

Exhaled Breath Condensate: Pilot Study of the Method and Initial Experience in Healthy Subjects

Eva Peterová^{1,2,*}, Jaroslav Chládek³, Darina Kohoutová¹, Veronika Knoblochová¹, Paula Morávková¹, Jaroslava Vávrová⁴, Martina Řezáčová², Jan Bureš¹

ABSTRACT

Analysis of Exhaled breath condensate (EBC) is a re-discovered approach to monitoring the course of the disease and reduce invasive methods of patient investigation. However, the major disadvantage and shortcoming of the EBC is lack of reliable and reproducible standardization of the method. Despite many articles published on EBC, until now there is no clear consensus on whether the analysis of EBC can provide a clue to diagnosis of the diseases. The purpose of this paper is to investigate our own method, to search for possible standardization and to obtain our own initial experience. Thirty healthy volunteers provided the EBC, in which we monitored the density, pH, protein, chloride and urea concentration. Our results show that EBC pH is influenced by smoking, and urea concentrations are affected by the gender of subjects. Age of subjects does not play a role. The smallest coefficient of variation between individual volunteers is for density determination. Current limitations of EBC measurements are the low concentration of many biomarkers. Standardization needs to be specific for each individual biomarker, with focusing on optimal condensate collection. EBC analysis has a potential become diagnostic test, not only for lung diseases.

KEYWORDS

exhaled breath condensate; standardization; healthy subjects

AUTHOR AFFILIATIONS

¹ 2nd Department of Internal Medicine – Gastroenterology, Charles University, Faculty of Medicine in Hradec Králové, University Hospital Hradec Králové, Czech Republic

² Department of Medical Biochemistry, Charles University, Faculty of Medicine in Hradec Králové, Czech Republic

³ Department of Pharmacology, Charles University, Faculty of Medicine in Hradec Králové, Czech Republic

⁴ Institute of Clinical Biochemistry and Diagnostics, Charles University, Faculty of Medicine in Hradec Králové, University Hospital Hradec Králové, Czech Republic

* Corresponding author: 2nd Department of Internal Medicine – Gastroenterology, Sokolská 581, 500 05 Hradec Králové, Czech Republic; e-mail: peterove@lfhk.cuni.cz

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INTRODUCTION

Exhaled breath condensate (EBC) is a biological fluid that consists mainly of water, but it also contains small droplets of airway lining fluid (36). Condensate carries molecules <65 kDa (25). EBC contains both, volatile and non-volatile compounds (35). Adenosine, ammonia, hydrogen peroxide, isoprostanes, leukotrienes, nitrogen oxides, peptides, cytokines belong to the compounds detected in EBC (42). In the clinical setting, non-volatile components, such as cytokines, are used for diagnostics and for monitoring of disease progression (87, 61, 27).

First reports on the EBC collection were published in early 1980s. The report on the EBC collection from the 1990s shows that the condensate was obtained by passing expired gas through tubing submerged in an ice-water bath (7). The first EBC studies documented, that the exhaled breath condensate test is simple, non-invasive and easy to perform. Homemade and commercially manufactured condensers are available today. Various homemade devices have been described, for example a Teflon or a Polypropylen tube, dipped in a bucket filled with ice, a double glass layer container or a device, where exhaled air condensation takes place between the two layers (70, 83, 102). Commercially manufactured condensers are also available, for example EcoScreen, Turbodeccs, ANACON or RTube (90, 26, 85, 14).

Nowadays, there is an increased interest in non-invasive diagnostics and investigation. Collection of EBC fulfils requirements of a non-invasive, repeatable test and thus applicable in the pediatric population (20, 88, 100) and in adult patients, especially in those, who have to control parameters daily. EBC has the potential to become a routine method used for diagnostics, mainly for lung diseases such as bronchial asthma (98, 33, 92, 16, 74), cystic fibrosis (101), idiopathic pulmonary fibrosis (82), bronchiectasia (64), tuberculosis (69), lung carcinoma (2, 46), acute lung injury and acute respiratory distress syndrome (19), chronic obstructive pulmonary disease (59), scleroderma with pulmonary involvement (38, 67), sleep apnea syndrome (18, 89), silicosis (77, 60) and other occupational lung diseases (24, 76, 75, 80, 92). Recently, EBC has been used in monitoring gastrointestinal diseases, such as gastroesophageal reflux disease (91, 81, 39, 58), inflammatory bowel disease (56, 52, 44), coeliac disease (5, 43). Other studies were carried out to monitor systemic sclerosis (28, 93), liver diseases (6), abdominal surgery (66, 84, 65), obese population (13), impacts of oxidative stress (78, 94, 63) or for toxicity screening (34, 79, 57).

If the breath is captured and analyzed correctly, it can be used to provide information of the current health status with a potential to predict future outcomes progression of the disease (50). Nowadays, the PubMed (at <https://www.ncbi.nlm.nih.gov/pubmed>) registers more than 1,300 scientific papers on EBC. However, several methodological issues, including standardization of EBC technique and validation of analytical methods, need to be addressed before this approach can be considered and taken into real practice. EBC composition may be influenced the time of exhalation, condenser temperature, use of nose clips, temperature and duration of condensate storage, saliva con-

tamination, smoking, eating, drinking of coffee may influence composition of EBC. The American Thoracic Society and European Respiratory Society developed guidelines for EBC collection and measurement of exhaled biomarkers, to suggest recommendations on the possible use and limits of exhaled biomarkers and to highlight those areas where further research is required (42).

Nevertheless, the major disadvantage and shortcoming of the EBC is lack of reliable and reproducible standardization of the method (37, 70, 54).

The aim of this study was to introduce our own method, to search for possible standardization and to obtain our own initial experience in healthy volunteers.

METHODS

SUBJECTS

Thirty healthy volunteers between 25–69 years of age were included in the study. The volunteers were the staff of the Faculty Hospital and the Faculty of Medicine of the Charles University in Hradec Králové. The exclusion criteria were the history of chronic lung disease or other serious chronic illness or respiratory infection within 2 weeks preceding the study. The group contained 14 smokers and 16 non-smokers. The project was carried out according to the Declaration of Helsinki, and was approved by the Ethics Committee (201706S11P) of the University Hospital in Hradec Králové. All participants signed an informed consent.

CONDENSATION OF EXHALED BREATH

The condenser EcoScreen (Jaeger, Hoechberg, Germany) is a system in which a mouthpiece with a one-way valve and a refrigerated collecting system are connected to a power supply by an extendable arm. Due to the function of the valve (which is connected to the mouthpiece), inspiratory and expiratory air are separated. The collecting system is connected to the valve block and is placed in a cooling thermoblock. During exhalation, air flows through the lamellar condenser, becomes liquid, and drops into the collecting vial.

All parts of the collection kit were rinsed with ethanol and deionised water and were air dried before each use. The subjects rinsed their mouth with infant water ($\text{Cl}^- < 5 \text{ mg/L}$) with a defined composition. The subject did not smoke, did not eat and did not drink coffee or sweet lemonade at least one hour before sampling. The subjects were not exposed to increased physical activity for at least 30 minutes before sampling. The sampling occurred between 8:00 and 11:00 a.m. The EcoScreen was cooled to -10°C , based on the information provided by the manufacturers. EBC was collected during 10 minutes of exhalation. After 10 minutes of quiet breathing, 1–3 mL of EBC were collected from adult individuals. Volume of EBC is proportional to the total exhaled volume and breathing frequency. EBC samples were immediately transferred to polypropylene tubes and were frozen at -80°C .

In order to examine repeatability of the measurements over a moderate period of time, the collection of EBC was repeated after two weeks.

DENSITY DETERMINATION

Total amount of 500 μL of EBC was transferred by a validated pipette to a polypropylene tube of defined weight. Thereafter, the condensate tube was weighed on Sartorius analytical balances (Germany). Accuracy of measurement was at 0.01 mg and was repeated three times. The measurement was run at 25 $^{\circ}\text{C}$.

PROTEIN DETERMINATION

Protein content was determined with a diagnostic reagent for quantitative *in vitro* determination of Bio-La-Test Total Protein kit (Erba Lachema, Czech Republic). Named values were verified by determination Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich). The measurement was run at 37 $^{\circ}\text{C}$ and was repeated three times.

UREA DETERMINATION:

Urea content was determined with a diagnostic reagent for quantitative *in vitro* determination of Bio-La-Test Urea kit (Erba Lachema, Czech Republic). The measurement was run at 37 $^{\circ}\text{C}$ and was repeated three times.

CHLORIDE DETERMINATION

Chloride content was determined with a Ion-selective chloride electrode (Fisher Scientific, Czech Republic). The measurement was run at 25 $^{\circ}\text{C}$ and was repeated three times.

PH DETERMINATION

All samples were deaerated in an ultrasonic bath. pH were determined with a pH electrode (Fisher Scientific, Czech Republic). The measurement was run at 25 $^{\circ}\text{C}$ and was repeated three times.

STATISTIC ANALYSIS

Results had normal distribution and therefore are presented as mean \pm SD or median (interquartile range). Data

obtained were tested statistically by means of non-paired t-test. All statistics was performed using SigmaStat software (Jandel Scientific, Eckhardt, Germany, Version 3.1).

RESULTS

SUBJECTS

Thirty healthy volunteers, 24 females and 6 males, were included in the study. The mean age of the female and male subjects was comparable (40.4 years, range: 25–58 and 39.7 years, range: 28–69, $P = 0.93$). The group included 14 smokers and 16 non-smokers. The proportions of smokers among females and males were 10/24 (42%) and 4/6 (67%), respectively ($P = 0.38$). The mean age of non-smokers and smokers did not differ (40.8 years, range: 25–58 vs. 39.6 years, range: 25–69, $P = 0.93$).

TWO-WEEK REPEATABILITY OF EBC MEASUREMENTS

The repeated measures analysis of variance was used to calculate the average intra-individual and inter-individual coefficients of variation of duplicate measurements performed two weeks apart as well as the intraclass correlation coefficient (Table 1). The mean intra-individual variabilities are considerable for the concentration of total protein, chloride and urea. On the contrary, the measurements of density and pH shows the lowest $\%CV_{\text{intra}}$. The intraclass correlation coefficient was good for pH ($ICC = 0.81$) and moderate for urea measurement ($ICC = 0.52$), whereas its negative values indicated that the test-retest variability ($\%CV_{\text{intra}}$) of density and total protein was higher than the interindividual variability ($\%CV_{\text{inter}}$) (Table 1). The repeatability of EBC pH and urea measurements is visualized with the help of Bland-Altman plots in Figure 1.

DENSITY

Density of EBC sample was 1007.2 ± 4.7 g/L (Table 1). Density of EBC was not dependent on smoking, gender or age of subjects. There was no statistically significant differ-

Tab. 1 Descriptive statistic of measured analytes and physical quantity.

	Density (g/L)	pH	Total Protein ($\mu\text{g}/\text{mL}$)	Chlorides (mmol/L)	Urea ($\mu\text{mol}/\text{L}$)
Mean \pm SD	1007 \pm 4.7	6.8 \pm 0.8	1.6 \pm 1.2	3.1 \pm 1.8	199 \pm 156
SD/Mean (%)	0.5	11.5	74.8	58.0	77.2
Median	1007	7.0	1.3	3.4	173
IQR	1004–1010	6.1–7.4	0.7–2.4	1.2–4.6	83.9–291
Range	996–1016	5.5–8.1	0.1–4.5	0.4–6.2	1.0–610
CV_{intra} (%)	1.5	5.3	61.2	57.7	61.3
CV_{inter} (%)	1.2	12.0	52.0	62.4	88.6
ICC	-0.06	0.81	-0.19	0.20	0.52

The descriptive statistics of measured analytes and physical quantity were calculated from the results of second measurements; SD – standard deviation, IQR – interquartile range. The CV_{intra} (%) and CV_{inter} (%) refer to the mean intra-individual and inter-individual coefficients of variation of duplicate measurements performed two weeks apart; ICC – intraclass correlation coefficient of repeated measurements.

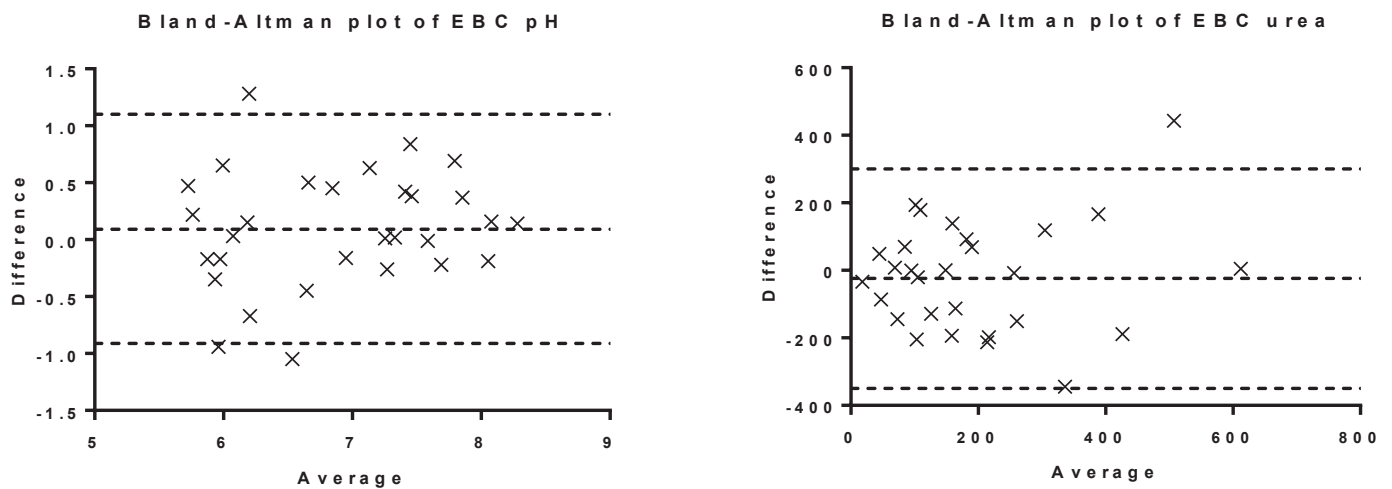


Fig. 1 The repeatability of EBC pH and urea measurements between visits 1 and 2 separated by two weeks. Bland Altman difference vs. average plots with the mean difference and 95% limits of agreement visualized as the broken lines.

Tab. 2 Pearson Product Moment Correlation between variables of interest.

		Age	Density	Chlorides	pH	TP	Urea
Age	r ²	x	0.12	0.12	0.10	-0.05	-0.02
	P	x	0.52	0.54	0.58	0.78	0.80
Density	r ²	-	x	0.07	-0.19	0.11	0.05
	P	-	x	0.73	0.31	0.55	0.41
Cl-	r ²	-	-	x	0.16	-0.18	-0.22
	P	-	-	x	0.40	0.36	0.25
pH	r ²	-	-	-	x	-0.33	0.013
	P	-	-	-	x	0.08	0.95
TP	r ²	-	-	-	-	x	0.013
	P	-	-	-	-	x	0.95

Coefficients of determination and two-tailed P-values are listed. The existence of correlation was not proved between any of the pairs of variables ($P > 0.05$).

Tab. 3 Univariate analyses of the effects of smoking, age and gender on measured analytes and physical quantity.

	Density	pH	Total Protein	Chlorides	Urea
	(g/L)		($\mu\text{g/mL}$)	(mmol/L)	($\mu\text{mol/L}$)
Smokers, N = 14	1008 \pm 3.4	6.2 \pm 0.5	1.6 \pm 1.5	2.8 \pm 2.0	229 \pm 178
Nonsmokers, N = 16	1006 \pm 5.7	7.4 \pm 0.5	1.9 \pm 1.2	3.4 \pm 1.7	172 \pm 134
P	0.32	< 0.001	0.13	0.41	0.33
Younger (25-40 yr), N = 14	1008 \pm 4.9	6.9 \pm 0.8	1.6 \pm 1.5	3.2 \pm 2.1	227 \pm 181
Older (41-70 yr), N = 16	1007 \pm 4.8	7.0 \pm 0.8	1.3 \pm 1.2	3.0 \pm 1.7	174 \pm 131
P	0.60	0.37	0.82	0.79	0.36
Women, N = 24	1007 \pm 5.0	6.9 \pm 0.8	1.5 \pm 0.9	3.1 \pm 1.9	164 \pm 125
Men, N = 6	1009 \pm 2.8	6.5 \pm 0.7	2.1 \pm 2.1	3.2 \pm 1.7	336 \pm 201
P	0.19	0.25	0.26	0.95	< 0.02

ence between non-smokers and smokers, between females and males and between age groups (Table 3). There were no significant relationships between density and other investigated parameters (Table 2).

PH

EBC pH was 6.8 ± 0.8 (Table 1). pH was dependent on smoking (Figure 2). EBC pH in smokers was lower than in non-smokers. Gender and age of subjects did not influ-

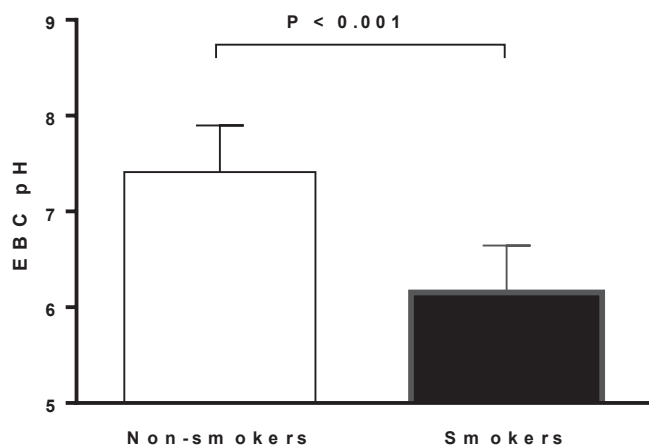


Fig. 2 Exhaled breath condensate pH in non-smokers and smokers. Each bar represents mean \pm SD.

ence pH values (Table 3). There were no significant relationships between pH and other investigated parameters or physical quantity (Table 2).

TOTAL PROTEIN

Total protein concentration were $1.6 \pm 1.2 \mu\text{g/mL}$ (Table 1). Our results show that total protein level is not dependent on smoking, gender and age of subjects. There were no statistically significant differences between non-smokers and smokers, between females and males and between age groups (Table 3). There were no significant relationships between total protein levels and other investigated parameters (Table 2).

CHLORIDE

Chloride ion concentration in EBC samples was $3.1 \pm 1.8 \text{ mmol/L}$ (Table 1). Chloride content was not dependent on smoking, gender and age of subjects. There were no statistically significant differences between non-smokers and smokers, females and males and between age groups (Table 3). There were no significant relationships between chloride ion levels and other investigated parameters (Table 2).

UREA

Urea concentration was $199 \pm 156 \mu\text{mol/L}$ (Table 1). Urea levels were dependent on gender (Figure 3). Urea concentration in females was lower than in males. Smoking and age do not have any impact on urea levels (Table 3). There were no significant relationships between urea levels and other investigated parameters or physical quantity (Table 2).

DISCUSSION

First attempts on breath diagnostics go back to Hippocrates who described foetor ex ore and foetor hepaticus in his treatise on breath smell analysis. The modern era of breath testing was initiated in 1971, when Pauling analyzed volatile organic compounds from breath trapped in

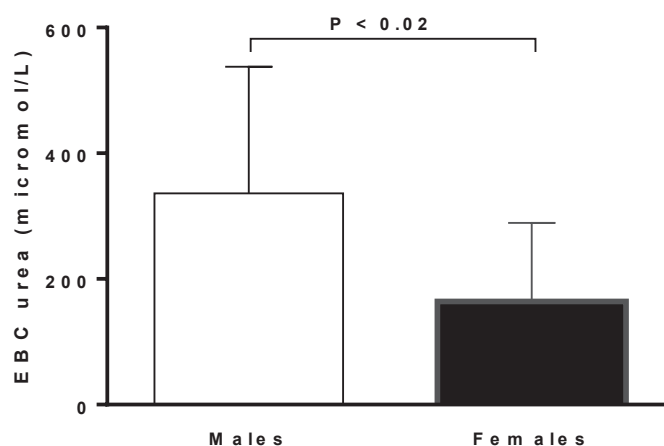


Fig. 3 Concentration of urea in the breath condensate exhaled by men and women. Each bar represents mean \pm SD.

a cooled stainless steel tube and found out that normal human breath contains more than 250 different volatile organic compounds (73, 54). At present, breath analysis is divided into two main directions. The first area of breath analysis deals with the detection of volatile organic compounds. The second areas looks into the aqueous part of breath, which contains mainly non-volatile compounds and water soluble volatiles. There is a lot of studies with both exhaled air analysis and/or, EBC (8, 72).

EBC represents one of the most accessible biological materials, which can be obtained by a non-invasive way. Yet, lack of reliable and reproducible standardization of the method is the major problem at present. Despite multiple articles having been published on EBC, there is no clear consensus at present on whether the analysis of EBC can provide a definite diagnosis of the diseases. There seems to be a high risk of pre-analytical and analytical errors and based on this, interpretation of EBC biomarkers should be taken with a lot of precaution.

There are numerous possible source of pre-analytical errors can be miscellaneous. Collection devices are an important source of variability of EBC biomarkers (86, 40, 21). The other principal factors of variability include cooling temperature (26) and condenser materials (86). Diet, smoking, medication and physical activity influenced EBC results (55, 15, 10, 9). Use of a nose clip is another unsolved question. When using a nose clip, the subject is forced to exhale only through the mouth, preventing thus accidental exhalation through the nose. Yet, the use of a nose clip may affect composition of EBC (95). Currently, there are devices developed by Loccioni Gruppo Humancare (Angeli di Rosora, Italy) which help to assess collection parameters, when the EBC is being obtained from human individuals. These devices provide continuous visual feedback to the subjects to control breathing patterns (99).

The method of determination may be the source of variability in the analytical phase. EBC can be analysed for example by ionex chromatography (47), plasma mass spectrometry (1), Liquid chromatography-mass spectrometry (48) or Polymerase chain reaction (62). We decided to use colorimetric assays and ion-selective electrodes in our study. The possible sources of variability errors are given for the relevant analytes and physical quantities.

The aim of our current study was to search for markers that could serve as a standard that several markers could be related to. Investigated the following parameters: density, pH, total protein level, chloride level and urea level. Some authors suggested that data normalization to the internal control is not necessary, because the levels of analytes during the disease increase many times (17, 23). Carter and colleagues have different view on normalization. Their study showed that standardization should be specific for each biomarker, as a more general model would not optimize collection of all compounds (21).

Our results showed that the density of EBC could be used for standardization. Density meets the requirements in the sense, that it reaches almost the same value across the across the different EBC samples as well as a good test-retest reproducibility of the result. It is not influenced by smoking, gender or age of subjects. Condensed water vapour enriched with trace amounts of volatile and non-volatile components is the major component of the EBC (99, 3). Therefore, the EBC density is close to 1 g/mL and the other dissolved minor components do not change the density. The larger content of dissolved volatile compounds such as ammonia (53) or air trapping in the sample would decrease density. Thus, density could help to compensate for the influence of such factors during sampling but it will unlikely fulfill all expectations for an internal standard. Namely, density is unrelated to the efficiency of the transfer of a particular molecule/ion into exhaled air and, to the completeness of its trapping during cooling.

The interpretation of EBC pH values is complex. EBC pH reflects acidity of the airway lining fluid. However, its value is from a part affected by volatile airway acids (CO_2) and bases (NH_3) which show a variable content upon standing of the EBC sample. The attempts to eliminate CO_2 as a confounding factor included either its removal from the sample by deaeration using an inert gas or saturation of the sample with CO_2 to a stable pH (41). In our study, we used deaeration using ultrasonic baths that secures that CO_2 was removed. Vaughan et al were able to document that EBC pH reflects lung homeostasis. The pH of the airway in health has been reported to be slightly alkaline while acidification of EBC occurs during disease states (96). The pH also depends on temperature of sampling and volume of NH_4^+ . EBC collected at -70°C were more acidic than those collected at -20°C (26) We assume that we eliminated this variability by using Ecosreen and followed the same conditions in all sampling. The literature shows that pH is influenced by diet (55). Ćalušić et al showed that consuming a standardized meal constituting 101.3 g of water, 34 g of protein, 11.2 g of fat, and 47.3 g of saccharides has no effect on pH (15). Hunt et al. found EBC pH decreased significantly after 50 ml sweet limonade and returned to baseline after 15 min (Hunt et al. 2006). We have eliminated the effect of food and beverages by avoiding least one hours before the sampling. The subjects were also not exposed to increased physical activity for at least 30 minutes before sampling. Bikov et al. found that following exercise EBC pH decreased in exercise-induced bronchoconstriction positive group although in healthy subjects this

decline was not demonstrated (11). Thus, amount of NH_4^+ found in the EBC reflects the contamination with salivary NH_3 , some of which is derived from bacterial degradation of urea (29). Volume NH_4^+ can be reduced by washing the mouth with infant water.

Smoking is another factor which influences pH. According to several studies, EBC pH may be lower in smokers compared to non-smokers (4, 51, 40). We confirmed this, too. The measurement of pH with a glass electrode is technically simple. The intraclass correlation coefficient of EBC pH was the highest of all analytes examined in this study. However, the influence of general factors on the EBC pH including the unstable content of volatile acids/bases, hydration of the airways, smoking, exercise-induced bronchoconstriction etc., precludes its use for standardization of the EBC measurements.

We also revealed, that there was a substantial variability in total protein concentration among investigated subjects. Our results are consistent with a study by Bloemen et al. Their study showed that total protein concentration of EBC differed significantly between healthy individuals. Unlike we did, she showed a correlation between the age of subjects and total protein concentration (12). The variability in total protein values might be caused by protease activity (49). The addition of protease inhibitors may further prevent degradation of proteins, however, protease inhibitors can interfere with protein analysis. EBC storage also plays its role. Storage in polyethylene tubes minimizes protein adhesion. (22). Elevated inflammatory markers in respiratory disease increase total protein levels in EBC (68). Type of material of the inner part of the condensers may also play its role. Ecosreen had greater albumin retention compared to glass or Teflon coatings (86).

Our results showed that chlorides are not suitable for standardization. This observation is consistent with the hypothesis that EBC chloride correlates with plasma chloride levels (30). Physical exercise can alter ion regulation by increasing chloride secretion to a significant and similar degree compared to the individuals with cystic fibrosis (97). EBC of patients with chronic persistent cough is more acidic and has lower chloride levels than EBC of non-coughing healthy subjects (71). We did not find any correlation between pH and chloride levels.

Urea concentrations in the EBC are quite low and more variable than the other evaluated parameters, i.e. density and pH. It is believed that the EBC urea value correlates with plasma urea levels (32) as well as chloride levels. NH_4^+ can also interfere with urea analysis (31).

CONCLUSIONS

EBC analysis has a potential become a safe and a non-invasive diagnostic test, not only for lung diseases. In the future it could replace some of more invasive methods. In order to address the issues regarding standardization, it is necessary to consider the matter of collection devices and techniques, modes and conditions. Standardization needs to be specific for each individual biomarker, with future investigations focusing on optimal collection.

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