about the origin of the biomarkers and cannot exclude the possibility that some biomarkers are antibiotic drug metabolites. The origin of these biomarkers would be an interesting and important line of research to pursue, but accurate and reliable peak identification is paramount in explaining the metabolic pathways related to volatile biomarkers. Thus, chemical standards and retention index references are needed to confirm the results reported in this work.

IV. Conclusions

Our pilot study from the breath of patients with NTM disease reported a panel of nine features, which enabled us to distinguish between pre-treatment and post-treatment samples. This work is the stepping stone to developing a culture-independent tool for tracking the response to the treatment of NTM pulmonary disease. Considering the limitations of this pilot study, a larger cohort is needed to validate the performance of the biomarkers in a clinical context. Additionally, using authentic chemical standards or high-resolution analytical tools should be employed to confirm the biomarker identity.

Conflict of Interest

The authors declare that they have no conflict of interest.

State of Human Rights

The study was approved by the National Jewish Health Institutional Review Board and Research Ethics Boards at the University of British Columbia.

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