RESEARCH ARTICLE

# Assessing asthma-specific breath markers in preschool children using remote breath collection

Rémy **Bourgeois** [1, ¤], Katharina **Rohrbach** [1, ¤], Yvette **Baumann** [1], Nathan **Perkins** [2], Elias **Seidl** [1], Srdjan **Micic** [1, ¤, *], Alexander **Moeller** [1, ¤, *]

*** Correspondence to:**

alexander.moeller@kispi.uzh.ch; ORCID: https://orcid.org/0000-0001-7284-4251

micic.math@gmail.com; ORCID: https://orcid.org/0000-0002-6887-2417

## ABSTRACT

Asthma is the most common chronic respiratory disease in children. However, distinguishing asthma from other respiratory complaints in preschool age remains a diagnostic challenge. Secondary electrospray ionization high resolution mass spectrometry (SESI-HRMS) together with a remote breath sample collecting method is a promising tool to overcome this diagnostic problem. This study investigated whether previously identified asthma-specific volatile organic compounds (VOCs) can be reidentified in preschool children by SESI-HRMS using an offline breath collection device.

A total of 30 patients presenting with chronic respiratory symptoms (CRS) and referred for the investigation of preschool asthma as well as 32 healthy controls between 3 and 6 years of age were recruited from the outpatient clinic at the University Children's Hospital in Zurich, Switzerland. Participants were instructed to exhale into an offline breath collection device entailing a Nalophan bag. Breath samples were transferred to SESI-HRMS in a heated box at body temperature. Samples were pumped into SESI-HRMS using a pressurized box. Detection of previously identified asthma-specific markers (mass-to-charge ratio (m/z) tolerance of 0.002 Da) and statistical analysis were performed.

Of 375 previously identified, asthma-specific m/z features, 125 were detected again in preschool children with the remote breath collection method. 16 detected m/z features showed statistically significant differences between patients with CRS and healthy controls (Wilcoxon rank-sum test, adjusted p-value <0.05). Eight of those 16 significant m/z features had been identified chemically as representatives of monosaccharide and fatty acid metabolism, lysine degradation and aldehydes in a previous study. Predictive performance of confirmed markers for distinguishing patients with CRS from healthy patients, using random forest algorithm in a repeated cross-validation, resulted in an AUC of 0.77 (95% CI: 0.60-0.93).

This is the first study to successfully reidentify asthma-specific VOCs in preschool children by SESI-HRMS using a breath collection device that entails Nalophan. Despite loss of m/z features, significant asthma-specific VOCs were identified, emphasizing the potential of this remote breath collection method for diagnosing asthma in preschool children.

**Doi**

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[1] Department of Respiratory Medicine, University Children's Hospital Zurich, Zurich, Switzerland and Zurich Exhalomics

[2] Department of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Zurich, Switzerland

[¤] These Authors have equally contributed to this work and RB and KR should be considered joint first; SM and AM should be considered joint last Authors.

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**KEY WORDS**

*Preschool; asthma; breath analysis.*

 

## HIGHLIGHTS BOX

**What is already known about this topic?** In the past few decades, different breath markers for asthma in the form of VOCs have been identified but results could not be replicated in preschool children. **What does this article add to our knowledge?** This is the first study to successfully reidentify asthma-specific breath markers in preschool children by SESI-HRMS using a remote breath collection device. Our findings emphasize the potential for targeted breath analysis in preschool children. **How does this study impact current management guidelines?** Current asthma diagnosis guidelines request objective tests, but these are challenging in preschool children. This study lays another foundation to develop an easy to perform and reliable breath test for asthma diagnosis in younger children.

## INTRODUCTION

Asthma is the most common chronic respiratory disease in children, affecting over 5 million children in Europe (1). The diagnosis remains challenging and there is no single diagnostic gold standard to diagnose asthma in children (2-4). In children aged 5-16 years, the diagnosis is usually based on a diagnostic algorithm that entails different tests, *i.e.* spirometry, bronchodilator responsiveness (BDR) testing and the measurement of exhaled nitric oxide (FeNO) (3). In preschool children, the proper diagnosis of asthma is generally not possible, since cognitively demanding breathing maneuvers necessary for spirometry and BDR testing are usually too complex to perform in this age group (5, 6). Therefore, children with a high suspicion of asthma, that are not able to perform pulmonary function testing according to current standards are usually referred as children with CRS (3). There is a need for alternative methods that are non-invasive, easy to apply and do not require complex cooperation to diagnose asthma in preschool children. A growing number of studies were published suggesting that breath analysis may offer such a possibility (2, 7).

Several different breath collecting methods for remote analysis have been developed in the last two decades (2). Some studies successfully detected asthma-specific breath markers in children using Tedlar sampling bags together with different exhalation maneuvers and analysis techniques (2). More recently, Decrue *et al*. successfully deployed a remote collection method using Nalophan bags and SESI-HRMS for breath analysis in infants (8). Nalophan proved itself as a reliable component of breath collecting devices in previous studies (9, 10), even if the breath sample was stored at room temperature over a longer period of time prior to measurement (9). Therefore, Nalophan is a reliable part of breath collecting devices for remote analysis.

In the past few decades, different breath markers for asthma in the form of volatile organic compounds (VOCs) have been identified (2, 11, 12). Typically, VOCs associated with oxidative stress, *e.g.* aldehydes, polysaturated fatty acids, ethane and pentane were identified in breath samples of asthma patients due to the inflammatory nature of the disease (13-22). Our research group more specifically demonstrated upregulated metabolisms of lysine, tyrosine, fatty acids, 2-oxocarboxylic acid and monosaccharides as well as downregulated metabolisms of arginine, proline, linoleic acid, palmitoylethanolamide (PEA) and aldehydes in school-aged children with allergic asthma (7). Thus, the above-mentioned VOCs are promising targets of asthma-related research using SESI-HRMS.

The aim of this study was to investigate whether 1) a remote breath collecting method entailing Nalophan is feasible to be used in SESI-HRMS analytics and 2) previously identified asthma-specific VOCs can be reidentified in preschool children with CRS distinguishing them from healthy controls (7).

## METHODS

### Study population

The EXPEDIA study (EXhalomics in PEDIatric Asthma) is a cross-sectional observational study that aims to assess the possibility of using exhaled volatile organic

compounds to diagnose asthma in children using online mass spectrometry.

For this study, 30 patients referred for the investigation of preschool asthma and presenting with CRS, *i.e.* wheezing and/or dry cough, as well as 32 healthy controls (HC) between 3 and 6 years of age were recruited from the outpatient clinic at the University Children's Hospital in Zurich, Switzerland. Children who had acute respiratory infections within the last 6 weeks, with other chronic respiratory or cardiac diseases or any condition restricting the proper execution of the breath sampling were excluded from the study.

### Breath collection and transportation

Remote breath collection was conducted using a custom-made offline breath collecting device (**Figure 1**) consisting of a 0.7-liter Nalophan bag (Kalle GmbH, Wiesbaden, Germany) attached to a sealable valve piece (Swagelok Company, Ohio, OH, USA) with zip ties, a custom-made connecting tube and a mouthpiece (QuinTron Instrument Company Inc., Milwaukee, WI, USA). Children were instructed to take deep inspirations and exhale directly into the Nalophan bag through the mouthpiece until the bag was fully inflated. Whole breath samples were collected without filtering or fractioning the exhalation. A VOC filter was not used, as it was deemed impractical for preschool children (23). Participants were not strictly restricted from medication usage or eating, and physical activity was naturally limited by the hospital setting of the doctor's visit. The filled bags were sealed and transferred to SESI-HRMS in a heated box at body temperature (37 °C) within 15 minutes (**Figure 2**) to prevent condensation of the sample (24, 25).

### Breath analysis

Breath analysis was conducted using a SESI source (SuperSESI, FIT FossilionTech, Madrid, Spain) combined with a high-resolution time-of-flight mass spectrometer (TripleTOF 5600+, AB Sciex, Concord, ON, Canada). Instrumental settings were previously published by our group (7, 26). In short, the sampling line (SL) temperature of the ion source was set at 130 °C, with temperatures of the ionization chambers 1 and 2 (IC1 and IC2) both at 100 °C. Ion spray voltage floating (ISVF) was set at +4500 V in positive mode and -4500 V in negative mode, respectively. For electrospray generation, Sharp Singularity™ nanoESI emitters (FIT FossilionTech, Madrid, Spain) with 20 µm inner diameter were used. The electrospray solution was 0.1% formic acid in water. During each measurement, the contents of the Nalophan bags were continuously infused into the SESI using a custom-made pressurized box (**Figure 2**) at a flow rate of 0.3 l/min for a standardized duration of 30 seconds. Data acquisition was performed in full scan mode in both positive and negative ionization mode. The mass range scanned was from 50 to 500 Da, with a scan time of 0.5 seconds per spectrum.

### Data preprocessing

The recorded raw mass spectra were aligned using PeakView 2.2 (AB Sciex, Concord, ON, Canada) and converted into the mzXML file format using MSConvert (ProteoWizard v3.0.2) (27). The processing of the converted mass spectral data was performed in R (version

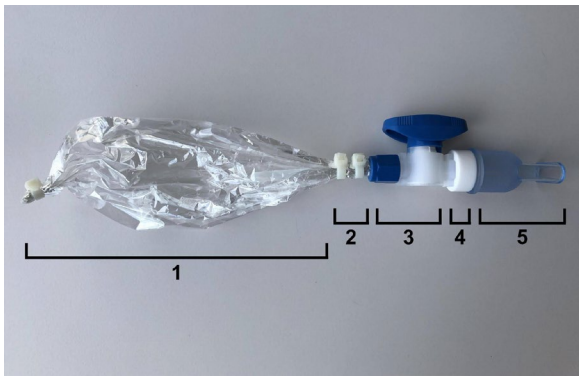


***Figure 1.*** *Offline breath collecting device. The device consists of a Nalophan bag (1) attached to a sealable valve piece (3) with zip ties (2), a connecting tube (4) and a mouthpiece (5). The collected breath air is trapped inside the bag after closing the valve piece.*

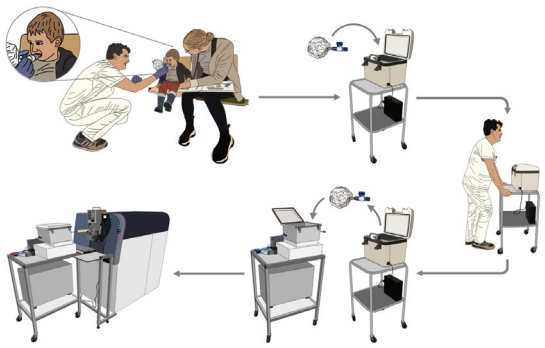

***Figure 2.*** *Remote breath collection, transportation and measurement. Breath collection was conducted remotely using a breath collecting device (**Figure 1**). The contents were then transferred to SESI-HRMS within 15 minutes in a heated box at body temperature. Each sample was pumped into SESI using a pressurized box.*

4.2.1, R Foundation for Statistical Computing, Vienna, Austria) following the methodology outlined in detail elsewhere (7, 26). To summarize, the mass spectra was fitted onto a linearly spaced mass-to-charge ratio (m/z) axis (step size: 0.0005, 900'000 data points, 50-500 m/z range) through piecewise cubic Hermite interpolation. For each measurement, the interpolated mass spectra associated with the infusion maneuvers into the instrument were averaged. Peak picking was then performed on the averaged mass spectra using a height filter of 10 counts per second, followed by centroiding via trapezoidal integration to determine the signal intensities of m/z features. Subsequently, for further analysis, the m/z feature intensities were normalized to the total ion current (TIC), log2-transformed and organized into a data matrix representing breath profiles.

### Statistical analysis

Markers previously linked to allergic asthma from our study (7) were used to filter the features within the remotely collected breath profiles of preschool children. An m/z tolerance of 0.002 Da was set for accurate mass matching. The Wilcoxon rank-sum test was used to assess the differential abundance of these features between the CRS and control groups (28). To account for multiple hypothesis testing, p-values were corrected using Storey's procedure (29). A significance level of adjusted p-value <0.05 was applied. Furthermore, to assess the predictive ability of the detected features in classifying the preschool children as healthy or with CRS, random forest algorithm was trained and tested in a 5 times repeated 10-fold cross-validation (30). To avoid training the classifiers on all detected signals, feature selection was performed in each cross-validation iteration using Meinshausen and Bühlmann stability selection as previously applied in our SESI-HRMS study (31, 32). In short, within each cross-validation loop, stability selection was performed using random 80% training data subsamples, Wilcoxon rank-sum p-values for feature ranking, and a threshold of the 20$^{th}$ percentile (0.2-quantile) of p-values across 100 repetitions. To retain the most important features, only those features with p-values below the 0.2-quantile threshold in at least 90 out of 100 repetitions were selected. More detailed information on statistical analysis is provided in the supplementary material.

### Ethics

This study was approved by the Ethics Committee of the Canton of Zurich (KEK-ZH-Nr. 2018-00441) and written informed parental consent and oral assent of the participants were obtained prior to study participation.

## RESULTS

Remotely collected exhaled breath samples of 30 children with CRS and 32 healthy controls were included in this study. The two groups had comparable age and sex distributions. Demographic and clinical characteristics of the two study cohorts are shown in **Table 1**.

In our previous exploratory SESI-HRMS study, 375 m/z features were found to be potentially associated with allergic asthma in school-aged children (7). From those features, 125 were detected again (<0.002 Da) in remotely collected breath samples of preschool children (**Table S1**). Among the detected features, 16 showed statistically significant differences between the control and CRS group (Wilcoxon rank-sum test, adjusted p-value <0.05, **Figure 3**), replicating the significance observed in the previous study between healthy and asthmatic children. Additionally, eight of the 16 significant features were chemically identified in our earlier work (7). These identified compounds belong to various metabolic pathways and chemical families, including monosaccharides and metabolites (glucuronate, glucarate), aldehydes (4-hydroxy-2-octenal, 4-hydroxy-2-hexenal), fatty acid metabolism (6-hydroxyhexanoic acid), and lysine degradation (glutarate). A complete list of all 16 significant features is provided in **Table 2**. Boxplots for the eight chemically identified markers are presented in **Figure 4** for further visualization.

The assessment of the confirmed markers in classifying samples as HC or CRS, using random forest algorithm in a 5-fold repeated 10-fold cross-validation, resulted in an area under the ROC curve (AUC) of 0.77 (95% CI: 0.60-0.93) (**Figure 5A**). The average classification accuracy reached 72.6% (CI: 59.2%-85%) with sensitivity of 72.9% (CI: 51.7%-94.6%) and specificity of 72.3% (CI: 52.7%-90.9%). On average, 14 ± 1.1 markers were selected in each cross-validation training data set. Markers and their selection frequencies used for classifier training are shown in **Figure 5B** and **Table S1**.

***Table 1.*** *Demographic and clinical characteristics of the participants.*

| Parameter | CRS group (n = 30) | HC group (n = 32) | Significance |
|---|---|---|---|
| Age [y] | 5.5 ± 1.1 | 5 ± 1.2 | n.s. |
| Female [n] | 10 (33.3%) | 11 (34.4%) | n.s. |
| Height [cm] | 111.7 ± 7 | 109.2 ± 9.1 | n.s. |
| Height z-score | -0.22 ± 0.98 | -0.02 ± 1.08 | n.s. |
| Weight [kg] | 19.8 ± 4.1 | 18.8 ± 3.3 | n.s. |
| Weight z-score | -0.02 ± 1.33 | 0.11 ± 0.95 | n.s. |
| BMI [kg/m²] | 15.7 ± 2.23 | 15.7 ± 1.4 | n.s. |
| BMI z-score | 0.09 ± 1.45 | 0.17 ± 1.03 | n.s. |
| Diagnosis | | | |
| Allergic asthma [n] | 12 (30%) | - | - |
| Suspected allergic asthma [n] | 5 (16.7%) | - | - |
| Non-allergic asthma [n] | 3 (10%) | - | - |
| Suspected non-allergic asthma [n] | 3 (10%) | - | - |
| Recurrent obstructive episodes [n] | 7 (23.3%) | - | - |
| Allergy [n] | 20 (66.7%) | 4 (12.5%) | *** |
| Food allergy [n] | 6 (20%) | 4 (12.5%) | n.s. |
| Allergy to aeroallergens [n] | 18 (60%) | 2 (6.3%) | *** |
| Hay fever [n] | 11 (36.7%) | 2 (6.3%) | ** |
| GINA management Step | | | |
| Step 1 [n] | 17 (56.7%) | - | - |
| Step 2 [n] | 5 (16.7%) | - | - |
| Step 3 [n] | 5 (16.7%) | - | - |
| No treatment [n] | 3 (10%) | | |
| Inhaled corticosteroid (ICS) therapy [n] | 11 (36.7%) | - | - |
| Bronchodilator use | | | |
| Salbutamol (100 µg/dose, prn) [n] | 25 (83.3%) | - | - |

*Continuous variables are presented as mean ± standard deviation (SD) and categorical variables as counts (%). The group differences were tested with a two-sample t-test for continuous variables and a Fisher's exact test for the categorical variables. Significance levels: n.s.: >0.05, *: <0.05, **: <0.01, ***: <0.001.*

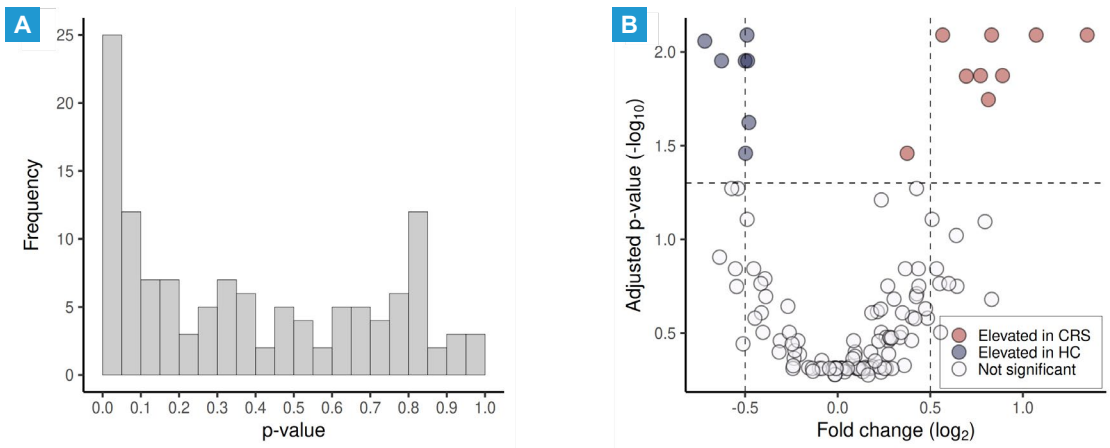


***Figure 3.*** *Statistical analysis of detected metabolites between CRS and HC groups.* ***(A)*** *Distribution of p-values according to the Wilcoxon rank-sum test of all 125 detected m/z features;* ***(B)*** *Volcano plot for all detected m/z features. X-axis: log2 fold change (CRS vs. HC). Y-axis: -log10 of adjusted p-value. Markers are colored according to significance and fold change: red (elevated in CRS, adj. p-value <0.05), blue (elevated in HC, adj. p-value <0.05), and white (not significant).*

***Table 2.*** *Significant asthma-associated markers from our previous study (7) detected in preschool children via remote breath collection.*

| m/z | Charge | Elevated in | log2FC | p-value | Adj. p-value | Compound (mol. formula) | Metabolic pathway/chemical family |
|---|---|---|---|---|---|---|---|
| 193.0355 | neg | CRS | 0.83 | 0.0003 | 0.0081 | Glucuronate C6H10O7 | Monosaccharides and metabolites |
| 194.0305 | neg | CRS | 0.57 | 0.0006 | 0.0081 | - | |
| 209.0305 | neg | CRS | 1.07 | 0.0006 | 0.0081 | Glucarate C6H10O8 | Monosaccharides and metabolites |
| 227.041 | neg | CRS | 1.35 | 0.0002 | 0.0081 | - | |
| 83.0335 | pos | HC | -0.49 | 0.0005 | 0.0081 | - | |
| 136.021 | pos | HC | -0.72 | 0.0008 | 0.0088 | Benzothiazole C7H5NS | Heterocyclic compounds |
| 115.075 | pos | HC | -0.50 | 0.0015 | 0.0111 | 4-Hydroxy-2-hexenal C6H10O2 | Aldehydes |
| 135.101 | pos | HC | -0.63 | 0.0013 | 0.0111 | - | |
| 143.106 | pos | HC | -0.49 | 0.0014 | 0.0111 | 4-Hydroxy-2-octenal C8H14O2 | Aldehydes |
| 196.0465 | neg | CRS | 0.77 | 0.0022 | 0.0134 | - | |
| 226.033 | neg | CRS | 0.89 | 0.0022 | 0.0134 | - | |
| 167.068 | pos | CRS | 0.69 | 0.0024 | 0.0135 | 3-Ethoxybenzoate C9H10O3 | Aromatic compound |
| 183.051 | neg | CRS | 0.81 | 0.0035 | 0.0179 | - | |
| 151.096 | pos | HC | -0.48 | 0.0050 | 0.0238 | 6-Hydroxyhexanoic acid C6H12O3 | Fatty acid metabolism |
| 131.035 | neg | CRS | 0.37 | 0.0083 | 0.0347 | Glutarate (pentanedioic acid) C5H8O4 | Lysine degradation |
| 191.164 | pos | HC | -0.50 | 0.0079 | 0.0347 | - | |

*Ordered by significance in the current study (adj. p-value). m/z: mass-to-charge ratio, charge: ionization mode, log2FC: log2-fold-change, Adj. p-value: adjusted p-value (28), Compound and metabolic pathway/chemical family: putatively identified compound in our previous work (7) with the associated metabolic pathway or chemical family. Dash "-" was used in case of unknown compounds.*

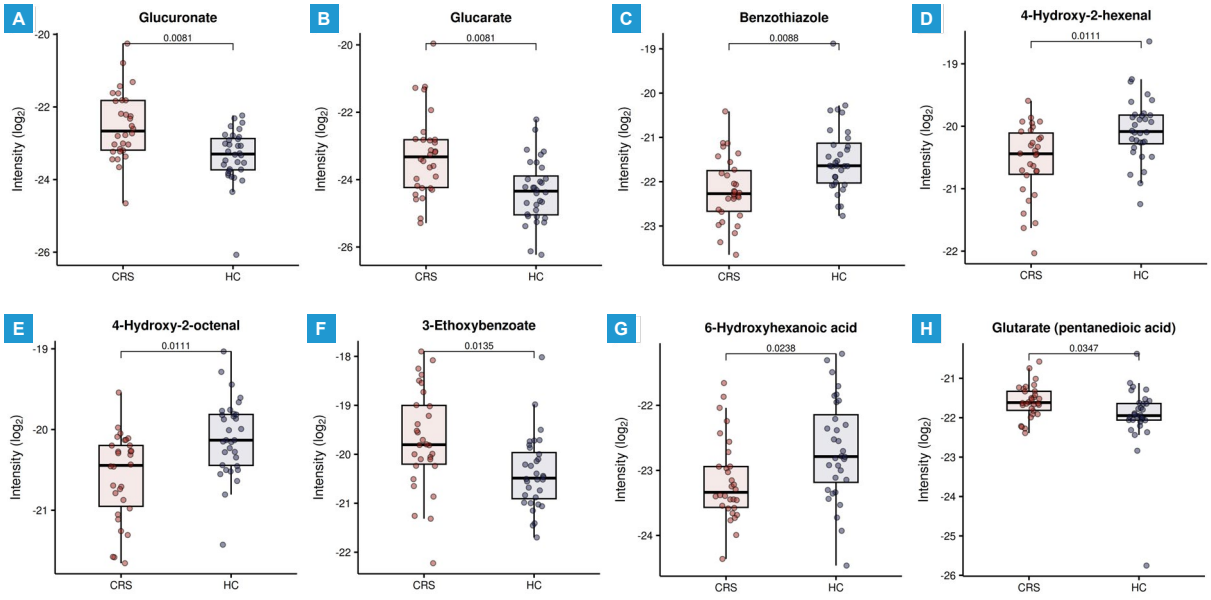


***Figure 4.*** *Boxplots of breath molecules with significant differences between HC preschool children and those with CRS. These molecules correspond to the previously chemically identified compounds in our study using SESI-HRMS, which were associated with allergic asthma in school-aged children (7).*

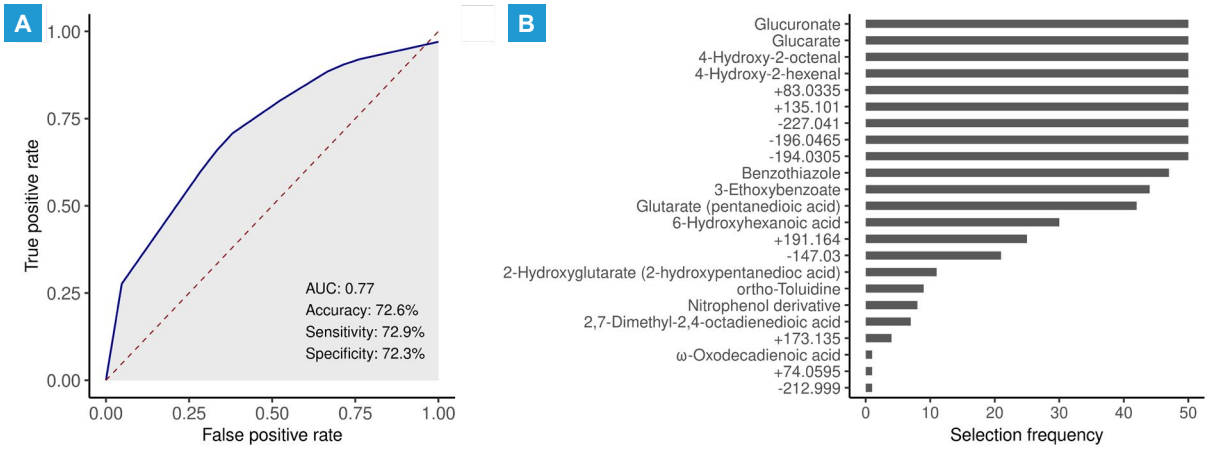


***Figure 5.*** *Classification performance.* ***(A).*** *Average receiver operating characteristic (ROC) curve for classifying CRS and HC control samples, averaged over all cross-validation iterations (33).The diagonal red dashed line represents the line of no discrimination, the blue line represents the average ROC curve, and the gray shaded area indicates the AUC;* ***(B)*** *selection frequencies of the markers used for classification in each cross-validation iteration. The markers are ordered from top to bottom by their selection frequency. Selection frequency represents how often a marker was selected during cross-validation. Compound names were used, when possible, while unidentified markers were represented by their m/z values with the corresponding ionization mode (+/-).*

## DISCUSSION

This study validates that an offline breath collecting device entailing Nalophan can be used in SESI-HRMS analytics to detect VOCs in preschool children. Furthermore, for the first time, we were able to reidentify asthma-specific VOCs in preschool children presenting with CRS, distinguishing them from healthy controls.

In a previous online breath analysis study, our research group identified 375 m/z features potentially associated with asthma in school-aged children (7). In the present study, we detected 125 of those m/z features again in a cohort of preschool children using our remote breath collection method. Of these, 16 m/z features showed statistically significant differences between children referred for the investigation of preschool asthma, presenting with wheezing and/or dry cough (CRS) and healthy control children, and 8 m/z features had previously been identified chemically (7).

Among the 8 significant, chemically reidentified markers were glucuronate and glucarate, which are both part of monosaccharide metabolism. It has been previously described that due to inflammation, airway hyperresponsiveness, hypoxia and increased breathing effort there is an increased energy demand in asthma patients leading to an altered energy metabolism (34).Depicting this alteration, an affected carbohydrate metabolism has been demonstrated both in the lungs of murine models and sera of human asthma patients (35, 36). Therefore, altered levels of glucuronate and glucarate in our preschool cohort correspond to previous findings.

Two other significant, chemically reidentified markers were 4-hydroxy-2-octenal and 4-hydroxy-2-hexenal, which both belong to the chemical group of aldehydes. Aldehydes were described as a product of lipid peroxidation in environments of oxidative stress (37), similarly observed in asthma patients. Remarkably, demonstrating the same trend as the asthma group in our study with school-aged children (7), the CRS group of the preschool cohort showed decreased instead of increased intensities for both 4-hydroxy-2-octenal and 4-hydroxy-2-hexenal. These contradictory results fall in line with the varying results of previous studies that observed both increased and decreased or even unchanged levels of aldehydes in patients with chronic respiratory symptoms compared to healthy controls (38-44).

With 6-hydroxyhexanoic acid, a representative of fatty acid metabolism was significantly different between children with CRS and healthy controls. Similar to aldehydes, the presence of fatty acid metabolites is an expression of oxidative stress (19), as observed in patients with chronic respiratory inflammation. A previous study analyzed breath profiles in patients with COPD exacerbations that showed decreased levels of fatty acids, indicating an upregulation of ω-oxidation-pathways (45). Similarly in our study population, a decreased signal intensity of

6-hydroxyhexanoic acid in CRS patients compared to healthy controls was observed, potentially explained by oxidative stress present in CRS patients.

Another significant, chemically reidentified marker was glutarate which is part of lysine degradation. Although the role of lysine degradation in the pathophysiology of asthma is not fully understood, it has been hypothesized that lysine degradation and thus accumulation of glutarate reflect the generation of energy due to increased breathing efforts in asthma patients (35). Supporting this hypothesis, elevated levels of glutarate in the sera, urine and breath of children were associated with pediatric asthma in previous studies (35, 46, 47). Therefore, elevated levels of glutarate in CRS patients of our study population correspond to preliminary findings.

Finally, two other significant, chemically reidentified m/z features were 3-Ethoxybenzoate and Benzothiazole. We interpreted these features as environmental contaminants since these substances do not occur naturally in any human metabolic pathway (48, 49). The reason for the statistically significant intensity difference for these substances between the CRS children and healthy controls is unclear. A potential reason for this difference may be differences in metabolization and elimination of environmental substances between children suffering from CRS and health controls due to an altered metabolic state underlying the chronic respiratory disease, analogous to the principles mentioned above. This hypothesis, however, should be subject of further research.

Among the remaining 109 non-significant features ($p > 0.05$), the majority were linked to endogenous pathways, such as fatty acid metabolism, lysine degradation, and aldehyde formation (**Table S2**). However, we also found three exogenous markers within this set: ortho-Toluidine (m/z 108.08), 4-Methyl-2-nitrophenol (m/z 152.0335), and a Nitrophenol derivative (m/z 166.0475). While these features did not show statistically significant differences between groups, their redetection aligns with previous observations, reflecting the complexity of breath profiles and potential environmental influences (7).

Assessment of classification performance based on machine learning resulted in an average AUC of 0.77 and an average classification accuracy of approximately 70%. Among the fourteen markers selected at least once during cross-validation, some chemically identified markers were consistently or very frequently selected (**Figure 5B**, **Table S1**). These included the endogenous compounds associated with energy metabolism (glucuronate and glucarate), aldehydes (4-hydroxy-2-hexenal and 4-hydroxy-2-octenal), and lysine degradation (glutarate), highlighting their potential contribution in classifying patients with wheezing and dry cough (CRS). However, the results indicate moderate discriminative power of the identified breath markers for differentiating between healthy controls and individuals with CRS. It is important to note that the machine learning pipeline employed in this study relied on internal validation through repeated cross-validation. External validation with an independent dataset is needed to confirm the model's performance in future studies.

Of 375 asthma-specific m/z features found in school-aged children (7), 250 were not detected again in our preschool cohort. In contrast to the previous study, that included allergic asthmatic school-aged children (7), here we present data from preschool children referred for the investigation of preschool asthma, presenting with chronic or recurrent respiratory symptoms, *i.e.* wheezing and/or dry cough, which is, a mixed population of children with confirmed and non-confirmed allergic or non-allergic asthma or recurrent obstructive bronchitis. These population differences may have contributed to differences in detected breath markers. Another potential reason may be overall metabolic changes over the course of childhood (50), leading to partially different breath patterns between preschool and school-aged children. To investigate these changes of breath patterns in children, longitudinal studies are needed.

Additional limitations to the comparability between the preschool and school-aged group were differences in quality and quantity of the exhalations. Samples from school-aged children were exhaled directly into SESI-HRMS minimizing the potential loss or alteration of volatile compounds between exhalation and detection (7). On the other hand, samples of the younger preschool children were collected using our breath collecting device and were analyzed with minimal delay. Although exhalation into our device was easier to understand and perform by preschool children compared to online breath analysis, the collection of a sample of comparable size and effort turned out to be difficult in some cases. Furthermore, the study aimed to reflect realistic condi-

tions by not strictly controlling potential confounders such as medication use, food intake or physical activity. This approach may introduce variability in VOC profiles. However, imposing such restrictions would have been impractical and ethically challenging, potentially limiting participation and compromising data collection. Further loss of asthma-specific markers may have occurred in the bag system itself. Although Nalophan showed less adsorption of VOCs compared to other materials in a previous study (10), it is likely that the intensity of a substantial number of markers was reduced due to their adhesion properties, as observed in other work (8, 9, 24). In the specific case of aldehydes, it has been shown that the functional group has an influence on the adhesion properties of the aldehyde to Nalophan (8). Furthermore, storage time between breath collection and measurement potentially facilitates oxidation processes of VOCs lowering their signal intensities (51). Finally, chemical modification of VOCs may also have occurred due to temperature changes, *e.g.* during transfer from the patient to the heated box, and humidity changes as a result of diffusion of water through Nalophan, as these processes were described in previous studies (9, 52, 53).

Despite the above-mentioned limiting factors, we were still able to detect 125 asthma-specific m/z features again in preschool children using our breath collecting device in a realistic clinical scenario. Our findings emphasize the potential of remote breath collection methods together with SESI-HRMS for targeted breath analysis in preschool children.

## CONCLUSIONS

This is the first study to successfully reidentify asthma-specific breath markers in preschool children by SESI-HRMS using a breath collection device that entails Nalophan. This study lays another foundation to develop an easy to perform and reliable offline breath test in order to lower the age for diagnosing asthma in children.

## COMPLIANCE WITH ETHICAL STANDARDS

### Conflict of interests

The Authors have declared no conflict of interests.

### Financial support

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### Author contributions

Study design and concept: JM, AM. Data acquisition: RB, KR, YB. Data processing and analysis: SM, NP. Data evaluation and interpretation: AM, SM, RB, NP. Drafting of the manuscript: RB, SM, AM, ES. Funding acquisition: AM. Review and editing of the manuscript: all Authors.

### Ethical approval

*Human studies and subjects*

The study followed the ethical standards established in the Declaration of Helsinki. It was approved by the Ethics Committee of the Canton of Zurich (KEK-ZH-Nr. 2018-00441) and written informed parental consent and oral assent of the participants were obtained prior to study participation.

### Data sharing and data accessibility

Data are available upon motivated request to the Corresponding Author.

### Publication ethics

*Plagiarism*

Authors declare no potentially overlapping publications with the content of this manuscript and all original studies are cited as appropriate.

*Data falsification and fabrication*

All the data corresponds to the real.

## REFERENCES

1. van den Akker-van Marle ME, Bruil J, Detmar SB. Evaluation of cost of disease: assessing the burden to society of asthma in children in the European Union. Allergy. 2005;60(2):140-9. doi: 10.1111/j.1398-9995.2005.00692.x.
2. Neerincx AH, Vijverberg SJH, Bos LDJ, Brinkman P, van der Schee MP, de Vries R, et al. Breathomics from exhaled volatile organic compounds in pediatric asthma. Pediatr Pulmonol. 2017;52(12):1616-27. doi: 10.1002/ppul.23785.
3. Gaillard EA, Kuehni CE, Turner S, Goutaki M, Holden KA, de Jong CCM, et al. European Respiratory Society clinical practice guidelines for the diagnosis of asthma in children aged 5-16 years. Eur Respir J. 2021;58(5):2004173. doi: 10.1183/13993003.04173-2020.
4. Brand PLP, Caudri D, Eber E, Gaillard EA, Garcia-Marcos L, Hedlin G, et al. Classification and pharmacological treatment of preschool wheezing: changes since 2008. Eur Respir J. 2014;43(4):1172-7. doi: 10.1183/09031936.00199913.

5. Crenesse D, Berlioz M, Bourrier T, Albertini M. Spirometry in children aged 3 to 5 years: reliability of forced expiratory maneuvers. Pediatr Pulmonol. 2001;32(1):56-61. doi: 10.1002/ppul.1089.
6. Kanengiser S, Dozor AJ. Forced expiratory maneuvers in children aged 3 to 5 years. Pediatr Pulmonol. 1994;18(3):144-9. doi: 10.1002/ppul.1950180305.
7. Weber R, Streckenbach B, Welti L, Inci D, Kohler M, Perkins N, et al. Online breath analysis with SESI/HRMS for metabolic signatures in children with allergic asthma. Front Mol Biosci. 2023;10:1154536. doi: 10.3389/fmolb.2023.1154536.
8. Decrue F, Singh KD, Gisler A, Awchi M, Zeng J, Usemann J, et al. Combination of Exhaled Breath Analysis with Parallel Lung Function and FeNO Measurements in Infants. Anal Chem. 2021;93(47):15579-83. doi: 10.1021/acs.analchem.1c02036.
9. Sola-Martínez RA, Zeng J, Awchi M, Gisler A, Arnold K, Singh KD, et al. Preservation of exhaled breath samples for analysis by off-line SESI-HRMS: proof-of-concept study. J Breath Res. 2023;18(1). doi: 10.1088/1752-7163/ad10e1.
10. Liu C, Zeng J, Sinues P, Fang M, Zhou Z, Li X. Quantification of volatile organic compounds by secondary electrospray ionization-high resolution mass spectrometry. Anal Chim Acta. 2021;1180:338876. doi: 10.1016/j.aca.2021.338876.
11. Azim A, Barber C, Dennison P, Riley J, Howarth P. Exhaled volatile organic compounds in adult asthma: a systematic review. Eur Respir J. 2019;54(3). doi: 10.1183/13993003.00056-2019.m.
12. Sola-Martínez RA, Lozano-Terol G, Gallego-Jara J, Morales E, Cantero-Cano E, Sanchez-Solis M, et al. Exhaled volatilome analysis as a useful tool to discriminate asthma with other coexisting atopic diseases in women of childbearing age. Sci Rep. 2021;11(1):13823. doi: 10.1038/s41598-021-92933-2.
13. Rahman I. The role of oxidative stress in the pathogenesis of COPD: implications for therapy. Treat Respir Med. 2005;4(3):175-200. doi: 10.2165/00151829-200504030-00003.
14. Alary J, Guéraud F, Cravedi JP. Fate of 4-hydroxynonenal in vivo: disposition and metabolic pathways. Mol Aspects Med. 2003;24(4-5):177-87. doi: 10.1016/s0098-2997(03)00012-8.
15. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med. 1991;11(1):81-128. doi: 10.1016/0891-5849(91)90192-6.
16. Flor AC, Kron SJ. Lipid-derived reactive aldehydes link oxidative stress to cell senescence. Cell Death Dis. 2016;7(9):e2366. doi: 10.1038/cddis.2016.275.
17. Rahman I, Biswas SK. Non-invasive biomarkers of oxidative stress: reproducibility and methodological issues. Redox Rep. 2004;9(3):125-43. doi: 10.1179/135100004225005219.
18. Schneider C, Porter NA, Brash AR. Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation. J Biol Chem. 2008;283(23):15539-43. doi: 10.1074/jbc.R800001200.
19. Dallinga JW, Robroeks CMHHT, van Berkel JJBN, Moonen EJC, Godschalk RWL, Jöbsis Q, et al. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. Clin Exp Allergy. 2010;40(1):68-76. doi: 10.1111/j.1365-2222.2009.03343.x.
20. Olopade CO, Zakkar M, Swedler WI, Rubinstein I. Exhaled pentane levels in acute asthma. Chest. 1997;111(4):862-5 doi: 10.1378/chest.111.4.862.
21. Paredi P, Kharitonov SA, Leak D, Shah PL, Cramer D, Hodson ME, et al. Exhaled Ethane Is Elevated in Cystic Fibrosis and Correlates with Carbon Monoxide Levels and Airway Obstruction. Am J Respir Crit Care Med. 2000;161(4):1247-51. doi: 10.1164/ajrccm.161.4.9906122.
22. Paredi P, Kharitonov SA, Barnes PJ. Analysis of expired air for oxidation products. Am J Respir Crit Care Med. 2002;166(12 Pt 2):S31-7. doi: 10.1164/rccm.2206012.
23. Weber R, Kaeslin J, Moeller S, Perkins N, Micic S, Moeller A. Effects of a Volatile Organic Compound Filter on Breath Profiles Measured by Secondary Electrospray High-Resolution Mass Spectrometry. Molecules. 2022;28(1):45. doi: doi: 10.3390/molecules28010045.
24. Gilchrist FJ, Razavi C, Webb AK, Jones AM, Spaněl P, Smith D, et al. An investigation of suitable bag materials for the collection and storage of breath samples containing hydrogen cyanide. J Breath Res. 2012;6(3):036004. doi: 10.1088/1752-7155/6/3/036004.
25. Mochalski P, King J, Unterkofler K, Amann A. Stability of selected volatile breath constituents in Tedlar, Kynar and Flexfilm sampling bags. Analyst. 2013;138(5):1405-18. doi: 10.1039/c2an36193k.
26. Kaeslin J, Micic S, Weber R, Müller S, Perkins N, Berger C, et al. Differentiation of Cystic Fibrosis-Related Pathogens by Volatile Organic Compound Analysis with Secondary Electrospray Ionization Mass Spectrometry. Metabolites. 2021;11(11). doi: 10.3390/metabo11110773
27. Kessner D, Chambers M, Burke R, Agus D, Mallick P. ProteoWizard: open-source software for rapid proteomics tools development. Bioinformatics. 2008;24(21):2534-6. doi: 10.1093/bioinformatics/btn323.
28. Mann HB, Whitney DR. On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other. Ann Math Stat. 1947;18(1):50-60. doi: 10.1214/aoms/1177730491.
29. Storey JD. A direct approach to false discovery rates. J R Stat Soc Series B Stat Methodol. 2002;64(3):479-98. doi: 10.1111/1467-9868.00346.
30. Breiman L. Random Forests. Mach Learn. 2001;45(1):5-32. doi: 10.1023/A:1010933404324.
31. Weber R, Haas N, Baghdasaryan A, Bruderer T, Inci D, Micic S, et al. Volatile organic compound breath signatures of children with cystic fibrosis by real-time

SESI-HRMS. ERJ Open Res [Internet]. 2020;6(1). doi: 10.1183/23120541.00171-2019.

32. Meinshausen N, Bühlmann P. Stability selection. J R Stat Soc Series B Stat Methodol. 2010;72(4):417-73. doi: 10.1111/j.1467-9868.2010.00740.x.
33. Fawcett T. An introduction to ROC analysis. Pattern Recognit Lett. 2006;27(8):861-74.
34. Papamichael MM, Katsardis C, Sarandi E, Georgaki S, Frima ES, Varvarigou A, et al. Application of Metabolomics in Pediatric Asthma: Prediction, Diagnosis and Personalized Treatment. Metabolites. 2021;11(4). doi: 10.3390/metabo11040251.
35. Chang C, Guo ZG, He B, Yao WZ. Metabolic alterations in the sera of Chinese patients with mild persistent asthma: a GC-MS-based metabolomics analysis. Acta Pharmacol Sin. 2015;36(11):1356-66. doi: 10.1038/aps.2015.102.
36. Ho WE, Xu YJ, Xu F, Cheng C, Peh HY, Tannenbaum SR, et al. Metabolomics reveals altered metabolic pathways in experimental asthma. Am J Respir Cell Mol Biol. 2013;48(2):204-11. doi: 10.1165/rcmb.2012-0246OC.
37. Jesenak M, Zelieskova M, Babusikova E. Oxidative Stress and Bronchial Asthma in Children-Causes or Consequences? Front Pediatr. 2017;5:162. doi: 10.3389/fped.2017.00162.
38. Ibrahim B, Basanta M, Cadden P, Singh D, Douce D, Woodcock A, et al. Non-invasive phenotyping using exhaled volatile organic compounds in asthma. Thorax. 2011;66(9):804-9. doi: 10.1136/thx.2010.156695.
39. Sagdic A, Sener O, Bulucu F, Karadurmus N, Özel HE, Yamanel L, et al. Oxidative stress status and plasma trace elements in patients with asthma or allergic rhinitis. Allergol Immunopathol. 2011;39(4):200-5. doi: 10.1016/j.aller.2010.07.006.
40. Caldeira M, Perestrelo R, Barros AS, Bilelo MJ, Morête A, Câmara JS, et al. Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. J Chromatogr A. 2012;1254:87-97. doi: 10.1016/j.chroma.2012.07.023.
41. Riscassi S, Corradi M, Andreoli R, Maccari C, Mercolini F, Pescollderungg L, et al. Nitric oxide products and aldehydes in exhaled breath condensate in children with asthma. Clin Exp Allergy. 2022;52(4):561-4. doi: 10.1111/cea.14066.
42. Gahleitner F, Guallar-Hoyas C, Beardsmore CS, Pandya HC, Thomas CP. Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath. Bioanalysis. 2013;5(18):2239-47. doi: 10.4155/bio.13.184.
43. van de Kant KDG, van Berkel JJBN, Jöbsis Q, Lima Passos V, Klaassen EMM, van der Sande L, et al. Exhaled breath profiling in diagnosing wheezy preschool children. Eur Respir J. 2013;41(1):183-8. doi: 10.1183/09031936.00122411.
44. Smolinska A, Klaassen EMM, Dallinga JW, van de Kant KDG, Jobsis Q, Moonen EJC, et al. Profiling of volatile organic compounds in exhaled breath as a strategy to find early predictive signatures of asthma in children. PLoS One. 2014;9(4):e95668. doi: 10.1371/journal.pone.0095668.
45. Gaugg MT, Nussbaumer-Ochsner Y, Bregy L, Engler A, Stebler N, Gaisl T, et al. Real-Time Breath Analysis Reveals Specific Metabolic Signatures of COPD Exacerbations. Chest. 2019;156(2):269-76. doi: 10.1016/j.chest.2018.12.023.
46. Saude EJ, Skappak CD, Regush S, Cook K, Ben-Zvi A, Becker A, et al. Metabolomic profiling of asthma: diagnostic utility of urine nuclear magnetic resonance spectroscopy. J Allergy Clin Immunol. 2011;127(3):757-64.e1–6. doi: 10.1016/j.jaci.2010.12.1077.
47. Carraro S, Bozzetto S, Giordano G, El Mazloum D, Stocchero M, Pirillo P, et al. Wheezing preschool children with early-onset asthma reveal a specific metabolomic profile. Pediatr Allergy Immunol. 2018;29(4):375-82. doi: 10.1111/pai.12879.
48. PubChem Compound Summary for CID 6951193, 3-Ethoxybenzoate. National Center for Biotechnology Information. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/3-Ethoxybenzoate. Accessed: May 26, 2024.
49. PubChem Compound Summary for CID 7222, Benzothiazole [Internet]. National Center for Biotechnology Information. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Benzothiazole. Accessed: May 26, 2024.
50. Cominetti O, Hosking J, Jeffery A, Pinkney J, Martin FP. Contributions of Fat and Carbohydrate Metabolism to Glucose Homeostasis in Childhood Change With Age and Puberty: A 12-Years Cohort Study (EARLYBIRD 77). Frontiers in Nutrition. 2020;7. Available from: https://www.frontiersin.org/articles/10.3389/fnut.2020.00139. Accessed: Jan 31, 2025.
51. Harshman SW, Mani N, Geier BA, Kwak J, Shepard P, Fan M, et al. Storage stability of exhaled breath on Tenax TA. J Breath Res. 2016;10(4):046008. doi: 10.1088/1752-7155/10/4/046008.
52. Ghimenti S, Lomonaco T, Bellagambi FG, Tabucchi S, Onor M, Trivella MG, et al. Comparison of sampling bags for the analysis of volatile organic compounds in breath. J Breath Res. 2015;9(4):047110. doi: 10.1088/1752-7155/9/4/047110.
53. Beauchamp J, Herbig J, Gutmann R, Hansel A. On the use of Tedlar® bags for breath-gas sampling and analysis. J Breath Res. 2008;2(4):046001. doi: 10.1088/1752-7155/2/4/046001.