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Methodological and physiological aspects of exhaled breath analysis

PhD thesis

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1. List of Abbreviations

ALF	airway lining fluid
ANOVA	analysis of variance
CF	cystic fibrosis
cmH_2O	centimetre of water
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
EBC	exhaled breath condensate
EIB	exercise-induced bronchoconstriction
E-nose	electronic nose
FE_{NO}	fractional exhaled nitric oxide
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
GC-MS	gas-chromatography mass-spectrometry
GINA	global initiative for asthma
ICS	inhaled corticosteroid
ILD	interstitial lung disease
HLA	human leukocyte antigen
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
kPa	kilopascal
L/min	litre/minute
Mbar	millibar
MHC	major histocompatibility complex
mL/sec	millilitre/second
µL	microlitre
mM	millimole/Litre
µM	micromole/Litre
µS/cm	micro Siemens/centimetre
N_2	nitrogen

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NH_3	ammonia
NH_4^+	ammonium ion
NH_4OH	ammonium hydroxide
O_2	oxygen
OSA	obstructive sleep apnoea
PC	principal component
Ppb	particles per billion
Ppm	particles per million
Ppt	particles per trillion
PTR-MS	proton-transfer-reaction mass-spectrometry
SD	standard deviation
SEM	standard error of the mean
VOC	volatile organic compound

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2. Introduction

2.1. The potential of exhaled breath analysis in medicine

Acute and chronic disorders of human body represent huge financial burden on governmental budgets. Primary, secondary and tertiary prevention are suggested to reduce the risks, the occurrence and complications of disorders. For this purpose, the identification of biomarkers which are robust and could be easily measured is warranted. In addition, for screening purposes the measurements must be cheap, preferably non-invasive or exert limited side effects. Exhaled breath analysis has a unique advantage that it is completely non-invasive therefore carry no risks for side effects.

The examination of diseases by smelling the breath has been widely used in general medicine since ancient times, as it is well known that certain disorders such as uncontrolled diabetes, liver and renal failure, lung abscess, and diseases of the oral cavity are accompanied with an unpleasant breath.

The sensitive olfaction of animals has been long exploited in various walks of life including medicine. It is widely known, that dogs are able to identify individuals or track drugs and explosive chemicals by their scent [1], while honeybees can detect land mines using their smell [2]. Dogs could distinguish breath samples of patients with lung cancer with a sensitivity and specificity of 99 and 99 percents, respectively [3]. Interestingly, in the same experiment canine smell could discriminate breast cancer with 88% sensitivity and 98% specificity [3]. In another study, dogs could identify colorectal cancer in breath samples with a sensitivity of 97% and 99% [4]. However, in a larger cohort of subjects (N=220) dogs were able to discriminate lung cancer breath samples from healthy individuals and patients with chronic obstructive pulmonary disease (COPD), but the sensitivity and specificity (71% and 93%, respectively) were moderate [5]. Although the use of animals in early cancer detection would be desirable because of the promising results, one possible limitation of animals is that their olfaction depends on their behaviour, species and exhaustion [6]. The inter-assay variability of animals is also poorer than that of machines, but the most considerable peril is that animals cannot provide a quantitative measure.

The principles of the modern machine-based human breath analysis were established by Linus Pauling et al. who identified numerous volatile substances in exhaled breath and suggested that the analysis of volatile compounds may have a diagnostic value in identifying disorders [7].

Breath analysis has an advantage over liquid biological matrices such as blood, sputum, urine or sweat that the liberation of volatile particles is not necessary and the time consuming preparations can be avoided. It is not invasive, hence has no side effects and the sampling does not influence the levels of body mediators *per se*.

However, the number of available breath measurements in clinical practice is limited. One possible explanation for this can be that the biological role and function of exhaled particles are not fully known. This is essential if we want to consider them as surrogate markers for a specific condition. A further reason for the limited use of breath tests is the lack of methodological studies including the assessment of physiological and methodological factors which can influence breath composition.

2.2. Composition of human breath and its clinical relevance

Human breath consists of thousands of molecules with various origins. Some of them are inhaled from the environment and exhaled immediately or are taken up by the body and expired later after diffusion through the alveolar membrane. Other compounds which are produced and metabolised in the body may be excreted through the alveoli. Breath biomolecules are also generated in the airway tract. Their production is associated with local cell metabolism and oxidative stress in addition to the airway inflammation.

The most prominent molecule in exhaled breath is the inert nitrogen (N_2) gas. In atmospheric ambient pressures it does not diffuse through the alveolar membrane, and the assessment of its levels in exhaled breath can be used to estimate dead-space and ventilation heterogeneity in the airway tract [8, 9].

The simultaneous measurement of oxygen (O_2) with parallel of carbon dioxide (CO_2) might reveal the metabolic state of the human body and provide an important estimate about exercise capacity [10].

Exhaled breath also contains a high but variable amount of water. The scientific importance of this fact became evident when Sidorenko successfully condensed exhaled vapour and measured non-volatile, but water-soluble substances in exhaled breath

condensate (EBC) [11]. The measurement of molecules in EBC was proven to be very promising in the assessment of the airways pathology [12].

Exhaled ammonia [13], acetone [14], and sulphur-containing compounds [15], such as hydrogen sulphide (H_2S) are measured in lower levels (ppm-particles per million) and related to the function of kidneys, pancreas and liver. Furthermore, exhaled H_2 measurements have been applied for the diagnosis of carbohydrate malabsorption [16], and $^{13/14}$C-urea breath test is widely used for detection of *Helicobacter pylori* infections in the gut [17].

Exhaled carbon monoxide (CO) is found in around 1-10 ppm concentrations and it increases with upregulation of the heme oxygenase expression in the airways [18], therefore its breath levels are elevated in smokers and patients with airways disease [19].

The concentration of exhaled nitric oxide is measured in ppb (particles per billion) and it is elevated in atopic diseases, including asthma and allergic rhinitis [19]. It took nearly two decades following introduction that fractional exhaled nitric oxide (FE_{NO}) measurement became the part of asthma guidelines. The role of FE_{NO} is established in asthma management as it is a non-invasive marker for eosinophilic airway inflammation and it is related to treatment responsiveness.

Exhaled breath further contains thousands of molecules which are found mainly in the ppb-ppt (particles per trillion) range. These organic molecules, called volatile organic compounds (VOCs) became of particular interest in the last three decades, as their quality and quantity change in several systemic and respiratory disorders, and VOC measurements might aid the early detection of respiratory disorders such as lung cancer [20].

Other modalities of exhaled breath can be also investigated. For instance, exhaled breath temperature is suggested as a surrogate marker for bronchial blood flow [21], and was found altered in airway disease [22, 23].

Two modalities of breath analysis, EBC and measurements of VOCs, which were applied in the presented Ph.D work, are further detailed in the forthcoming sections.

2.3. Exhaled breath condensate (EBC)

A possible approach to measure non-volatile compounds of the airway lining fluid (ALF) is to condense the moisture of exhaled breath. During 5-10 minutes of tidal

breathing, small droplets arise from the ALF and condense while directed through a cooled chamber [12]. During this process, water-soluble molecules dissolve into the forming condensate fluid. However, exhaled breath condensate (EBC) contains not only non-volatile molecules released from the ALF. Other, volatile molecules, such as ammonia may also dissolve into EBC depending on their water-solubility. Although, unfortunately, the upper airway origin of some molecules cannot be completely excluded, it was shown that EBC samples usually do not contain amylase, which suggests that salivary droplets are not mixed with condensate fluid [24]. In addition, as the ratio of water vapour diluting the ALF droplets is unpredictable, a dilution indicator has also to be estimated to predict the ALF concentration of a certain molecule from its EBC levels [25].

Nevertheless, EBC can give an idea of airway inflammatory processes, and various biomarkers have been analysed successfully in EBC previously, including its pH [26-28], ammonia [26], lipid mediators [29], adenosine triphosphate [24, 30], hydrogen peroxide [31], proteins [32, 33], etc. However, due to some methodological pitfalls, this technique is not ready for the daily clinical use, yet [12].

2.3.1. EBC pH

Impaired regulation of airway acid-base status plays an important role in the pathophysiology of airway diseases. Low airway pH was associated with bronchoconstriction [34], impaired ciliary function [35] and enhanced airway inflammation [36]. Therefore, it is not surprising that one of the most studied biomarker in EBC is pH. Acidity is relatively easy to estimate by the means of indicator dyes, pH-probes or blood-gas analyser. Furthermore, contrarily to other analytical methods for other markers where applicability in EBC is restricted by limited assay sensitivity, condensate pH is reliably measured with current methods. Indeed, pioneer studies found low EBC pH associated with various respiratory disorders, including asthma, COPD and cystic fibrosis (CF) [37]. Therefore, the assessment of airway pH using a non-invasive method became of interest.

EBC pH might be a non-invasive marker for airway acidification; however it is influenced by numerous physiological factors. Firstly, as EBC is a very dilute liquid, its pH is strongly affected by the end-tidal CO_2 concentration. As the latter may vary between collections depending on systemic metabolic (exercise, digestion) or

pathological (respiratory failure) factors, neat EBC pH may not fully represent the intrathoracic airway pH. In addition, room CO_2 can modify EBC pH very rapidly, which tends to increase in untreated samples as the post collection EBC CO_2 (5%) starts to equilibrate with the atmospheric CO_2 (approximately 0.03 %). The effect of CO_2 can be reduced by two main methods; CO_2 can be flushed away by purging with an inert gas (argon or nitrogen) [26] or EBC can be loaded with CO_2 gas, thus titrating the sample. The latter, CO_2-loading method is more accurate; however its use is limited by the availability of special blood-gas analysers capable to measure in the range of 5.5-7.5 pH [28].

Secondly, EBC pH may be influenced by volatile acids and bases which dissolve into the generated condensate fluid throughout the respiratory tract, including the oral cavity. The importance of one particular volatile base, the ammonia was highlighted by Effros et al. The authors suggested that as ammonia is the main anion in EBC and it is predominantly produced orally, EBC pH cannot represent airway pH [25]. On response, Wells et al. showed that high EBC ammonia is not necessary for high EBC pH [26]. The importance of volatile acids and bases and their effect on EBC pH are still controversial.

A third, previously not investigated factor is the influence of respiratory droplet dilution on EBC pH. It is well known, that the amount of airway water diluting respiratory particles is variable and the adjustment to respiratory droplet dilution may more precisely estimate mediator concentrations in the airways from the EBC levels [25]. For this a dilution indicator is needed, which is measurable in condensate fluid and has a known concentration in the airways. As the airway lining fluid is theoretically isotonic and the diluting water contains no ions, the measurement of non-volatile individual ions or net conductivity may provide the dilution of liberated AFL droplets in EBC. Our workgroup has previously developed and validated a method to estimate dilution factor based on the conductivity measurements of vacuum-treated samples [24, 30]. Although the airway levels of some specific, non-volatile molecules can easily be calculated from EBC concentrations, the utility of such a method to derive airway pH from EBC pH is ambiguous. There are three main reasons for this; firstly, EBC pH is not only determined by the pH of ALF droplets but also by volatile acids and bases. Secondly, contrarily to other metabolites, pH is expressed on a logarithmic scale, therefore

calculations involving dilution factor may be more complicated. Thirdly, in proximal gastric reflux, gastric particles with low pH may mix with EBC droplets in the pharynx and oral cavity, increasing its acidity. However, it would be still important to study the potential influence of dilution factor on EBC pH.

2.4. Exhaled volatile compounds

Apart from non-volatile molecules, exhaled breath contains thousands of volatile particles. It is known that some of them are not only inactive derivates of systemic and airway processes but also have an active biological role. For instance, nitric oxide and carbon monoxide, markers of airway inflammation and oxidative stress, act on airway smooth muscles [19]. Breath H_2S levels have been associated with oral and hepatic malodour for a long time [15]. Recently, this molecule has become of certain interest as a biological transmitter and it is suggested to play a role in neutrophil involvement in the airways [38]. However, the biological function of most volatile compounds is still unclear. They may be active or inactive metabolites of inflammatory and oxidative stress-related processes. Therefore, the analysis of those molecules may be even more promising as their levels are associated with accelerated systemic and airway metabolism and oxidative stress. It is not surprising that altered compositions of volatile compounds were found in various systemic (diabetes, liver and renal failure) and respiratory (asthma, COPD, lung cancer) disorders. The analytical approaches to measure volatile compounds developed in two main ways. On one hand, a possible technique is to measure only one particular molecule, thus increasing the specificity of the measurement. The measurement of exhaled monoxides, such as carbon monoxide or nitric oxide is now routinely used in respiratory medicine [19, 39]. Exhaled breath ethane was related to airways oxidative stress, and elevated levels of exhaled ethane were found in asthma [40], COPD [41], CF [42], and interstitial lung diseases (ILDs) [43]. In non-respiratory medicine, exhaled ethanol is utilised as a surrogate marker for high blood alcohol [44], and the analysis of exhaled acetone might be useful for the assessment of uncontrolled diabetes [14].

On the other hand, complex systemic processes modify several different pathophysiological pathways, hence they cannot be analysed completely at the levels of single molecules. In addition, small changes during physiological processes can be hidden because of analytical or statistical factors, while analysing the pattern of

molecules together using powerful bioinformatics might reveal miniscule, but still important alterations. Finally, multivariate statistical approaches might also identify casual relationships between molecules contrarily to univariate methods which can only describe associations. The idea of "omics" technology in breath (breathomics) originates from Pauling et al. who suggested that the pattern of exhaled metabolites (metabolomics) can be specifically related to a disorder [7]. Pattern analysis of molecules in exhaled breath is frequently performed in medical research and the wide spectrum of analytical techniques covers highly sophisticated methods.

2.4.1. Measurement of exhaled breath volatile compound patterns

There are various methods available to analyse the volatile substance composition of exhaled breath. The coupling of gas chromatography (GC) with mass spectrometry (MS) is the gold-standard approach. However, this technique is expensive and requires special analytical knowledge; therefore these devices are settled in special laboratories. The main advantage of GC-MS is that it identifies and quantifies molecules with high selectivity. Contrarily, its main disadvantage is that it is applicable in static or very slowly changing gas mixtures. Mass spectrometers use electric impact ionisation; however without coupling with GC they have lower specificity for molecules compared to GC-MS. Still, they are cheaper, require less space and their use is relatively easier than GC-MS. Proton-transfer-reaction mass-spectrometers (PTR-MS) are more specific approaches compared to MS. Instead of electric ionisation, PTR-MS uses chemical ionisation with H_3O^+. H_3O^+ ions reacts only with molecules which have larger proton affinities than H_2O, thus primary molecules of exhaled breath (O_2, N_2, CO_2) do not produce signal. In addition, the technique allows rapid measurement, therefore applicable for on-line breath analysis [45].

The electronic nose (E-nose) is a composite of sensor arrays which mimics the discrimination of the mammalian olfactory system [6]. It is known that the mammalian olfactory organ consists of an array of cross-reactive receptors, in which each odorant molecule induces a response; hence each receptor reacts to a mixture of molecules [46, 47]. Similarly to mammalian noses, E-noses also use cross-reactive sensors in addition to a pattern recognition method [47]. It should be noted that E-noses cannot quantify or qualify the molecules of the analysed gas mixtures but able to distinguish two gas composites based on the pattern of substances. The main advantages of E-nose

technology over more sophisticated techniques, such as GC-MS, that it is relatively cheap, hand-held and easy-to-use. The application field of these devices is not limited to the medical research, but they can be found also in military, security, food processing approaches [6].

Although the method is theoretically similar to the human nose, there are some differences between the two. Firstly, it is known that the electronic nose is less specific for odorant substances, as a highest specificity demands irreversible interaction between the receptor and the molecule. Human nasal receptors; therefore have a lifetime of a few weeks [48]. Secondly, the selectivity of electronic nose for volatile molecules from human nose is different, as the latter is known to be insensitive for some molecules which are found in high concentrations in the ambient air, such as carbon dioxide or water vapour [6]. This represent a considerable challenge for the technique, as electronic noses may have difficulties to differentiate the signal molecules from the background noise. The selectivity of E-noses for volatile compounds is determined by the material of the in-built sensors.

Numerous E-noses with different working principles are currently available. The idea for the E-nose approach was first introduced by Dodd and Persaud who used three different metal oxide sensors to identify volatile substances [49]. In another setup, optical sensor systems are based on the modulation of light properties such as the absorbance of the target gas [50]. Ion mobility spectrometry separates the molecules based on the principles of their ion mobilites [51]. Infrared spectroscopy applies molecular vibrations and the absorption of them is characteristic for different molecules [52]. Conducting polymer sensor arrays are based on electrical resistance changes from steady-state induced by the volatile particle attachment to the sensor [47]. Thermal and gravimetric sensor arrays are also known [53]. It is also possible to combine these techniques with each other or with GC-MS. Although this increases the sensitivity and selectivity of volatile compounds measurements, the methods would become more complicated and expensive, while the main advantage of the E-nose principle is its easy usability.

Still, the most criticized point of the E-nose use in breath research is that the equipments provide an unspecific signal. Without the definite knowledge of which volatile compounds are related to the signal pattern, the differences in E-nose results between

health and disease may be false. For instance, marked alterations in exhaled breath of lung cancer patients might be associated not with the disease itself, but with behavioural (cigarette smoking), environmental (passive smoking) or methodological (different breathing technique) issues.

While the E-nose studies carried out on exhaled breath samples seem promising, unfortunately less is known about certain methodological and physiological factors which may influence the results.

2.4.1.1. The use of E-noses in medicine

E-noses were used in the identification of infections, such as ulcers [54], bacterial vaginosis [55-57], urinary tract infections [58, 59], upper airway infections [60, 61], pulmonary tuberculosis [62, 63]. Altered exhaled breath volatile compound profiles ("breathprints") were associated with diabetes [64], renal [65], hepatic [66] and heart [67] diseases, during heart rejection [68] and breast cancer [69]. In respiratory medicine E-noses were able to discriminate breath samples of lung cancer [70-72], malignant mesothelioma [73, 74], asthma [75-77], COPD [76], sarcoidosis [78], Alpha 1-antitrypsin deficient [79] patients or those with pulmonary embolism [80] from healthy subjects.

2.4.1.2. Cyranose 320 in breath research

The Cyranose 320 is a conducting polymer sensor array containing 32 carbon black sensors. During the measurement, an in-built pump is sucking the sample, and volatile molecules of the specimen attach to the surface of the polymer and induce swelling of the polymer film. The swelling increases the electrical resistance of the composite which generates an electrical signal. Different molecules produce specific responses based on their chemical features including molecular shape, size, volume, dipole moment and hydrogen bonding capacity [47]. It was shown that the sensor responses are linearly related to the concentration of the volatile substance [81] and independent from the background [82]. An example for a Cyranose 320 reading on a breath sample is shown on Figure 1.

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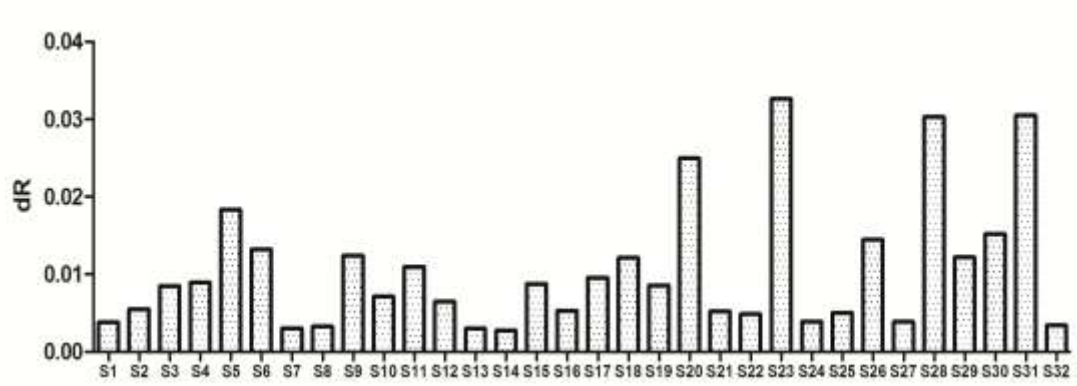

Figure 1. An example for a signal pattern obtained with Cyranose 320 in a breath sample of a healthy volunteer (own data)
Exhaled breath volatile substances induce responses (changes in resistance, dR) in each sensor (S 1-32). The signal is calculated by using the following formula: dR= (Rs-R)/R, where Rs is the response to the sampled gas and R is the response to the background gas (baseline reading).

Cyranose 320 is the most widely used E-nose in respiratory medicine. Most notably, Cyranose 320 was chosen among the analytical techniques which are used in an ongoing European Union Innovative Medicine Initiative cohort, the U-BIOPRED which aims to identify biomarkers for severe asthma [83]. The results of studies using this equipment in breath research are summarised in Table 1.

Disease	Number of subjects	Sensitivity (%)	Specificity (%)	Cross-validation value (%)	Remarks	Reference
Lung Cancer (LC)	N=62 LC N=14 no LC	71	92	ND	LC patients had more severe smoking history compared to controls	[70]
LC	N=10 LC N=10 COPD N=10 H	ND	ND	85	LC vs. COPD	[71]
				80	LC vs. H	
Malignant mesothelioma (MM)	N=13 MM N=13 asbestosis N=13 H	92	86	81	MM vs. asbestosis	[73]
		92	69	85	MM vs. H	

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MM	N=20 MM N=42 H	90	91	90	The discrimination was less accurate when patients with asbestosis (N=20) were included	[74]
Asthma (A)	N=20 A N=20 H	ND	ND	100	N=10 mild A vs. N=10 young H	[75]
		ND	ND	NS	N=10 severe A vs. N=10 old H	
Asthma, COPD, Smoker (S)	N=20 A N=30 COPD N=20 non-S N=20 S	ND	ND	96	A vs. COPD	[76]
		ND	ND	95	A vs. non-S	
		ND	ND	93	A vs. S	
		ND	ND	66	COPD vs. S	
		ND	ND	NS	COPD vs. non-S	
Asthma, COPD	N=60 A N=40 COPD	85	90	88	COPD vs. fixed A (N=21)	[84]
		91	90	83	COPD vs. reversible A (N=39)	
Alpha 1-antitripsin deficiency (AAT)	N=10 COPD with AAT N=23 COPD without AAT N=10 H	ND	ND	82	AAT vs. non-AAT	[79]
		ND	ND	81	non-AAT vs. H	
		ND	ND	60	AAT vs. H	
Pulmonary embolism (PE)	N=20 PE N=20 non-PE	ND	ND	NS	Patients with comorbidities	[80]
		ND	ND	85	Patients without comorbidities	
Obstructive sleep apnoea (OSA)	N=28 OSA N=10 H	93	70	80		[85]

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Pulmonary tubercolosis (TB)	N=56 TB N=22 H	84	73	ND		[86]
Sarcoidosis (SR)	N=63 SR N=32 H	ND	ND	64	All subjects	[87]
		ND	ND	83	Active SR vs. H	
SR	N=31 SR N=25 H	ND	ND	83	Treated SR vs. H	[78]
		ND	ND	NS	Untreated SR vs. H	
Cystic fibrosis (CF), Primary Ciliary Dyskenesia (PCD)	N=25 CF N=20 PCD N=23 H	84	65	ND	H vs. CF	[88]
		80	57	ND	H vs. PCD	
		75	56	ND	CF vs. PCD Borderline discrimination (p=0.055)	
Lung transplantation (LTx)	N=15 LTx N=33 H	63	75	73		[89]
Smoking (S)	N=7 S N=8 Non-S	ND	ND	ND	Training group	[90]
	N=15 S N=24 Non-S	100	92	95	Validation vs. training	
Virus infection (VI)	N=15 VI N=6 H	ND	ND	ND	In wheezing children, subjects with VI differed from those who had no VI	[91]
Gastro-oesophageal reflux disease (G)	N=11 A N=9 A+G N=8 COPD N=9 COPD+G	ND	ND	85	A vs. A+G	[92]
		ND	ND	65	COPD vs. COPD+G	
Breast cancer (BC)	N=10 BC N=10 H	ND	ND	75		[93]
Diabetes mellitus (DM)	N=50 DM N=25 H	ND	ND	ND	Sensors were analysed separately, and were related to blood HbA1c	[94]

Table 1. Clinical studies conducted on human breath samples using Cyranose 320.

A-asthma, AAT- Alpha 1-antitripsin deficiency, BC-breast cancer, CF-cystic fibrosis, COPD-chronic obstructive pulmonary disease, DM-diabetes mellitus, G-gastro-oesophageal reflux disease, H-healthy, LC-lung cancer, LTx-Lung transplantation, MM-malignant mesothelioma, ND-not determined, NS-not significant, OSA-obstructive sleep apnoea, PCD-primary ciliary dyskenesia, PE-pulmonary embolism, S-smoker, SR-sarcoidosis, VI-virus infection

In line with the E-nose technology, Cyranose 320 applies an unspecific approach for volatile substance measurement and compares volatile compound patterns ("breathprints") rather than individual molecules. Still, it would be important to know what molecules are measured during E-nose analysis. This depends on the quantities and qualitative characteristics of volatile substances.

A moderately sensitive lower detection limit of the carbon black polymers (0.1-100 ppm depending on the water vapour content of the specimen was previously estimated for various volatile substances such as organic acids (including n-propanoic acid, n-butanoic acid, n-pentanoic acid, n-hexanoic acid, n-heptanoic acid, isobutanoic acid, isopentanoic acid and isohexanoic acid), alcohols (including ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 2-pentanol and 3-pentanol), esters (including isopentyl acetate, isopentyl propionate, isopentyl butanoate, isopentyl pentanoate, isopentyl hexanoate, ethyl acetate, n-propyl acetate, n-butyl acetate, n-pentyl acetate, n-hexyl acetate, n-octyl acetate, n-decyl acetate, isopropyl acetate and isobutyl acetate) and alkanes (including n-pentane, n-hexane, n-heptane, n-octane and n-nonane) [95, 96]. Very similar detection limits were found recently for ethanol (0.1 ppm), ethyl acetate (0.26 ppm) and hexane (0.25 ppm) [97]. Using their in house produced carbon black sensors similar to those in Cyranose 320, Kang et al. found similar lower detection limits for some typical breath VOCs, including acetic acid (0.1 ppm), toluene (1.2 ppm), ethanol (0.6 ppm), acetone (5 ppm), pentane (20 ppm) and isoprene (11 ppm) [98]. Furthermore, Cyranose 320 could distinguish gaseous samples based on their volatile sulphur compound levels [99] and it is also sensitive for ammonia in above 1 ppm concentration [100] with the most selective sensors being 6 and 23. In addition, Cyranose 320 was able to detect NO_2 and Cl_2 gas, however in that experiment the lowest concentration of those gases was 5 ppm [101]. In conclusion,

Cyranose 320 can detect volatile substances between approximately 100 ppb and 100 ppm. Based on the qualitative and quantitative characteristics of human breath VOCs, one can assume certain VOCs responsible for Cyranose 320 results.

In a previous study, quantifying breath VOCs with GC-MS, Barker et al. found only a limited number of volatiles (methanol, ethanol, acetone and isoprene) in the 100 ppb-100 ppm range [102]. Another study using solid phase microextraction revealed that pentane, xylene and acetonitrile may also be found in a concentration above 100 ppb in breath samples of some healthy subjects [103]. Unfortunately, that study did not analyse the healthy smoker subjects separately, but higher breath levels of methanol, isoprene, xylene and acetonitrile were associated with smoking in other studies [104-106]. In a large, but unselected population of subjects (N=344) Moser et al determined concentrations of some selected VOCs in exhaled breath using PTR-MS. In addition to methanol and acetone the authors found that the median levels of benzene were also above 100 ppb [107]. Of note, elevated levels of exhaled benzene were also associated with smoking [105, 106]. The most prominent volatile sulphur compounds in breath are hydrogen sulphide, methanethiol and dimethyl sulphide, found in variable concentrations, but their physiological thresholds were estimated to be 100 ppb, 12 ppb and 24 ppb, respectively [108]. In addition to the aforementioned VOCs, breath ammonia is found in 1-10 ppm levels [13].

2.4.1.3. The current status of exhaled breath analysis using Cyranose 320 electronic nose.

Surprisingly, despite the lack of validation studies, various results show that the diagnostic potential of Cyranose 320 is excellent (classification accuracy of 95-100%) to identify asthma as well as OSA, good (classification accuracy of 90-95%) to identify malignant mesothelioma and fair (classification accuracy of 85-90%) to identify lung cancer from exhaled breath samples. Nevertheless, these results should be interpreted with caution:

1. The statistical methods for data reduction (principal component analysis) may overestimate the classification accuracy as it separates the groups on the basis of largest differences, which are not necessarily related only to the disorders themselves but also to other unknown factors.

2. These studies were performed in relatively small cohorts (N<100 in all cases) and should be validated in further cohorts.
3. The fact that qualitative and quantitative characteristics of volatile substance in mixtures driving the differences are not determined one cannot exclude that instead of disease false and not disease-specific signals are captured. For instance, some studies showed that breath samples in lung cancer differed from health [70, 71], however another study showed that smoking may itself alter the volatile compound pattern [90].

While human breath contains hundreds of different volatile molecules, the number of potential candidates which may drive E-nose signals is limited because of their quantitative and qualitative modalities in exhaled breath. Taking into account the studies establishing VOC levels in human breath, acetone, methanol, ethanol, isoprene, ammonia, pentane, hydrogen sulphide, xylene, acetonitrile and benzene are those which may produce a measurable Cyranose 320 signal. However, there is a possibility that an aberrant molecule found in lower concentrations in the breath of healthy individuals can be extremely overproduced in disorders. Unfortunately, it is not very likely that such a molecule exist, as in this case there would be no need for an unspecific platform, and studies should focus on developing sensitive and selective sensors to measure that specific molecule instead.

Comparing the results of previous GC-MS studies to those with electronic nose, it seems plausible that the differences observed by E-noses are caused by not a mixture of many and not only a couple of molecules. In line with this, altered levels of acetone [109-112], isoprene [109-111, 113], benzene [20], xylene [109], pentane [114], ethanol [112] and methanol [111] were found in lung cancer, nonetheless methanol, isoprene [106] and benzene [106, 109, 114] were associated with smoking itself. Elevated levels of pentane [115] and reduced levels of ammonia [116, 117] were linked with asthma. Altered levels of breath pentane were found in OSA [118] and malignant mesothelioma [119].

In spite of the promising results of case-control studies, only a limited number of experiments investigated the methodological factors that may influence Cyranose 320 results.

One possible factor which may affect E-nose results is the temporal drift of the sensors, meaning that the steady-state E-nose responses change along time with usage. The temporal drift can be short or long term. It is not known for conducting polymers whether repetitive measurements influence sensor reactivity when volatile substances do not detach from the composite fast enough and sensors cannot regain baseline conformation. Knobloch et al. repeated sampling every two hours in total for 6 hours using a commercially available conducting polymer E-nose (ST214, Scensive Tech. Ltd.), and showed a significant short term drift [120]. However, contrarily, initial studies on carbon black sensors showed that the time for desorption is less than 1 second [47]. A study by Lazar et al. supported these findings showing no change in exhaled volatile compound pattern 90 minutes after baseline [121]. The oxidation of the polymers contributes to the long term drift [53]. Supporting this, Nake et al. exposed Cyanose 320 to CH_3SH and assessed results along 8 days. E-nose responses correlated highly with the elapsed time ($r=0.99$), suggesting a considerable effect of sensor drift [122]. Nevertheless, long term stability of the exhaled breath E-nose signal has been reported in few studies [73, 74, 76, 121, 123], indicating that Cyranose 320 results are reproducible within 6 weeks.

Exhaled breath contains a variable amount of water which may affect the E-nose result [124]. Although it was shown that the selectivity of carbon black polymer sensors for volatile substances is not influenced by humidity [82], the increase in vapour pressure decreases sensor sensitivity [47]. There are two possible solutions to reduce this confounding factor. It was shown that sensors 5, 6, 23, 31 are the most sensitive water sensors [100], and the manufacturer suggest excluding them to prevent this effect. The other technique is to lead exhaled breath through a silica gel during collection which adsorbs most of the humidity [75, 76], however one study suggested that this method is ineffective [124]. The main disadvantage of the former technique is that the excluded sensors may be specific for other important molecules besides water. Silica gels reduce water content with an unpredictable and possibly varying ratio making sensor signals inconsistent as sensor sensitivity for volatile molecules is dependent on ambient water content [47]. Notably, the workgroup which introduced the application of silica gel in breath sampling later decided to dismiss it and instead began to exclude the water sensitive sensors [86]. Conducting polymer arrays are also influenced by the

temperature of the sample gas, therefore a temperature-adjustment is required during analysis [120].

E-nose volatile pattern differences between healthy individuals and patients with obstructive lung diseases may not be due to airway inflammation or oxidative stress, but the magnitude of airway narrowing which may modify the kinetics of exhaled particles. However, this effect is still contradictory, as Lazar et al. showed that the acute changes in airway calibre do not affect the E-nose results [121], while Biller et al. concluded that Cyranose 320 results are related to the lung function parameters [125].

Assessing the relationship between exhaled volatile compound pattern and systemic inflammatory parameters, Biller et al. showed that a significant relationship existed between Cyranose 320 data and blood total leukocyte number, as well as neutrophils, lymphocytes, monocyte and eosinophil percentages in healthy subjects [125]. In addition, Fens et al. showed that the E-nose pattern is associated with airway inflammation measured by sputum eosinophil cationic protein and myeloperoxidase levels in COPD subjects [126].

Complex physiological circumstances may affect the exhaled volatile compound levels in a more elaborate way. These changes cannot be derived from the sum of different factors, but need to be investigated using powerful bioinformatics. For instance, food and beverage intake may influence the exhaled volatile compound measurements [127]. Following meal, volatile substances might originate from the oral cavity, from stomach, or from blood after complete digestion. It is widely known that alcohol ingestion increases exhaled ethanol levels [44]. Breath acetone concentration is not only directly influenced by the consumed food, but also by the systemic metabolic state [128]. Other volatile compounds levels, like that of exhaled carbon monoxide are also increased following sugar intake, which may be related to the enhanced systemic oxidative stress [129]. Unfortunately, a general recommendation how long the subject should fasten before volatile compound measurements has not been put forward. Preliminary measurements are warranted for as many target volatile molecules as possible. In general, subjects fast for 2-3 hours when breath is analyzed.

Other important influencing conditions are physical exercise and physiological pregnancy.

2.4.2. Effect of physical exercise on exhaled volatile compounds

Exercise may alter exhaled volatile particle levels through several mechanisms.

Physical challenge enhances the metabolism of carbohydrates, lipids and amino acids and elevates the cardiac output. Hence, the excretion of blood borne volatile compounds, such as acetone or isoprene through exhaled breath increases with exercise [130].

Hyperventilation during exercise may wash out the volatile molecules which are produced in the airways [131]. For instance, despite the increased production of nitric oxide in the airways, the concentration of exhaled nitric oxide decreases during physical challenge [132].

Hyperventilation and the consequent drying of airway mucosa may induce inflammatory response, especially in asthmatic subjects. We have previously shown that exercise increases exhaled inflammatory markers of adenosine [133] and cysteinyl-leukotrienes [29] in subjects with exercise-induced bronchoconstriction. In addition, the levels of oxidative stress markers, such as EBC H_2O_2 [134-136] and malondialdehyde [137] as well as inflammatory markers, such as EBC PGE_2 and TXB_2 [138] increase after exercise in healthy subjects. Nevertheless, the effect of exercise on oxidative stress markers needs further evaluation, as in other studies no significant changes were found in EBC H_2O_2 or tiobarbituric acid reactive substance levels following moderate cycling [139] or in EBC 8-isoprostane concentration after swimming [140]. The effect of exercise on EBC oxidative stress markers could be work load dependent, as Araneda et al. found an increase in EBC H_2O_2 and NO_2^- levels after 21.1 km and 42.2 km running, but not after 10 km in elite long distance runners [141]. Chronic training at high altitude (2,800 m) increased EBC 8-isoprostane and H_2O_2 levels in healthy subjects [142]. As airway inflammation and oxidative stress is related to the exhaled volatile compound levels, their levels may be altered after exercise.

Exhaled breath pH increases following exercise, as shown by various workgroups [143, 144]. It is known that the entrapment of some acids and bases in condensate in their non-volatile form is based on condensate acidity. For instance, decreasing EBC pH increases its non-volatile ammonium (NH_4^+) concentrations [25], which presumably reduces the levels of exhaled ammonia (NH_3), the volatile base form of this molecule. In line with this, elevated NH_3 and reduced propionic acid breath levels were measured in

parallel with EBC alkalisation following physical exercise in healthy subjects [143]. However, experiments by Marek et al. contradicts other studies that showed EBC alkalisation following exercise, as these authors described an increase in EBC lactate concentration [135, 145]. Since lactate measured in EBC correlated with its blood levels [145], it may suggest that the change in EBC pH following exercise depends on aerobic/anaerobic threshold during exercise challenge. Similarly to oxidative stress markers, changes in EBC pH may also be workload dependent, as in elite runners EBC pH values tended to increase at 10 km, but showed a tendency to decrease at 21.1 and 42.2 km (changes were not significant at any timepoints) [141]. Interestingly, studies investigating systemic changes of oxidative stress markers are also contradictory and seem to be related to work load [146, 147].

2.4.3. Effect of pregnancy on exhaled volatile compounds

Physiological pregnancy activates numerous pathways which may influence exhaled biomarkers. Haemodynamical changes during gestation might alter the excretion rate of some blood borne volatile substances. The levels of hormones and metabolites change in pregnancy. In addition, pregnancy is characterised by accelerated metabolism [148], increased systemic oxidative stress [149] and immune tolerance [150]. These changes may all lead to altered exhaled volatile compound composition.

The number of studies measuring exhaled biomolecule levels in pregnancy is low. Stolarek et al. reported decreased levels of EBC H_2O_2 during pregnancy [151], Shin et al. showed that exhaled pentane increases during delivery [152], while Zusterzeel et al. concluded no differences in exhaled ethene concentration in pregnant women [153]. We aimed to measure EBC vascular endothelial growth factor in pregnancy, however could not detect this molecule in the majority of samples [33]. Supporting the previous results [154], we have found no difference in exhaled nitric oxide in healthy pregnant women [155]. More importantly, pregnancy did not alter the fractional exhaled nitric oxide levels in asthmatic women either [155], therefore this variable can be used as a potential biomarker for the assessment of airway and possibly systemic inflammation [156] and thus asthma control in pregnancy [157]. A study, investigating a profile of 107 VOCs showed that the contour of breath methylated alkanes was altered in preeclampsia [158], without a difference in physiological pregnancy compared to healthy, non-pregnant women.

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3. Objectives

This PhD work aimed to investigate methodological and physiological factors which may affect the exhaled breath measurements. These were the following:

3.1. The effect of respiratory droplet dilution on EBC pH

- The main aim was to assess the relationship between EBC dilution and EBC pH values between healthy and asthmatic subjects.
- The other aims included:
    - To study the reproducibility of EBC pH and EBC dilution measurements
    - To compare EBC dilution and EBC pH values between healthy and asthmatic subjects.
    - To compare EBC pH and dilution values with various variables of asthma control, including lung function, exhaled nitric oxide and inhaled steroid use.

3.2. Reproducibility of Cyranose 320 measurements

- We aimed to assess the short term reproducibility of exhaled volatile compound pattern assessed with Cyranose 320 within a day.
- We aimed to assess the long term reproducibility of exhaled volatile compound pattern assessed with Cyranose 320 over 8 weeks.

3.3. EBC pH and exhaled volatile compound pattern during physical exercise

- We aimed to assess the effect of physical exercise on EBC pH and exhaled volatile compound pattern in healthy subjects
- We aimed to compare exercise-induced EBC pH and exhaled volatile compound pattern changes, thus investigating the relationship between the two variables.

3.4. Effect of physiological pregnancy on exhaled breath volatile compounds

- The main aim was to compare exhaled volatile compound pattern between pregnant and non-pregnant women.

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- In addition we aimed to assess:
    - The effect of accompanying disorders and smoking on discrimination model set on pregnant and non-pregnant subjects.
    - The relationship between exhaled volatile compound pattern and gestational age in pregnant women.

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4. Methods

4.1. The effect of respiratory droplet dilution on exhaled breath condensate pH

4.1.1. Study design

The study had a case-control design in which exhaled breath condensate dilution and pH values were compared in 112 (55 asthmatic and 57 healthy) individuals. After filling informed consent, exhaled breath condensate was collected from all subjects for pH and respiratory droplet dilution measurements. In asthmatic subjects additional exhaled nitric oxide and lung function measurements were performed according to the latest guidelines [159, 160]. Exhaled breath condensate was collected during tidal breathing using Rtube (Respiratory Research, Charlottesville, VI, USA) for 10 minutes without wearing a nose clip with a chilling tube that was previously cooled at -80 °C. Following the collection samples were divided for pH (250 µL) and dilution (600 µL) measurements and stored at -80 °C until analysis.

4.1.2. Study subjects

55 asthmatic (32±9 years) and 57 healthy (29±7 years) volunteers were recruited. Asthma was defined using the latest Global Initiative for Asthma (GINA) guidelines [75], and confirmed by >12% and 200 mL increase in FEV_1 after administration of 400 µg salbutamol. According to the GINA, 19 asthmatics were considered well controlled, 20 partially controlled and 16 uncontrolled. Asthmatic patients were recruited at the outpatient clinic of Department of Pulmonology and none of the subjects was hospitalised due to asthma exacerbation in the last year. Twenty-three asthmatic patients used inhaled corticosteroids (ICS) either alone or in a combination with long acting beta agonist and twenty-two were considered steroid-naive. Healthy subjects were recruited among workers and students of Semmelweis University. None of the subjects had respiratory tract infection within 2 months prior to the study. The volunteers were asked to avoid consuming food or beverages 2 hours prior to the breath measurements.

To measure EBC dilution, we used the vacuum evaporation method validated by our workgroup previously [24, 30]. To investigate the effect of vacuum evaporation on EBC

conductivity, further seven healthy subjects were involved and EBC conductivity was estimated before and after vacuum treatment.

Furthermore, to study the analytical reproducibility of EBC pH and conductivity measurements twelve and seven healthy volunteers were recruited for pH and conductivity analysis, respectively. Their samples were divided into two and measured in parallel.

4.1.3. Exhaled breath condensate pH measurements

EBC pH was estimated using a glass pH electrode (SV 20 Seveneasy, Mettler Toledo, Schwerzenbach, Switzerland) after 10 minutes of de-aeration with argon gas. During this approach the majority of CO_2 is eliminated resulting in a more reproducible EBC pH than of an untreated sample [26].

4.1.4. Exhaled breath condensate dilution factor measurements

EBC dilution factor was estimated by conductivity measurements in vacuum treated samples. During 12 hours of vacuum evaporation at 700 mbar and room temperature all the water and volatile constituents of EBC are removed and only non-volatile ions remain. Evaporation was performed on 600 µL serially diluted 150 mM NaCl standards (1/1000, 1/2000, 1/4000, 1/8000 and 1/16000), distilled water (serving as 0 mM NaCl) as well as EBC samples. After evaporation standards/samples were reconstituted in 600 µL distilled water (conductivity 3.3±0.9 µS/cm) and conductivity was measured with GMH 3410 conductivity meter (Greisinger Electronic GmbH, Regenstauf, Germany). The detection limit of conductivity measurements was estimated at 6.85 µM NaCl [30].

To study the potential of vacuum evaporation to eliminate ammonia, the main volatile ion in EBC, conductivity was measured in serially diluted NH_4OH solutions (178.5 mM, 89.3 mM, 44.6 mM and 14.9 mM) before and after vacuum-treatment.

4.1.5. Statistical analysis

The sample size (N=112) was estimated to investigate the relationship between EBC pH and respiratory droplet dilution with an effect size of 0.3, the power of 90% and the probability of α error of 0.05.

Statistica 8.0 (Stat Soft, Inc., Tusla, OK, USA) was used for statistical analysis. The normality of data was estimated with Kolmogorov-Smirnov test. Wilcoxon test was used to compare pre and post vacuum-treatment conductivity. Mann-Whitney test was

used to compare EBC pH and dilution values between healthy and asthmatic groups. The relationship between EBC pH and dilution was examined by Spearman-correlation. Stepwise-multiple regression was used to assess the relationship between EBC pH, dilution factors and clinical variables. The asthmatic group was divided into low pH (≤7.2) and normal pH (>7.2) groups and exhaled nitric oxide, lung function values and asthma control were compared between the two subgroups using logistic regression. General linear model was used to compare EBC pH between the three asthma control groups with and without adjustment on respiratory droplet dilution. Data are expressed as median (25-75 % inter-quartile range). A $p<0.05$ value was considered significant.

4.2. Reproducibility of Cyranose 320 measurements

4.2.1. Study design and subjects

To study the short-term variability of "breathprints" obtained with Cyranose 320 (Smiths Detection, Pasadena, USA), fifty-six healthy subjects (40±13 years, 20 men) were involved. Exhaled breath samples were collected from all subjects and processed instantly with Cyranose 320. A second sample was also collected immediately after the instrument has finished the previous analysis and processed using the same algorithm. Because electronic nose analysis took around 10 minutes, the two measurements were performed 10 minutes apart. None of the subjects were smokers or had respiratory tract infection within 2 weeks prior to the study. The volunteers were asked to avoid consuming food or beverages 2 hours prior to the breath measurements.

The long-term variability was assessed in 12 healthy subjects (30±5 years, 3 men). Exhaled breath was collected at baseline as well as 30 minutes, 60 minutes, 120 minutes, 1 day, 1 week, 4 week and 8 weeks after the first measurement. None of the subjects were smokers or had respiratory tract infection within 2 weeks prior to the study. The volunteers were asked to avoid consuming food or beverages 2 hours prior to the breath measurements.

4.2.2. Exhaled breath collection and measurements

Exhaled breath collection was performed in the same way in all cases. After a deep inhalation through a VOC filter to total lung capacity, subjects exhaled at controlled flow rate (50 mL/sec) against resistance (15-20 cmH_2O) to residual volume. The first 500 mL of exhaled air, representing the anatomical dead space was discarded by leading

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through a T-valve and the second part, representing alveolar air was collected into a Teflon-coated Mylar bag, which is chemically inert with respect to most compounds in the breath [161] (Image 1, EcoMedics, Dürnten, Switzerland). The bags were attached to the E-nose and processed immediately. After auto scale normalisation, sensor responses (dR) were calculated using formula dR=(Rs-R)/R, where Rs is the response to the sampled gas and R is the response to the baseline reading, the reference gas being the VOC-filtered room air. Cyranose 320 contains 32 sensors but to avoid the confounding effect of water vapour exhaled breath volatile compound pattern was analysed using only 28 sensors (the four water sensitive sensors 5, 6, 23, and 31 were excluded). The raw data were stored in the onboard database and then transferred to an offline database for further analysis. Between each collection, Mylar bags were purged using 99.999% N_2 gas (Linde, Budapest, Hungary).

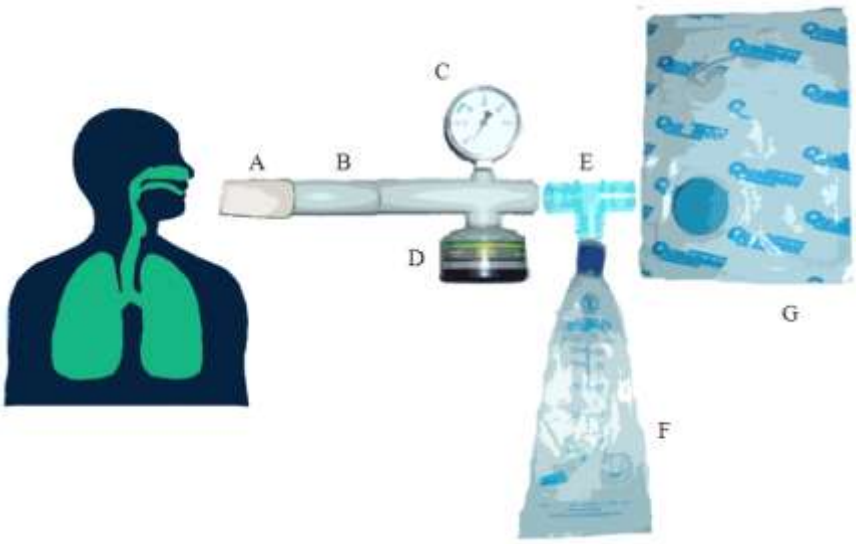

Image 1. Setup for collection for Cyranose 320 measurements
After inhaling through a VOC-filter (D) to total lung capacity, the subject exhales through a mouthpiece (A) and a bacteria filter (B) while the exhalation flow rate is being controlled with a flow-meter (C). The first part of the exhaled breath, representing the dead space, is discarded in a dead space bag (F) via a T-valve (E) and the alveolar air is collected in a Teflon-coated Mylar bag (G).

4.2.3. Statistical analysis

The statistical analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). To reduce the dimensionality of the data set, principal component analysis (PCA), an exploratory technique was applied to investigate how the data cluster in the multi-sensor space. The responses of 28 sensors underwent data reduction (PCA) and

the principal components were sorted by their Initial Eigen value sizes and the first 3 principal components (PCs, capturing 99% of the variances between the datasets) were used in further analyses.

Assessing short-term variability PCs were correlated using Pearson-test and compared using paired t-test.

To study long-term variability intra-class correlations between sensor responses were calculated by the Pearson-test and repeated measures analysis of variance (ANOVA), followed by the Dunnett's post hoc tests were used to assess the temporal changes in PCs. A p value <0.05 was considered significant.

4.3. EBC pH and exhaled volatile compound pattern during physical exercise

4.3.1. Study design

Exhaled breath volatile compounds as well as exhaled breath condensate were collected and lung function test was performed before the 6-minute outdoor running test at 14.5±5.7 °C. The cycles of breath collection and spirometry were repeated 0, 15, 30 and 60 minutes following the exercise.

4.3.2. Study subjects

Ten healthy subjects (22±4 years, 6 men) participated in the study. None of them had any chronic disorder or respiratory tract infection in the 6 weeks prior to the study. None of them were smokers and were asked to avoid consuming food or beverages and physical exercise 2 hours before the measurements.

4.3.3. Exhaled breath volatile compounds collection and analysis

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 using Cyranose 320 (Smiths Detection, Pasadena, USA). After exclusion of the four water-sensitive sensors, 28 responses underwent data reduction (principal component analysis), the principal components were sorted by their Eigenvalue sizes and the highest 4 of them (which represented 98.9% of total variances) were used for further analysis.

4.3.4. Exhaled breath condensate collection and pH analysis

Exhaled breath condensate was collected using the Rtube device (Respiratory Research, Charlottesville, VI, USA). The chilling tube was held at -80 °C and EBC was collected for 10 minutes of tidal breathing without wearing a nose clip. The samples were stored for no longer than 1 month at -80 °C.

EBC pH was estimated using the CO_2-loading method described by our workgroup, previously [28]. Briefly, condensate samples were perfused with CO_2 gas for 1 second, and pCO_2 was estimated together with pH. This procedure was repeated 3 times and the pH-pCO_2 plot was created from the results. A pH belonging to pCO_2 of 5.33 kPa was calculated using logarithmical regression analysis. This method had a coefficient of variation of 3.3% as described previously [28].

4.3.5. Lung function

Lung function tests were performed using PDD-301/s electronic spirometer (Piston, Budapest, Hungary) according to the latest guidelines [160]. Three technically acceptable manoeuvres were performed and the highest values were used. Forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) values were used for further analysis.

4.3.6. Statistical analysis

SPSS 15.0 and Graph Pad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis. Data normality was estimated using Kolmogorov-Smirnov test. Temporal changes of principal components, EBC pH and lung function parameters were assessed using mixed linear model and post hoc tests. The relationship between EBC pH and PCs was assessed with Pearson correlation. Data are presented as mean±SEM. A $p<0.05$ was considered significant.

4.4. Exhaled breath volatile compound pattern (“breathprint”) during pregnancy

4.4.1. Study design

The study had two, the model setting and the validation parts.

In the model setting part “breathprints” of 48 pregnant and 25 non-pregnant women were compared. In addition, we also compared volatile compound patterns only in

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healthy, non-smoking volunteers (20 pregnant and 18 non-pregnant) to exclude the effect of smoking and various disorders. Discrimination models were built in all (N=73) and healthy (N=38) subjects. We also compared non-pregnant women in their follicular (days 0-14 in their menstrual cycle) or luteal (days 15-28 in their menstrual cycle) phases.

In the validation part 15 healthy non-pregnant subjects, 15 healthy pregnant individuals in their 2nd trimester and 15 healthy pregnant women in their 3rd trimester were classified externally by the model set in the previous part.

4.4.2. Study subjects

In total, 78 pregnant and 40 non-pregnant women participated in the study. Pregnant women were recruited at their scheduled visits at the outpatient clinic of the First Department of Obstetrics and Gynecology, Semmelweis University. Control subjects were students or workers of Semmelweis University, and none of them delivered baby the year before the breath test or used oral contraceptives. None of the subjects had upper or lower respiratory tract infection in the past four weeks. Subjects with bronchial asthma were also excluded. The subjects were asked to avoid consuming food or beverages 2 hours before breath measurements.

48 pregnant (31±5 years) women in their 3rd trimester (37.4±2.3 gestational weeks) and 25 non-pregnant control volunteers (31±8 years) participated in the model setting part. Based on their medical history 20 pregnant and 18 non-pregnant women were considered healthy. The characteristics of these subjects are listed in Table 2.

	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%
Hypothyreoidism	8%	0%
Other (anti-phospholipid syndrome, uterine myoma)	4%	0%

Table 2. Subjects' characteristics
Proportions of healthy subjects and each disease are listed as percentages

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In pregnant subjects the labour was uncomplicated in all cases (N=27 by transvaginal delivery and N=21 by Caesarean section). In ten cases the Caesarean section was elective, and in eleven cases the reasons for the operation included of oxytocin-resistant dystocia in 6 cases, abnormal foetal presentation in 3 cases, umbilical cord prolapse and threatening intrauterine hypoxia in 1-1 cases. 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.

In the validation part, 15 non-pregnant women (30±8 years), 15 pregnant subjects in their 2nd trimester (32±4 years, 22.3±3.5 gestational weeks) and 15 pregnant volunteers in their 3rd trimester (32±4 years, 37.6±2.7 gestational weeks) were recruited. None of these subjects were smokers, had respiratory tract infection in the last 4 weeks or suffered from any chronic disease including allergy, asthma, diabetes mellitus, liver or renal disease, or any obstetrical complications. They were also asked to avoid consuming food or beverages 2 hours before breath collection.

4.4.3. Exhaled breath collection and analysis

Exhaled breath collection and analysis were performed as it was described at section 3.2.2.

4.4.4. Statistical analysis

Commercially available statistical software (SPSS 15) was used for statistical analysis. Principal component analysis (see section 3.2.3) was applied for data reduction and the highest four principal components (capturing 99% of the variances within the dataset) were used for further analyses. The groups were compared applying the Mahalanobis-distance [162], a stepwise classification technique to classify cases into categorical division using the four principal components. Pearson's test was used to compare PCs with gestational age as well as the day of the menstrual cycle. In the validation part, the Mahalanobis-distance was used for the classification of newly sampled data into the preset groups. A $p<0.05$ value was considered significant.

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5. Results

5.1. Effect of respiratory droplet dilution on EBC pH

5.1.1. The effect of vacuum evaporation on NH_4OH and EBC conductivity

After vacuum evaporation the conductivity of serially diluted NH_4OH decreased by approximately 99% (from 486.15 to 5.78 µS/cm, from 347.15 to 5.715 µS/cm, from 306.6 to 1.43 µS/cm and from 172.65 to 1.275 µS/cm; 178.5, 89.3, 44.6 and 14.9 mM NH_4OH, respectively).

Furthermore, conductivity was also reduced in EBC samples of the seven healthy individuals from 52.65 (36.45-111.80) µS/cm to 6.22 (3.98-8.93) µS/cm (p=0.01) representing an 88% decrease.

5.1.2. EBC pH

There was no difference in pH between the duplicate EBC samples (7.91 (7.23-8.30) and 7.83 (7.23-8.12), p=0.48). The mean coefficient of variation was 3%.

There was no significant difference in EBC pH between asthmatic patients (7.91 (7.31-8.04)) and healthy subjects (7.83 (7.54-8.05); p=0.76, Figure 2). Similarly, no difference in EBC pH was found when ICS-naive asthmatic subjects (7.81 (6.81-8.00) were compared to controls (p=0.27).

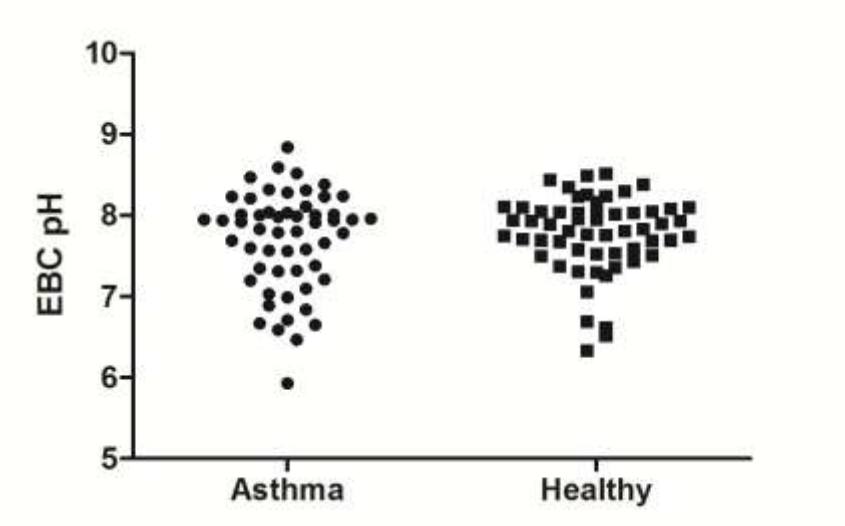

Figure 2. Comparison of EBC pH between asthma and health
No significant difference was observed in EBC pH between asthmatic patients and healthy subjects (p=0.76).

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5.1.3. Respiratory droplet dilution

There was no difference between the conductivity of the two parallel vacuum-treated samples (5.92 (5.87-8.42) µS/cm and 5.54 (4.48-9.44) µS/cm, p=0.58) representing estimated dilution rates of 2535 (1781-2558) and 2705 (1589-3349) (p=0.58). The mean coefficient of variation was 12%.

We could not find any significant difference in the conductivity of asthmatic (10.8 (5.6-17.1) µS/cm) and control (9.7 (6.4-15.7) µS/cm) samples. In line with this, respiratory droplet dilution rates were similar (2326 (1460-4630) vs. 2646 (1435-3969), p=0.41; asthma vs. control, respectively, Figure 3). Furthermore, there was no difference between ICS-naive asthmatic (2495 (1469-6411)) and healthy control EBC respiratory droplet dilution values, either (p=0.80).

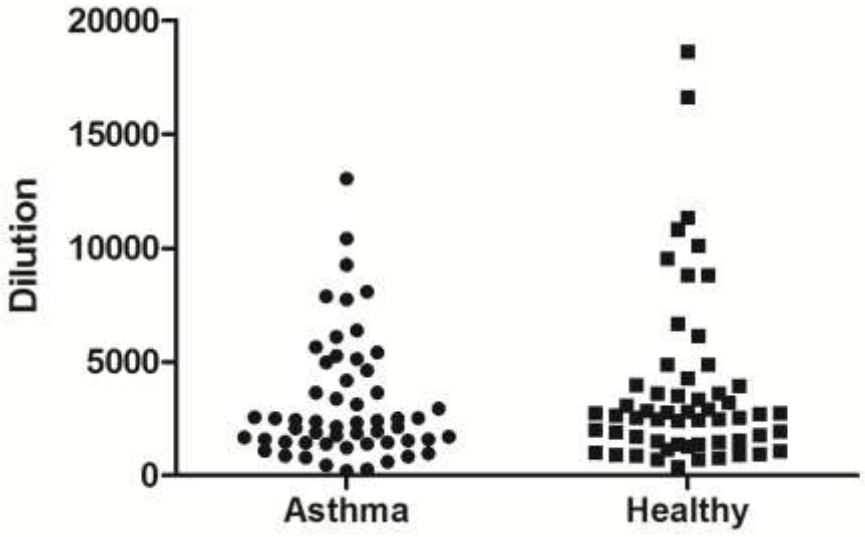

Figure 3. Comparison of EBC respiratory droplet dilution between asthma and health

No significant difference was observed in EBC respiratory droplet dilution between the asthmatic and healthy groups (p=0.41).

5.1.4. Relationship between EBC pH and respiratory droplet dilution

There was a significant negative relationship between EBC pH and respiratory droplet dilution both in asthmatic (p=0.01, r=-0.35, Figure 4a) and the healthy groups (p=0.02, r=-0.29, Figure 4b). Therefore, it was not surprising that the relationship was also significant when all subjects were analysed together (p<0.001, r=-0.32, Figure 4c). The relationship between EBC pH and respiratory droplet dilution was still significant after the adjustment to FEV_1, FE_{NO}, asthma control or ICS use (p<0.05 in each case).

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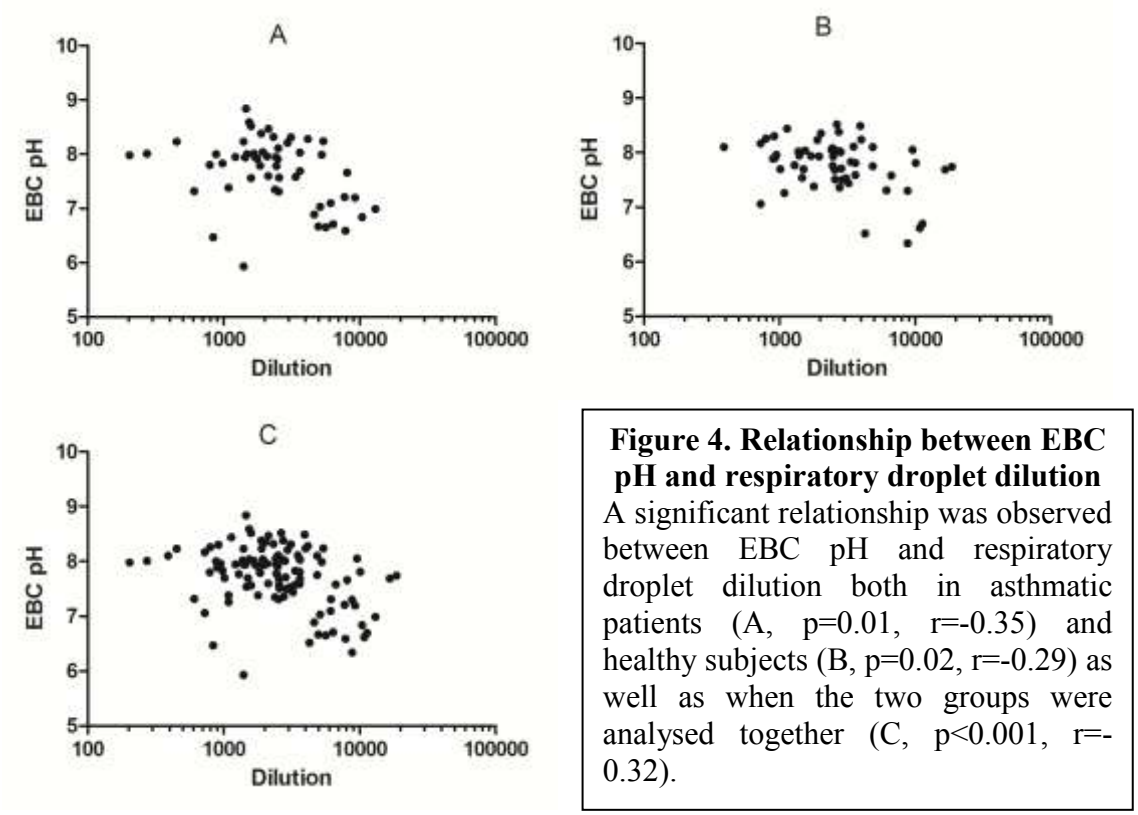

Figure 4. Relationship between EBC pH and respiratory droplet dilution
A significant relationship was observed between EBC pH and respiratory droplet dilution both in asthmatic patients (A, p=0.01, r=-0.35) and healthy subjects (B, p=0.02, r=-0.29) as well as when the two groups were analysed together (C, p<0.001, r=-0.32).

5.1.5. Relationship between EBC pH and clinical variables

EBC pH did not correlate with any of the clinical variables, including FEV_1, FVC or FE_{NO} either when analysed before or after adjustment to respiratory droplet dilution (p>0.05). There was no difference in EBC pH when controlled, partially controlled and uncontrolled asthmatic patients were compared either before (p=0.71) or after (p=0.87) adjustment to respiratory droplet dilution. There was no difference in EBC pH between ICS-naive (7.81 (6.81-8.00)) and steroid-treated (7.92 (7.37-8.23)) asthmatics (p=0.23). Furthermore, low EBC pH (≤7.2) was not associated with lower FEV_1, higher FE_{NO} or poorer asthma control (p>0.05).

5.2. Reproducibility of Cyranose 320 measurements

5.2.1. Short-term variability of Cyranose 320 measurements

There was no difference in Cyranose 320 results between the two subsequent measurements assessed with paired t-tests (p=0.85, 0.41 and 0.63, PC1, PC2 and PC3, respectively). There was a significant intra-class correlation between the two

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measurements in PC1, PC2 and PC3 ($p<0.05$, $r=0.51$, 0.75, and $r=0.37$, PC1, PC2 and PC3, respectively, Figure 5).

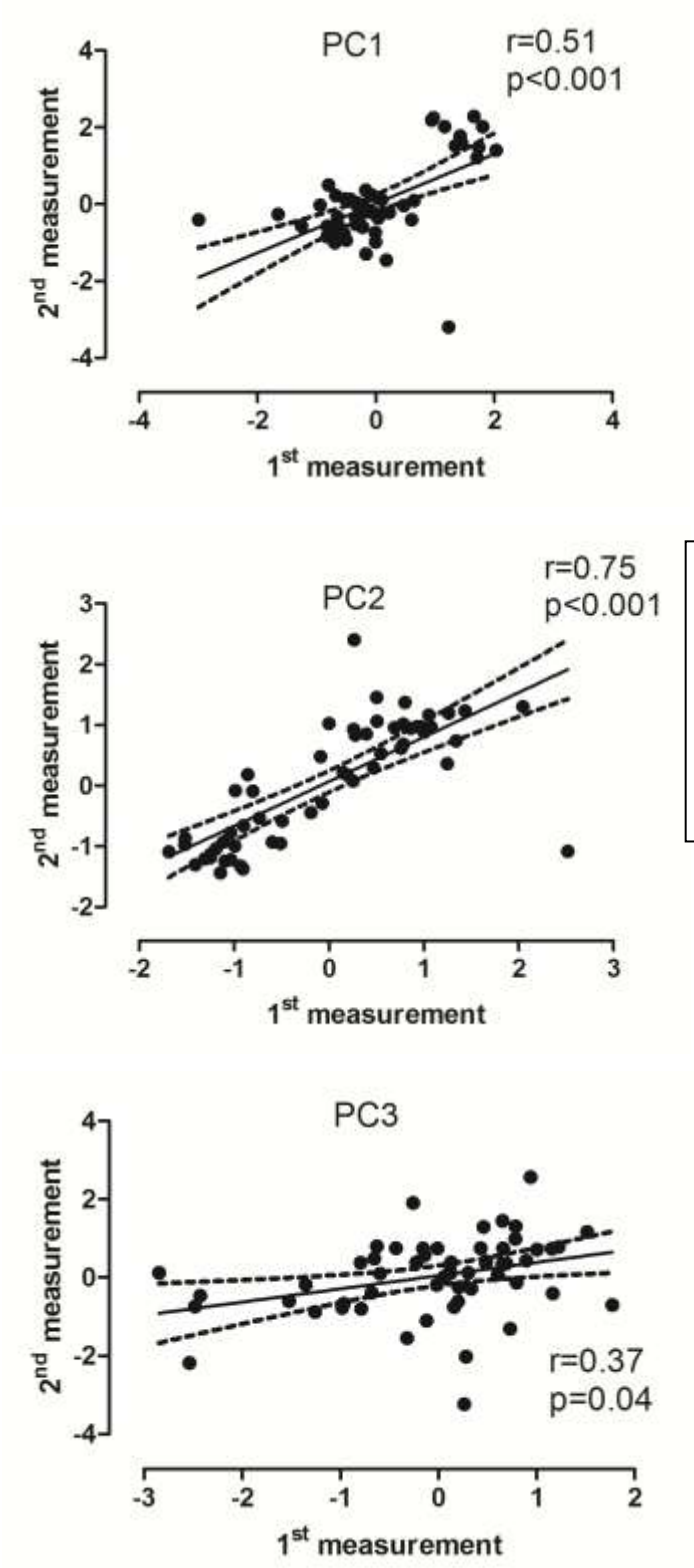

Figure 5. Intra-class correlation between two subsequent Cyranose 320 measurements.
The two measurements correlated significantly in PC1, PC2 and PC3.

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5.2.2. Long-term variability of Cyranose 320 measurements

We found significant intra-class correlations between the sensor responses within and between days with correlation coefficient (r) ranging between 0.7-0.9. There was also a significant relationship between baseline and 30 minutes, baseline and 1 day, baseline and 1 week, baseline and 4 weeks as well as baseline and 8 weeks measurements assessing the principal components (Table 3).

Measurement compared with baseline	PC1	PC2	PC3
30 minutes	**0.73**	0.11	0.24
60 minutes	0.05	0.16	0.24
120 minutes	0.25	0.06	0.29
1 day	**0.70**	0.08	0.36
1 week	**0.54**	0.28	0.34
4 week	0.52	0.26	**0.52**
8 week	**0.53**	0.33	0.32

Table 3. Correlation coefficient between baseline measurements and measurements at different timepoints
Correlation coefficients are expressed, significant relationships ($p<0.05$) are in bold.

Using repeated measures ANOVA, we did not find any significant difference in PCs along time ($p=0.13$, $p=0.15$ and $p=0.08$; PC1, PC2 and PC3, respectively).
However, analysing only within day changes (baseline, 30, 60 and 120 minutes) there was a tendency for changes assessed by PC1 ($p=0.07$), suggesting short term drift in E-nose readings (Figure 6).

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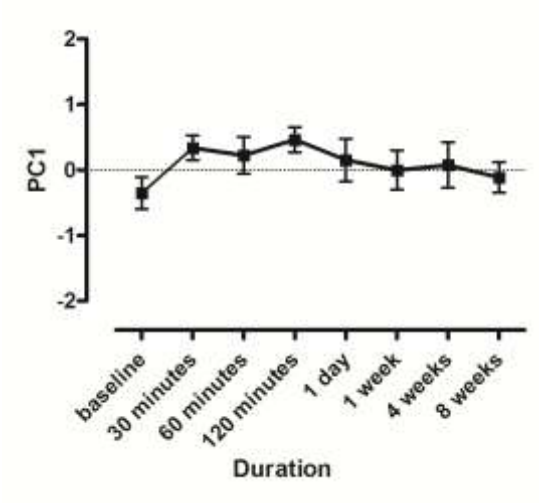
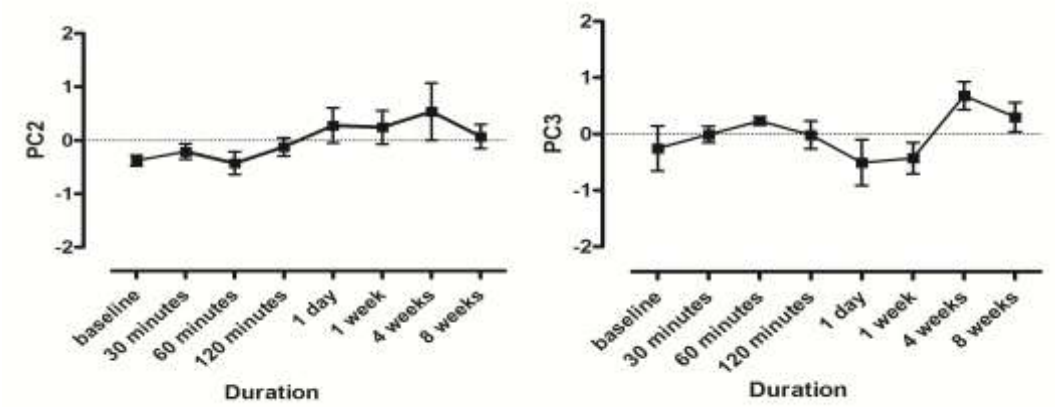


Figure 6. Long-term reproducibility of Cyranose 320 measurements
There was no change in PCs in time as assessed with repeated measures ANOVA. Data are expressed as mean±SD.



5.3. EBC pH and exhaled volatile compound pattern during physical exercise

No change was observed either in FEV_1 or FVC values following exercise ($p>0.05$).

5.3.1. Effect of physical exercise on volatile compound pattern

Exercise caused significant alterations in PC3 ($p=0.04$, mixed linear model, Figure 7), suggesting that this variable is responsible for exercise-related volatile compound changes. The differences were significant at 15 minutes ($p<0.01$), 30 minutes ($p=0.03$) and 60 minutes ($p=0.03$) following exercise compared to baseline, while no change was observed immediately post exercise ($p>0.05$). There was no difference between the post exercise time points.

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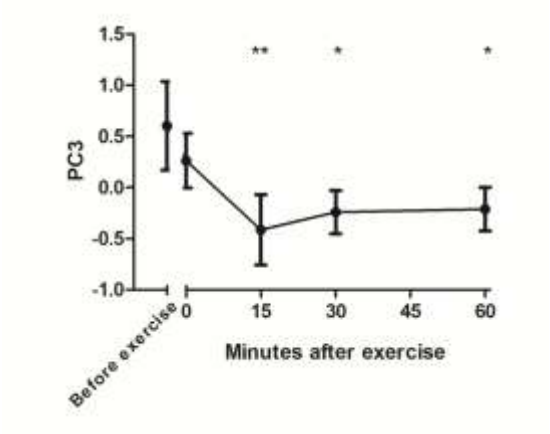

Figure 7. Effect of exercise on exhaled breath volatile compound pattern
Exercise caused significant alterations in principal component 3 (PC3, p=0.04). Significant differences were observed at 15, 30 and 60 minutes after exercise.
Data are expressed as mean±SEM
*-p<0.05, **-p<0.01

5.3.2. Effect of exercise on EBC pH

Exercise caused significant increase in EBC pH (p=0.01, mixed linear model, Figure 8). Significant differences were observed at 15, 30 and 60 minutes post exercise compared to the baseline value (p=0.04 in all cases). No difference was observed among the post exercise EBC pH values (p>0.05).

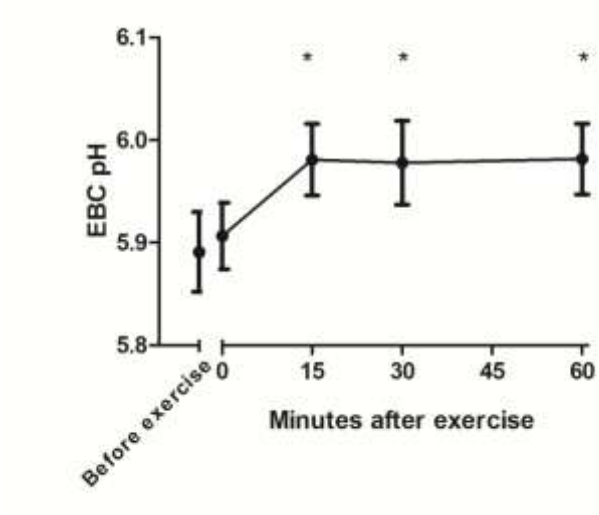

Figure 8. Effect of exercise on exhaled breath condensate pH
Exercise caused significant increase in exhaled breath condensate (EBC) pH (p=0.01). Significant differences were observed at 15, 30 and 60 minutes after exercise.
Data are expressed as mean±SEM
*-p<0.05

5.3.3. Relationship between exhaled breath volatile compounds and EBC pH

A significant relationship was observed between exhaled breath volatile compound pattern and EBC pH (p=0.01, r=-0.34, Figure 9).

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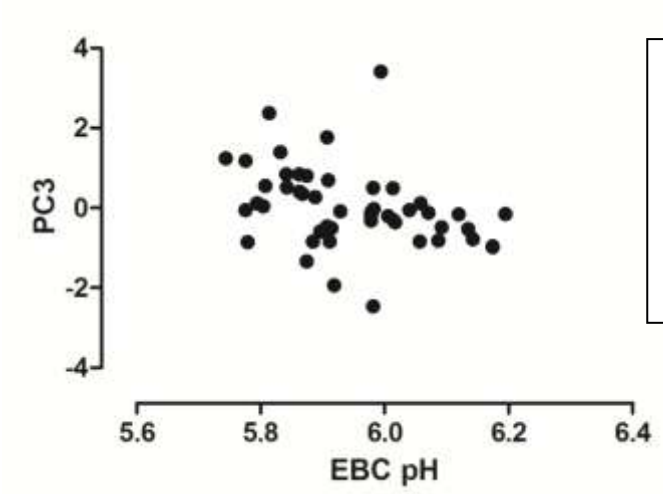

Figure 9. Relationship between exhaled breath volatile compound pattern and EBC pH
A significant correlation was found between EBC pH and PC3 when all samplings were plotted (n=50; p=0.01, r=–0.34)

5.4. Exhaled breath volatile compound pattern during pregnancy

5.4.1. Comparison of breathprints in pregnant and non-pregnant subjects

Exhaled breath volatile compound patterns ("breathprints") were significantly different in pregnant versus non-pregnant women (N=73, p=0.015, Figure 10).

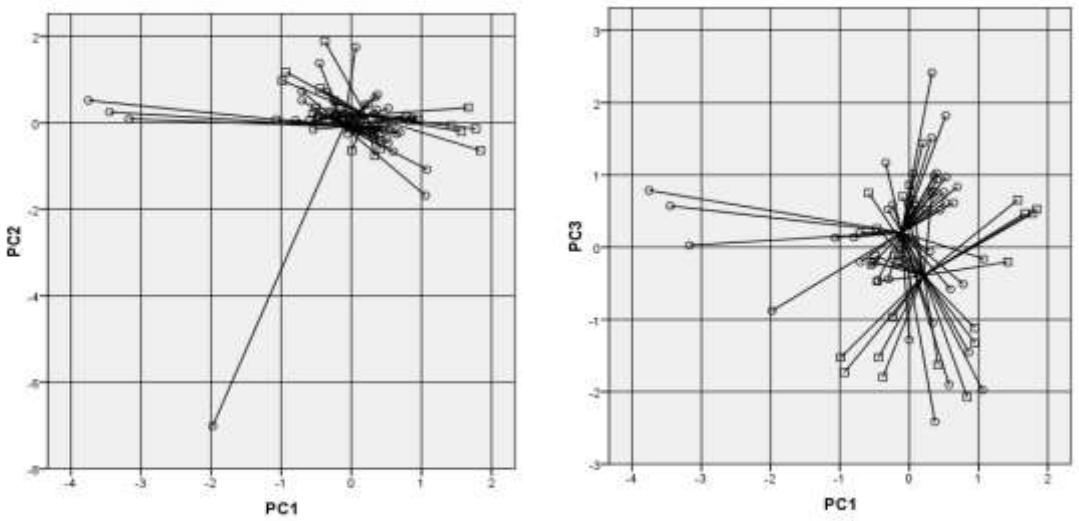

Figure 10. Comparison of "breathprints" between pregnant and non-pregnant subjects (N=73, principal component analysis plot)
Principal components 1, 2 and 3 are plotted against each other. The plot shows discrimination between pregnant (circles, N=48) and non-pregnant (squares, N=25) women along discriminate composite principal factors (p=0.015). The difference was still significant when the groups were compared without the four outlier individuals (p=0.017).

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Comparing only healthy subjects (N=38) the difference was also significant (p=0.001, Figure 11). In this case the two groups were separated better than in all subjects, therefore only healthy subjects were used to build up model for validation part.

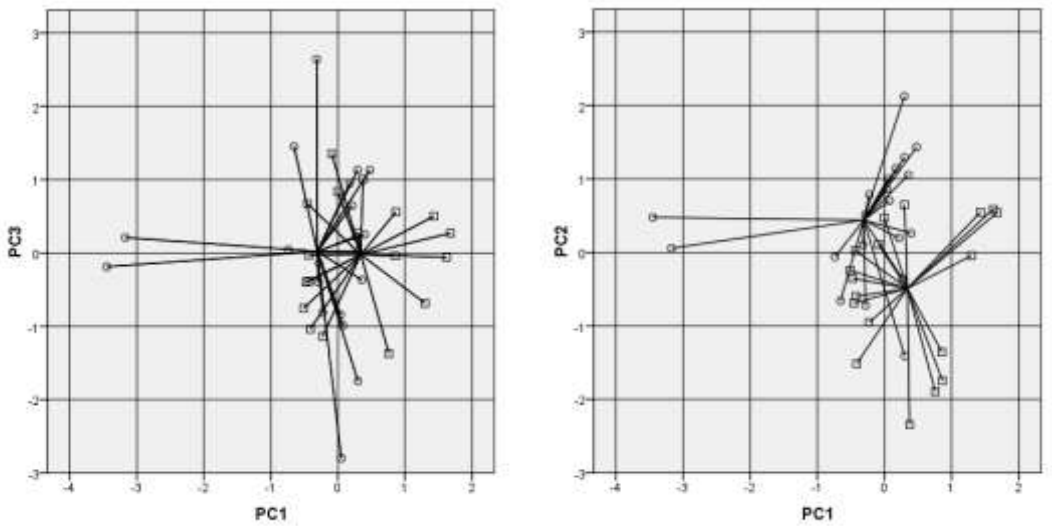

Figure 11. Comparison of "breathprints" between healthy pregnant and non-pregnant subjects (N=38, principal component analysis plot)
Principal components 1, 2 and 3 are plotted against each other. The plot shows discrimination between pregnant (circles, N=20) and non-pregnant (squares, N=18) women along discriminate composite principal factors (p=0.001).

5.4.2. The relationships between the breathprint and gestational age or the day and phase of menstrual cycle

We found significant differences in "breathprints" between the pregnant and non-pregnant groups in PC3, suggesting that this factor reflects the pregnancy-related changes. Therefore, we used this value for further analysis. We found a significant relationship between the "breathprint" and gestational weeks (p=0.01, r=-0.36, n=48, Figure 12).

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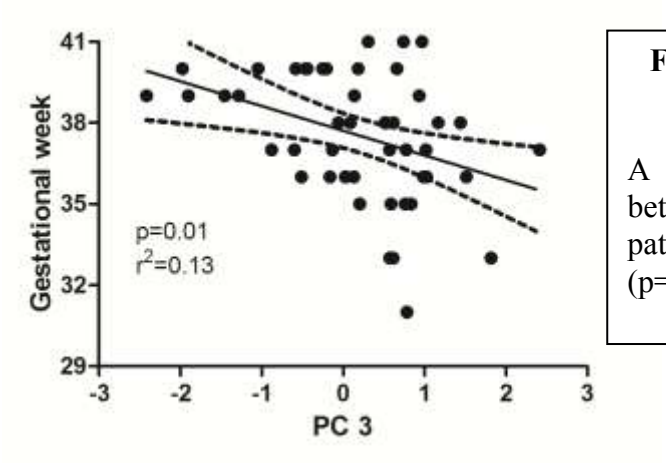

Figure 12. Relationship between "breathprint" and gestational weeks

A significant correlation was found between exhaled volatile compound pattern and the gestational weeks (p=0.01, r=-0.36).

However, there was no correlation between the "breathprint" and the day of menstrual cycle (p=0.38, r=-0.18, n=25). In addition, comparing women in their follicular (n=15) and luteal (n=10) phases, no difference was found between the two groups (p=0.66, Mahalanobis-distance).

5.4.3. Validation part

Similarly to the model setting part, comparing the 15 healthy pregnant women in their 3rd trimester with 15 healthy non-pregnant subjects we found significant differences (p=0.02). However, the 15 2nd trimester individuals could not be discriminated from either group (p>0.05).

Based on the model on healthy women (n=38), subjects in the validation part could be discriminated well (Mahalanobis distance, cross-validation value 80%). 13 of the 15 3rd trimester pregnant subjects and 11 healthy controls were classified correctly (87% sensitivity, 73 % specificity, 76 % positive predictive value and 84% negative predictive value, Figure 13). However, the 15 subjects in the 2nd trimester were classified poorly (47% sensitivity).

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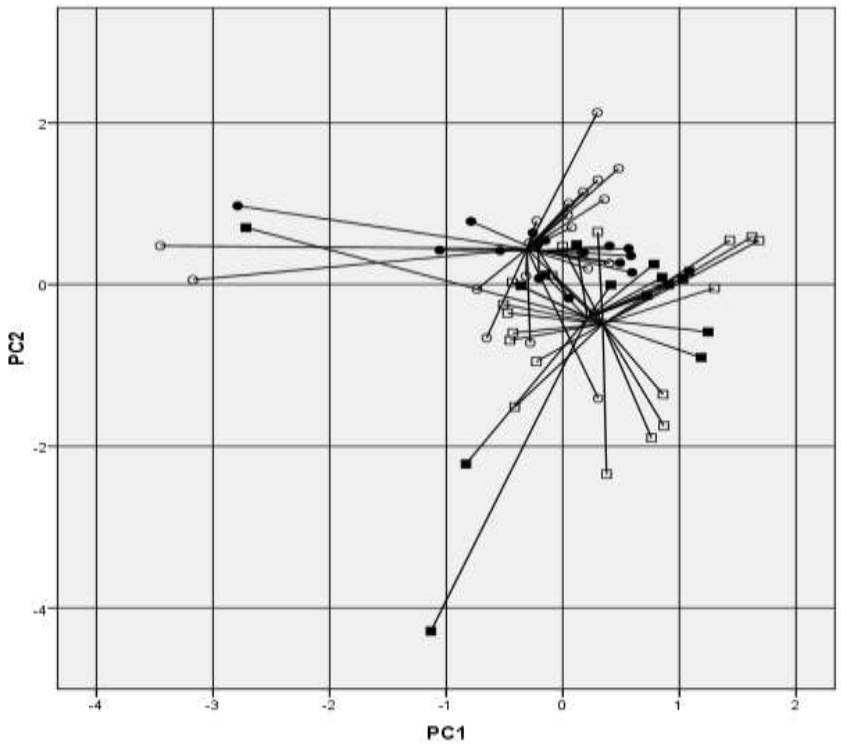

Figure 13. Validated two-dimensional principal component analysis plot on healthy subjects

Principal components 2 is plotted against principal component 1. The plot shows the classification of blindly collected pregnant (full circles) and non-pregnant (full squares) volunteers to the preset original groups (open circles-pregnant, open squares-non pregnant). The Mahalanobis-distance could correctly classify the new subjects with 87% sensitivity and 73% specificity.

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6. Discussion

6.1. The effect of respiratory droplet dilution on exhaled breath condensate pH

We have studied the influence of an important methodological factor, the respiratory droplet dilution on EBC pH. We have shown that there is a significant, albeit weak association between EBC pH and respiratory droplet dilution which may help the general interpretation of the EBC pH results.

pH is the most studied biomarker in EBC. Early studies suggested that by analysing EBC acidity one can estimate airway pH [27]. However, latter studies confirmed that EBC pH is also determined and influenced by countless of physiological and methodological factors [37]. These may explain the discrepancies between the early [27] and more recent [163] studies of the same workgroup.

It is well known that the EBC mediator concentrations cannot directly be extrapolated to airway mediator levels because the volume of water diluting airway droplet levels is variable. To resolve this error, a respiratory droplet dilution indicator is warranted. This indicator component must have a known concentration in the airways but cannot originate from the alveoli (the main source of diluting water). As measuring metabolites in airway lining fluid is difficult, we need constituents which have a known concentration in serum, diffuses through the cell membranes and well measurable in EBC. Several molecules, including proteins, urea, ions have been suggested as a dilution indicator [12], but the easiest solution is to measure the conductivity of ammonia-free condensate samples. Ammonia is the most prominent volatile which determines EBC conductivity, but it is produced mainly in the mouth, therefore needs to be eliminated for precise dilution calculations [25]. This can be achieved by lyophilisation [164, 165] or vacuum treatment [24, 30, 166] of EBC samples. It was shown that lyophilisation eliminates 99% of ammonia decreasing the conductivity of EBC by 90% [164]. In our present work we have shown that vacuum evaporation has a very similar efficacy. Of note, dilution estimates seem to be lower with the vacuum-treatment method than with lyophilisation [166]. Although values determined after

lyophilisation may be more reproducible, this technique needs approximately 1 hour of sampling which does not seem feasible in clinical settings [164].

The correction for a dilution factor has improved the scientific interpretation of results on EBC non-volatile compounds. However, it has been debated if respiratory droplet dilution influences the concentrations of EBC volatile molecules [167]. It is known that EBC pH is not only determined by the acidity of airway droplets, but also by volatile molecules dissolving into condensate fluid [37, 168].

Our study was the first attempt to investigate the association between EBC pH and respiratory droplet dilution. We found a significant, but weak, negative relationship between the two parameters. The precise reason for our findings is not known. A possible explanation could be that respiratory droplets are slightly alkaline [37], but during condensation and subsequent mixing with volatile gases and gastric droplets EBC pH may decrease. A higher dilution rate may reduce the buffer capacity of EBC by reducing the non-volatile buffer content, hence increasing the instability of EBC pH toward dissolution of airway volatiles.

6.2. Reproducibility of Cyranose 320 measurements

In this part we aimed to assess the reproducibility of the Cyranose 320 measurements. The sampling was controlled in all cases in order to investigate the effect of possible drift. We also tried to minimise the effect of environmental factors by asking the subjects not to consume food or beverages prior to the measurements.

We found no significant temporal change in exhaled volatile compound pattern measured by Cyranose 320 over short (within a day) or long (two months) period, however there was a within-day tendency for changes in the highest principal component (PC1). This might suggest a possible short term drift of Cyranose 320 sensors, and this has to be investigated. Nevertheless, our results confirm the previous findings that exhaled volatile compound pattern measurements are reproducible. Diskin et al. measured exhaled ammonia, acetone, isoprene and ethanol levels over 30 days. They found stable levels for acetone and ammonia, while ethanol and isoprene were more variable, but the latter two molecules were in a ten-fold lower concentration than ammonia and acetone [169]. Another study investigating a number of VOCs over 16 days supported these findings, showing that exhaled acetone levels are stable over days, while VOCs in a lower concentrations <100 ppb are less reproducible [170]. This

suggests that the relative stability of Cyranose 320 results might be due the relatively constant levels of to ammonia and acetone in the breath.

6.3. EBC pH and exhaled volatile compound pattern during physical exercise

We showed that physical exercise altered exhaled breath volatile compound pattern as well as exhaled breath pH. These exercise-caused changes maintained for at least one hour following the challenge. In addition, we observed a significant relationship between EBC pH and breath volatile compound pattern.

Physical exercise may modify the volatile composition of exhaled breath via several mechanisms. It is well-known that the physical challenge is associated with certain physiological alterations, such as the increase in cardiac output and ventilation rate, which can consequently influence the concentration of exhaled biomarkers [130, 132]. In addition, the airway production of some endogen metabolites can change during exercise in the airways as shown previously for H_2O_2 [134]. Finally, the alkalisation of airway tract may also alter the production of some exhaled volatile acids and bases by decreasing acids and increasing bases [143].

The major disadvantage of the unspecific E-nose approach is that we cannot investigate the individual volatiles responsible for the exercise-caused changes. It is known that carbon black sensors found in the Cyranose 320 are reactive to alkanes, esters, alcohols and volatile acids [95, 96]. Furthermore, Cyranose was able to measure sulphur-containing volatile compounds [122]. It is also known that breath volatile substances are measurable in a concentration higher than 100 ppb [96], therefore the potential candidates contributing to E-nose pattern are acetone, methanol, ethanol, isoprene, ammonia, pentane, hydrogen sulphide, xylene, acetonitrile and benzene. However, the last three substances were associated with smoking therefore they not seem to be related to the differences observed in our study [104, 105].

Isoprene and acetone are blood borne volatile substances, the levels of which are therefore elevated with increased cardiac output during exercise [130]. However, this seems to be an unlikely explanation for the differences observed in our experiment, as we found alterations in exhaled breath volatile compound pattern later than 15 minutes following exercise, when cardiac output presumably returned to resting level. Interestingly, in another study, where healthy subjects were instructed to cycle for 30

minutes, the increase in exhaled acetone and isoprene levels occurred in the first 5 minutes with a further constant decrease, even below the baseline levels [171]. Hence, the alteration in exhaled volatile compound pattern in our study might reflect the reduction of blood borne volatiles. Unfortunately, neither of the two studies [130, 171] assessed exhaled metabolite levels following the exercise challenge.

The effect of exercise on exhaled pentane, a marker of oxidative stress was investigated in healthy individuals as well as patients with renal failure [172]. The authors showed that pentane output increased during exercise and elevated further (20-fold increase compared to baseline) at 5 minutes following the challenge in healthy subjects and also in patients. Supposing that the potential concentration of pentane in exhaled breath is 1-100 ppb [103] changes of such a magnitude are presumably detected by Cyranose 320. However, no study has investigated the time course of breath pentane levels for 1 hour after exercise.

Breath levels of ethanol or methanol, other possible contributors to E-nose pattern have not been investigated during exercise. It is known that hyperventilation reduces the breath levels of both molecules [131]. However, this effect does not seem plausible explanation for our observations, as we detected alterations in exhaled volatile compound pattern 15 minutes following exercise, when the ventilation rate presumably returned to baseline.

Another possible explanation for the changes in exhaled breath volatile compound pattern may be the altered production of some airway volatile acids and bases. Propionic acid was previously found reduced in EBC following exercise [143]. Cyranose is sensitive for this compound, but its concentrations in EBC (12 μM to 8 μM, pre- and post-exercise, respectively) cannot be translated into volatile levels easily; therefore the exercise-induced propionic acid changes are not surely assessable with Cyranose 320. On contrary, ammonia in its volatile form is measurable in relatively high (ppm level) concentrations in exhaled breath, and therefore exercise-induced changes (184 μM to 253 μM in EBC) [143] could be measured with Cyranose 320 [100]. However, the most ammonia-sensitive sensors (6 and 23) were excluded in our experiment. Similarly to propionic acid, it is not known if the ammonia excess in exhaled breath is maintained for at least 1 hour. Nevertheless, the hypothesis that exhaled volatile acids and bases are responsible for “breathprint” alterations in our study is supported by the fact that EBC

pH is also altered and stays elevated following exercise. The relationship between exercise-induced EBC pH as well as propionic acid and ammonia changes was previously described by Greenwald et al. [143] and supported by our observations, namely that exhaled volatile compound pattern correlated with EBC pH. The cause and effect relationship between EBC pH and exhaled volatile acids and bases is not fully known. Besides the influence of volatiles, other factors can also affect EBC pH. Firstly, droplets which release from the ALF contain non-volatile ions which determine ALF acidity. Secondly, gastric acid droplets may mix with condensate in the pharynx and oral cavity reducing EBC pH. However, the opposite effect is equally true; the rate of how volatiles trap into condensate in their non-volatile form is determined by the EBC pH.

The exact reason for EBC alkalisation, although supported also by two independent workgroups [143, 144] has to be investigated.

The analysis of exhaled breath is the most feasible method to assess exercise-related changes in the airways, as it is non-invasive, carries no risks for side-effects, therefore can be repeated in short time intervals. It is particularly important when investigating the pathophysiology of exercise-induced bronchoconstriction (EIB), which is the temporary narrowing of the airways caused by physical exercise. Exhaled breath analysis has already provided useful information about changes ongoing in the airways during the development of EIB. We have shown that EBC adenosine [133] and cysteinyl-leukotrienes [29] levels increase during EIB. In addition, similar changes were shown for EBC endothelin-1 [173], eotaxin [174], C-reactive protein [175] and RANTES [176] in EIB patients following exercise. These studies facilitated our understanding of this disorder. Our findings suggest that exhaled volatile substances alter during exercise even in healthy subjects. These results are particularly important when assessing exhaled volatile compounds in EIB.

6.4. Exhaled breath volatile compound pattern during pregnancy

We found that the exhaled breath volatile compound pattern is altered in physiological pregnancy during the 3rd trimester compared to non-pregnant women. However, these differences were not present in the 2nd trimester. This and the relationship between gestational age and volatile compound pattern suggest that the production of volatile substances changes during the course of pregnancy. This is not surprising, as gestation-

related alterations of physiological processes, such as systemic metabolism and oxidative stress also show temporal patterns.

Possible volatile substances which may drive changes in Cyranose 320 results are acetone, methanol, ethanol, isoprene, ammonia, pentane, hydrogen sulphide, xylene, acetonitrile and benzene. Among these molecules only pentane has been investigated in breath samples during gestation, previously. Shin et al. showed that exhaled pentane increased during delivery; however, that study did not compare baseline pre-labour levels to non-pregnant women [152]. Nevertheless, there are some possible explanations for the observed differences.

It is known that systemic metabolism is accelerated during pregnancy [148]. This might change the output of certain blood borne volatile substances. In addition, increased systemic oxidative stress is associated with healthy pregnancy [149]. Contrarily, reduced levels of H_2O_2 were found in exhaled breath samples during gestation [151], however exhaled ethene, another marker of oxidative stress did not differ from levels measured in non-pregnant women [153]. A study monitoring oxidative stress in healthy pregnant women showed that systemic oxidative stress is elevated at the beginning and tends to decrease towards the end of gestation [177]. This study suggests that systemic oxidative stress is not the most plausible reason for our results, as we did not find differences at the 2nd trimester when oxidative stress is presumably more elevated compared to the 3rd trimester, when it tends to be more similar to the non-pregnant state. However, interestingly, an inverse relationship has been found between systemic and exhaled oxidative stress markers, previously [151]. This might suggest that despite the "normalisation" of systemic oxidative stress towards the end of pregnancy, the magnitude of airway oxidative stress-related differences between pregnant and non-pregnant women may increase in breath samples.

Pregnancy is associated with altered hormone levels, and one study found a significant relationship between serum 17β-oestradiol and exhaled H_2O_2 levels suggesting that hormones may affect exhaled substances [151]. Supporting this, acetone release in urine is affected by menstrual cycle [178]. While no study investigated the effect of menstrual cycle on exhaled acetone levels, it is known that breath acetone concentration is related to acetone in other biological matrices including urine [179]. Ovulation also increases breath volatile sulphur compounds [180]. On the contrary, menstrual cycle did not

affect breath ethanol kinetics [181]. Nevertheless, we did not find any alterations in exhaled volatile compound pattern between non-pregnant women in their follicular or luteal phases. This may suggest that the change in hormone levels is not a plausible reason for the observed differences, either.

Pregnancy is also described by immune tolerance [150]. Systemic [125] and airways [126] inflammatory markers were related to exhaled "breathprint" measured by Cyranose 320. Major histocompatibility complex (MHC) genes can produce volatiles [182]. The soluble form of these human leukocyte antigen (HLA) molecules can be present in blood and urine samples of mice and detectable by a quartz crystal microbalance-semiconducting metal-oxide sensor electronic nose [183]. So far, no studies have been conducted to assess these molecules in human breath, but foetal HLA molecules might be present in breath, and an altered mixture of maternal and paternal volatiles by the foetus can provide an explanation for our findings.

The production of volatile substances, the so called pheromones were shown to be altered during pregnancy both in animal [184] and human [185] studies. In humans, the following substances were identified from para-axillary and nipple-areola sweat patch samples: 1-dodecanol, 1-1'-oxybis octane, isocurcumenol, alpha-hexyl-cinnamic aldehyde, and isopropyl myristate [185]. The chemical structure of these molecules allows detection by Cyranose 320, however it is not known whether they are present in human breath and in a measurable concentrations. In addition, the role and function of pheromones in humans are also debated [185].

Finally, we have recently shown that exhaled breath condensate pH is also altered in pregnancy, as we found higher EBC pH in healthy pregnant vs. healthy non-pregnant women [186]. As the production of volatile compounds measured with Cyranose 320 is associated with EBC pH, as shown during exercise, changes in airway acidity during pregnancy might partly explain our results.

While the differences between pregnant and non-pregnant women were obvious irrespective of their medical conditions, we showed that the discriminative function of exhaled breath analysis was greater when only the healthy subjects were studied. These results are similar to findings of Fens et al. who showed that the differences between healthy subjects and patients with pulmonary embolism diminished when patients with co-morbidities were also analysed [80]. While our study was not powered to investigate

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this effect, confounding factors such as smoking might most probably influence the electronic nose readings as shown before [90].

7. Conclusions

7.1. The effect of respiratory droplet dilution on exhaled breath condensate pH

1. There is a significant negative relationship between EBC pH and dilution factor both in asthmatics and healthy subjects.
2. EBC pH and dilution factor measurements are reproducible.
3. There is no difference in EBC pH or dilution factor between patients with stable asthma and healthy controls.
4. EBC pH and dilution are not associated with lung function, exhaled nitric oxide or inhaled corticosteroid use.

7.2. Reproducibility of Cyranose 320 measurements

1. "Breathprints" obtained with Cyranose 320 are reproducible within a day.
2. "Breathprints" obtained with Cyranose 320 are reproducible within 8 weeks.

7.3. EBC pH and exhaled volatile compound pattern during physical exercise

1. Exercise alters exhaled breath condensate pH and exhaled volatile compound levels, therefore subjects should avoid physical exercise at least 1 hour before breath sampling.
2. There is a significant association between EBC pH and exhaled "breathprint" obtained with Cyranose 320.

7.4. Exhaled breath volatile compound pattern during pregnancy

1. Pregnancy alters exhaled volatile compound pattern ("breathprint") as it was shown before and after validation of discrimination model.
2. Accompanying disorders and smoking affect the discrimination ability of electronic nose to detect pregnancy.
3. Changes in exhaled "breathprint" are related to the stage of pregnancy.

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8. Summary

Exhaled breath analysis has a promising, still undiscovered potential in the diagnosis and the monitoring of systemic and respiratory disorders. It is completely non-invasive and harmless and the collection of samples does not interfere with the studied metabolic and pathologic processes.

Exhaled breath condensate analysis and exhaled volatile compound measurements showed promising results in discriminating common, in some cases severe respiratory diseases such as asthma, COPD or lung cancer. However, these approaches still remained only research tools and were not introduced into clinical practice. This may be due to so far unidentified methodological and physiological factors which hamper the widespread clinical use of exhaled breath analysis. The main objective of this PhD work was to study the effect of various methodological and physiological factors, thus facilitating the understanding of exhaled biomarker results.

We were the first to show that exhaled breath condensate pH is negatively influenced by respiratory droplet dilution. Our results suggest that dilution measurements must be performed in parallel with EBC pH analysis.

We have also shown that exhaled volatile compound measurements with Cyranose 320 are reproducible and E-nose measurements do not show a temporal change. This is important when using this method in follow-up studies of patients with respiratory disorders.

We demonstrated that physical exercise influences both EBC pH and the levels of exhaled volatile gases. This information could alert researches when conducting studies and aid the interpretation of previous results.

Finally, we found that in pregnancy a condition where non-invasive diagnostics are preferred, the levels of exhaled volatile compounds are altered. This may be important when investigating changes of respiratory physiology during gestation and also when conducting exhaled breath studies in pathological pregnancies.

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9. Összefoglalás

A kilégzett levegő vizsgálata ígéretes, de még nem teljesen feltárt lehetőségeket rejt a szisztémás és légúti betegségek diagnosztikájában és követésében. A módszer legfőbb előnyei közé tartozik, hogy teljesen ártalmatlan és a mintagyűjtés önmagában nem befolyásolja a vizsgált metabolikus és patológiás folyamatokat.

A kilégzett levegő kondenzátum és a kilégzett illékony anyagok vizsgálata biztató eredményeket mutattak a gyakori, esetenként súlyos légúti betegségek, mint az asztma, a COPD és a tüdőrák elkülönítésében, ugyanakkor ezek a módszerek továbbra is csak kutatási fázisban vannak és még nem részei a klinikai gyakorlatnak. Ennek oka az eddig nem feltárt metodikai és élettani tényezőknek tulajdonítható, melyek hátráltatják a módszerek széles körű klinikai elterjedését. A jelen PhD munka legfőbb törekvése az volt, hogy tanulmányozza a különféle metodikai és élettani tényezőket, hogy ezáltal jobban megérthessük és pontosabban értelmezhessük a kilégzett biomarker eredményeket.

Elsőként mutattuk ki, hogy a kilégzett levegő kondenzátum pH-ját negatívan befolyásolja a légúti folyadékcseppek hígulása. Eredményeink azt jelentik, hogy a hígulás mérését párhuzamosan minden EBC pH méréssel el kell végezni.

Azt is kimutattuk, hogy a kilégzett illékony anyagok Cyranose 320 elektromos orral történő mérése jól reprodukálható és az E-orr eredmények nem változnak az idővel. Ez fontos tanulság, ha a módszert a légúti betegségek követésében szeretnénk használni.

Kimutattuk, hogy a fizikai terhelés befolyásolja mind az EBC pH-t, mind a kilégzett illékony anyagok koncentrációját. Eredményeink figyelmeztethetik a kutatókat a vizsgálatuk megtervezésénél és segíthetnek a korábbi eredményeik értelmezésében.

Végül kimutattuk, hogy a terhesség, ahol a non-invazív diagnosztikát különösen előnyben részesítjük, önmagában megváltoztatja a kilégzett illékony gázok szintjét. Ezt figyelembe kell venni a terhesség alatti légúti élettani folyamatok tanulmányozása során illetve, amikor a kilégzett levegőt patológiás terhesség vizsgálatára kívánjuk használni.

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11. The bibliography of the candidate's publications

11.1. Publications related to the theme of the PhD 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.
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- **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.
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11.2. Other publications

- 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.
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