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Technical Note

Combination of Exhaled Breath Analysis with Parallel Lung Function and FeNO Measurements in Infants

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ABSTRACT: Breath analysis by secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) offers the possibility to measure comprehensive metabolic profiles. The technology is currently being deployed in several clinical settings in Switzerland and China. However, patients are required to exhale directly into the device located in a dedicated room. Consequently, clinical implementation in patients incapable of performing necessary exhalation maneuvers (e.g., infants) or immobile (e.g., too weak, elderly, or in intensive care) remains a challenge. The aim of this study was to develop a method to extend such breath analysis capabilities to this subpopulation of patients by collecting breath samples remotely (offline) and promptly (within 10 min) transfer them to SESI-HRMS for chemical analysis. We initially assessed the method in adults by comparing breath mass spectra collected offline with Nalophan bags against spectra of breath samples collected in real time. In total, 13 adults provided 176 pairs of real-time and offline measurements. Lin's concordance correlation coefficient (CCC) was used to estimate the agreement between offline and real-time analyses. Here, 1249 mass spectral features (55% of total detected) exhibited Lin's CCC > 0.6. Subsequently, the method was successfully deployed to analyze breath samples from infants (n = 16), obtaining as a result SESI-HRMS breath profiles. To demonstrate the clinical feasibility of the method, we measured in parallel other clinical variables: (i) lung function, which characterizes the breathing patterns, and (ii) nitric oxide, which is a surrogate marker of airway inflammation. As a showcase, we focused our analysis on the exhaled oxidative stress marker 4-hydroxynonenal and its association with nitric oxide and minute ventilation.

INTRODUCTION

Breath analysis by mass spectrometry (MS), as a noninvasive technique to capture metabolic profiles of patients, has been subject to research over the last decades.[1,2] Its noninvasive nature makes it very attractive for clinical diagnosis and therapeutic monitoring.[3,4] Within the plethora of available MS-based methods, broadly speaking, there exist two main strategies: real-time and offline techniques. The former covers several ionization variants, including proton transfer reaction-MS (PTR-MS),[5] selected ion flow tube-MS (SIFT-MS),[6] and secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS). Real-time breath analysis entails several advantages. Mostly, it is fast (allowing for large screening), and because there is no sample manipulation, it is less prone to alterations (i.e., analytes undergoing further chemical transformations during analysis[7]). All three MS-based real-time breath analysis platforms have been deployed in clinical settings.[8,9] However, there are cases in which, even if the MS is located in the hospital, real-time breath analysis of patients is not feasible. This includes, for example, compromised patients that cannot approach the MS facility or noncooperative patients like infants or hospitalized and cognitive disabled elderly. In such cases, hybrid approaches whereby samples are collected in a recipient and analyzed shortly afterward by real-time mass spectrometry are the only available option.[10,11] Much work has been done in the field to determine the impact of collection of volatile organic compounds in bags for subsequent mass spectrometric analysis.[12,13] However, this has never been determined for SESI-HRMS, whereby hundreds of mass spectral features are typically detected. The aim of this study was to perform such an assessment, showcasing the feasibility of the developed method to analyze exhaled metabolites in infants in a real life but controlled clinical settings.

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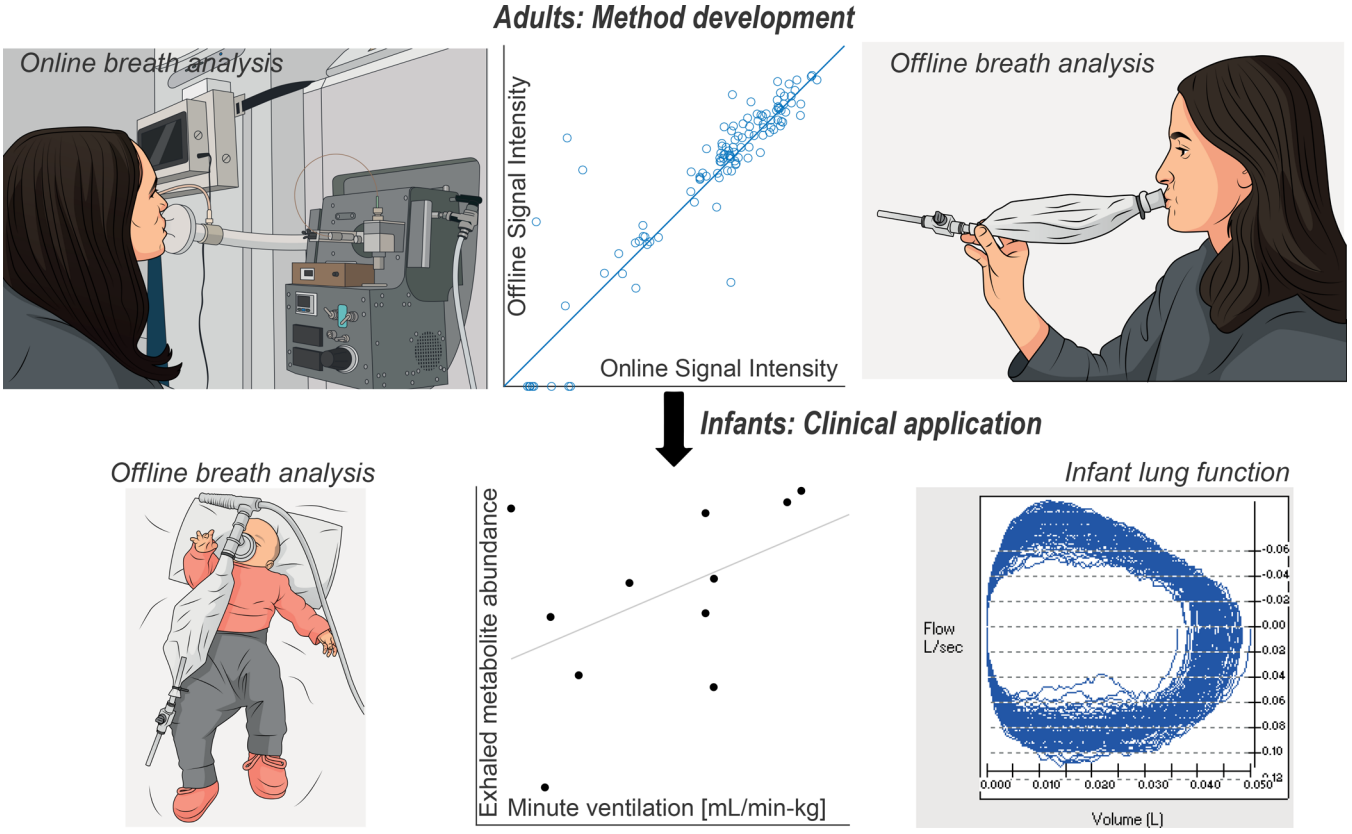

Figure 1. Experimental setup for adults' and infants' breath analyses. Initially, adults provided real-time and offline breath samples for comparison of the newly developed offline technique against the preexisting real-time technique. Once validated, we deployed the method to measure infants during unsedated sleep. Additionally, infant lung function and FeNO measurements were performed in parallel and correlated to exhaled target metabolites.

EXPERIMENTAL SECTION

Figure 1 shows a schematic of the workflow. Detailed information on study participants, breath analysis, quality protocol, and data analysis, including tables and figures, are given in the Supporting Information.

RESULTS AND DISCUSSION

Comparison between Real-Time and Offline Exhaled Metabolic Profiles in Adults. During the first phase of this study, we aimed to develop and assess the performance of the offline collection method as compared to the real-time approach. The assessment of the agreement between real-time and offline methods was performed by computing the Lin's concordance correlation coefficient (Lin's CCC) for each mass spectral feature.[14] Lin's CCC ranges from −1 to +1 (i.e., agreement between two methods). The histogram of the distribution of Lin's CCC for the 2284 measured features is shown in Figure S1a. Out of 2284 features, 845 showed a Lin's CCC > 0.8; additionally, 404 features had Lin's CCC between 0.6 and 0.8. Hence, 55% of the features exhibited a Lin's CCC > 0.6. A similar moderate agreement between real-time and offline collection methods have been observed for SIFT-MS.[15] Figure S1b shows the distribution of the location shift for the portion of features with a Lin's CCC > 0.6. It shows a tendency toward negative values, indicating that the signals tend to be reduced in the offline measurements, suggesting a general trend of adsorption and losses of the metabolites onto the surface of Nalophan bag. Positive values probably correspond to impurities released by the Nalophan bags, some of which have been previously characterized.[16]

We further investigated whether there was a trend for the heavier species——generally less volatile——to exhibit a stronger tendency to be lost in the offline collection process. Figures S2−S4 show the location shift as a function of m/z. No apparent trend was observed. Further insights on the Lin's CCC analysis are shown in Figures S5−S7. We then investigated whether some chemical families of exhaled metabolites exhibited a systematic greater or lower Lin's CCC. To do so, we concentrated in a series of aldehydes previously identified in exhaled breath by SESI-HRMS.[17] Figure S1c displays Lin's CCC vs chain length for three homologous series of aldehydes: 2-alkenals, 4-hydroxy-2,6-alkadienals, and 4-hydroxy-2-alkenals. It shows that there is no clear dependency of the quality of the bag measurement (i.e., Lin's CCC) on the chain length, but it rather suggests that the functional groups in the molecular structure of the metabolites dictate how well the signals are preserved in the bag. For example, the overall Lin's CCC for the 2-alkenals family is lower than for other two series of aldehydes containing a hydroxyl group, suggesting that the former family has a greater affinity for Nalophan (i.e., "sticky" nature). Some outliers are particularly evident. For example, 4-hydroxy-2-dodecenal's Lin's CCC falls well below the overall trend of the family. This may be rationalized by the presence of several isomeric species——hence with different affinities for Nalophan——which are simultaneously detected. This is one limitation of the SESI-HRMS technique, as the lack of chromatography implies

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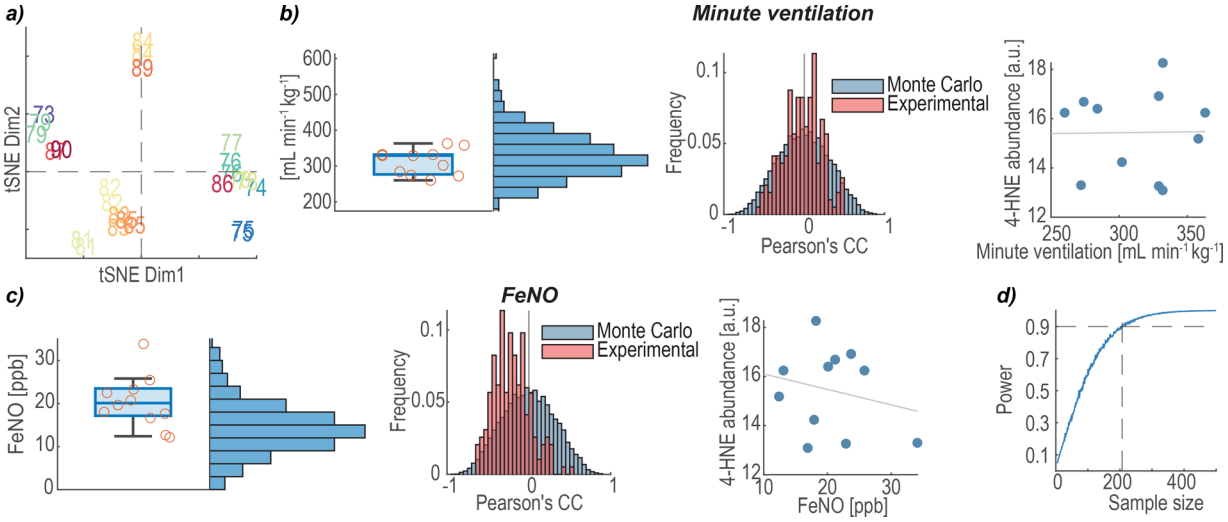

Figure 2. (a) Results from t-SNE analysis for breath mass spectra detected for infants (each color represents an individual); patients' number are represented in the scatter plot, note how replicate measurements cluster together. (b) Distribution of minute ventilation/kg body weight of infants enrolled in this study; data fall within previously measured values in a large cohort (histogram of data from Fuchs et al.[25] shown for reference). Histogram of experimental correlation coefficients and of Monte Carlo simulations of the expected distribution of correlations for minute ventilation/kg with the MS data, with a relationship for 4-HNE vs minute ventilation/kg (r = 0.01; p = 0.97). (c) Distribution of measured FeNO of infants enrolled in this study and histogram of normative data on FeNO in a birth cohort of infants. Histogram of experimental correlation coefficients and of Monte Carlo simulations of the expected distribution of correlations for FeNO with the MS data, with a relationship for 4-HNE vs FeNO (r = 0.22; p = 0.51). (d) Estimation of number of patients needed in a follow-up study.

that isomers cannot be resolved. An additional limitation that should be noted is that the majority of the metabolites detected by this technique remain to be identified; hence, further insights on whether certain metabolites and or functional groups are particularly penalized during the offline collection process remain to be investigated.

Within the latter family of aldehydes, we paid particular attention to 4-hydroxynonenal (4-HNE). 4-HNE is a major product of n-6 fatty acid oxidation. It has been shown to be involved in a great number of pathologies such as metabolic diseases, neurodegenerative diseases, and cancers.[18] We have recently found evidence that preterm infants are subject to increased oxidative stress;[19] hence, we hypothesize that 4-HNE may be altered in this preterm population, which accounts to around 10% of births worldwide.[20]

Figure S1d shows the real-time vs offline signal intensity for all pairs of measurements for 4-HNE. Lin's CCC for this metabolite was 0.61, which is in the lower end of the cutoff we considered in this study. Another common representation for method comparison is so-called Bland–Altman plots (Figure S1e). It shows that the mean difference between the two pairs of measurements (i.e., real-time minus offline) is below zero, indicating that 4-HNE tends to be retained in the bags, although the effect is not dramatic. It is also noticeable that outliers tend to appear at the weakest signal intensities (mean of two measurements less than 13 au), whereas for stronger signal intensities the information is more faithfully preserved. Overall, although far from perfect (i.e., Lin's CCC = 1), the collection of breath samples in such Nalophan bags allows for the analysis of 4-HNE with a reasonable degree of confidence (i.e., Lin's CCC > 0.6).[21] Please note that this is within the time frame of 10 min, which is approximately the time it requires to transport the sample within the hospital facilities. Longer storing times at room temperature may probably compromise the quality of the measurement. Alternative sample collection methods such as solid-phase microextraction (SPME) have been widely used for breath analysis.[22,23] These devices have shown to be robust and easy to handle. However, one limitation is the different compound selectivity depending on the SPME fiber. This is a fundamental difference with the approach of collecting the entire gas sample in a container (in our case a bag) and delivering it as intact as possible for direct MS analysis. In addition, the need of a further vaporization step to release the sample from the SPME fiber would introduce further practical complications in the interfacing with the SESI source. For these reasons, we believe that bags are better suited for offline SESI-HRMS analysis of breath. However, a systematic head-to-head comparison would be needed to confirm this hypothesis.

Deployment of Offline Method in Infants. *Repeatability.* Once the offline technique in adults was validated against the real-time analysis approach, we extended the method to the analysis of exhaled metabolites in infants, while in parallel lung function tests and FeNO measurements were carried out. On the basis of the validation results, we further considered only the mass spectral features with a Lin's CCC > 0.6. In order to summarize the information contained in the high-dimensional space of the breath mass spectra, we performed t-SNE analysis.[24] Figure 2a shows the resulting scatter plot (last two numbers of patient ID shown). The plot shows that, for those infants where two replicate samples could be collected, the measurements cluster together. This further reassures the quality of the measurement. The plot suggests four main clusters of patients. We did verify whether this could be influenced by a batch effect, but this was not the case. For example, patients BILD4386 and BILD4387 were twins measured on the same day, yet they tended to occupy different spaces in the t-SNE domain. Taken together, this provides an initial indication that the method can provide a true metabolic read out from the exhaled breath of infants.

Exhaled Metabolites vs Lung Function. Subsequently, we further explored the data to evaluate whether the breath

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metabolites may be associated with the lung function parameters. For the first time, we were capable of collecting parallel breath specimens from infants who were subjected to routine clinical examinations of the respiratory system (n = 11). Figure 1 shows an example of a flow-volume loop for one of the participants. Figure 2b shows distribution of the minute ventilation per kg body weight. The data fall within previously measured values in a large cohort (histogram of data from Fuchs et al.[25] shown for reference), suggesting that these patients are a representative sample of a typical infant population. To better understand the associations between breathing mechanics and the exhaled metabolites, we then computed Pearson's correlation coefficients. Figure 2b shows the histogram of the distribution of correlation coefficients between minute ventilation and all mass spectral features with Lin's CCC > 0.6. The distribution is centered around zero, suggesting an independence of the vast majority of the exhaled metabolites from the breathing pattern (for reference, random associations resulting from a Monte Carlo simulation are overlaid). In real-life clinical settings, infants' breathing patterns can vary. Such variability—as assessed by minute ventilation—can potentially have an impact in the read out of exhaled metabolites. Figure 2b suggests that this is not the case for 4-HNE because, despite our very limited sample size, we found no correlation (r = 0.01; p = 0.97) between 4-HNE and minute ventilation.

Exhaled Metabolites vs Inflammation Marker FeNO. The FeNO values observed in our small cohort were also within the expected range according to previous studies, albeit leaning toward highest values of the spectrum (Figure 2c). In contrast to minute ventilation, we found a rather clear trend toward negative correlations between FeNO and exhaled metabolites, including 4-HNE (Figure 2c). The reasons why negative associations may exist between exhaled 4-HNE and FeNO are at this point unclear, and drawing any conclusion from this limited data set would be premature. However, FeNO has been associated with a wide range of pathophysiological processes (mainly inflammation of the airways), whereas 4-HNE is regarded as an oxidative stress marker. Hence, it is reasonable to think that they will provide complementary information.

Our goal in the coming years is to identify significant associations between markers of oxidative stress such as 4-HNE and clinical characteristics using the methodology presented in this technical note. For this reason, we then took advantage of this pilot study to estimate the number of patients that we will require to enroll in a follow-up study. The results of the calculation are shown in Figure 2d (see Supporting Information for details). Assuming a significance level of 5%, with 90% power, the goal of detecting an absolute correlation of at least r = 0.22 (like HNE vs FeNO) will require a sample size of n = 210 participants.

Clinical Relevance. Adding a metabolic dimension to lung function and clinical tests via exhaled breath analysis holds promise toward a more personalized approach. Extending this possibility to noncooperative patients (e.g., children or cognitive disabled patients) and immobile patients due to the nature of their disease or treatment (e.g., infectious disease or treatment in intensive care unit) opens new possibilities for therapeutic drug monitoring and diagnosis of disease, especially in pediatric medicine.

CONCLUSION

We have developed an offline collection and subsequent high-resolution mass spectrometric analysis method for breath in a clinical setting. The characterization of the agreement of the offline and real-time analysis revealed over 1000 mass spectral features of moderate to high quality. Among these metabolites there were relevant ones such as 4-HNE, which could be measured for the first time in infants, along with lung function characteristics and FeNO as a clinical outcome.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c02036.

Additional experimental details, materials, and methods, including tables and figures (PDF)

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Notes

The authors declare the following competing financial interest(s): P.S. is co-founder of Deep Breath Initiative A.G. (Switzerland), which develops breath-based diagnostic tools. K.D.S. is a consultant for Deep Breath Initiative A.G. (Switzerland).

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