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Within- and between-person variability of exhaled breath condensate pH and NH_4^+ in never and current smokers

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Summary
Recent studies have suggested that the collection of exhaled breath condensate (EBC) may be a viable method in occupational field studies to sample secretions of the lower airway because it is simple to perform and non-invasive. However, there are unresolved questions about whether certain laboratory conditions may influence the analysis of EBC biomarker measurements. A total of 12 subjects performed 116 EBC tests. The effect of short and long-term sample storage and sample volume on two biomarkers of acid stress, pH and NH_4^+, in EBC were investigated and did not significantly influence either marker measurement after argon deaeration. We also investigated the variability and the effect of smoking on the biomarkers by collecting six samples each from five adult never smokers and five adult current smokers over a period of 1 month ($n = 60$ total). For pH, the within-person and between-person variability was larger in current smokers compared to never smokers. Similar results were found for NH_4^+. Cigarette packs smoked per day now was also associated with both pH ($p = 0.01$) and NH_4^+ ($p = 0.04$) using mixed effects regression analysis. The variability and smoking results suggest that repeated measurements of EBC pH and NH_4^+ from the same individual may accurately predict the biological state of the airways of current smokers when compared to never smokers.

Background

Exhaled breath condensate (EBC) may be used to determine the components of airway lining fluid environment,[1,2] measure airway inflammation in the respiratory tract and

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diagnose different lung diseases,[3,4] and/or monitor responses to therapy.[5] The collection and analysis of biomarkers in EBC has shown promise as a simpler alternative to more invasive techniques such as sputum induction, bronchoscopy or bronchoalveolar lavage (BAL) for sampling the epithelial fluid lining the respiratory tract. The procedure for EBC collection is non-invasive, does not irritate the airways and therefore does not have an impact on lung function or airway inflammation. EBC collection does not require specialized facilities to perform and thus is an attractive method to use in the field for occupational exposure studies.[6] EBC collection is performed by having the subject breathe through a device that cools the exhaled air to produce a condensed liquid exhalate.[7]

Despite the promise that EBC collection offers, there are still unresolved questions surrounding the utility of EBC, including controversies regarding the origins of solutes in EBC (i.e. lower respiratory tract or upper respiratory tract),[8,9] concerns about the current assays not being sensitive enough to detect solutes of interest,[10,11] varying methodologies between studies in the collection, storage, and processing of the EBC assay[10] and questions pertaining to the variability of repeated measurements in individuals.[12] In the present study, we have addressed the latter two concerns by first examining the robustness of the assay by investigating the reproducibility of two biomarkers of acid stress, pH and NH_4^+, and the effect of short-term/long-term sample storage conditions and volume. Secondly, we determined the within-person (WPV) and between-person variability (BPV), as well as the effect of smoking, on these biomarker measurements in never smokers and current smokers.

Methods

The study was approved by the Clinical Research Ethics Board of the University of British Columbia. Subjects gave informed consent for participation in the study and personal identifiers were not released. The subjects were instructed to not eat acidic foods or drink alcohol 2 hours (h) prior to sample collection. Subjects participated in a short respiratory health questionnaire that was adapted from the standardized American Thoracic Society questionnaire,[13] where acute smoking amount (number of cigarette packs smoked per day now), cumulative smoking amount (pack-years: average cigarette packs smoked per day over the entire time smoked multiplied by number of years smoked regularly), smoking status and asthma status were determined. The collection of EBC was performed using the Rtube™ with filter, obtained from Respiratory Research, Inc. (Charlottesville, VA, USA) as described by Hunt et al.[14] The subjects breathed through the EBC device for 15 min at a tidal breathing rate, while not wearing noseclips. Argon deaeration was performed on the samples at 350 μL/min for 10 min immediately upon collection. pH of EBC samples were measured using an Orion model 720A pH meter (Orion, Boston MA) with an Accumet microprobe (Fisher Scientific, Pittsburgh PA). pH measurements were taken three times consecutively and the values averaged.

NH_4^+ concentrations of EBC were measured using high performance liquid ion chromatography (Dionex, Sunnyvale CA) with conductivity detection. A 125 μL aliquot of each sample was transferred to a 0.5 mL Dionex Poly Vial and diluted with the addition of 575 μL of de-ionized MilliQ water (total volume of 700 μL). A six-point quadratic calibration curve of ammonium choride (NH_4Cl) standards was used for quantitation of samples (reference method: NIOSH Method 6016). The calibration standards were included at the beginning and the end of each batch. EBC samples and calibration standards were placed in the HP-IC autosampler; for every 12 samples, a single calibration standard (quality control run) was repeated to check the performance of the HP-IC during the batch. Duplicate samples were run during the batch analysis for every 18 samples to check method accuracy. The limit of detection of the method was 0.50 μmol/L for NH_4^+.

Unless otherwise specified, the natural logarithm of NH_4^+ was taken in all instances to produce a normal-like distribution. The effects of sample volume on pH and NH_4^+, as well as the variability results were analyzed using a mixed effects model to account for repeated measurements from the same subject (proc mixed procedure, SAS for Windows, version 9.1, (SAS Institute, Cary, NC). Statistical significance was set at $p \leqslant 0.05$.

Results

The procedures of the methodological questions investigated in this study are outlined in Table 1. A total of 12 subjects participated in the study, generating 116 EBC samples (Table 2). There was a very high compliance rate with the subjects in successfully performing the EBC test (>99%). A significant correlation was found between pH and NH_4^+ (μM) (Figure 1, $r = 0.37$, $p < 0.0001$, $n = 116$ total). A higher correlation was found between EBC pH and the natural logarithm of NH_4^+ (ln(μM)) ($r = 0.63$, $p < 0.0001$, $n = 116$ total). We investigated the reproducibility of the pH assay by examining the short-term variability of pH using three measurements taken consecutively. The short-term variability of pH was minimal (mean coefficient of variation 0.17%, S.D. 0.16 with a range ~0–1.2%). In both comparisons, low pH values only had low NH_4^+ values, but low levels of NH_4^+ were seen in both low and high pH samples.

Storage conditions and volume

Long-term storage of the samples did not have a large influence on the pH measurements as there was good concordance between the pH measurements taken initially and after long-term storage of the samples (20–24 months at −80 °C) ($n = 96$, mean pH difference −0.007, S.D. 0.35, range 1.56). Short-term storage conditions were investigated by measuring the pH prior to initial sample freezing and storing the sample in a −20 °C freezer for 8 h (Figure 2). There was no significant effect of different storage methods on either pH or NH_4^+ by itself or when adjusting for smoking status (all $p > 0.05$). In addition, no association was found between EBC volume and either pH or NH_4^+ (Figure 3) (both $p > 0.05$).

Table 1 Methodological considerations investigated in the study.

Methodological question	Number of subjects	Procedure	EBC parameters investigated
Reproducibility of pH measurements	12 (5 males, 7 females)	pH measured 3 times ($n=116$ total)	Short-term variability of EBC pH
Short-term sample storage	10 (4 males, 6 females)	4 tests each; 2 tests immediately measured and 2 tests stored in −20 °C freezer for 8 hours before measurement ($n=40$ total)	Storage method, EBC pH and ln NH_4^+
Long-term sample storage	12 (5 males, 7 females)	Several tests performed on a different day over a period of ~1 month ($n=96$ total)	Fresh pH measurements and pH measurements after 20–24 months of −80 °C storage
Effect of sample volume on EBC pH and NH_4^+	10 (4 males, 6 females)	6 tests each; each test performed on a different day over a period of ~1 month ($n=60$ total)	Sample volume, EBC pH and ln NH_4^+
Within- and between-person variability of EBC pH and NH_4^+	10 (male: 2 never smokers, 2 current smokers; female: 3 never smoker, 3 current smokers)	6 tests each; each test performed on a different day over a period of ~1 month ($n=60$ total)	Within- and between-person variability of EBC pH and ln NH_4^+
Effect of smoking on EBC pH and NH_4^+	10 (male: 2 never smokers, 2 current smokers; female: 3 never smoker, 3 current smokers)	6 tests each; each test performed on a different day over a period of ~1 month ($n=60$ total)	Smoking variables, EBC pH and ln NH_4^+

Table 2 Descriptive characteristics of study population.

	Never smokers	Current smokers	Other
Subjects (n)	5	5	2
Age (years)	33.6±9.4	22.6±7.0	22.5±0.7
Male (n)	2	2	1
Samples (n)	40	40	36
EBC volume (mL, mean±S.D.)	1.9±0.3	2.0±0.4	2.0±0.07
EBC average pH (mean±S.D.)	7.6±0.2	7.3±0.9	7.7±0.02
EBC NH_4^+ (μM, mean±S.D.)	526.9±357.0	455.7±354.6	545.0±80.9

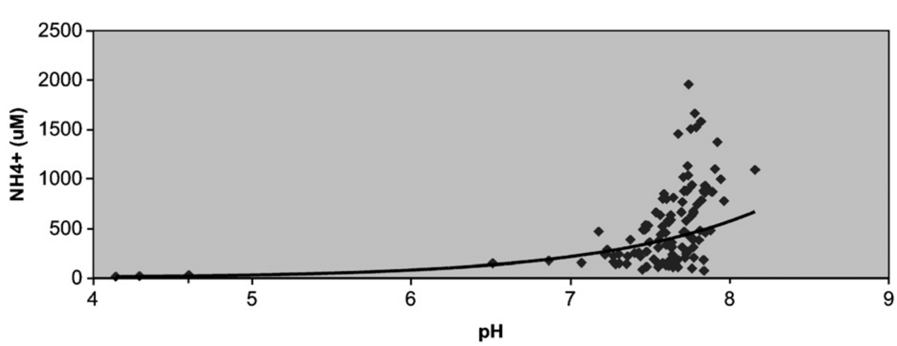

Figure 1 Correlation of pH and NH_4^+. Scatter-plot of pH and NH_4^+ values with an exponential trendline.

Variability

Variance components for EBC pH was investigated in five never smokers and five current smokers ($n=60$ total) (Figure 4). For current smokers, the average cigarette packs smoked per day was 0.30 (S.D. 0.12), and average pack-years was 0.92 (S.D. 0.59). Two of the 10 subjects were former asthmatics. The BPV (variance 0.4, S.E. 0.4, $p>0.05$)

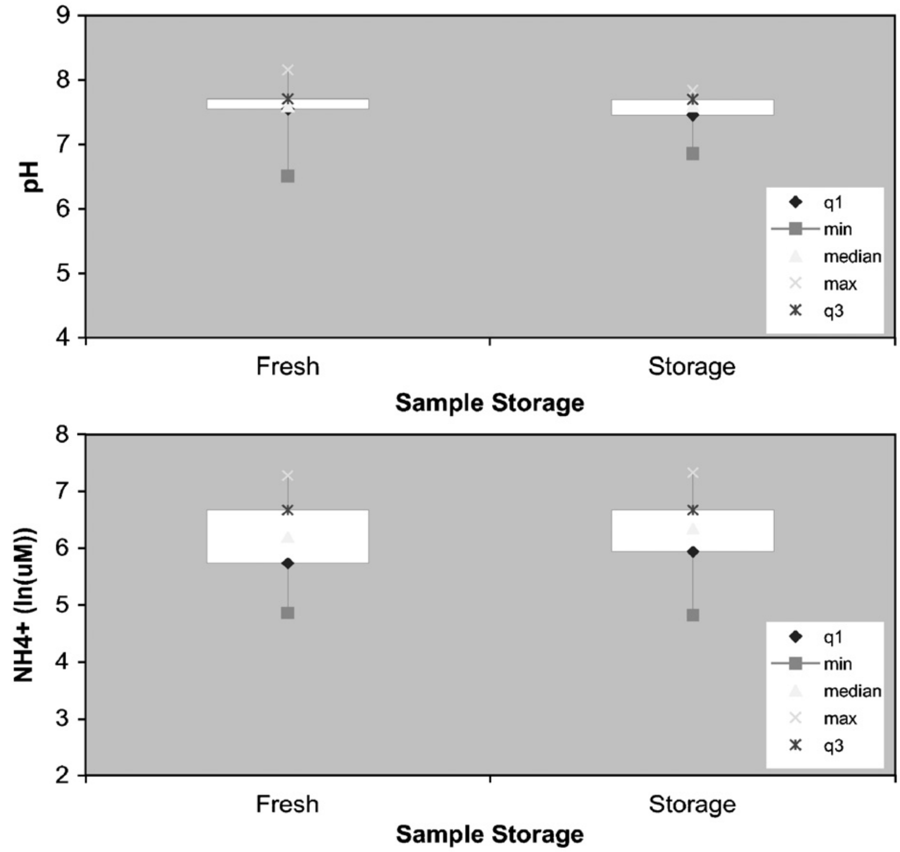

Figure 2 Effect of short-term storage conditions on pH and NH_4^+. Fresh indicates samples that were measured immediately, while storage indicates samples that were stored at −20 °C for 8 h before measurement.

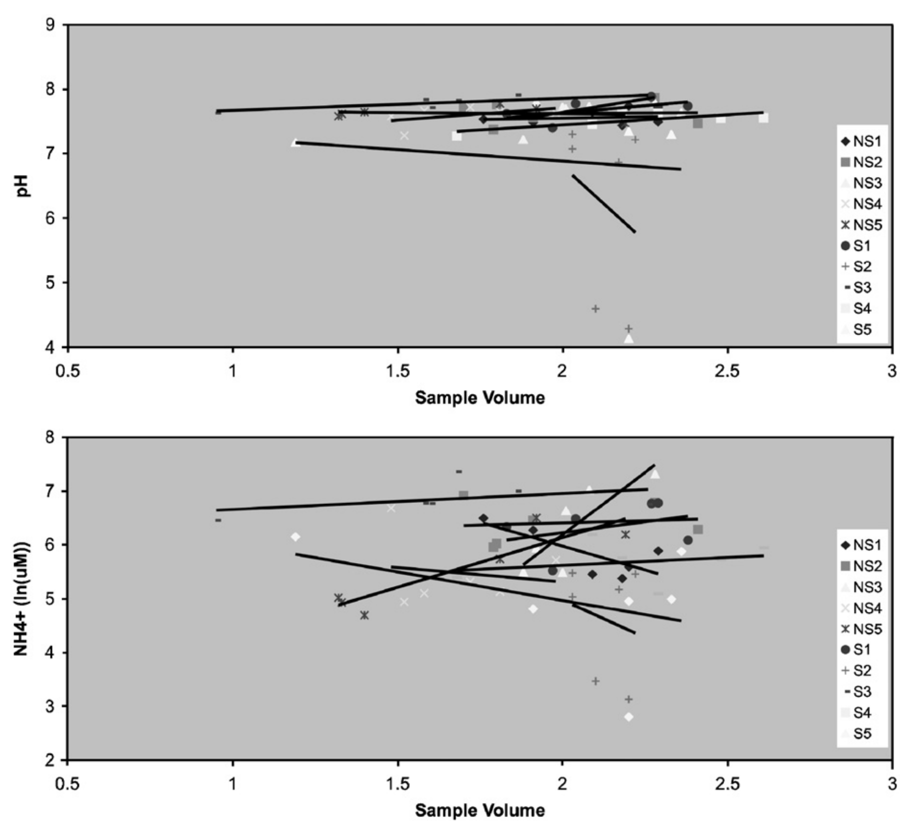

Figure 3 Effect of sample volume (mL) on pH and NH_4^+. Scatter-plot of sample volume and pH or NH_4^+. Regression lines were fitted based on values from each individual ($n = 10$).

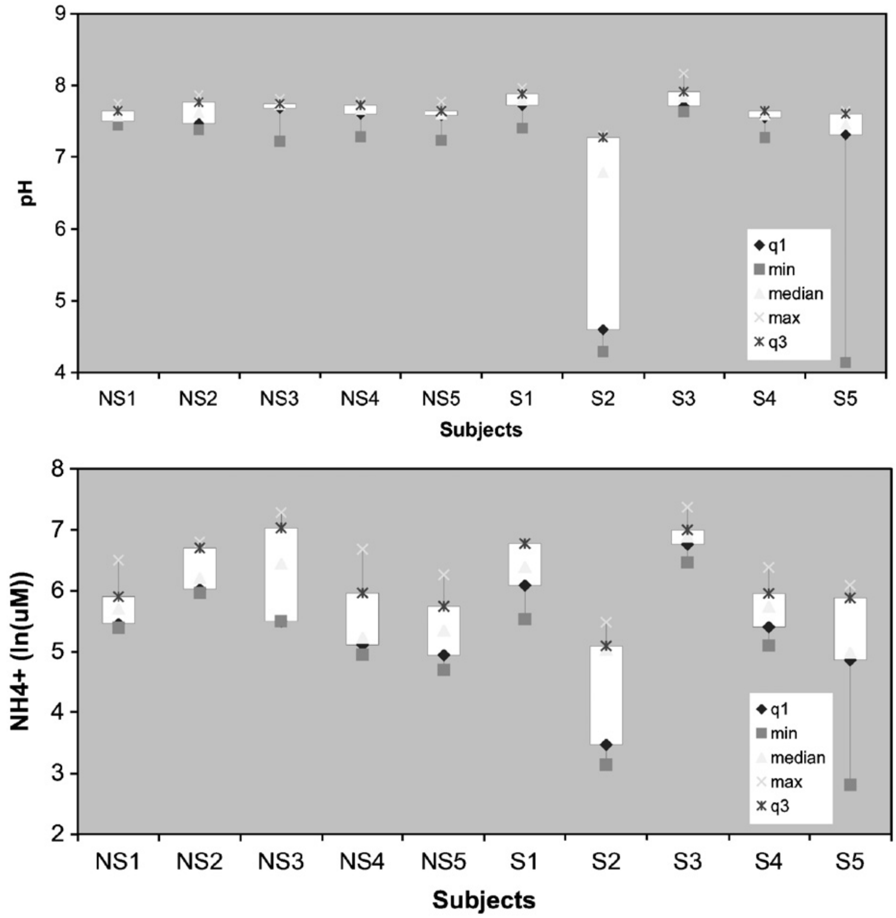

Figure 4 Within- and between-person variabilitity of pH and NH_4^+ in never smokers and current smokers. Boxplots of repeated measurements ($n = 6$) of pH/NH_4^+ from 10 individuals over a one month period. NS indicates never smoker, while S indicates current smoker.

Table 3 Mixed effects regression results for smoking and pH and NH_4^+.

Variable	pH coefficient (S.E.)	p	NH_4^+ (ln(μM)) coefficient (S.E.)	p
Smoking status	—	NS	—	NS
Cumulative smoking amount	—	NS	−0.9 (0.4)	0.05
Acute smoking amount	−0.3 (1.0)	0.01	−4.2 (1.7)	0.04

Each smoking variable analyzed separately, after adjusting for asthma status. Acute smoking amount: number of cigarette packs smoked per day now; Cumulative smoking amount: pack-years, average cigarette packs smoked per day over the entire time smoked multiplied by number of years smoked regularly; S.E.: standard error; NS: not significant.

and the WPV (variance 0.8, S.E. 0.2, $p = 0.0002$) for current smokers were both larger than that seen in never smokers (BPV: ~0; WPV: variance 0.03, S.E. 0.007, $p = 0.00007$). Similar results were found for NH_4^+ (current smokers: BPV-variance 0.8, S.E. 0.7, $p > 0.05$ and WPV-variance 0.6, S.E. 0.2, $p = 0.0002$; never smokers: BPV-variance 0.1, S.E. 0.1, $p > 0.05$ and WPV-variance 0.4, S.E. 0.1, $p = 0.0002$).

In mixed effects regression analyses, acute smoking amount was associated with both pH ($p = 0.01$) and NH_4^+ ($p = 0.04$) after adjusting for asthma status. Similar results were found for cumulative smoking amount and NH_4^+ ($p = 0.05$) only, while no association was found for smoking status and either biomarker measurement (Table 3). The global mean pH for never smokers was higher than the global mean for never smokers (7.6 (S.E. 0.03) vs. 7.3 (S.E. 0.3), respectively). This was also seen for NH_4^+ (global mean for never smokers 5.9 (S.E. 0.2) vs. current smokers: 5.7 (S.E. 0.4)).

Discussion

In EBC, carbon dioxide (CO_2) is a major volatile compound that can combine with H_2O to form H^+ and bicarbonate (HCO_3^-). This can influence EBC pH. Since fresh samples generally involve unstable pH readings, the often-used protocol of argon deaeration at 350 μL/min for 10 min was performed in the study to stabilize the pH. Ammonia measurements on the other hand, appear not to be influenced by argon deaeration.[15] There are concerns about the effects of CO_2 on pH, since it has been reported that residual CO_2 remains even after argon bubbling.[16] In the present study, PCO_2 levels were not measured and therefore we were unable to assess the influence of CO_2 levels on our marker measurements. However, the high reproducibility of our measurements and the high concordance rate found between initial pH measurements and pH measurements after 20–24 months suggest that the pH assay is robust and stable after argon deaeration.

The mechanisms leading to dysfunction in the acid-base equilibrium of the airways is unknown, although regulation is generally maintained by the production and release of acids. Some acids present in EBC include lactate[17] and nitric oxide metabolites (nitrite and nitrate),[18] while bases that have been measured in EBC include albumin[19] and bicarbonates.[17] Our results of a moderate correlation of pH and NH_4^+ is consistent with the results of previous studies that have investigated the acid–base equilibrium in healthy subjects that had undergone asthmatic exacerbations,[20] in stable allergic asthmatic children,[21] and in randomly selected subjects.[22]

For occupational studies that require collection of field samples, it can be difficult to obtain EBC marker measurements at the site. Therefore, storage of the samples at the field site before being transported to the laboratory at the end of the day may be required. Our marker measurements were not influenced by either short-term or long-term storage of the samples, which is consistent with a previous finding from Vaughan et al.[23] who reported a high correlation coefficient of 0.97 between fresh pH measurements and pH measurements of samples stored for either 1 year ($r = 0.97$, $p < 0.001$, $n = 24$) or 2 years ($r = 0.98$, $p < 0.001$, $n = 11$) at $-20\,^{\circ}C$. In addition, the lack of association of volume on either marker measurements in our study suggests that increased EBC volume does coincide with the enhanced collection of respiratory solutes. This finding is supported by McCafferty et al.[24] who noted that a reduced tidal volume was associated with reduced water vapor availability but did not affect solute dilution.

The variability of EBC pH and NH_4^+ measurements taken from repeated samples in the same individual, as well as the role of acid stress in the airways in response to tobacco smoke inhalation is not completely understood. In this study, we have seen differences in both pH and NH_4^+ measurements between never smokers and current smokers. The small within-person variability for pH and NH_4^+ among never smokers in this study suggest that the pH assay is highly reproducible. The larger within-person and between-person variability seen in current smokers may reflect the effects of tobacco smoke inhalation on the acid–base equilibrium of the airways.

Smoking has been shown to be associated with EBC markers of inflammation[25,26] and oxidative stress.[27] Although EBC pH/NH_4^+ have been documented to differ in asthmatic[4,14,21] and COPD subjects,[4,28] this is the first report to show an association of smoking with markers of EBC acid stress in otherwise healthy subjects. It is possible that smoking may induce acid stress events via acid reflux[29] or via other mechanisms linked to inflammation or oxidative stress.

The results from this study show that EBC pH and NH_4^+ is a simple, robust, and reproducible assay as it is not influenced by storage conditions or sample volume. The variability and smoking findings suggest that the measurement of pH and NH_4^+ in repeated EBC samples in the same individual may be a good predictor of the biological state of the airways of current smokers when compared to never smokers.

Competing interests

The authors declare that they have no competing interests.

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