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RESEARCH ARTICLE

Exhaled breath volatile alterations in pregnancy assessed with electronic nose

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Abstract

Context: Pregnancy-linked accelerated metabolism and oxidative stress may alter the exhaled volatile compound pattern ("breathprint"). Electronic noses can distinguish "breathprints" associated with different disorders.

Objective: This is the first study assessing alterations in "breathprint" during gestation.

Material and methods: 130 women participated in our study (78 pregnant vs. 52 non-pregnant). Breath samples were processed by an electronic nose and analyzed using principal component analysis.

Results: Significant differences were found in exhaled breath pattern between pregnant and non-pregnant women ($p = 0.001$).

Conclusion: Pregnancy-induced changes in exhaled gases need to be considered when pregnant women with respiratory disorders carry out breath tests.

Key words: Pregnancy, breath test, electronic nose

Introduction

Human breath contains thousands of volatile compounds with 20–30 of them being present in all subjects (Horvath et al. 2009). The origin of these molecules is two-fold: exogenous volatiles that are inhaled and exhaled, and endogenously produced derivates formed in the airways or originating from the alveoli. Volatiles taken up by the circulatory system from different organs may appear in breath samples by penetrating from the capillaries to the alveoli. Although the exact role of these volatiles is not completely understood, they have been thought to be markers of oxidative stress, and could indicate an altered systemic and cellular metabolism. Several studies have focused on the changes of exhaled volatile patterns in relation to different diseases, limited not only to those of the lung, but also including the kidneys (Peng et al. 2009b), the liver (Probert et al. 2009, Van den Velde et al. 2008) and the heart (Phillips et al. 2004). Less attention has been given to the physiological changes/conditions in the body that are known for profound changes in metabolism. This, however, is of great importance for two distinct reasons. One is that the exhaled biomarker profile could potentially serve as a non-invasive marker of the condition, and the other is that if the exhaled "breathprint" is altered by physiological changes, this modification needs to be taken into account when the breath test is carried out in subjects with such condition.

Pregnancy represents an important example of physiological conditions associated with major changes in metabolism and oxidative stress. Increased systemic metabolism (Duggleby & Jackson 2002) and oxidative stress (Cargnoni et al. 1994), as well as altered maternal systemic immunity (McCracken et al. 2004) are well-known features of pregnancy. In addition, there is growing evidence that volatiles excreted by the maternal body may be of importance in the early mother-infant recognition and communication, not only in animals, but also in humans (Vaglio et al. 2009). It is also well known that the magnitude of these alterations

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varies during pregnancy with a peak near the terminus. These phenomena could be observed not only in the blood or urine samples, but possibly also in exhaled breath. Nevertheless, the number of studies on exhaled compounds in pregnant subjects is low, and generally limited to a single molecule (Stolarek et al. 2008, Shin et al. 1997, Tamasi et al. 2009, Morris et al. 1995, Zusterzeel et al. 2002). Pregnancy-related changes recruit various molecular and cellular pathways, which are presumably interrelated and cannot be fully investigated at the level of single molecules. Systems biology is a novel approach to describe complex processes by studying a spectrum of molecules simultaneously. It generates information by pattern recognition algorithms on an array of signals using powerful bioinformatics. These methods may unravel metabolic networks, which may be hidden by analysis of individual compounds.

The use of electronic noses represents a relatively new method for exhaled breath volatile pattern measurement (Di Natale et al. 2003). These devices are composites of nanosensor arrays and a built-in processor with different learning algorithms. Opposite to gas chromatography and mass spectrometry, the noses cannot chemically characterize or directly quantify individual molecular components, but are able to separate and recognize different gas mixtures by the distinction of "breathprints" (a technique called "breathomics"). This unspecific sensor approach is analogue to the mammalian olfactory system (Buck & Axel 1991). Electronic noses have successfully been used to distinguish exhaled "breathprints" of healthy subjects/patients with lung cancer (D'Amico et al. 2010, Dragonieri et al. 2009, Machado et al. 2005), asthma (Dragonieri et al. 2007, Montuschi et al. 2010), chronic obstructive pulmonary disease (COPD) (Dragonieri et al. 2009, Fens et al. 2009), or upper (Bruno et al. 2008, Mohamed et al. 2003, Preti et al. 2009) and lower airway infections (Hockstein et al. 2004, Hockstein et al. 2005). However, methodological studies regarding the "breathprint" analysis using electronic nose (Montuschi et al. 2010) are still lacking.

In this study, our aim was to investigate if metabolic changes relating to the late phase of pregnancy are reflected in an altered exhaled breath volatile pattern, using an electronic nose.

Methods

Subjects

In total, 78 pregnant and 52 non-pregnant women participated in this study. Pregnant women appearing at scheduled visits at the outpatient clinic of the First Department of Obstetrics and Gynecology, Semmelweis University were recruited. Control subjects were age-matched students and workers of the University, and none had delivered a baby the year before the breath test or used hormonal contraceptives. Subjects did not present upper or lower respiratory tract infection in the past four weeks. We divided subjects into three groups according to study parts (model setting part, validation part and variability part).

Model setting part

A total of 48 pregnant women (31 ± 5 years third trimester, 37.4 ± 2.3 gestational weeks) and 25 non-pregnant control subjects (31 ± 8 years) participated. In pregnant women the labor was uncomplicated in all cases (n = 27 by transvaginal delivery and n = 21 by Caesarean section). In ten cases Caesarean section was electively repeated, while other reasons for the operation included oxytocin-resistant dystocia in six cases, abnormal foetal presentation in three cases, umbilical cord prolapse and threatening intrauterine asphyxia in one case, respectively. The mothers gave birth to healthy children at 39.2 ± 1.1 gestational weeks. The average birth weight was 3455 ± 479 grams with a 0- and 5-minute Apgar score of 9.1 ± 0.8 and 9.9 ± 0.4, respectively. None of the newborns had a congenital malformation. Based on their medical history, 20 pregnant and 18 control subjects were considered healthy (without any chronic illnesses or smoking in the medical history). Disorders and smoking habit of subjects are listed in Table 1.

Validation part

We recruited 30 healthy pregnant (15 women in the second trimester, 32 ± 4 years, 22.3 ± 3.5 gestational weeks; 15 women in the third trimester, 32 ± 4 years, 37.6 ± 2.7 gestational weeks) and 15 healthy non-pregnant women (30 ± 8 years). Control subjects were recruited among co-workers at the First Department of Obstetrics and Gynecology (same collection site as for pregnant women) to avoid the environmental effect on exhaled volatiles. None of the patients suffered from any chronic disease including allergy, asthma, diabetes mellitus, liver and renal diseases or any obstetrical disorders as assessed by an obstetrician specialist. All subjects were non-smokers.

Variability part

Twelve healthy, non-smoking volunteers (30 ± 5 years) participated. None of them were pregnant, asthmatic, or diabetic.

Study design

In the model setting part (cross-sectional, case control), we compared "breathprints" obtained from pregnant and non-pregnant women. This part was powered to detect possible "breathprint" alterations in pregnancy, irrespective of any accompanying disease. In a subgroup

Table 1. Subject characteristics.

	Pregnant N = 48	Non-pregnant N = 25
Healthy, never smoker	42%	72%
Allergic rhinitis	19%	16%
Smoking (current/ex)	13/2%	12/0%
Gestational diabetes (controlled with diet)	13%	0%
Hypothyroidism	8%	0%
Other (anti-phospholipid syndrome, uterine myoma)	4%	0%

analysis, we compared volatile patterns only in healthy, non-smoking subjects (20 pregnant and 18 non-pregnant) to exclude the effects of different disorders. Non-pregnant volunteers were subdivided into subjects at the follicular (days 0–14 in the menstrual cycle) or the luteal phases (days 15–28 in the menstrual cycle).

In the validation part, the obtained "breathprints" were analyzed in the model established in the model setting part. Cross-validation values were calculated to show how accurate the classification of pregnant and control subjects recruited was.

In the variability part, subjects gave a breath sample at baseline, at one hour and the following day, to determine the reproducibility of the E-nose measurements.

All the subjects were asked to refrain from exercising, eating, and drinking for at least two hours prior to the exhaled breath collection. All of the current smokers in the model setting part were restricted from smoking on the day of the measurement. After completing the informed consent and questionnaire, exhaled breath was collected. The measurements were done at room air from 8 to 12 am. The research was carried out according to the Declaration of Helsinki. The protocol was approved by the local ethics committee (Semmelweis University TUKEB 110/2007), and written informed consent was obtained from each subject prior to the study.

Collection of exhaled breath for electronic nose analysis

After inspiring volatile organic compound (VOC)-filtered air by a single deep inspiratory capacity manoeuvre, subjects exhaled at a controlled flow-rate (50 ml/sec) against resistance (15–20 cmH_2O) using the meter designed for collecting exhaled breath for the off-line measurement of exhaled nitric oxide (EcoMedics, Dürnten, Switzerland). Exhaled air representing the dead space was discarded and samples representing the alveolar region were collected in a Teflon-coated Mylar bag (EcoMedics, Dürnten, Switzerland). Breath samples were then examined by a commercially available electronic nose containing 32 resistance-sensitive sensors (Cyranose 320; Smiths Detection, Pasadena, US). After auto scale normalization, sensor responses (dR) were calculated using formula: $dR=(R_S-R)/R$, where R_S is the response to the sampled gas and R is the response to the baseline reading, the reference gas being the VOC-filtered ambient room air. "Breathprints" were analyzed using the signals from 28 sensors (water-sensitive sensors as reported by the manufacturer were excluded). The raw data were stored in the onboard database, and then copied to an offline database and used for further analysis. Between collections, Mylar bags were purified using 99.999% N_2 gas (Linde, Budapest, Hungary).

Statistical analysis

To reduce the dimensionality of the data set, the Principal Component Analysis (PCA), an exploratory technique was applied to investigate how the data cluster in the multi-sensor space was used to analyze the pattern of the sensor response (SPSS 15.0). The spectra of sensor responses underwent data reduction, and the principal components (PCs) were sorted by Initial Eigen value sizes, after which the highest four PCs (capturing 99% of the variances within the dataset) were used for further analyses. The Mahalanobis distance (De Maesschalck et al. 2000), a stepwise classification technique was applied to classify cases into categorical division using the four PCs. Principal components were compared using unpaired post-hoc *t*-tests, and the Pearson's -test was employed for the assessment of the correlation between exhaled breath volatiles and the day of the menstrual cycle or the gestational age. In the validation part, we used Mahalanobis distance for the classification of the newly sampled data into the two preset groups. In the variability part, intraclass correlations between sensors were calculated by the Pearson's test, and repeated measures ANOVA was used to assess the temporal changes in PCs. A $p<0.05$ value was considered significant.

Results

Model setting part

Comparison of "breathprints" in pregnant and non-pregnant subjects

The "breathprints" of pregnant subjects differed significantly from the "breathprints" of controls ($N=73$, $p=0.015$, Figure 1A and B). Pregnancy caused significant alterations in the principal component 3 ($p=0.02$) suggesting that this factor reflects the pregnancy-related changes in "breathprint". Therefore, we used this variable for further investigations.

Comparison of "breathprints" in healthy pregnant and non-pregnant subjects

In the subgroup analysis on 20 pregnant and 18 non-pregnant subjects, Mahalanobis distance showed a significant difference ($p=0.001$, Figure 1C and D) in the "breathprint" between the two groups. As this model separated the pregnant and non-pregnant women better than the model where all the subjects were included, we decided to use the two healthy subgroups as preset groups in the validation part.

Relation between "breathprint" and gestational age or the day and phase of menstrual cycle

There was a significant correlation between principal component 3 and gestational age ($p=0.01$, $r=-0.36$, $n=48$, Figure 2). However, no correlation was found between the day of the menstrual cycle and the PC ($p=0.38$; $r=-0.18$, $n=25$). No difference in the "breathprint" was found between non-pregnant women at the follicular ($N=15$) and luteal ($N=10$) phases of the menstrual cycle by analyzing principal components ($p=0.66$, Mahalanobis distance).

Validation part

There was a significant difference in the "breathprint" between subjects during the third trimester of

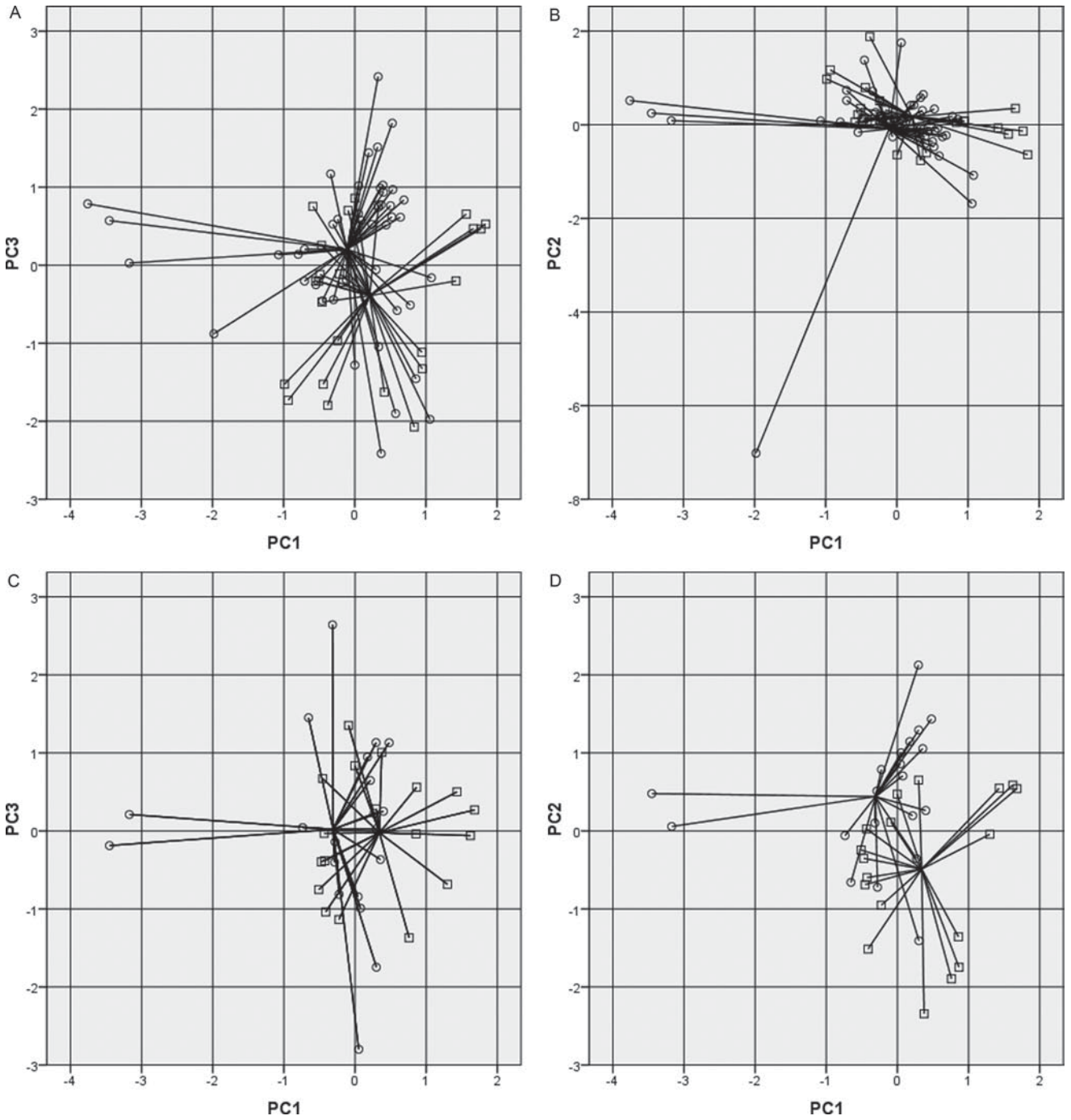

Figure 1. (A,B) Two-dimensional principal component analysis (PCA) plots on all subjects. Principal component 3 (PC3) is plotted against principal component 1 (PC1) (A) and principal component 2 (PC2) is plotted against principal component 1 (PC1) (B). The plot shows the discrimination of "breathprints" between pregnant (circles, $n = 48$) and non-pregnant control subjects (squares, $n = 25$) along discriminative composite principal factors ($p = 0.015$). (C,D) Two-dimensional principal component analysis (PCA) plots on healthy subjects. Principal component 3 (PC3) is plotted against principal component 1 (PC1) (C) and principal component 2 (PC2) is plotted against principal component 1 (PC1) (D). The plot shows the discrimination of "breathprints" between healthy pregnant (circles, $n = 20$) and non-pregnant control subjects (squares, $n = 18$) along discriminative composite principal factors ($p = 0.001$).

pregnancy and healthy controls ($p = 0.02$); however, the 15 second trimester-pregnant women could not be discriminated from either group ($p > 0.05$, Mahalanobis distance).

Based on the model established on healthy subjects ($N = 38$) in the model setting part, subjects in the validation part could be discriminated well (Mahalanobis distance, cross-validation value 80%), 13 of 15 third trimester-pregnant subjects and 11 healthy controls were classified correctly (87% sensitivity, 73% specificity, 76% positive predictive value and 84% negative predictive value, Figure 3). However, the pregnant subjects in the second trimester were poorly classified (47 % sensitivity).

Variability part

There was no difference between the baseline vs. 1 hour (within-day) and baseline vs. next day (between-day) "breathprints" (Mahalanobis distance, $p > 0.05$, Figures 4A, 4B, 5A and 5B). Similarly, no difference was observed in PCs at the three occasions assessed by repeated measures ANOVA ($p > 0.05$). There was a significant intra-class correlation in the within-day and between-day responses of all sensors ($p < 0.05$).

Discussion

In this study we investigated if pregnancy alters the exhaled breath volatile pattern. We showed that "breathprints" obtained in late pregnancy differ from that of the non-pregnant control group.

We included pregnant and age-matched non-pregnant women in this study. Our main aim was to investigate alterations in breath samples of pregnant women

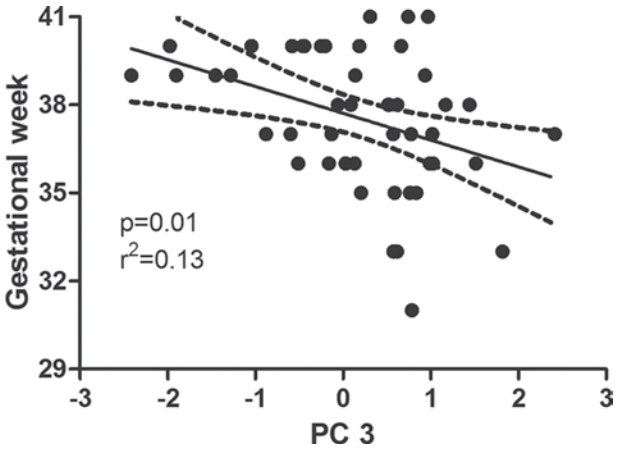

Figure 2. Relation between exhaled breath "breathprint" and gestational age. Gestational weeks are plotted against principal component 3 (PC3) values showing a significant relationship between them ($p = 0.01$, $r = -0.36$). Regression line together with 95% confidence limits is shown.

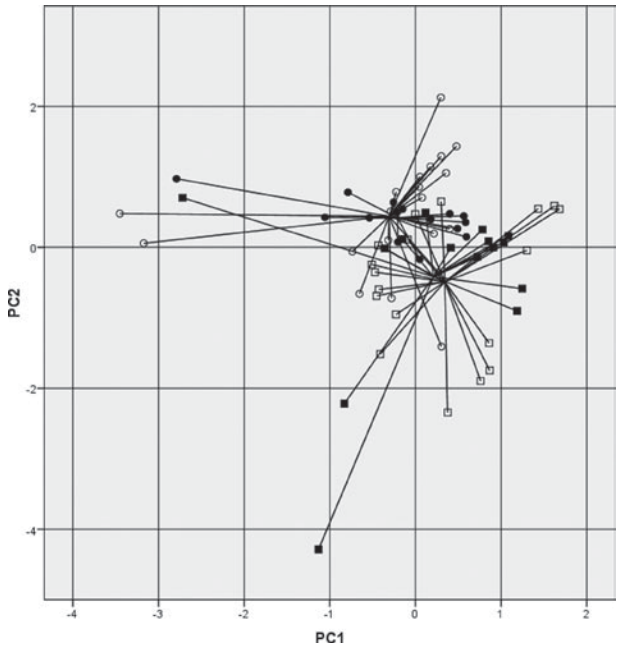

Figure 3. Validated two-dimensional principal component analysis (PCA) plot on healthy subjects. Principal component 2 (PC2) is plotted against principal component 1 (PC1). The plot shows the classification of blindly collected pregnant (full circles) and non-pregnant (full squares) subjects to the preset original groups (open circles-pregnant, open squares-non pregnant). The Mahalanobis distance could correctly classify these two groups (87% sensitivity, 73% specificity).

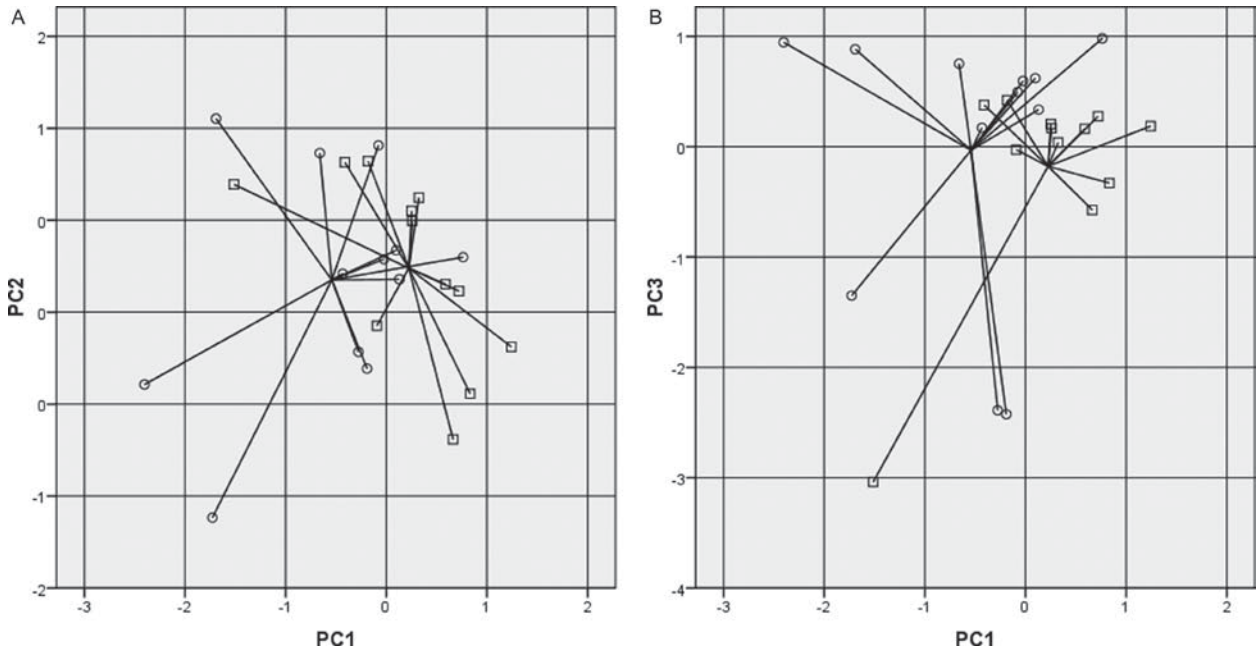

Figure 4. (A,B) Two-dimensional principal component analysis plots on within-day measurements. Principal component 2 (PC2) is plotted against principal component 1 (PC1) (A) and principal component 3 (PC3) is plotted against principal component 1 (PC1) (B). No difference was observed between "breathprints" obtained at baseline (circles) and at one hour (squares).

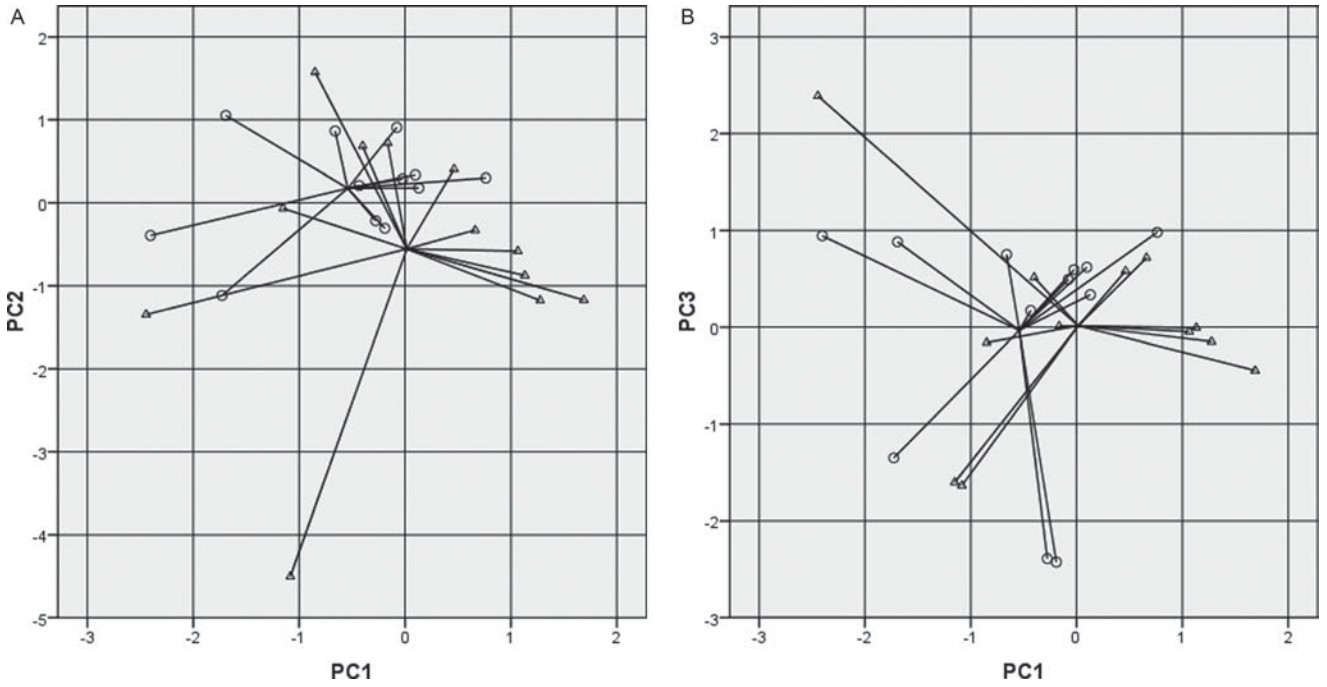

Figure 5. (A,B). Two-dimensional principal component analysis plots on between-day measurements. Principal component 2 (PC2) is plotted against principal component 1 (PC1) (A) and principal component 3 (PC3) is plotted against principal component 1 (PC1) (B). No difference was observed between "breathprints" obtained at baseline (circles) and the next day (triangles).

regardless of any possible influencing factor (e.g. smoking, allergic rhinitis or gestational diabetes); however, all of the accompanying diseases were considered to be well controlled. In 73 subjects we received a significant discrimination between the two groups. Analyzing only healthy women without any confounding factors, we achieved a higher level of significance ($p = 0.001$ vs. $p = 0.015$). This suggests that any underlying disorder or condition, mentioned above, may influence "breathprints"; however analyzing this effect was not our goal in this study.

This is the first study examining the pattern of a broad number of volatiles in the exhaled breath of pregnant subjects, as the number of studies investigating exhaled biomarkers in pregnancy till date is limited. Stolarek et al. found a decreased level of exhaled breath condensate hydrogen-peroxide (H_2O_2) in pregnant women compared to non-pregnant controls (Stolarek et al. 2008), while Shin et al. reported an increase in exhaled breath pentane concentration during labor (Shin et al. 1997). However, no difference was found in exhaled nitric oxide (Tamasi et al., 2009, Morris et al., 1995) or ethene (Zusterzeel et al. 2002) concentrations between pregnant and non-pregnant groups. Only one study has previously measured a number of exhaled volatiles together and concluded that the profile of 107 molecules (breath methylated alkane contour) differs in preeclampsia compared to physiological pregnancy and non-pregnant controls without a difference between healthy pregnant and non-pregnant subjects (Moretti et al. 2004). Our study demonstrated that when examining a broad volatile pattern of the exhaled breath, differences can be observed between healthy pregnant and non-pregnant women using a promising new tool, the electronic nose.

Electronic noses represent a cheap and possible easy way to detect small differences between various gas mixtures analyzing not only a limited number of molecules, but also a wide spectrum of gas particles. Components of the gas mixture induce a specific response on the built-in sensors according to the mean of its mass, shape, dipole moment and hydrogen binding capacity, providing the measured gas mixture's specific spectrum of sensor signals, which is analyzed by means of various pattern recognition algorithms (Lewis 2004, Scott 2007). The material of the sensors determines the sensitivity of the electronic noses on the volatile compounds. It is suggested that conducting polymer sensors in Cyranose 320 are sensitive to polar compounds such as alcohols and organic acids (Schaller et al. 1998). While this approach has an advantage to indentify volatiles associated with oxidative stress, the possible effect of environmental contaminants in hospital (such as isopropanol, ethanol, formaldehyde and mid-chain ketones) cannot be completely eliminated. Therefore, in the validation part we took special care for the same environmental conditions.

The electronic nose technique by definition implies that there are several different sensors constructed on the array with differing numbers of sensors in electronic noses. To analyze the responses (variables) of a range of sensors together, multivariate tests are required, but in order to avoid model over-fitting, data reduction techniques are needed and the principal component analysis (PCA) is the most commonly used among them.

The PCA has an advantage over other methods i.e. the generated variables, called principal components by definition do not correlate with each other. In this study, the Mahalanobis distance was used as a discrimination technique (De Maesschalck et al. 2000), which can be calculated in the principal component space. In this case the distance does not need to be corrected for the covariances between variables, since Principal Components are orthogonal (uncorrelated). There are various discrimination methods applied in electronic nose studies; however there is still no consensus on which statistical method is to be used.

In this study, we used a polymer composite sensor array electronic nose, which has previously been used successfully (Fens et al. 2009, Dragonieri et al. 2007, Dragonieri et al. 2009) for discriminating "breathprints" in airway disorders without the need to identify molecules driving the disorders. It is known that breath contains thousands of different volatiles; however, the lower detection limit of this instrument is presumably higher than some particle concentration in exhaled air (James, 2005). Therefore, results ("breathprint") obtained by an electronic nose do not fully represent the whole VOC mixture of exhaled air, but show a vast number of volatiles, which give a pattern discriminative in various diseases. As this technique is non-invasive and provides a rapid, inexpensive analysis without requiring specialist technicians, electronic noses may have the potential as a diagnostic tool.

Although studies on exhaled breath analysis using electronic noses are promising in the diagnostics of various diseases, a growing amount of factors, which can influence the data, should be taken into consideration. It has been previously shown that upper and lower airway diseases, including bronchial asthma (Dragonieri et al. 2007, Montuschi et al. 2010), COPD (Dragonieri et al. 2009, Fens et al. 2009), lung cancer (D'Amico et al. 2010, Dragonieri et al. 2009, Machado et al. 2005) and infections (Hockstein et al. 2004, Hockstein et al. 2005) may alter the exhaled "breathprint". Systemic endocrine and metabolic diseases should also be taken into account as confounding factors when data are analyzed (Van den Velde et al. 2008, Mohamed et al. 2002, Voss et al. 2005, Lee et al. 2009). Moreover, smoking (Buszewski et al. 2009, Basanta et al. 2010) and ingestion of food and beverages together with sampling conditions might also affect breath results (Kischkel et al. 2010, Pleil 2009, Mitsubayashi et al. 2005), albeit methodological studies are still lacking regarding exhaled breath electronic nose measurements (Amann et al. 2010). Therefore, in our study, special caution was taken when collecting breath samples. We used a standard sampling procedure that was developed for off-line exhaled nitric oxide measurement by using forced exhalation against resistance (to close the soft palate) with a fixed airflow. Although this method may not be optimal for "breathprint" determination and the assessment of volatiles originating from the alveoli, it ensures us with well standardized and reproducible sampling conditions (Peng et al. 2009a).

We included subjects in late pregnancy as changes in systemic metabolism and oxidative stress are more stable in this period. The highly accelerated systemic metabolism, together with the increased oxidative stress is a well-known marker of pregnancy, and both phenomena can modify exhaled air volatile pattern (Horvath et al. 2009). In addition, hormonal and immunological changes are also well known features of pregnancy, and some of them may be represented by changes in volatile metabolites. The fact that a weak, but significant relationship was found between gestational age and "breathprints" suggests that the production of volatiles changes during pregnancy. This was supported by the validation study which showed that "breathprint" is altered significantly only in the third trimester of pregnancy. This suggests that analysis of breath volatiles in pregnancy may not be an additional tool for detection of gestation, but may provide useful information about complex metabolic processes occurring during the late phase of pregnancy. Pregnancy is characterized with elevated levels of sexual hormones. There are no data available if these hormones are present in the airways or *per se* cause "breathprint" changes; however, progesterone and 17β-estradiol enhance minute ventilation in healthy women (Slatkovska et al. 2006), resulting in hyperventilation in pregnancy and in the luteal phase of the menstrual cycle (Saaresranta and Polo 2002). Furthermore, an increased 17β-estradiol concentration was associated with a decreased level of exhaled H_2O_2 in healthy pregnancy, suggesting that its level might affect oxidative stress in the lungs (Stolarek et al. 2008). However, in our study, we did not find any correlation between exhaled breath volatile pattern and the day of the menstrual cycle. Furthermore, there were no differences between the follicular and luteal phases in non-pregnant subjects despite the well-known differences between the hormonal levels of the two periods. Therefore, the unique role of the above mentioned hormonal changes, is implausible in the observed "breathprint" differences in pregnant vs. non-pregnant healthy women.

Immune tolerance of the maternal body in the presence of different foetal antigens is a key factor for successful pregnancy (Munn et al. 1998, McCracken et al. 2004). It is known that major histocompatibility complex (MHC) genes produce volatiles. These human leukocyte antigen (HLA) molecules have soluble forms that are present in blood and urine, and can be detected by the electronic nose. Furthermore, these molecules were shown to be driving factors in the sexual behavior in mice (Montag et al. 2001) and changes in their concentration might modify the sexual drive in pregnant animals. There are no data to show whether these foetal HLA molecules are present in the exhaled breath, but the production of a mixture of maternal and paternal volatiles by the foetus can be a plausible explanation for the alterations of exhaled breath pattern in pregnancy.

Studies, mainly based on animal experiments have shown that the volatile molecules, so called pheromones, present in the urine and glandular secretions, are

modified during pregnancy and become involved in the mother-infant recognition after birth (Apfelbach et al. 2005, Dominguez-Salazar et al. 2002). A special apparatus, the vomeronasal system was developed (Doving and Trotier, 1998) to detect pheromones; however, the existence of that region in humans is under debate (Vaglio et al. 2009). Some studies support the presence of pheromones even in humans, being steroids and oestrogen derivates in females (Stern and McClintock 1998) and androgen derivates in males (Grosser et al. 2000). These molecules were linked to mother-infant recognition in human behavior studies (Porter and Winberg 1999), and Vaglio et al demonstrated that the levels of five identified volatiles secreted in the para-axillary and nipple-areola regions were increased in pregnancy, and some were still present, even 6 months after the labor (Vaglio et al. 2009).

Conclusion

In conclusion, this study showed that gestation alters the exhaled breath volatile pattern, and "breathprints" are discriminative for subjects in the late phase of pregnancy. However, the reason for the observed pregnancy-induced alterations in "breathprint" is not completely understood; besides the increased metabolic rate and oxidative stress, the observed changes may reflect hormonal, immunological and pheromonal pathways. To unravel the volatiles that drive the distinctive patterns, gas chromatography/mass spectrometry or nuclear magnetic resonance spectroscopy would be necessary (Van Berkel et al. 2008). These results also prove relevant for studies on exhaled breath volatiles in pregnant and non-pregnant females.

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Declaration of interest

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