Methodological and physiological aspects of exhaled breath analysis

Doctoral theses

András Bikov

Semmelweis University
Doctoral School of Clinical Medicine

Supervisor: Dr. Ildikó Horváth, professor, D.Sc.

Opponents: Dr. Zoltán Novák, associate professor, Ph.D.
Dr. Gábor Pavlik, professor, D.Sc.

Head of doctoral committee: Dr. Endre Cserháti, professor, D.Sc.
Members of doctoral committee: Dr. Györgyi Mezei, assistant professor, Ph.D.
Dr. Andor Hirschberg, chief physician, Ph.D.

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1. Introduction

The analysis of exhaled breath has been used in medicine since ancient times for the diagnosis of various disorders. In 1970s Linus Pauling has shown that exhaled breath contains thousands of volatile molecules which levels are strongly related to airway and systemic metabolism and oxidative stress. In 1980s Sidorenko et al. condensed the expired air and successfully isolated proteins from exhaled breath condensate (EBC) fluid, thus inventing a method to analyse the non-volatile compounds of the airways. In the last decades, numerous studies proved that the composition of exhaled breath is altered in various pulmonary and extrapulmonary diseases. However, the number of breath test used in daily routine is low. The main reason for this is that the methodological and physiological factors affecting the exhaled breath results were not investigated thoroughly.

The most studied biomarker in EBC is pH. It is suggested that the acidity of EBC is not only determined by the pH of airway droplets but by volatile acids and bases dissolving into the condensate. Moreover, the interpretation of EBC results is complicated by a variable (1:50-1:50000) amount of alveolar water diluting the airway droplets. Our workgroup has previously validated a method to detect the magnitude of dilution by the measurement of conductivity of vacuum treated EBC samples; however the effect of dilution on EBC pH has not been investigated before.

The electronic nose is a potential technique to study exhaled volatile compounds. Similarly to mammalian olfaction, it is not able to analyse the qualitative or quantitative modalities of the gas mixture, but can distinguish different gas composites based on their

molecule pattern. Cyranose 320 is the most widely used electronic nose in respiratory research, and it could differentiate exhaled breath of patients with asthma, COPD or lung cancer from those of healthy individuals. Nonetheless, only few studies have investigated the short and long term reproducibility of Cyranose 320.

Similarly to diseases, physiological processes, such as physical exercise or physiological pregnancy could also modify the levels of exhaled biomarkers.

Physical exercise increases cardiac output and minute ventilation, changes airway and systemic metabolism as well as oxidative stress and it induces airway inflammatory response.

Physiological pregnancy is characterised by accelerated metabolism, elevated systemic oxidative stress, altered hormone levels, immune tolerance and increased production of pheromones.

The hereby presented research was dedicated to investigate the importance of previously not studied methodological and physiological factors on exhaled biomarkers, thus facilitating the clinical application of exhaled breath analysis.

2. Objectives

1. To study the relationship between EBC pH and respiratory droplet dilution.
2. To study the short and long term reproducibility of Cyranose 320.
3. To study the effect of physical exercise on exhaled volatile compounds and EBC pH.
4. To study the effect of pregnancy on exhaled volatile compounds.

3. Methods

3.1. Study subjects

In total, 308 volunteers participated in the four studies.

The asthmatic subjects were recruited at the asthma outpatient clinic of the Department of Pulmonology, Semmelweis University. Asthma was diagnosed according to the Global Initiative for Asthma (GINA) guideline and confirmed by >12% and 200 mL increase in FEV_1 after administration of 400 μg salbutamol. None of the subjects was hospitalised due to asthma exacerbation in the last year, indicating that the patients were considered stable.

The pregnant individuals were recruited at the 1st Department of Obstetrics and Gynecology, Semmelweis University. In total, 78 pregnant women were investigated, 63 in the 3rd trimester and 15 in the 2nd trimester.

The healthy controls were recruited among students and workers of Semmelweis University.

All subjects enrolled signed an informed consent prior to the experiments.

3.2. Study designs

The relationship between EBC pH and dilution was investigated in 55 healthy and 57 asthmatic subjects. Exhaled breath condensate was collected in all individuals for pH and dilution measurements. The two parameters were correlated within the asthmatic and the healthy groups as well as all together.

Investigating the short term reproducibility of Cyranose 320, exhaled breath samples were analysed at baseline and also 10 minutes after in 56 healthy subjects. The long

term reproducibility was analysed in 12 healthy volunteers who donated exhaled breath samples for 8 weeks.
The effect of physical exercise on exhaled biomarkers was studied in 10 healthy individuals. Before as well as 0, 15, 30 and 60 minutes after an outdoor exercise challenge test exhaled breath samples were collected for volatile compound and pH measurements.
The effect of pregnancy was analysed in 118 women. In the first phase of the study, exhaled breath samples were compared between 48 3rd trimester pregnant women and 25 non-pregnant women. Based on the statistical model set in the first phase, breath samples of 30 pregnant (15 2nd trimester and 15 3rd trimester) and 15 non-pregnant women were categorised into the preset groups.

3.3. Exhaled breath tests

Exhaled breath condensate was collected for 10 minutes using the Rtube device (Respiratory Research, Charlottesville, VI, USA). The subjects were breathing with tidal volumes and were not wearing nose clip. The chilling tube was previously cooled at -80 °C. The samples were stored at -80 °C until analysis.
To collect volatile compounds Teflon coated and poly-vinyl-chloride bags were used. After inhalation of VOC-filtered air, subjects exhaled to residual volume at controlled expiratory flow (50 mL/sec) against resistance (15-20 cmH_2O). The first 500 mL of exhaled air, representing the anatomical dead space was discarded and the remaining alveolar space air was analysed. The collection protocol during physical exercise was different. Subjects were asked to inhale through their nose and exhale into a three-litre poly-vinyl-chloride bag three times. During the exhalation the dead space was not

discarded and the expiratory flow was not controlled. After the third exhalation the bag was closed with a clip and analysed immediately.
The EBC pH was determined using the argon purging and the carbon dioxide loading methods.
The dilution factor was estimated following 24-hour vacuum evaporation of condensate as well as serially diluted saline samples. After reconstitution with distilled water conductivity was measured in each condensate and saline sample.
The Cyranose 320 electronic nose (Smiths Detection, Pasadena, USA) was used for exhaled volatile compound analysis. The confounding effect of water vapour was eliminated after exclusion of 4 water-sensitive sensors (5,6,23,31). The 28 sensor data were generated by the ratio of samples responses and the background (VOC-filtered room air in our measurements). The data underwent data reduction using the prinical component analysis (PCA). The highest 3 principal components (PCs) were used for further analysis.

3.4. Statistical analysis

We used commercially available statistical programs (Statistica 8.0, SPSS 15, Graph Pad Prism 5.03) for statistical analyses.
Mann-Whitney test was used to compare EBC pH and dilution values between asthmatics and healthy subjects. The relationship between EBC pH and dilution was analysed with Spearman correlation.
Investigating the short term reproducibility of Cyranose 320, the principal components representing the baseline and 10-minute measurements were compared with paired t-test and Pearson test. Analysing the long term

reproducibility principal components were compared using ANOVA and Pearson correlation.

The effect of physical exercise on exhaled biomarkers was studied using mixed-linear model. EBC pH was correlated to principal components using the Pearson test. Mahalanobis distance, a discrimination technique was used to compare pregnant and non-pregnant breath samples. We also used Mahalanobis distance to categorise the new 45 samples into the previously preset groups. Pearson test was used to investigate the relationship between the principal components and gestational age.

4. Results

4.1. Relationship between EBC pH and dilution

We did not find any significant difference in EBC pH (7.91 (7.31-8.04) and 7.83 (7.54-8.05)) or dilution (2326 (1460-4630) vs. 2646 (1435-3969)) between the asthmatic and healthy groups ($p>0.05$).

There was a negative correlation between EBC pH and dilution in the asthmatic ($r=-0.35$, Figure 1A) as well as healthy groups ($r=-0.29$, Figure 1B)

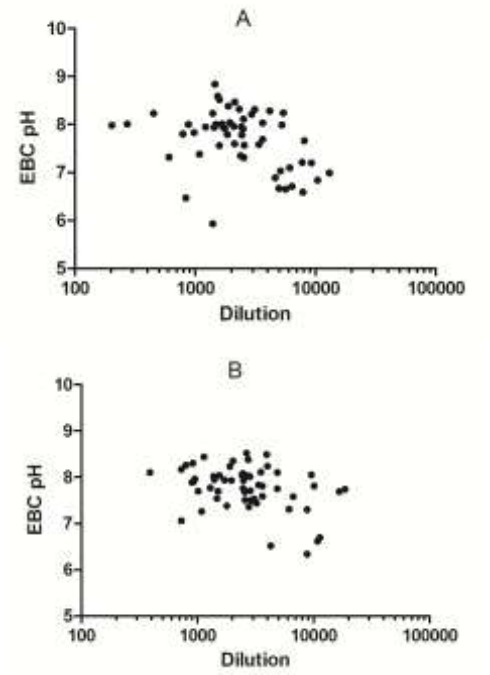

Figure 1.
The relationship between EBC pH and dilution in the asthmatic (A) and healthy (B) subjects

4.2. Reproducibility of Cyranose 320

We did not find any significant difference between the two breath samples collected at baseline and after 10 minutes assessing the principal components ($p=0.85$, 0.41 and 0.63, PC1, PC2 and PC3, respectively). Nonetheless, there was a significant relationship between the two samples ($r=0.51$, 0.75, and $r=0.37$, PC1, PC2 and PC3, respectively, all $p<0.05$).

There was no difference in electronic nose results during the 8 weeks of follow up ($p>0.05$).

4.3. Effect of physical exercise on exhaled biomarkers

Physical exercise significantly increased EBC pH and altered exhaled volatile compound pattern (Figure 2).

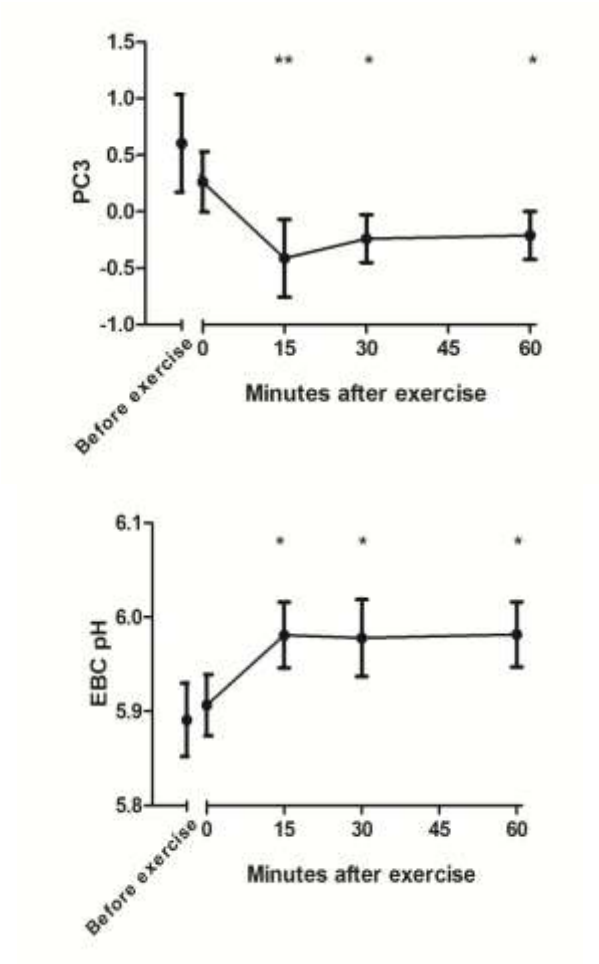

Figure 2
Effect of exercise on exhaled volatile compound pattern and EBC pH

There was a significant relationship between EBC pH and exhaled volatile compound pattern ($p=0.01$, $r=-0.34$).

4.4. Effect of pregnancy on exhaled volatile compounds

We found a significant difference comparing exhaled breath samples obtained from 48 pregnant and 25 non-pregnant volunteers ($p=0.015$). After excluding subjects with accompanying disorders (hay fever, diabetes, hypothyreosis) and smokers, the level of discrimination was higher ($p=0.001$).

There was a significant relationship between exhaled volatile compound pattern and the gestational weeks ($p=0.01$, $r=-0.36$).

Comparing 15 pregnant women in the 3rd trimester and 15 non-pregnant subjects to the preset model based on 20 pregnant and 18 non-pregnant healthy non-smoker subjects the electronic nose could discriminate the new volunteers with 87% sensitivity, 73% specificity, 76% positive predictive value and 84% negative predictive value (Figure 3). However, the 15 2nd trimester women were classified poorly.

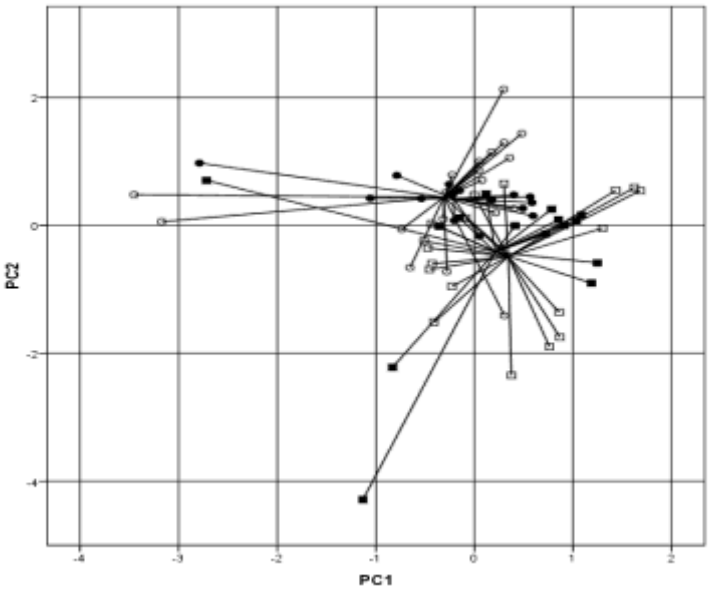

Figure 3
Circles represent pregnant, squares represent non-pregnant samples. The open signs represent the original (20 pregnant and 18 non-pregnant) the full signs represent the new (15 pregnant and 15 non-pregnant) groups.

5. Conclusions

5.1. Relationship between EBC pH and dilution

- There is no significant difference in EBC pH or EBC dilution between subjects with stable asthma and healthy controls.
- EBC pH is influenced by respiratory droplet dilution.

5.2. Reproducibility of Cyranose 320

- The electronic nose results are reproducible within short (within a day) and long term (8 weeks).

5.3. Effect of physical exercise on exhaled biomarkers

- Physical exercise increases EBC pH and alters exhaled volatile compound pattern.
- EBC pH is influenced by exhaled volatile compounds.

5.4. Effect of pregnancy on exhaled volatile compounds

- Pregnancy alters the exhaled volatile compound pattern.
- The production of exhaled volatile compounds changes during pregnancy.

6. List of publications

6.1. Publications related to the thesis

- Kovacs D, **Bikov A**, Losonczy G, Murakozy G, Ildiko Horvath. (2013) Follow up of lung transplant recipients using electronic nose. J Breath Res, 7: 017117.
  Impact factor: 2.541 (2011)
- **Bikov A,** Galffy G, Tamasi L, Lazar Z, Losonczy G, Horvath I. (2012) Exhaled breath condensate pH is influenced by respiratory droplet dilution. J Breath Res, 6: 046002.
  Impact factor: 2.541 (2011)
- **Bikov A**, Pako J, Kovacs D, Tamasi L, Lazar Z, Rigo J, Losonczy G, Horvath I. (2011) Exhaled breath volatile alterations in pregnancy assessed with electronic nose. Biomarkers, 16: 476-84.
  Impact factor: 2.215
- **Bikov A**, Lazar Z, Schandl K, Antus B, Losonczy G, Horvath I. (2011) Exercise changes volatiles in exhaled breath assessed by an electronic nose. Acta Phys Hung, 98: 321-328.
  Impact factor: 0.821

  Σ Impact factor: 8.118

6.2. Publications not related to the thesis

- Eszes N, **Bikov A**, Lazar Z, Bohacs A, Muller V, Stenczer B, Rigo J, Losonczy G, Horvath I, Tamasi L. (2013) Changes in exhaled breath condensate pH in healthy and asthmatic pregnant women. Acta Obstet Gynecol Scand, 92: 591-597.
  Impact factor: 1.771 (2011)
- Weiszhar Z, **Bikov A**, Galffy G, Tamasi L, Ungvari I, Szalai C, Losonczy G, Horvath I. (2013) Elevated Complement Factor H Levels in Asthmatic Sputa. J Clin Immunol, 33: 496-505.
  Impact factor: 3.077 (2011)
- **Bikov A**, Bohacs A, Eszes N, Weiszhar Z, Ivancso I, Muller V, Rigo J, Losoncyz G, Tamasi L, Horvath I. (2012) Circulating and exhaled vascular endothelial growth factor in asthmatic pregnancy. Biomarkers, 17: 648-54.
  Impact factor: 2.215 (2011)
- Eszes N, Bohács A, Cseh Á, Toldi G, **Bikov A**, Ivancsó I, Müller V, Horváth I, Rigó J, Vásárhelyi B, Losonczy G, Tamási L. (2012) Relation of circulating T cell profiles to airway inflammation and asthma control in asthmatic pregnancy. Acta Phys Hung, 99: 302-10.
  Impact factor: 0.821 (2011)
- Ungvári I, Hullám G, Antal P, Kiszel P, Gézsi A, Hadadi É, Virág V, Hajós G, Millinghoffer A, Nagy A, Kiss A, Semsei Á, Temesi G, Melegh B, Kisfali P, Széll M, **Bikov A**, Gállfy G, Tamási L, Falus A, Szalai C. (2012) Evaluation of a partial genome screening of two asthma susceptibility regions using Bayesian network based Bayesian multilevel analysis of relevance. Plos One, 7: e33573.

Impact factor: 4.092 (2011)

- Ungvári I, Hadadi E, Virág V, **Bikov A**, Nagy A, Semsei A, Gálffy G, Tamási L, Horváth I, Szalai C. (2012) Implication of BIRC5 in asthma pathogenesis. Int Immun, 24: 293-301.

  Impact factor: 3.415 (2011)
- Toldi G, Molvarec A, Stenczer B, Müller V, Eszes N, Bohács A, **Bikov A**, Rigó J, Vásárhelyi B, Losonczy G, Tamási L. (2011) Peripheral Thelper1/Thelper2/Thelper17/ regulatory T cell balance in asthmatic pregnancy. Int Immun, 23: 669-77.

  Impact factor: 3.415
- Gajdocsi R, **Bikov A**, Antus B, Horvath I, Barnes PJ, Kharitonov SA. (2011) Assessment of Reproducibility of Exhaled Hydrogen Peroxide Concentration and the Effect of Breathing Pattern in Healthy Subjects. J Aerosol Med Pulm Drug Deliv, 24: 271-275.

  Impact factor: 2.200
- **Bikov A**, Gajdócsi R, Huszar É, Szili B, Antus B, Losonczy G, Horváth I. (2010) Exercise increases exhaled breath condensate cysteinyl leukotriene concentration in asthmatic patients. J Asthma, 47:1057-62.

  Impact factor: 1.341
- Lázár Z, Cervenak L, Orosz M, Gálffy G, Komlósi ZI, **Bikov A**, Losonczy G, Horváth I. (2010) Adenosine triphosphate concentration of exhaled breath condensate in asthma. Chest, 138: 536-42.

  Impact factor: 6.519
- Tamasi L, Bohács A, **Bikov A**, Andorka Cs, Rigó J Jr. Losonczy Gy, Horváth I. (2009) Exhaled nitric

oxide in pregnant healthy and asthmatic women. J Asthma, 46: 786-791.
Impact factor: 1.372

- Lázár Z, Huszár É, Kullmann T, Barta I, Antus B, **Bikov A**, Kollai M, Horváth I. (2008) Adenosine triphosphate in exhaled breath condensate of healthy subjects and patients with chronic obstructive pulmonary disease. Infl Res, 57: 367-373.
Impact factor: 1.457

Cumulative impact factor: 39.813