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Exhaled breath condensate pH is a robust and reproducible assay of airway acidity

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ABSTRACT: Exhaled breath condensate (EBC) pH is low in several lung diseases and it normalises with therapy.

The current study examined factors relevant to EBC pH monitoring. Intraday and intraweek variability were studied in 76 subjects. The pH of EBC collected orally and from isolated lower airways was compared in an additional 32 subjects. Effects of ventilatory pattern (hyperventilation/hypoventilation), airway obstruction after methacholine, temperature (-44 to +13°C) and duration of collection (2–7 min), and duration of sample storage (up to 2 yrs) were examined. All samples were collected with a disposable condensing device, and de-aerated with argon until pH measurement stabilised.

Mean EBC pH (n=76 subjects, total samples=741) was 7.7±0.49 (mean±SD). Mean intraweek and intraday coefficients of variation were 4.5% and 3.5%. Control of EBC pH appears to be at the level of the lower airway. Temperature of collection, duration of collection and storage, acute airway obstruction, subject age, saliva pH, and profound hyperventilation and hypoventilation had no effect on EBC pH.

The current authors conclude that in health, exhaled breath condensate pH is slightly alkaline, held in a narrow range, and is controlled by lower airway source fluid. Measurement of exhaled breath condensate pH is a simple, robust, reproducible and relevant marker of disease.

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Exhaled breath condensate pH is a noninvasive, simple and inexpensive assay that can be performed repeatedly without adversely affecting a patient, and can be collected safely both orally and from endotracheal and tracheostomy tubes. Importantly, exhaled breath condensate pH correlates well with other indices of airway inflammation, specifically-induced sputum eosinophilia and neutrophilia [1]. Further, a low exhaled breath condensate (EBC) pH is found during acute exacerbations of asthma [2], chronic obstructive pulmonary disease (COPD) [3], and cystic fibrosis [4], and also in stable COPD, bronchiectasis and moderate asthma [1]. The possibility exists that this simple assay may be a useful surrogate for invasive or more complicated attempts at assessment of airway inflammation, such as biopsy, bronchoalveolar lavage or induced sputum. Therefore, in the present study the technique has been broadly examined to identify potential technical confounders in EBC collection and assay.

Methods

Subjects

Healthy nonsmoking subjects without history of significant chronic respiratory disease were recruited from the area around Charlottesville, VA, and within the hospital and clinics of the University of Virginia Health Sciences System. Subjects undergoing intubation were recruited from the preoperative suite. Informed consent was obtained from all subjects, and the studies were approved by the Human Investigation Committee at the University of Virginia.

Sample collection

Exhaled breath condensate collection was performed using the RTube™ EBC collection system (Respiratory Research, Inc, Charlottesville, Virginia, USA; fig. 1a) at temperatures and durations as noted for each experiment. Nose clips were not worn, because they are somewhat uncomfortable and in preliminary experiments it was determined that they had no effect on EBC pH. Measurement of pH was performed after deaeration by bubbling argon [2] through the sample while monitoring pH until the reading stabilised completely.

Protocols

Intraweek and intraday variability in health. Seventy-six subjects (24 male, mean age 21 yrs, range 18–48 yrs) recruited for a parent study performed EBC collections for 10 min before breakfast in their homes for 7 days. On the seventh day, they performed three additional collections: before lunch, before dinner and before bedtime. The subjects were instructed to perform the collections at least 1 h after the last ingestion of any food or drink. Some of the subjects were receiving

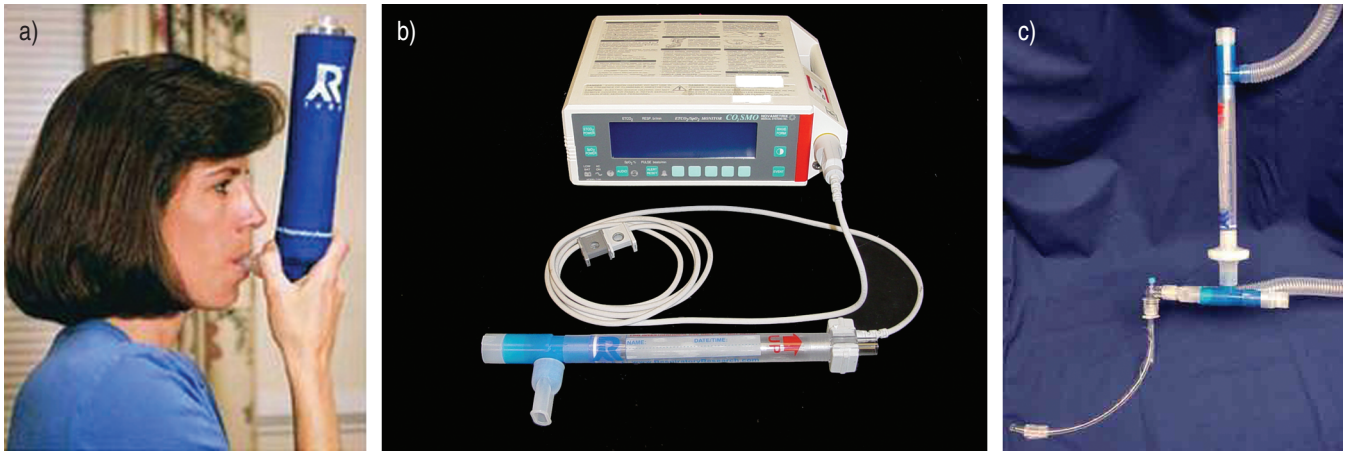

Fig. 1. – Exhaled breath condensate collection system. a) Used in standard fashion, the subject breathed at tidal volumes through the mouth. Ambient air was inhaled through a one-way valve at the bottom of the device. Exhaled air was channeled through another one-way valve incorporated within the condenser chamber atop the mouthpiece. Condensate was formed and collected from the condenser wall with a built-in syringe-style plunger. b) For monitoring ventilation, an end-tidal carbon dioxide monitor was attached to the top of the standard-sized condenser chamber. One-way flow of air assured accurate measures. c) The same collection system was incorporated into a ventilator circuit. A cuffed endotracheal tube was used to isolate the lower airway. The condenser was placed early in the exhalation limb of the circuit.

preparations of Echinacea in a blinded fashion as part of the parent trial, but otherwise no medications were being taken. No screening was performed for atopy, although medications for asthma excluded subjects. Sample collection temperatures were approximately that of the subject′s home freezers (-4°C to -17°C). Samples were stored in their home freezers and delivered to the laboratory personnel the day after the last collection.

Temperature of collection. Ten adult subjects (five male, mean age 30 yrs; range 18–42 yrs) performed consecutive EBC collections at various temperatures using RTubes with modified cooling jackets that allowed for ranges of relatively constant temperatures to be maintained in the condenser during the collection process. Temperatures of the inside of the condenser rose by several degrees during all collections. Average temperatures of collection were +13°C, -6°C, -17°C, and -44°C. All samples were processed for pH assay in batch.

Duration of collection. Six adult subjects (three male, mean age 31 yrs, range 22–42 yrs) performed EBC collections for 2 min each, followed immediately by collections for 7 min each. All samples were collected at -17°C. Samples were assayed concurrently for pH.

Effects of ventilatory pattern. Eleven adult subjects (seven male, mean age 33 yrs, range 21–40 yrs) performed EBC sampling at normal tidal breathing, and again while hyperventilating. End tidal carbon dioxide ($ETCO_2$) monitoring was performed by attaching a Novametrix 7100 (Wallingford, CT, USA) $ETCO_2$ monitor directly into the distal end of the RTube condensing chamber (fig. 1b). Excellent waveforms were obtained. Subjects began rapid respiratory rate/relatively high tidal volume hyperventilation prior to initiating the second collection, and then continued rapid breathing through the RTube condenser. $ETCO_2$ values were maintained at below 27 torr by biofeedback. Immediately thereafter, EBC collections were performed in five of the subjects while they shallowly and slowly breathed a 5% CO_2, 95% oxygen mixture to attain an $ETCO_2$ of 46. All collections were performed for 3 min using the standard RTube condensation equipment, with temperature of -17°C at start of collection.

Effects of airway obstruction. Six subjects (3 male, mean age 31, range 21–40 yrs) with stable asthma and suspected bronchial hyperreactivity underwent staged methacholine challenge tests using a Rosenthal dosimeter (PDS Instrumentation, Louisville, CO, USA). Subjects performed baseline spirometry and 90 s EBC collections before the first methacholine dose was given. After achieving >20% reduction in their baseline forced expiratory volume in one second (FEV1), another 90 s EBC collection was performed prior to reversal with albuterol.

Concurrent measurements of exhaled breath condensate pH and saliva pH. Twenty subjects (9 male, mean age 34, range 21–56 yrs) performed 10-min oral EBC collections while depositing saliva in a test tube. Salivary pH was assessed before and after de-aeration with argon.

Measurement of exhaled breath condensate from the isolated lower airway. Thirty-two subjects (13 male, mean age 53, range 29–72 yrs) with pending elective surgical procedures (28/32 were neurosurgical patients), but no history of chronic respiratory disease, were enrolled. Subjects initially provided a 10-minute collection of EBC collected in the standard fashion (breathing into a mouthpiece). Anaesthesia was induced with a volatile anaesthetic and the airway was stabilised and secured with a cuffed endotracheal tube. Immediately upon confirmation of placement by $ETCO_2$ monitoring, EBC was again collected for 10 min by inserting the RTube into the exhalation circuit as close as possible to the endotracheal tube (fig. 1c).

Effects of sample storage. Assays of EBC pH were performed on samples (n=24) that had been stored at -20°C for >1 yr (maximum 27 months). Samples studied included controls and samples from subjects with acute asthma exacerbations (and thus low pH). Measurements obtained immediately after collection were compared to repeat measurements 12–27 months later. Samples were de-aerated with argon prior to each measurement.

Statistical considerations

Intraday and intraweek reproducibility of EBC pH assays were evaluated using mean coefficient of variation. Comparisons of sampling conditions (different temperatures and duration of collection, effect of hyperventilation,

hypoventilation, airway obstruction and anatomic source) were compared by paired t-testing or repeated measures analysis of variance. Correlation coefficients were determined and Bland Altman analysis performed for assays repeated after 1 or 2 yrs. Data are presented as mean±SD. p-Values <0.05 were considered significant.

Results

Reproducibility of exhaled breath condensate pH measurements

From this cohort of 76 healthy control subjects, 741 EBC samples were obtained. No subjects suffered any significant ill effects during sampling, although one subject experienced tingling around the mouth partway through collection that did not recur when asked to breath more gently. This was ascribed to probable hyperventilation. Out of 760 sample-days, only 19 samples were not available for assay (2.5%), revealing excellent compliance with the protocol despite the subjects using the devices completely unsupervised at home. Mean pH of all samples was 7.70±0.49. Evaluation of the reproducibility of EBC pH measurements from seven consecutive mornings revealed a mean coefficient of variation of 4.5% (full intrasubject range 0.9 to 20%). The mean intraday coefficient of variation was 3.5% (range 0.6 to 23%). There was no difference between the mean pH values of females and males.

Temperature of collection does not affect exhaled breath condensate pH in healthy subjects

Respectively, mean EBC pH collected at condenser temperatures of +13°C, -6°C, -17°C, and -44°C was 7.95±0.22, 7.83±0.34, 7.94±0.32, and 7.90±0.3 (fig. 2). There were no significant differences identified in the pH of EBC collected at different temperatures in these healthy subjects.

Duration of collection does not affect exhaled breath condensate pH

Six subjects performed EBC collections for 2 min, followed by a second sample collected for 7 min. Four of the subjects performed three consecutive collections at each collection duration. The range of sample volumes for 2-min collections was 250–350 μL and for 7-min collections was 800–1250 μL. For these back-to-back collections, the mean intrasubject coefficient of variation (CV) for 2-min collections for pH was 0.5%. Likewise for 7-min collections, the mean intrasubject CV for pH was 0.5%. The pH was the same for the 2-min collections (8.0±0.11) and the 7-min collections (8.1±0.18; p=ns).

Ventilatory pattern is not relevant to exhaled breath condensate pH

During tidal breathing, subjects maintained $ETCO_2$ levels of 38.4±1.1 torr. While hyperventilating, $ETCO_2$ was brought down to 23±0.7 torr (p<0.001; n=11). The five subjects who performed hypoventilation increased their $ETCO_2$ from baseline (38±0) to 46.8±1.1 torr (p<0.001). Exhaled breath condensate pH at tidal breathing in this healthy group of subjects was 8.19±0.13; during hyperventilation the mean pH was 8.20±0.14; and during hypoventilation the mean pH was 8.14±0.15 (p=ns). No subject had an EBC change by >0.2 units (fig. 3).

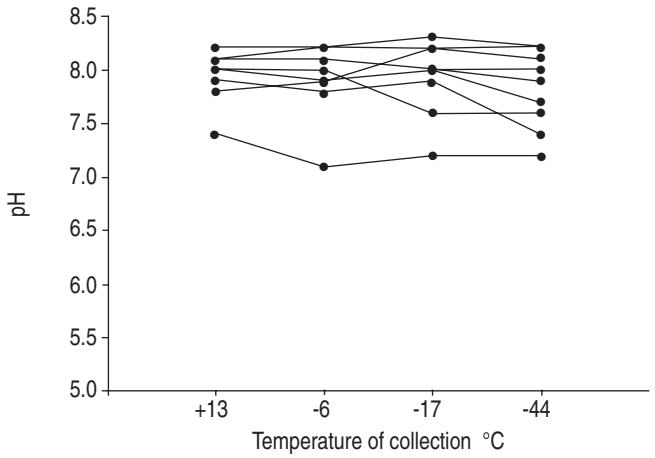

Fig. 2. – No effect of condenser temperature on exhaled breath condensate (EBC) pH. Subjects performed consecutive EBC collections at temperatures as noted. The condenser was modified by incorporation of liquids with relevant phase change characteristics to help stabilise temperature.

No effects of acute airflow obstruction on exhaled breath condensate pH assays

Methacholine challenge produced a mean per cent decline in FEV1 of 27% in these six asthmatic subjects, using mean dose of 2.25 mg to achieve the requisite degree of airway obstruction. Mean baseline pH was 8.03±0.1, and while obstructed the EBC pH was 8.00±0.06 (p=ns). No subject had >0.1 unit change in pH while their airways were obstructed.

No correlation between saliva pH and exhaled breath condensate pH

In 20 subjects, saliva pH after de-aeration was 7.26±0.54 compared to matched concurrently collected EBC samples

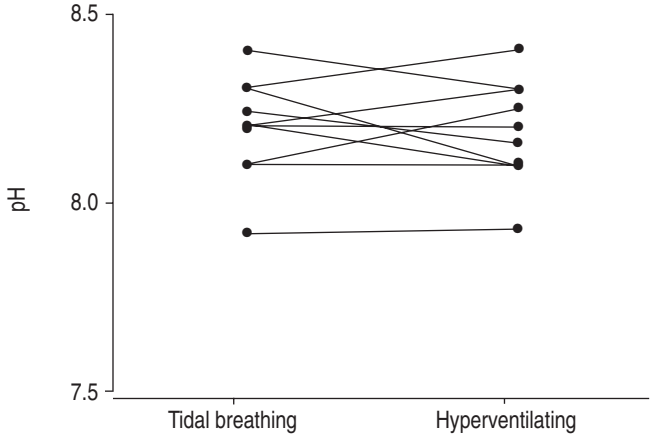

Fig. 3. – Degree of ventilation does not affect exhaled breath condensate (EBC) pH. Subjects performed consecutive collections for 3 min each while breathing at normal tidal volumes and then while hyperventilating. End-tidal carbon dioxide ($ETCO_2$) was monitored during collection by attachment to the top of the RTube condenser. By biofeedback, subjects maintained $ETCO_2$ below 27 torr (23±0.7 (mean±SD)) during the entire 3 min hyperventilation collection time.

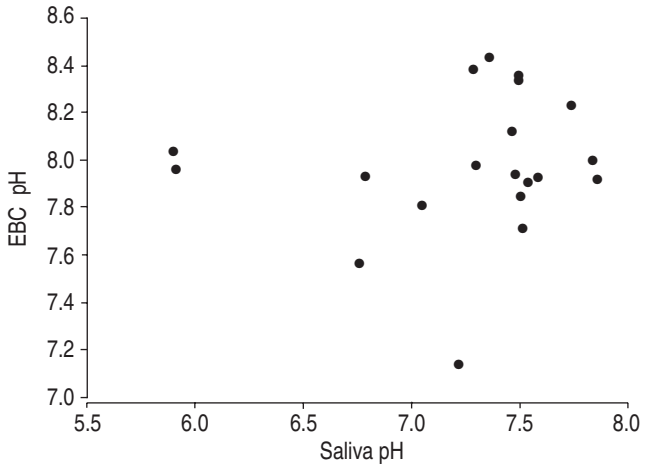

Fig. 4. – No correlation of salivary pH and exhaled breath condensate (EBC) pH. Twenty subjects provided concurrent EBC samples and saliva samples. R^2=0.02, p=NS.

which had a mean pH of 7.97±0.30. Linear regression revealed an R^2 of 0.02, p=NS (fig. 4). There was no correlation between EBC pH and either directly measured or deaerated saliva pH.

Isolated lower airway exhaled breath condensate pH assays

In 30/32 subjects studied there was minimal or no difference in the EBC pH obtained from oral collections and isolated lower airway collections. Mean pH before and after intubation in these subjects was 7.9±0.23 and 7.8±0.28 respectively (p=NS, n=30) (fig. 5). In two subjects the EBC pH was markedly acidic in the sample collected from the isolated lower airway, but not at baseline before intubation. Of note, the time to secure the airways of each of these two subjects was prolonged, although neither subject had symptomatic evidence of gross acid aspiration.

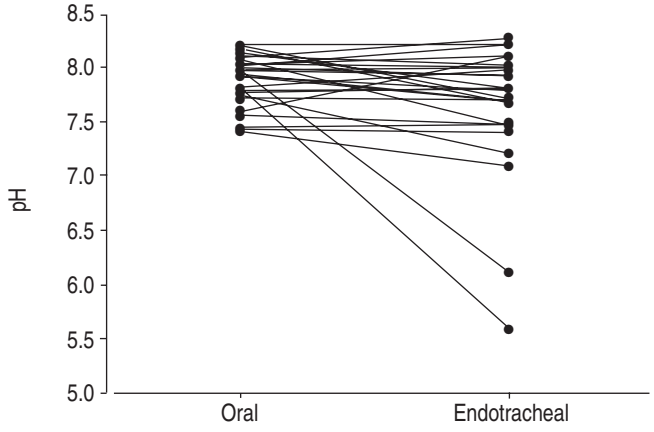

Fig. 5. – Exhaled breath condensate pH from isolated lower airways. Subjects performed exhaled breath condensate (EBC) collections in the standard fashion (using mouthpiece) in the pre-operative area just prior to induction of general anaesthesia. Immediately after the airway was secured by endotracheal intubation, an additional EBC sample was collected by inserting the RTube into the exhalation circuit adjacent to the endotracheal tube. Little difference was noted in the pH of samples between the two routes of collection, although two subjects had low EBC pH from the samples collected from the isolated lower airway, thought to be attributable to silent aspiration during prolonged intubation effort.

The pH of exhaled breath condensate is not affected by duration of sample storage

Prolonged storage had no effect on EBC pH assays regardless of whether the initial pH was normal or profoundly low. The correlation coefficient of paired EBC pH assays of samples studied after collection and re-assayed after >1 yr in -20°C storage was 0.97 (p<0.001, n=24). After 2 yrs in storage, the correlation coefficient was 0.98 (p<0.001, n=11; fig. 6a). By Bland-Altman analysis, the measure was reproducible at both normal and low pH values (fig. 6b). In this system, deaeration is performed before each assay, but it is not necessary to de-aerate sample prior to storage (data not shown).

Age of subject does not affect exhaled breath condensate pH

In subanalyses of each individual experiment and all the data together, no effect of subject age on EBC pH was identified.

Discussion

The possibility that EBC pH may serve as a noninvasive indicator of airway inflammation is supported by the tight correlations of EBC pH with inflammatory cell populations

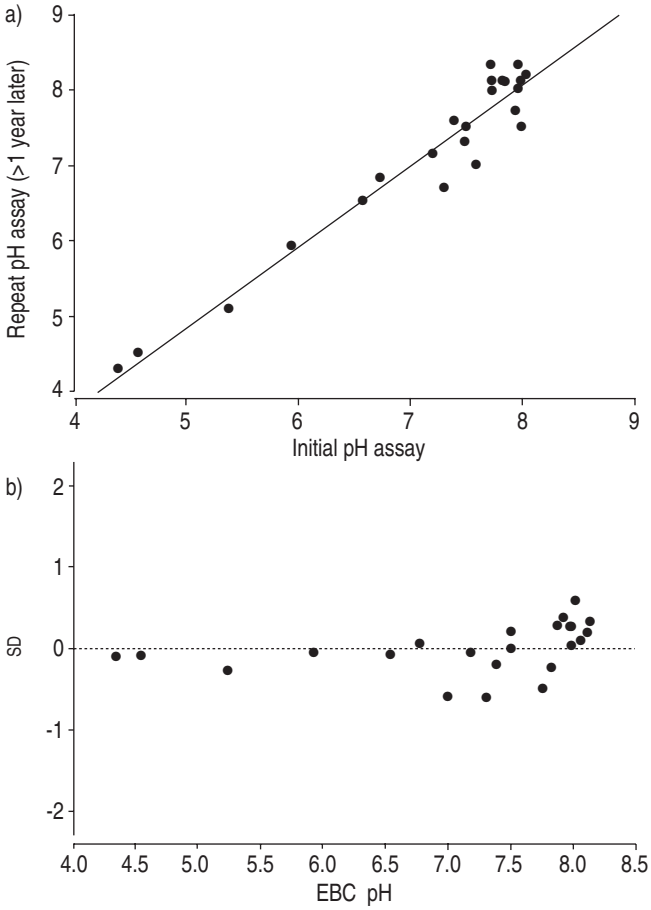

Fig. 6. – The pH of exhaled breath condensate (EBC) remains stable in storage. a) Sample was assayed for pH soon after collection. After >1 yr, the stored samples were de-aerated and assayed again. Results of assays >1 yr apart were nearly identical with a correlation coefficient >0.97. b) Bland-Altman plot shows no appreciable effect of the sample pH on the level of variability of the assay.

measured in induced sputum [1]. Perhaps more important is that EBC acidification could be representative of an airway pH homeostatic alteration that underlies some or much of the pathology of lung diseases, including inflammation. For both these reasons, it became necessary to determine if EBC pH abnormalities might be caused by simple airflow obstruction or by potential artifacts, so that the technique could be understood and applied to future research of airway biochemistry, immunology, and physiology. Further, the present authors wished to determine the major anatomic contributor to EBC pH and the overall reproducibility of the measurement.

A total of 930 EBC samples were collected in the several studies reported in this paper. This is by far the largest set of data reported on EBC to date. The current study reports that EBC pH measurements, after de-aeration with argon, are robust, highly reproducible over time, not affected by degree of ventilation, unaltered by prolonged storage of sample, and are not affected by the temperature or duration of collection. Neither subject age nor salivary pH affects EBC pH. The lower airway appears to be the dominant determinant of EBC pH. Further, the current study demonstrated that EBC pH is not deviated by marked airway obstruction alone, confirming the present authors′ previously reported observations [2] and suggesting that conditions underlying airway obstruction in asthma (inflammation and airway pH deviation itself) are the primary causes of EBC acidification.

The airway pH homeostatic process is not understood. The pH of the airway in health has been reported to be slightly alkaline (pH in the range of 7–8) [5–8]. In states of acute lung disease, where a knowledge of airway chemistry would be most beneficial, the invasiveness of most sampling techniques have precluded even experimental assessment of the airway environment. The pH of the airway in diseases such as acute asthma, COPD, and adult respiratory distress syndrome has therefore not been well documented by invasive measurements.

Exhaled breath condensate is now being used by centres throughout the world in an effort to gain insight into the airway environment in multiple lung diseases. It is a safe and simple procedure even in small children [9]. The fluid exhaled from the lungs and trapped in a condenser contains expired particles of airway lining fluid, aerosolised presumably by turbulent airflow. These particles are diluted by large amounts of condensed water. Importantly, EBC is an excellent trap for water-soluble volatile gases. These gases may be acidic or basic. Acidification of EBC, often pronounced, occurs during disease states and appears to reflect acidification of the source fluid (the airway lining fluid). In this regard, source fluid acidification enhances the volatility of acids, while decreasing volatility of bases, thus providing a ready explanation for acidification of EBC in that it can trap these water-soluble volatiles.

The pH of EBC has been found to be low in stable asthma, COPD, bronchiectasis [1], cystic fibrosis [4], and acute respiratory distress syndrome [10]. The pH is markedly low in exacerbations of asthma [2], COPD [11] and cystic fibrosis [4], with normalisation of pH occurring with steroid or antibiotic therapy. These findings have raised obvious questions about how airway function might be adversely affected by endogenous acidification.

Indeed, altering the pH of the airway environment is known to affect multiple aspects of airway function. Nebulised citric or acetic acids are used to trigger bronchoconstriction and cough for testing antitussive agents. These effects are at least in part mediated by acid (protons) triggering capsaicin sensitive neurons [12] and the release of tachykinins [13]. Chlorine gas is thought to cause wheezing and coughing in substantial part because of the rapid formation of hydrochloric and hypochlorous acids upon contact with the airway lining fluid [14]. Indeed in cats, instillation of 50 μL of 0.2 N hydrochloric acid into the trachea caused a 420% increase in airway resistance [15]. Dogs respond similarly [16].

Multiple additional effects of airway acidification are expected. Mild acidification (below pH 6.5) increases mucous viscosity [17], converting it from sol to gel [18] which is perhaps relevant to mucous plugging. Low environmental pH enhances inducible nitric oxide synthase 2 expression and activity in rat peritoneal macrophages through the action of tumour necrosis factor (TNF)-α and nuclear factor-κB [19].

Acidity affects airway cell protein expression. Guinea pig airway epithelial cells induce stress-related proteins including hsp72 in response to transient mild acidification [20]. Low pH alters both the synthesis and secretion of TNF-α by alveolar macrophages [21]. Acidic insult augments hyperoxic injury in the rat, in part because of a loss of antioxidants [22]. Acidification enhances oxidant and nitrative stresses by increasing reactivity of small inorganic molecules and by altering enzymatic antioxidant activities [2].

Although it has been suggested that ammonia derived from the mouth might prevent EBC pH assays from being useful, or that asthmatic hyperventilation might lead to *ex vivo* EBC acidification because of altered ammonia absorption [23], our data dispel these possibilities and obviate these arguments. Isolated lower airway EBC samples have the same pH as matched samples collected during oral breathing, despite oral ammonia being completely excluded. Likewise, even with voluntary profound hyperventilation, far greater than seen in asthma, the current authors have been unable to artifactually alter EBC pH.

However, ammonia appears to play an important role in airway pH in another manner altogether. The present authors have previously reported that glutaminase (an enzyme that produces two bases: ammonia and bicarbonate) is expressed in human airway epithelium *in vitro* and *in vivo* and is upregulated by mild acidic stress [24]. Failure of this pathway to respond to acidic stress would result in decreased production of base, and limited ability to normalise airway pH. Acidification in turn traps as ammonium any ammonia that is present in the airway environment, including inhaled ammonia from the oropharynx, preventing it from volatile egress from the lung. Combined, these complementary effects explain the greatly reduced levels of ammonia exhaled from subjects in whom the EBC is acidic [24].

The current authors have noted that being a cigarette smoker did not appear to affect EBC pH measures (unpublished observations), however confidence in this will be assessed after completion of a study, recently initiated, specifically to address the affect of acute and chronic cigarette smoking on EBC pH. The current authors have not yet evaluated the potential effects of inhaled particle pH, nor ambient temperature and humidity on EBC pH. The experiments have all been performed indoors with unmeasured, but not extreme, humidity levels and reasonably normal room temperatures. The current authors cannot rule out an effect of very dry cold air on EBC pH, although if an effect exists, it may be difficult to differentiate from the effect of such an insult on airway lining fluid acidity.

Nonvolatile components of EBC are derived from aerosolised airway lining fluid particles. Dilution by condensed water vapor affects the concentrations of nonvolatile components of EBC, a fact long-recognised but receiving increasing attention lately [25]. The pH of EBC is affected by volatile compounds. Acids tend to be volatile at low pH, and the converse applies to bases. The issues and concerns of variable dilution of droplets in EBC do not apply when the substances of interest are water-soluble volatiles. This may be one reason why EBC pH assays are particularly reproducible. Indeed in

back-to-back assays the coefficient of variation was only 0.5% and diurnal and interday coefficients of variation were both <5%.

The lack of effect of condenser temperatures on EBC pH assays needs to be interpreted cautiously. Specifically, these subjects were healthy, and as expected their EBC pH was normal (slightly alkaline). It is likely that if condenser temperatures are low enough for EBC to collect in solid form in the chamber (which occurs when starting temperatures are -20 or below in actual practice), water-soluble volatile components of exhaled air will not be as well trapped as when the condensation occurs in liquid phase at more modest temperatures. If certain volatile acids are exhaled only in disease states, when the source fluid (airway lining fluid) pH is low, then EBC collected at too cold a temperature may not reveal the differences between health and disease as well. For these reasons, as well as for ease of use, the present authors currently recommend collections of EBC at condenser temperatures reasonably close to zero when pH is of primary interest. Although volume of EBC collected is greater at lower condenser temperatures, volume is of much less interest than the nonvolatile and volatile constituents.

The current authors de-aerate the EBC samples with argon to normalise carbon dioxide. This is a surprisingly simple task. Argon is heavier than air, so sits atop the sample keeping atmospheric carbon dioxide (CO_2) from re-entering the sample during pH measurement. CO_2-free air is also suitable for de-aeration. As long as EBC pH stabilises completely during de-aeration, the assay is highly reproducible and time-independent. There is no rush to de-aerate and assay the sample. Other investigators do not de-aerate samples, and also find significant differences in EBC pH between health and disease. The initial pH values in the nondeaerated EBC from healthy subjects are lower than when deaerated, because of the differences in CO_2 content. Low pH samples, however are low no matter what technique is used, and indeed samples with a pH <5 barely change during deaeration.

Exhaled breath condensate pH assay is extremely simple to perform, inexpensive, and robust. Longitudinal data in subjects suggest that exhaled breath condensate pH is a useful assay in individual patients, as opposed to simply distinguishing group means. In that it seems to reflect acid-base disturbance and inflammation in the airway, exhaled breath condensate pH assays may provide important information for description, elucidation, diagnosis of illness, and medication titration in the clinical setting.

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