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# Exhaled breath condensate pH standardised for $CO_2$ partial pressure

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**Short title:** $CO_2$-standardised pH in EBC

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**ABSTRACT**

**Background:** Exhaled breath condensate pH is considered to reflect acid-base balance of the airways. Current pH measurements do not take into account the effect of $CO_2$. The aim of this study was to determine the effect of condensate $CO_2$ partial pressure on pH and to provide a more precise mode of EBC pH determination.

**Methods:** Condensate pH and $CO_2$ partial pressure were measured in parallel from 12 healthy volunteers and 12 asthmatics by blood gas analyser in neat, argon deaerated and $CO_2$ loaded samples. Regression analysis was used a) to test the relation between pH and $CO_2$, b) to calculate pH at 5.33 kPa $CO_2$ level (physiological alveolar $CO_2$ partial pressure). Reproducibility of different pH readings was compared by Bland-Altman test.

**Results:** Condensate $CO_2$ concentration was variable either in neat or argon deaerated samples. There was a close negative logarithmic relation between $CO_2$ and pH ($r^2>0.99$, $p<0.01$). Calculation of pH at 5.33 kPa $CO_2$ level provided approximately 6 times better reproducibility than the currently used measurements.

**Conclusions:** Condensate $CO_2$ partial pressure influences pH measurements. Determination of pH at a standard $CO_2$ level provides the most reproducible condensate pH values to date.

**Key words:** EBC, breath test, exhaled biomarkers, airway biology, airway inflammation

## INTRODUCTION

Exhaled breath condensate (EBC) analysis is a promising method for investigating airway pathology [1]. Easy repeatability and its non-invasive nature make EBC collection attractive to clinicians. However, the measurement of different exhaled biomarkers such as hydrogen peroxide, nitrogen oxides, cytokines, leukotrienes yields greatly variable results. The pH is currently considered to be the most robust variable of EBC [2-3]. Measurement of EBC pH has already proven valuable to determine the degree of acidification of EBC in patients with various inflammatory lung diseases [4-7], persons exposed to hypertonic saline solution inhalation [8] or acute lung injury [9].

It is acknowledged by the ATS/ERS Task Force that the pH of neat EBC samples is unstable [1]. Argon deaeration was suggested to improve reproducibility of pH readings [1-2]. In theory, inert gas removes all volatile components of EBC allowing the measurement of non-volatile acidity. When assayed continuously by a glass microelectrode, it has been observed that the pH of EBC stabilises after 8-10 min of bubbling with argon [2]. It is generally assumed that a stable pH marks the complete removal of $CO_2$ and other volatile components.

$CO_2$ is the major volatile component of EBC. In aqueous environment $CO_2$ forms $H^+$ and $HCO_3^-$ and profoundly affects the pH of dilute solutions such as EBC. Levels of $CO_2$ have not yet been systematically tested in EBC. Although argon deaeration causes a significant decrease in $CO_2$ partial pressure of the condensate ($p_{EBC}CO_2$), the remaining $CO_2$ could influence pH results [10]. Furthermore it is not clear if $NH_3$, another volatile component of EBC, is important in deaeration-induced changes in EBC pH [11-12].

The aim of this study was to determine the effect of $CO_2$ on condensate pH and to achieve a better reproducibility of pH readings by considering $p_{EBC}CO_2$ both in healthy subjects and in asthmatic patients.

## METHODS

### Subjects

Twelve healthy non-smoker individuals without any disease in their medical history (8 women, 4 men, mean age: 41 years, range 21-61 years, FVC>90%, $FEV_1$>80%, $FEV_1$/FVC>70%) and twelve atopic asthmatic subjects without upper airway disease in clinically stable condition (7 women, 5 men, mean age: 43 years (range: 25-64 years), FVC>90%, $FEV_1$>80%, $FEV_1$/FVC>70%, FENO<20 ppb), treated with short acting $\beta_2$-agonists and inhaled corticosteroids (400 μg/day budenoside) were enrolled in the study. The study was approved by the local ethics committee and participants gave their written informed consent.

### EBC collection

EBC was collected for 10 min with a commercially available condenser (EcoScreen, Jaeger, Würzburg, Germany). Nose clips were not worn. Subjects were asked to inhale through the nose and exhale through the mouth in their normal rhythm of breathing. This sampling method provides larger sample volume than that with using a nose-clip [13]. Furthermore there is no difference in exhaled biomarker concentration between the two types of sampling in subjects without upper airway disease [13,14].

From healthy subjects two EBC samples were collected on two consecutive days between 7 and 8 am. From both samples pH and $CO_2$ were determined in duplicates a) from neat samples within 10 minutes after sampling; b) after argon deaeration for 2.5, 5, 7.5 and 10 minutes; c) after $CO_2$ loading for 1, 2, 3 and 4 seconds.

### Handling of EBC samples

#### *Neat EBC samples*

Neat EBC samples were used for measurement immediately after sampling (all measurements were made within 10 minutes after sampling).

***Deaerated EBC samples***

The generally used argon deaeration method was chosen to obtain data comparable with published results.

Each EBC samples were divided into 250 µl aliquots in 8 plastic tubes. Aliquots were simultaneously bubbled with argon (Argon 4.6; Messer Hungarogáz Kft, Budapest, Hungary) using a purpose made bubbling device having 8 arms. The device assured the same argon flow (300 ml/min) in every plastic tube. Samples were deaerated in duplicates for 2.5, 5, 7.5 and 10 min and aliquots were taken for pH and $CO_2$ determination after each deaeration period.

***$CO_2$ loaded EBC samples***

$CO_2$ loading was achieved by bubbling $CO_2$ gas through the samples ($CO_2$ 4.5; Messer Hungarogáz Kft, Budapest, Hungary). Since this manoeuvre caused a rapid increase in $p_{EBC}CO_2$ very short intervals of bubbling (one seconds) were chosen to obtain a stepwise increase in $p_{EBC}CO_2$ concentration. $CO_2$ gas was bubbled through the EBC samples for one second intervals four times. After each one second bubbling period aliquots were taken for pH and $CO_2$ measurements (in other words each one second $CO_2$ bubbling was followed by approximately 10-15 seconds of aliquot taking when no gas was bubbled through the sample).

**pH and $CO_2$ measurement**

EBC samples were immediately transferred into glass capillaries. The closed capillaries were stored for no longer than 1 hour at room temperature before measurements.

pH and $p_{EBC}CO_2$ measurements were performed by means of a blood gas analyser (ABL 520, Radiometer, Copenhagen, Denmark). The reliability of the blood gas analyser in determining EBC pH was tested before the study by comparing it to a glass microelectrode (Radelkis, Budapest, Hungary) and by repeated measurement of a deaerated EBC sample for 10 times.

**Calculation of EBC pH at predetermined $p_{EBC}CO_2$**

The pH value at 5.33 kPa $p_{EBC}CO_2$ was calculated using data obtained from neat and $CO_2$ loaded samples by regression analysis.

**Comparison of $CO_2$ normalised EBC pH to other pH readings**

We tested the repeatability of the three different types of EBC pH determination (measurement in neat samples and in argon deaerated samples and the $CO_2$ normalised EBC pH), the day to day variability of EBC pH of healthy individuals and finally compared EBC pH of healthy persons with that of stable asthmatic patients by using all three types of EBC pH determination.

Repeatability of EBC pH readings was tested in EBC samples of healthy participants divided into two equal aliquots. From both parts of given EBC samples we performed 6 pH and $CO_2$ measurements, 1 from the neat sample, 1 after 10 min argon deaeration and 4 from $CO_2$ loaded samples following the 4 one second long loading period. .

Day to day variability of EBC pH was tested for the three pH reading methods in healthy participants.

Comparison of EBC pH of healthy and asthmatic persons was performed also with all the three different pH reading methods.

**Ammonia measurement**

Ammonia was measured spectrophotometrically in neat EBC samples and after argon deaeration for 10 min (Diagnostic ammonia assay kit, RANDOX, Ardmore, UK) in the same healthy participants.

**Statistical analysis**

A pH-$p_{EBC}CO_2$ plot was created for each sample using the data collected by the blood gas analyser. Logarithmic regression and coefficient of determination ($r^2$) were calculated. The pH and $CO_2$ values obtained in the neat sample and after $CO_2$ loading periods were used to calculate pH at a standardised $CO_2$ level ($p_{EBC}CO_2$ of 5.33 kPa).

Bland-Altman test was performed to compare repeatability of pH assessment of neatand argon deaerated samples with $CO_2$-normalised pH values. Paired t-test was applied for comparison of ammonia concentrations before and after deaeration and also for comparison of mean of differences. Data are given in mean±SD. GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses.

**RESULTS**

**$CO_2$ removal (argon deaeration)**

$CO_2$ level varied in a broad range in neat EBC samples: 4.31-0.67 kPa (mean±SD: 2.20±0.65). Corresponding pH values in neat EBC samples were between 6.17-7.19 (6.89±0.31).

The time course of $CO_2$ removal was not predictable. The $p_{EBC}CO_2$ could not be reduced to a standard level at a given time point, and the reduction of $p_{EBC}CO_2$ was not proportional to time. Representative curves of time courses of $p_{EBC}CO_2$ reduction (panel a) with the corresponding pH increase (panel b) obtained from two parallel aliquots of the same EBC sample are shown on Figure 1.

In EBC samples deaerated for 10 minutes $p_{EBC}CO_2$ was variable between samples in the range of 0.44-0.09 (0.22±0.1) with corresponding pH of 7.39-8.36 (7.91±0.31).

**$CO_2$ loading**

$CO_2$ bubbling raised $p_{EBC}CO_2$ very quickly. Each 1-second $CO_2$ bubbling period caused an approximately 5-10 kPa increase in $p_{EBC}CO_2$. (In 10 sec $p_{EBC}CO_2$ level reached 80-100 kPa and could not be further increased.)

**pH measurement and calculation**

The blood gas analyser gave the same pH values as the glass microelectrode. The mean pH value of argon-deaerated EBC samples after 10 min deaeration was 8.04 (range 7.91-8.11).

Loading samples with $CO_2$ revealed a close negative logarithmic correlation between pH and $p_{EBC}CO_2$ ($r^2>0.99$, $p<0.01$, Figure 2). This correlation allows the calculation of EBC pH at any standardised $p_{EBC}CO_2$. 5.33 kPa was chosen because it is suspected to be identical to the alveolar surface lining fluid (ASL) $CO_2$ level of healthy persons in physiologic conditions. The mean EBC pH standardised to 5.33 kPa $CO_2$ partial pressure was 6.54 (range 6.06-6.96).

For deaerated samples the correlation between pH and $p_{EBC}CO_2$ was slightly lower ($r^2>0.98$, $p<0.01$). As 5.33 kPa is outside of the measured interval in this setting, the deaeration protocol is not appropriate for the estimation of EBC pH at 5.33 kPa $p_{EBC}CO_2$.

*Repeatability of pH readings:* The limits of agreement for parallel samples determined by the Bland-Altman test were 0.27 for the argon deaerated, 0.25 for the neat samples and 0.04 for $CO_2$ standardisation (Figure 3). These results show that EBC pH standardised to

$p_{EBC}CO_2$ is approximately six times more precise than pH measurement of either neat or deaerated samples.

Coefficient of variation (CV) is not an appropriate statistical method to determine reproducibility of a method. Still we provide the coefficients of variation for the purpose of comparison with other studies. The CV was found to be 3.9% for the deaerated, 4.5% for the neat and 3.3% for the calculated values.

*Variability of EBC pH:* EBC pH showed daily variability when determined by either method. Mean difference of pH values between deaerated samples was 0.359, between neat samples 0.376 and between standardised pH values 0.278. Variability of standardised values is demonstrated on Figure 4. Standardised pH values showed normal distribution.

*EBC pH of asthmatic patients:* The close negative logarithmic correlation between pH and $p_{EBC}CO_2$ was detected in patients as well (Figure 2 panel b). The mean EBC pH of stable asthmatic subjects standardised to 5.33 kPa $CO_2$ pressure was 6.41 (range: 6.26-6.68). Calculated pH was as reproducible as that of healthy persons and no significant difference was found between EBC pH of stable asthmatic patients and healthy participants by any of the used pH reading methods.

**Ammonia measurement**

There was no significant difference between ammonia concentrations before and after deaeration by argon for 10 min (86±70 and 82±65 μM/l). There was no correlation between ammonia levels and pH, neither before ($r^2$=0.09) nor after deaeration ($r^2$=0.01) nor with the calculated values ($r^2$=0.15).

**DISCUSSION**

Exhaled breath condensate analysis is a promising topic of investigation. However low

reproducibility of measurements of different exhaled biomarkers limits its application. pH has been considered to be the most robust parameter of EBC [3]. EBC pH is determined by volatile and non-volatile components [11,12,15]. Volatile components have been suspected to cause a disturbing noise in EBC pH measurement. It was assumed that argon bubbling removes the volatile components of EBC almost completely. However, the assumption has never been tested.

Although the general suggestion of the ERS/ATS TaskForce Report is to use nose-clip for EBC sampling it acknowledged that samples could be collected without using it [1].

By measuring $CO_2$ partial pressure in EBC we found that $CO_2$ influences EBC pH to a great extent. However, $CO_2$ can neither be completely removed of EBC nor decreased to a standard level by bubbling even if continued as long as 20 min.

Quite high levels of $p_{EBC}CO_2$ were achieved by repeated $CO_2$ load compared to physiologic range and substantial increase of $p_{EBC}CO_2$ level was caused by just one second of $CO_2$ load. Lower $p_{EBC}CO_2$ levels may be created if the sample is let to stay after one episode of $CO_2$ load and aliquots for pH and $CO_2$ measurements are taken every some minutes (while $CO_2$ is diffusing out of EBC). We created some pH-$p_{EBC}CO_2$ curves from 8 points in the range of 2.5-15 kPa. The same close negative logarithmic correlation was found as in case of higher $CO_2$ levels. The repeated $CO_2$ loading version was chosen in the study because it was less time consuming. A negative logarithmic correlation was found between pH and $p_{EBC}CO_2$. The near perfect logarithmic correlation between EBC pH and $p_{EBC}CO_2$ found in the $CO_2$ loading protocol is a consequence of the Henderson-Hasselbach equation. Similarly, the very strong logarithmic correlation in the deaeration protocol means that argon bubbling mainly removes $CO_2$ and does not influence other components of the condensate. According to our findings argon deaeration does not change EBC ammonia concentration.

The close correlation allows the calculation of EBC pH at any standard $p_{EBC}CO_2$.

Standardisation to 5.33 kPa is justified as it is considered to correspond to ASL $CO_2$ level. EBC pH calculation at 5.33 kPa $p_{EBC}CO_2$ is six times more reproducible than pH measurements that do not take into account the $CO_2$ level. The reason of the better repeatability of the $CO_2$ standardisation method is that in either deaerated or neat pH measurements $CO_2$ level may vary and thus cause a significant change in pH. (Visually it means, that the standardised pH is read at a fixed point of the $CO_2$-pH regression line, while the neat and the deaerated pH value moves along the regression line.)

Repeatability of standardised EBC pH is not increased at the expense of a loss of ability to detect differences between groups. It may be visually demonstrated on Figure 2 showing that regression lines run almost in parallel.

Although our results allow the reliable calculation of EBC pH they do not give information about the identity of components that determine EBC pH and this could be an area of further investigation.

The possibility that EBC is contaminated with saliva is debated [1, 11-13]. The results of salivary contamination would be that estimated EBC pH does not correspond to ASL pH. Even if this were true it would not weaken the good repeatability of the method itself.The variability of EBC pH does not contradict good reproducibility either. In fact, reliable pH determination ensures that a change in pH corresponds to real variability instead of the uncertainty of the measurement.

The fact that our stable asthmatic patients had an EBC pH statistically similar to that of healthy persons does not exclude the possibility that patients in more severe state of disease or during exacerbations would have a lower EBC pH. Even though the statistical demonstration of EBC acidification in inflammatory airway diseases is interesting from a pathophysiological point of view, it only has clinical importance if a cut off value between healthy and pathologic pH values can be set.

A number of questions remain to be answered including the reason of the variability of EBC pH and the potential of EBC pH determination in clinical routine.

In summary by the parallel measurement of pH and $CO_2$ partial pressure in EBC we found that $CO_2$ affects condensate pH to great extent and that condensate $CO_2$ level cannot be standardised by the currently recommended deaeration. We found a near perfect negative logarithmic correlation between pH and $CO_2$ partial pressure in EBC. This correlation allows the calculation of pH at $CO_2$ partial pressure of 5.33 kPa. The calculated EBC pH of healthy adults shows variability in the interval of 6-7.

We conclude therefore that EBC $CO_2$ partial pressure is an important confounding factor of pH measurements. Determination of EBC pH standardised to $p_{EBC}CO_2$ provides the most reproducible EBC pH values to date.

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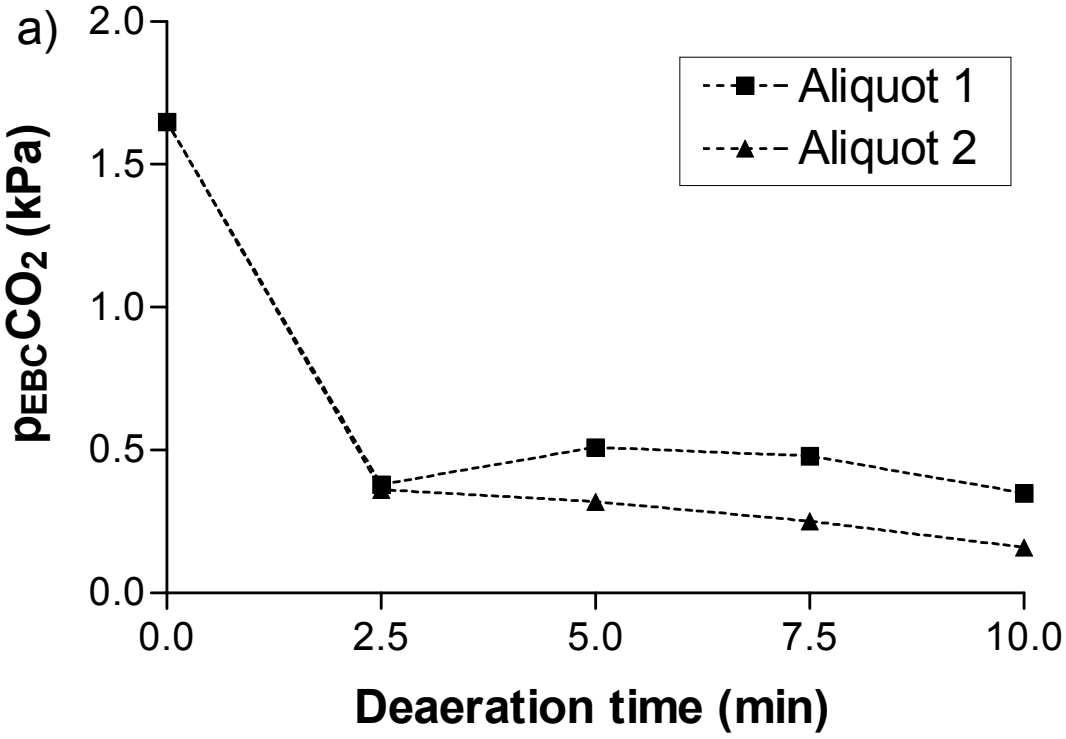
**Legends to figures**

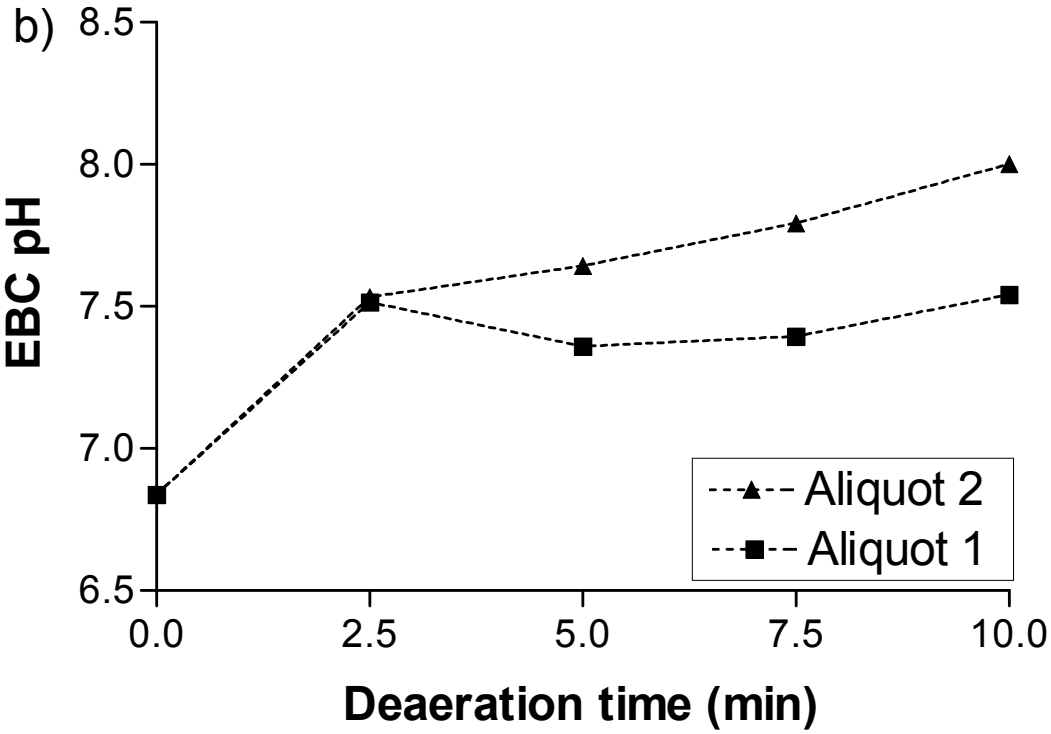
**Figure 1** Representative time course of $CO_2$ (panel a) and pH (panel b) during parallel argon deaeration of the same EBC sample.

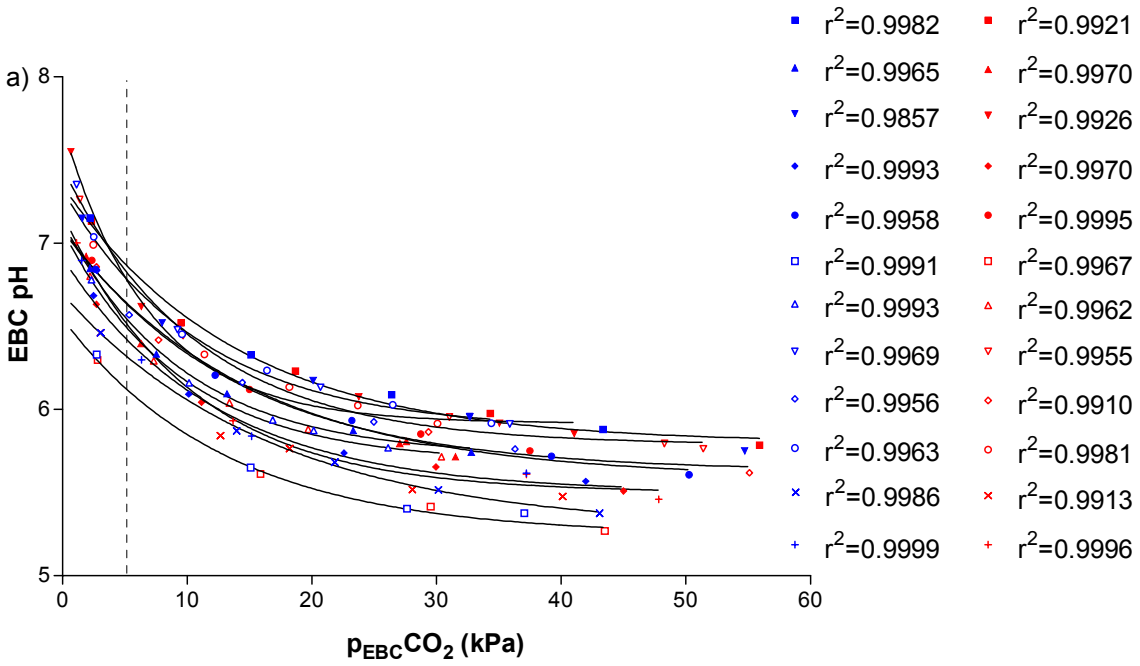
**Figure 2** Close negative logarithmic correlation between pH and $p_{EBC}CO_2$ upon $CO_2$ load in healthy (panel a) and asthmatic (panel b) persons. Spots with the same form but different colour represent duplicate values obtained from the same sample. Only one regression line is drawn for one sample divided in two for better visibility of the graph, as the two regression lines run almost in parallel. The dotted line stands for 5.33 kPa of $p_{EBC}CO_2$.

**Figure 3** Repeatability of pH measurement. Comparison of two values obtained from the same sample by deaeration (panel a), neat measurement (panel b) and calculation according to the $CO_2$ loading protocol (panel c) by the Bland-Altman test.

**Figure 4** Variability of EBC pH. Standardised pH values to 5.33 kPa $p_{EBC}CO_2$ of the healthy participants on two different days.


a)
2.0
1.5
1.0
0.5
0.0
$p_{EBC}CO_2$ (kPa)
Aliquot 1
Aliquot 2
0.0
2.5
5.0
7.5
10.0
Deaeration time (min)


b)
8.5
8.0
7.5
7.0
6.5
EBC pH
Aliquot 2
Aliquot 1
0.0
2.5
5.0
7.5
10.0
Deaeration time (min)


a)
EBC pH
$p_{EBC}CO_2$ (kPa)
$r^2$=0.9982
$r^2$=0.9921
$r^2$=0.9965
$r^2$=0.9970
$r^2$=0.9857
$r^2$=0.9926
$r^2$=0.9993
$r^2$=0.9970
$r^2$=0.9958
$r^2$=0.9995
$r^2$=0.9991
$r^2$=0.9967
$r^2$=0.9993
$r^2$=0.9962
$r^2$=0.9969
$r^2$=0.9955
$r^2$=0.9956
$r^2$=0.9910
$r^2$=0.9963
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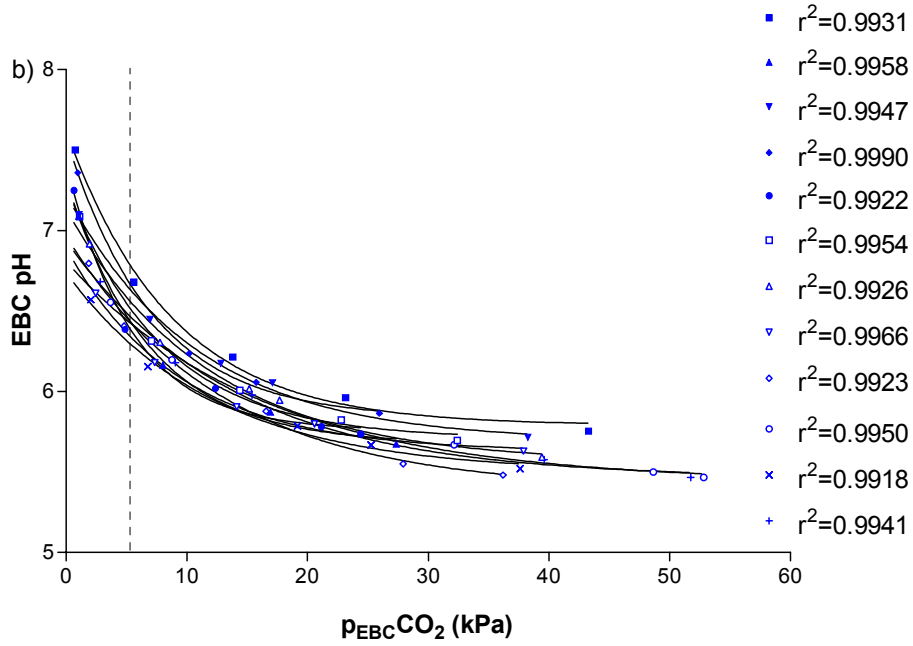
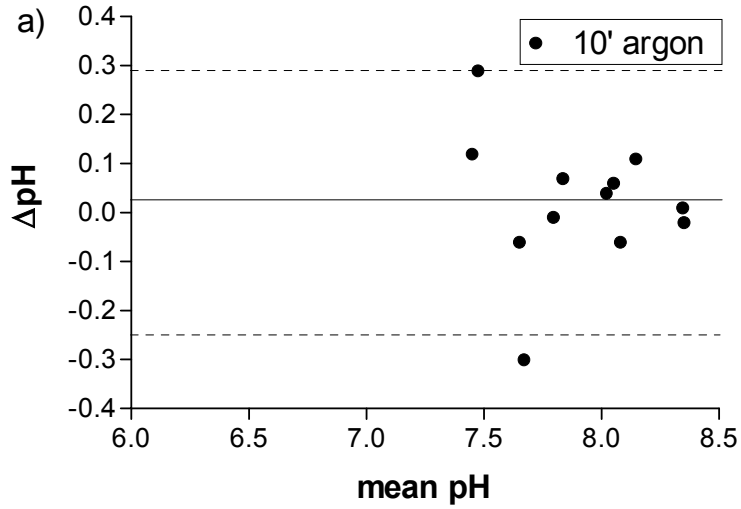
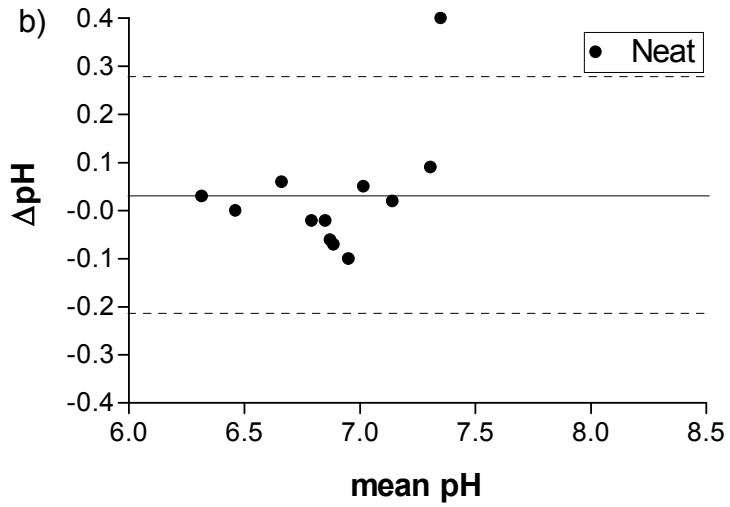
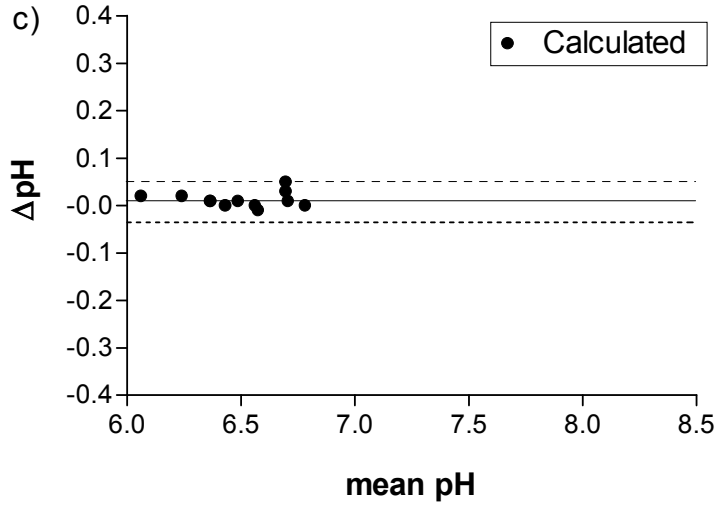
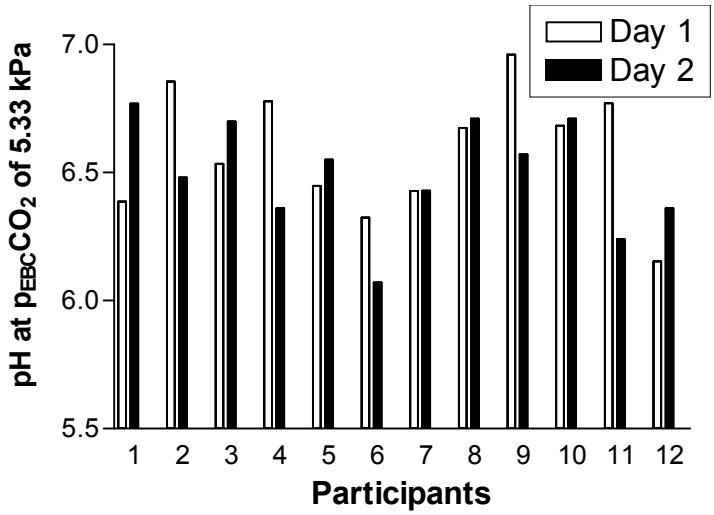
$r^2$=0.9913
$r^2$=0.9999
$r^2$=0.9996


b)
EBC pH
$p_{EBC}CO_2$ (kPa)
$r^2$=0.9931
$r^2$=0.9958
$r^2$=0.9947
$r^2$=0.9990
$r^2$=0.9922
$r^2$=0.9954
$r^2$=0.9926
$r^2$=0.9966
$r^2$=0.9923
$r^2$=0.9950
$r^2$=0.9918
$r^2$=0.9941


a)
10' argon
ΔpH
mean pH


b)
Neat
ΔpH
mean pH


c)
Calculated
ΔpH
mean pH


Day 1
Day 2
pH at $p_{EBC}CO_2$ of 5.33 kPa
7.0
6.5
6.0
5.5
1
2
3
4
5
6
7
8
9
10
11
12
Participants