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Breath biomarkers for lung cancer detection and assessment of smoking related effects — confounding variables, influence of normalization and statistical algorithms

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ABSTRACT

Background: Up to now, none of the breath biomarkers or marker sets proposed for cancer recognition has reached clinical relevance. Possible reasons are the lack of standardized methods of sampling, analysis and data processing and effects of environmental contaminants.
Methods: Concentration profiles of endogenous and exogenous breath markers were determined in exhaled breath of 31 lung cancer patients, 31 smokers and 31 healthy controls by means of SPME-GC-MS. Different correcting and normalization algorithms and a principal component analysis were applied to the data.
Results: Differences of exhalation profiles in cancer and non-cancer patients did not persist if physiology and confounding variables were taken into account. Smoking history, inspired substance concentrations, age and gender were recognized as the most important confounding variables. Normalization onto PCO_2 or BSA or correction for inspired concentrations only partially solved the problem. In contrast, previous smoking behaviour could be recognized unequivocally.
Conclusion: Exhaled substance concentrations may depend on a variety of parameters other than the disease under investigation. Normalization and correcting parameters have to be chosen with care as compensating effects may be different from one substance to the other. Only well-founded biomarker identification, normalization and data processing will provide clinically relevant information from breath analysis.

1. Introduction

More than 3000 different substances can be determined from human breath [1–3] by means of hyphenated analytical techniques [4,5]. Some of these substances have been described as being linked to lung disease, inflammatory and malignant processes in the body [6–9]. In addition, previous exposure to various chemical substances may be recognized in this way. As breath analysis is completely non-invasive it holds promise for screening purposes. Prognosis of malignant diseases such as bronchial carcinoma [10,11] could be significantly improved if early diagnosis was possible by means of non-invasive screening tests. Correlations between lung cancer and different exhaled breath biomarkers have been reported [12–18]. But up to now, none of the markers or marker sets proposed for cancer recognition reached clinical relevance in terms of reliable disease recognition and sufficient sensitivity and specificity. Crucial and still unsolved issues in breath analysis are ambient concentrations of potential biomarkers, prior intake and actual excretion of environmental contaminants and the lack of standardized and generally accepted methods of sampling, analysis and data processing.

Clear distinction of endogenous disease related biomarkers from contaminants originating from the actual environment or from prior uptake is indispensable for clinically relevant breath testing. Hence reliable methods for breath sampling, separation and identification of volatile substances have to be set up, and, finally, physiologically sound and smart algorithms for data processing have to be applied.

Within a clinical study in lung cancer patients, smokers and healthy non-smoking controls, we looked upon endogenous volatile substances, compounds occurring in cigarette smoke and contaminants from the clinical and laboratory environment. Finally, different algorithms were applied to the data in order to account for inspired concentrations and physiological variables such as body surface area (BSA) or minute ventilation.

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2. Materials and methods

2.1. Study design

Exhaled VOCs were measured in alveolar breath of lung cancer patients, healthy smokers and healthy non-smokers. In parallel, room air (inspired air) was collected for background correction. Physiological parameters such as body weight, body height, blood pressure, and heart rate were recorded.

2.2. Demographics

The study was approved by the local University Medical Centre Ethics Committee and all subjects gave their written informed consent.

93 individuals were enrolled into the study. 31 of them (23 male, 8 female) suffered from lung cancer, 31 were healthy smokers (9 male, 22 female) and 31 were healthy non-smoking controls (10 male, 21 female). Nineteen lung cancer patients had non-small cell lung cancer (NSCLC) and twelve had small cell lung cancer (SCLC). Patients and volunteers were classified according to smoking status and pack years; they were not on any special diet and did not consume food or cigarettes for at least one hour before breath sampling.

Lung cancer patients were recruited from the department of pneumology. They were in hospital for primary diagnosis, staging, surgical, radio- and/or chemotherapy.

Samples of expired air were collected before chemotherapy or radiation therapy was initiated. Two lung cancer patients had a history of smoking <4 pack-years and all others had a history of smoking between 15 and 60 pack-years. All lung cancer patients had a tumour stage >T2 according to TNM-Classification. Patients enrolled into the smoker group had to feature at least 6 pack-years of smoking history. Healthy volunteers came from the general population and had no history of cancer or any other chronic diseases. Demographic characteristic of patients and volunteers are summarized in Table 1.

2.3. Determination of cancer biomarkers in blood

Concentrations of neuron-specific enolase (NSE), carcino-embryonic antigen (CEA) and cytokeratine fragment 21-1 (Cyfra 21-1) were determined in the blood of all patients and volunteers. These biomarkers have currently been proposed for diagnosis and staging of lung cancers. Analysis of blood cancer biomarkers was performed in the central laboratory of Rostock University Hospital by means of commercially available kits using electrochemiluminescence immunoassays (ECLIA, Elecsys system 2010, Roche Diagnostics GmbH, Germany).

2.4. Breath gas sampling

Breath gas sampling was done in a separate room after patients or volunteers had been resting for 10 min. Controlled alveolar breath gas sampling based upon fast-responding mainstream CO_2 measurement (Capnogard, Novametrix, USA) was applied for all patients and volunteers as described before [19]. In brief, 10 mL exhaled alveolar air were drawn into a gastight syringe under visual control of expired PCO_2 and immediately transferred into an evacuated sealed 20 mL headspace vial. Inspired samples were taken from room air in parallel. All measurements were made in duplicate. All breath gas samples were processed within 6 h after sampling. End tidal PCO_2 concentrations and respiratory rates were recorded during sampling.

Table 1
Patients' demographics.

	Lung cancer (NSCLC/SCLC)	Smokers	Controls
Subjects (n)	31 (19/12)	31	31
Age (mean, years)	68	37	30
Sex (male/female)	23/8	9/22	10/21
Current smokers	–	31	4
Ex smokers	29	–	–
Never smokers	2	–	27
Pack-years	4–60	6–50	occasionally

Abbreviations: 1 pack-year = 20 cigarettes per day/year; NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer patients.

2.5. Standards and materials

C1–C6 standard mixtures, n-heptane, branched hydrocarbons, dimethyl sulfide, cyclohexanone, 2,5-dimethylfuran, dimethyl formamide were obtained from Sigma Aldrich (Steinheim, Germany). Aldehyde standard mixtures (C1 to C10 aldehydes, 2-propenal and 2-butenal) and a mixture of different volatile organic compounds (formaldehyde, acetaldehyde, methanol, ethanol, 2-methyl-1,3-butadiene, acetone, 2-propenal, acetonitrile, 2-butanone, benzene, 2-butenal, toluene, chlorobenzene, 1,2-dimethylbenzene, 4,7,7-trimethylbicyclo[3.1.1] hept-3-ene, 1,2-dichlorobenzene and 1,2,4-trichlorobenzene) stored in stainless steel canisters were purchased from Ionimed Analytik (Innsbruck, Austria).

Gas tight syringes were purchased from Hamilton (Bonaduz, Switzerland), and 0.1 L gas bulbs from Supelco (Bellefonte, CA, USA), 20 mL headspace vials, Teflon coated rubber septa in combination with magnetic crimp caps were purchased from Gerstel (Muelheim an der Ruhr, Germany) and Teflon coated butyl septa in combination with magnetic crimp caps were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Helium and nitrogen of purity 5.0 (i.e., 99.999%) were purchased from Linde (Vienna, Austria), and Tedlar bags came from SKC (Eighty Four, PA, USA).

2.6. Calibration solution

Liquid reference substances were transferred into a 100 mL evacuated gas sampling bulb by means of a 10 μL syringe. The gas sampling bulb was equilibrated with nitrogen. Discrete volumes of this gas mixture were then transferred into a Tedlar bag filled with nitrogen by means of a 1 mL syringe. In addition, reference gaseous standard mixtures were filled in the same bag. This stock mixture was appropriately diluted with nitrogen to obtain the desired concentration levels. Calibration samples containing adequate VOC concentrations were transferred into 20 mL evacuated sealed glass vials and equilibrated with nitrogen.

2.7. Analytical procedures

A selection of 42 volatile organic substances (hydrocarbons, ketones, aldehydes, alcohols, nitriles, amides, furanes, sulfide and aromatic compounds) was quantified in the breath samples. Each substance was identified by its mass spectrum and its retention time. Identification was confirmed by comparing the retention times and mass spectra with those of pure standard substances.

Linear range, limit of detection (LOD) and limit of quantification (LOQ) were determined using a seven point calibration (six repetitions) according to DIN 32645. Linearity of the method was assessed in the way that different concentration ranges of the compounds were examined.

2.8. SPME-GC-MS analysis

Volatile substances in the samples were preconcentrated by solid-phase micro extraction (SPME) using 75 μm Carboxen/PDMS coated fibres (Supelco, Bellefonte, PA, USA) as described before [20]. A CTC Combi PAL SPME autosampler was used for automated preconcentration and desorption of the volatile organic compounds. The sample vials were agitated for 3 min at 40 °C in the heating block of the CTC autosampler. Subsequently, the SPME fibres were inserted into the

vials for 7 min for preconcentration. Release of the substances from the SPME fibre was achieved by direct desorption in the heated injector of the GC in the splitless mode (60 s). The injector was equipped with a 0.75 mm I.D. SPME inlet liner (Supelco, Bellefonte, PA, USA) with a constant temperature of 290 °C. Prior to the next analysis fibres were preconditioned for 28 min in the heated injector. The GC-MS analysis was performed with a Varian Star 3900 CX gas chromatograph equipped with a Varian Saturn 2100 mass ion trap spectrometer. A CP PoraBond Q (25 m; 0.32 mm; 5 μm film thickness) capillary column from Varian was applied for substance separation. Initial inlet pressure was 64.33 kPa at 90 °C. The carrier gas was helium with a constant flow of 1.7 mL/min resulting in a flow velocity of 34.05 cm/s. The initial oven temperature was 90 °C for 6 min, then was raised by 15 °C/min to 120 °C and kept at this temperature for 1 min, then raised to 140 °C at a rate of 10 °C min and kept at this temperature for 7 min and finally raised to 260 °C at a rate of 15 °C/min and held there for 6 min. For mass spectrometry, total ion current with a mass range of 35–300 amu was monitored for all samples using electron-impact ionization (70 eV). A scan rate of 1 scan/s was applied. The ion source and transfer line temperature were maintained at 150 °C and 200 °C, respectively.

2.9. Statistical analysis

Data were processed by means of five different algorithms:

a) Expiratory concentrations in single breath alveolar samples were examined.
b) Alveolar concentrations were normalized to body surface area (BSA). Body surface area was calculated according to the Dubois formula:

$$BSA\left(m^2\right) = 0.007184 \times weight(\mathrm{kg})^{0.425} \times height(\mathrm{cm})^{0.725}.$$

c) Alveolar concentrations were normalized to end tidal PCO_2 ($P_{et}CO_2$).
d) Inspired VOC concentrations were subtracted from alveolar concentrations. Negative results were set to zero.
e) A principal component analysis (PCA) was done with different data (sub) sets.

Statistical calculations were done using SigmaStat 3.5/SigmaPlot 10.0 and using the software The Unscrambler® (CAMO). Mann–Whitney Rank test (two groups) Kruskal–Wallis One Way Analysis of Variance (>2 groups) and post hoc Student–Newman–Keuls or Dunn's Method were employed to detect significant differences between groups. Results are given as medians and 25th–75th percentiles or as means and standard error of the mean (SEM). A $p<0.05$ was considered to be statistically significant.

PCAs were validated by means of a leverage correction and were calculated with 20 components. For the PCA compounds were excluded if their concentrations were below LOQ in more than 50% of the patients. As some results were in counts and not in nmol/L and concentration ranges of the substances were quite large data were standardized. In this way, results with low means and consecutively low variance were equally weighted as results having large means. Four participants (1 lung cancer patient, 1 healthy smoker, 2 healthy non-smoking controls) were not included, since their results would have dominated the model too much.

3. Results

3.1. VOC analysis

Table 2 shows analytical details and quantitative results for 42 selected volatile organic compounds. 14 of these 42 VOCs were basically branched hydrocarbons and could not be detected in the samples.

Results were processed in five different ways:

a) expired concentrations were determined for single breath alveolar samples,
b) concentrations were normalized to body surface area and
c) end tidal PCO_2, and
d) expired concentrations were corrected (subtraction) for inspired values, negative results were set to zero.
e) In addition, a principal component analysis was done.

3.1.1. Univariant VOC data analysis

Table 3 summarizes exhaled concentrations in single alveolar breath (EX), exhaled concentrations normalized to BSA (EX/BSA) or $P_{et}CO_2$ ($EX/P_{et}CO_2$) and expired concentrations corrected for inspired concentrations (EX-INS) of 14 substances showing significant differences between lung cancer patients, smokers and non-smoking controls.

Significant differences between female and male subjects were found for exhaled 2-methyl-1,3-butadiene ($p=0.002$), exhaled propanal ($p<0.001$) and exhaled isopropanol ($p<0.001$) concentrations. Median values and 25th and 75th percentiles were 4.87 (2.99–6.70) nmol/L for 2-methyl-1,3-butadiene, 0.10 (0.00–0.34) nmol/L for propanal, 3.68 (1.50–9.53) nmol/L for isopropanol in males and 3.01 (1.96–4.86) nmol/L for 2-methyl-1,3-butadiene, 0.00 (0.00–0.00) nmol/L for propanal and 1.33 (0.61–2.12) nmol/L for isopropanol in females. Individuals younger than 40 years exhaled significantly less 2-methyl-1,3-butadiene than older ones ($p=0.003$). Median exhaled 2-methyl-1,3-butadiene concentrations and 25th and 75th percentiles were 4.84 (2.84–6.34) nmol/L for individuals older than 40 years and 3.05 (1.78–4.63) nmol/L for individuals below the age of 40 years.

Body surface area itself showed no differences between the investigated groups ($p=0.279$). Mean values and standard deviations were 1.89 (0.21) m^2 for lung cancer patients, 1.80 (0.22) m^2 for smokers and 1.83 (0.20) m^2 for non-smokers. End tidal PCO_2 showed significant differences when smokers, lung cancer patients and non-smoking controls were compared to each other ($p=0.004$). Mean values and standard deviations were 5.53 (0.73) kPa for smokers, 4.86 (0.69) kPa for lung cancer patients and 5.01 (0.97) kPa for healthy non-smokers.

Exhaled concentrations of 2-butanone, cyclohexanone, ethanol, acetaldehyde, pentanal, heptanal, 2-propenal, propane, pentane, hexane, heptane, 2-methylbutane and 1,2-dimethylbenzene did not show any statistical significances between the study groups. In addition, concentrations of exhaled VOCs showed no differences between NSCLC and SCLC patients.

3.1.2. Multivariate VOC data analysis

Fig. 1 shows results from the PCA based on (a) expired concentrations from single breath alveolar samples and (b) from expired concentrations corrected for inspired concentrations ($C_{EX}–C_{INS}$) where negative value had been set to zero. Smokers correlated positively on PC1 and non-smokers correlated negatively on PC1 regardless if correction for inspired concentrations was applied or not. The scores plot based on expired concentrations showed a tendency towards positive values on PC3 for lung cancer patients; healthy controls showed a tendency towards negative values. After correction for inspired substance concentrations no discrimination between lung cancer patients and healthy controls could be observed anymore. Table 4 shows loading values of PC1 and PC3 from the PCA based on uncorrected and corrected data. On PC1 2,5-dimethylfuran, acetonitrile, benzene and toluene showed the strongest positive correlation. Without correction, isopropanol and 1-propanol were the most positively correlated substances on PC3. After correction dimethyl formamide was the most positively correlated substance on PC3 and 2-butanone, hexanal and butane were the strongest negatively correlated substances. 38% of the

Table 2
Calibration: quantification ions, linear ranges, limits of detection and quantification and detection ranges for 42 volatile organic compounds.

Compound	Quan ion	Linear range (nmol/L)	Correlation coefficient r	LOD (nmol/L)	LOQ (nmol/L)	Detection range in exhalation samples (nmol/L)	Detection range in inhalation samples (nmol/L)
2-Methyl-1,3-butadiene	67	0.10–22.53	1.000	0.024	0.095	0.53–14.77	0.10–1.62
Acetone	43	0.99–23.65	1.000	0.266	0.985	0.99–129.70	0.99–21.36
2-Butanone	43	0.16–24.54	0.998	0.043	0.158	0.16–6.04	0.16–7.17
Cyclohexanone	55	1.25–32.17	0.999	0.214	1.252	1.25–1.40	1.25–1.43
Dimethyl sulfide	62	0.27–5.05	0.999	0.075	0.270	0.27–0.69	d.
Acetonitrile	41	1.72–23.20	0.994	0.492	1.716	1.72–10.26	d.
Ethanol	45	5.09–60.58	0.911	1.305	5.098	5.10–99.22	5.10–124.67
Isopropanol	45	0.72–50.20	0.995	0.205	0.717	0.72–103.02	0.72–117.62
Acetaldehyde	43	1.28–22.07	0.997	0.363	1.280	1.28–8.33	1.28–6.43
Propanal	57	0.34–5.41	0.999	0.094	0.341	0.34–6.82	0.34–2.98
Butanal	43	0.16–10.41	0.999	0.042	0.161	0.16–20.55	0.16–4.72
Pentanal	43	0.44–9.99	0.998	0.121	0.436	0.44–2.62	0.44–1.74
Hexanal	56	0.31–10.31	0.999	0.083	0.305	0.31–3.34	0.31–3.92
Heptanal	70	0.12–9.47	0.999	0.029	0.116	d.	d.
Octanal	56	0.37–8.43	0.999	0.099	0.365	n.d.	n.d.
2-Propenal	55	0.39–9.01	0.999	0.105	0.398	d.	d.
2-Butenal	39	0.87–9.15	0.999	0.235	0.872	n.d.	n.d.
Propane	43	0.35–10.09	0.999	0.096	0.350	0.35–5.09	0.35–4.31
Butane	43	0.13–9.99	1.000	0.034	0.131	0.13–8.78	0.13–2.28
Pentane	41	0.43–10.19	0.999	0.119	0.431	d.	d.
Hexane	41	0.11–9.99	0.999	0.029	0.114	0.11–0.34	0.11–0.26
Heptane	41	0.13–4.35	1.000	0.035	0.129	0.13–0.31	0.13–0.22
2-Methylbutane	41	0.19–4.11	0.999	0.053	0.191	0.19–0.58	0.19–0.85
2-Methylpropanal	41	0.42–4.36	0.998	0.119	0.422	n.d.	n.d.
2,2-Dimethylbutane	41	0.52–4.00	0.995	0.152	0.522	n.d.	n.d.
2,3-Dimethylbutane	41	0.33–4.08	0.999	0.091	0.327	n.d.	n.d.
2-Methylpentane	43	0.28–4.50	0.999	0.079	0.284	n.d.	n.d.
3-Methylpentane	56	0.18–4.09	1.000	0.047	0.177	n.d.	n.d.
2,2-Dimethylpentane	57	0.13–3.99	1.000	0.036	0.134	n.d.	n.d.
2,4-Dimethylpentane	41	0.11–3.55	1.000	0.028	0.108	n.d.	n.d.
3,3-Dimethylpentane	43	0.31–4.06	0.998	0.086	0.307	n.d.	n.d.
2-Methylhexane	41	0.09–3.57	1.000	0.023	0.087	n.d.	n.d.
Cyclohexane	56	0.62–4.94	0.996	0.179	0.619	n.d.	n.d.
Benzene	78	0.11–11.14	1.000	0.029	0.112	0.11–0.80	0.11–0.27
Toluene	91	0.19–5.02	1.000	0.055	0.199	0.20–3.64	0.20–3.45
Chlorobenzene	112	0.23–11.14	1.000	0.059	0.228	n.d.	n.d.
1,2-Dimethylbenzene	91	0.31–10.51	0.999	0.082	0.310	0.31–0.41	0.31–0.39
1,2-Dichlorobenzene	146	0.35–9.79	0.999	0.093	0.348	n.d	n.d.
Carbon disulfide[a]	76	–	–	–	–	0.07–37.53	0.21–42.68
Dimethyl formamide[a]	73	–	–	–	–	0.06–15.69	0.47–11.83
2,5-Dimethylfuran[a]	95	–	–	–	–	0.02–1.68	0.08–0.11
1-Propanol[a]	59	–	–	–	–	0.13–65.78	0.09–125.61

Abbreviations: LOD: limit of detection; LOQ: limit of quantification; n.d.: not detected (i.e. value was below LOD); d.: detected (i.e. value was between LOD and LOQ).
[a] Substance intensities were expressed in counts multiplied by 1000^{-1}.

total variance could be accounted for by means of the first 3 PCs extracted from expired concentrations in single breath alveolar samples. 35% of the total variance could be accounted for by means of the first 3 PCs extracted from corrected data (C_{EX}–C_{INS}). All other PCs did not show any significant differences between the study groups.

3.2. Serum biomarkers

Table 5 shows serum biomarker concentrations in lung cancer patients, healthy smokers and healthy non-smoking controls. Significant differences were found for Cyfra 21-1 between lung cancer patients and smokers and between lung cancer patients and non-smokers ($p \leq 0.001$). CEA concentrations were significantly different from each other in all three groups ($p \leq 0.001$), NSE concentrations were only significantly different between lung cancer patients and smokers ($p = 0.002$).

Table 6 shows differences between serum biomarker concentrations in patients with different types of lung cancer. NSE concentrations exhibited statistically significant differences between NSCLC and SCLC groups ($p = 0.001$). CEA and Cyfra 21-1 showed no significant differences between NSCLC and SCLC groups.

4. Discussion

The analytical method based upon controlled alveolar sampling and GC-MS analysis proved to be sufficiently sensitive and reproducible to determine substance concentrations in the low nmol/L range. Concentration profiles of typical endogenous and exogenous breath markers were determined and evaluated in terms of their ability to distinguish patients with lung cancer from healthy volunteers and active smokers from non-smokers. Different algorithms were used to account for inter-individual variation due to physiological parameters such as BSA or ventilation (PCO_2) and for inspired concentrations. Data reduction was achieved by means of principal component analysis. Depending on the kind of data processing, statistically significant differences in the profiles of exhaled substance concentrations could be identified in the study groups. Age, gender, smoking history and inspired substance concentrations were recognized as the most important confounding variables. In contrast to the characterisation of previous smoking behaviour it was not possible to characterize any unique breath VOC that could be used to identify lung cancer unequivocally and with reasonable reliability.

In this study, solid-phase micro extraction (SPME) in combination with controlled alveolar sampling was used for sample generation and

Table 3
VOC concentrations and statistically significant differences between study groups.

	Data processing	Lung cancer	Smokers	Controls	LC vs. S	LC vs. C	C vs. S	p-value
Acetonitrile	EX (nmol/L)	1.72 (1.72–1.72)	1.72 (1.72–2.33)	1.72 (1.72–1.72)	s.	n.s.	s.	<0.001
	EX/BSA (nmol/L m^{-2})	0.91 (0.75–0.95)	0.99 (0.89–1.29)	0.93 (0.75–1.07)	s.	n.s.	s.	0.005
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.34 (0.25–0.39)	0.33 (0.30–0.43)	0.34 (0.29–0.42)	n.s.	n.s.	n.s.	0.575
	EX-INS (nmol/L)	1.72 (1.72–1.72)	1.72 (1.72–2.33)	1.72 (1.72–1.72)	s.	n.s.	s.	<0.001
Benzene	EX (nmol/L)	0.11 (0.11–0.11)	0.12 (0.11–0.24)	0.11 (0.11–0.11)	s.	n.s.	s.	<0.001
	EX/BSA (nmol/L m^{-2})	0.06 (0.05–0.06)	0.07 (0.06–0.14)	0.06 (0.05–0.07)	s.	n.s.	s.	0.003
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.02 (0.02–0.03)	0.02 (0.02–0.04)	0.02 (0.02–0.03)	s.	n.s.	s.	0.011
	EX-INS (nmol/L)	0.00 (0.00–0.00)	0.07 (0.00–0.11)	0.00 (0.00–0.00)	s.	n.s.	s.	<0.001
2,5-Dimethyl furan	EX (counts)	0.00 (0.00–0.00)	284.50 (0.00–678.13)	0.00 (0.00–0.00)	s.	n.s.	s.	<0.001
	EX/BSA (counts m^{-2})	0.00 (0.00–0.00)	131.69 (0.00–423.43)	0.00 (0.00–0.00)	s.	n.s.	s.	<0.001
	EX/$P_{et}CO_2$ (counts kPa^{-1})	0.00 (0.00–0.00)	55.24 (0.00–137.58)	0.00 (0.00–0.00)	s.	n.s.	s.	<0.001
	EX-INS (counts)	0.00 (0.00–0.00)	284.50 (0.00–594.50)	0.00 (0.00–0.00)	s.	n.s.	s.	<0.001
Acetone	EX (nmol/L)	16.75 (11.61–26.12)	6.10 (4.18–10.30)	15.82 (9.82–22.15)	s.	n.s.	s.	<0.001
	EX/BSA (nmol/L m^{-2})	8.66 (5.94–13.88)	3.48 (2.13–6.37)	7.91 (5.61–13.07)	s.	n.s.	s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	3.67 (2.24–5.15)	1.07 (0.74–1.99)	3.29 (1.95–4.87)	s.	n.s.	s.	<0.001
	EX-INS (nmol/L)	13.88 (9.13–22.75)	4.93 (3.19–9.32)	10.43 (7.90–19.41)	s.	n.s.	s.	<0.001
Dimethyl sulfide	EX (nmol/L)	0.27 (0.00–0.27)	0.27 (0.27–0.27)	0.30 (0.27–0.31)	s.	s.	n.s.	0.002
	EX/BSA (nmol/L m^{-2})	0.12 (0.00–0.15)	0.15 (0.14–0.17)	0.19 (0.12–0.17)	s.	s.	n.s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.04 (0.00–0.06)	0.05 (0.04–0.06)	0.08 (0.05–0.08)	s.	s.	n.s.	0.022
	EX-INS (nmol/L)	0.27 (0.00–0.27)	0.27 (0.27–0.27)	0.30 (0.00–0.31)	s.	s.	n.s.	0.005
Dimethyl formamide	EX (counts)	5589.50 (3649.63–7715.38)	1403.00 (0.00–2601.63)	558.50 (0.00–4822.00)	s.	s.	n.s.	0.003
	EX/BSA (counts m^{-2})	3061.88 (1887.09–3985.59)	763.47 (0.00–1457.98)	287.91 (0.00–2550.43)	s.	s.	n.s.	0.005
	EX/$P_{et}CO_2$ (counts kPa^{-1})	1097.71 (739.90–1570.59)	258.84 (0.00–468.96)	99.75 (0.00–1023.19)	s.	s.	n.s.	0.002
	EX-INS (counts)	1855 (0.00–3340.88)	8.00 (0.00–695.88)	0.00 (0.00–2954.13)	s.	s.	n.s.	0.019
2-Methyl-1,3-butadiene	EX (nmol/L)	5.22 (0.54)	4.08 (0.59)	3.79 (0.36)	n.s.	n.s.	n.s.	0.115
	EX/BSA (nmol/L m^{-2})	2.63 (1.72–3.40)	1.50 (0.93–3.13)	1.96 (1.29–2.63)	n.s.	n.s.	n.s.	0.115
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.99 (0.64–1.29)	0.47 (0.30–1.08)	0.79 (0.45–1.07)	s.	s.	n.s.	0.012
	EX-INS (nmol/L)	4.60 (2.90–6.16)	2.92 (1.62–5.61)	3.50 (2.11–4.76)	n.s.	n.s.	n.s.	0.110
Toluene	EX (nmol/L)	0.39 (0.20–0.59)	0.25 (0.20–0.37)	0.27 (0.20–0.52)	n.s.	n.s.	n.s.	0.182
	EX/BSA (nmol/L m^{-2})	0.20 (0.11–0.32)	0.15 (0.11–0.19)	0.15 (0.12–0.29)	n.s.	n.s.	n.s.	0.251
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.08 (0.04–0.13)	0.05 (0.04–0.06)	0.06 (0.04–0.10)	s.	n.s.	s.	0.024
	EX-INS (nmol/L)	0.00 (0.00–0.01)	0.06 (0.00–0.14)	0.00 (0.00–0.00)	s.	n.s.	s.	0.003
Butane	EX (nmol/L)	0.15 (0.13–0.36)	0.13 (0.13–0.25)	0.31 (0.14–0.58)	n.s.	n.s.	n.s.	0.083
	EX/BSA (nmol/L m^{-2})	0.08 (0.07–0.19)	0.09 (0.07–0.13)	0.18 (0.08–0.35)	n.s.	n.s.	n.s.	0.105
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.03 (0.03–0.08)	0.03 (0.02–0.05)	0.06 (0.04–0.11)	s.	s.	n.s.	0.034
	EX-INS (nmol/L)	0.00 (0.00–0.11)	0.09 (0.00–0.13)	0.18 (0.00–0.52)	s.	s.	s.	0.002
Propanal	EX (nmol/L)	0.34 (0.00–0.37)	0.00 (0.00–0.34)	0.00 (0.00–0.00)	s.	s.	n.s.	<0.001
	EX/BSA (nmol/L m^{-2})	0.17 (0.00–0.20)	0.00 (0.00–0.16)	0.00 (0.00–0.00)	s.	s.	n.s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.07 (0.00–0.09)	0.00 (0.00–0.05)	0.00 (0.00–0.00)	s.	s.	n.s.	<0.001
	EX-INS (nmol/L)	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	n.s.	Do not test	Do not test	0.003
Butanal	EX (nmol/L)	1.81 (0.99–5.17)	0.16 (0.00–0.42)	2.23 (0.95–2.84)	s.	n.s.	s.	<0.001
	EX/BSA (nmol/L m^{-2})	0.99 (0.54–2.46)	0.09 (0.00–0.24)	1.02 (0.51–1.59)	s.	n.s.	s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.36 (0.20–1.14)	0.03 (0.00–0.08)	0.42 (0.22–0.69)	s.	n.s.	s.	<0.001
	EX-INS (nmol/L)	1.07 (0.38–3.51)	0.00 (0.00–0.23)	0.32 (0.00–1.40)	s.	s.	s.	<0.001
Hexanal	EX (nmol/L)	0.59 (0.38–0.86)	0.31 (0.31–0.31)	0.63 (0.38–2.32)	s.	n.s.	s.	<0.001
	EX/BSA (nmol/L m^{-2})	0.32 (0.20–0.53)	0.18 (0.16–0.19)	0.36 (0.20–1.16)	s.	n.s.	s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	0.12 (0.08–0.19)	0.06 (0.05–0.06)	0.11 (0.08–0.44)	s.	n.s.	s.	<0.001
	EX-INS (nmol/L)	0.00 (0.00–0.03)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	n.s.	Do not test	Do not test	0.038
Isopropanol	EX (nmol/L)	6.47 (3.31–13.40)	1.06 (0.18–1.88)	1.41 (0.72–2.32)	s.	s.	n.s.	<0.001
	EX/BSA (nmol/L m^{-2})	4.29 (1.76–7.20)	0.68 (0.08–0.97)	0.76 (0.43–1.28)	s.	s.	n.s.	<0.001
	EX/$P_{et}CO_2$ (nmol/L kPa^{-1})	1.33 (0.63–3.07)	0.19 (0.02–0.37)	0.29 (0.14–0.46)	s.	s.	n.s.	<0.001
	EX-INS (nmol/L)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.00 (0.00–0.00)	n.s.	Do not test	Do not test	0.025
1-Propanol	EX (counts)	2599.50 (1279.00–8536.25)	286.50 (0.00–1217.13)	434.50 (0.00–1295.88)	s.	s.	n.s.	<0.001
	EX/BSA (counts m^{-2})	1478.23 (575.33–4534.38)	158.14 (0.00–744.56)	236.18 (0.00–673.52)	s.	s.	n.s.	<0.001
	EX/$P_{et}CO_2$ (counts kPa^{-1})	519.90 (209.97–1952.69)	55.63 (0.00–216.14)	59.75 (0.00–226.74)	s.	s.	n.s.	<0.001
	EX-INS (counts)	0.00 (0.00–0.00)	0.00 (0.00–43.88)	0.00 (0.00–0.00)	Do not test	Do not test	n.s.	0.006

Concentrations expressed as median values (25th–75th percentile) or as mean values (standard error of mean). Non parametric test for independent measures (Kruskal–Wallis test followed by Student–Newman–Keuls method) was used to compare lung cancer patient, smoker and healthy controls.
Abbreviations: LC: lung cancer patients; S: smokers; C: non-smoking controls; s: significant ($p<0.05$); n.s: not significant ($p>0.05$).

preparation. Although sensitivity for some compounds is limited [21,22] SPME represents important advantages for clinical studies as most relevant substances can be determined in the low nmol/L range from a single breath with sample volumes as low as 10 to 15 mL. In addition, SPME samples can be processed automatically.

As bronchial carcinomas predominantly occur in older men in the population [10,11] sex and age distribution of the patient groups we investigated were different. To minimize the risk that "healthy smokers" had undiscovered lung carcinoma, we tolerated the lower mean age in this group. Healthy volunteers were deliberately chosen younger than carcinoma patients to minimize the occurrence of undiscovered carcinoma and chronic disease such as COPD.

Serum tumour markers play a role for staging and principally for monitoring of therapy as well as for secondary prevention. Even different combinations of serum markers could not reasonably be used for lung cancer screening [23] or primary diagnosis. In our study, they were determined as conventional biomarkers that potential breath markers could have been compared with.

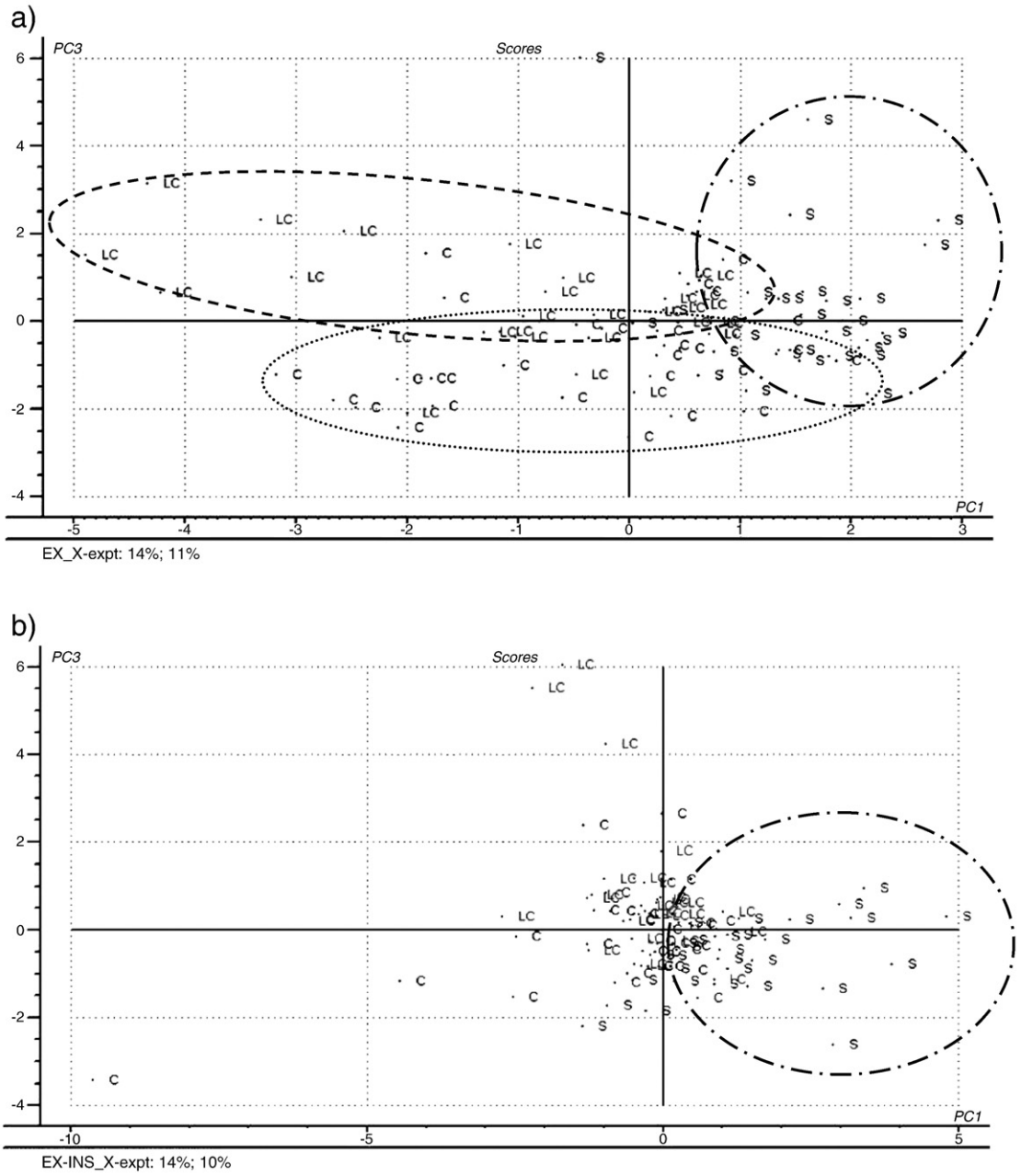

Fig. 1. Scores plot of (a) PC1 vs. PC3 based on expiratory concentrations in single breath alveolar samples without any correction and of (b) PC1 vs. PC3 based on expired concentrations corrected for inspired concentrations (C_{EX}–C_{INS}). PC1 described 14% of variance in each data set. PC3 described 11% of variance in the uncorrected (C_{EX}) data set and 10% of variance in the corrected (C_{EX}–C_{INS}) data set. Broken line: lung cancer patients; dotted line: healthy controls; dot-and-dashed line: smokers.

Significant concentration differences of breath VOCs were found between cancer patients and control groups when univariant and multivariate statistical methods were applied to uncorrected expired concentrations. Substances showing differences in the uncorrected data were predominantly exogenous compounds from the clinical or laboratory environment. These substances were often found in high ambient concentrations; sometimes inspired concentrations were even higher than expiratory concentrations. Substances like dimethyl formamide and carbon disulfide most probably represent analysis related contaminations. As there is evidence that these substances can be released from plastic materials such as GC-MS septa [14] concentration differences between the study groups have to be regarded as artificially generated.

Alcohols such as 1-propanol and isopropanol as well as aldehydes such as propanal and formaldehyde are typical ingredients of disinfectants. Due to their high ambient concentrations in the clinical environment lung cancer patients seemed to exhale significantly higher amounts of these compounds than smokers and non-smoking controls. As intake, storage and exhalation of exogenous substances represent complex processes [24] depending on substance solubility and other physiochemical properties this problem cannot be solved by simply subtracting inspired from expired concentrations [20,25]. Subtraction methods like “alveolar gradients” in which negative and positive values were generated [12,26,27] are difficult to interpret and statistical significance may be artificially generated in this way. Nevertheless, many of these substances, e.g. isopropanol have been described as breath biomarkers of disease and have been proposed for recognition of lung cancer [13,28]. Contradictory results of different studies [12,13] and the influence of inspired concentrations onto results as shown in our study firmly suggest that breath gas

Table 4
Loading values of PC1 and PC3 from uncorrected (expiratory concentrations) and corrected (C_{EX}–C_{INS}) data.

	$PC1_{EX}$	$PC3_{EX}$	$PC1_{EX-Ins}$	$PC3_{EX-Ins}$
2-Methyl-1,3-butadiene	−0.206	0.359	0.104	0.218
Acetone	−0.322	3.91E−02	−0.193	6.40E−02
Dimethyl sulfide	0.227	−2.51E−02	0.139	0.124
Acetonitrile	8.92E−02	0.161	0.125	−0.121
Benzene	8.48E−02	0.442	0.3	1.85E−02
Butane	−0.208	8.82E−02	−0.226	−0.24
Hexane	0.11	0.347	1.77E−02	−0.188
Heptane	−2.99E−02	0.346	0.151	0.337
Propanal	−0.11	0.153	6.35E−03	4.23E−02
Butanal	−0.252	5.33E−02	−0.12	0.476
Pentanal	−0.242	−0.133	−5.73E−02	0.508
Hexanal	−0.368	−0.283	−0.245	−0.215
2-Butanone	−0.32	−0.265	−0.389	−0.199
Ethanol	0.118	−4.33E−02	6.22E−02	−9.82E−02
Isopropanol	−0.33	0.217	0.107	−0.14
Toluene	−0.16	0.226	0.305	−4.14E−02
Trichloromethane	−0.216	6.85E−02	0.139	−0.114
2,5-Dimethylfuran	0.233	0.206	0.523	−2.20E−02
Dimethyl formamide	−0.126	0.169	−0.333	0.302
1-Propanol	−0.306	0.171	0.111	−4.94E−02

constituents having high inspired concentrations should be excluded as biomarkers regardless of the method of data interpretation. Only if inspired concentrations are lower than 5% of expired ones they can be neglected [20,25]. It is also important to notice that inspired concentrations may vary considerably depending on the specific environment.

Inspired concentrations of dimethyl sulfide were sufficiently low and expired concentrations showed significant differences between lung cancer patients and healthy controls. Dimethyl sulfide and other sulfur containing compounds have been identified as important contaminants originating from bacterial growth in gingiva or saliva [27,29]. Van den Velde described that dimethyl sulfide was significantly increased in persons with oral malodour when compared with healthy volunteers [29]. In our study, the concentration of dimethyl sulfide was lowest in lung cancer patients. I. e. the results obtained in our study may have been related to the dental status rather than to cancer specific effects. As on average cancer patients were older than the controls they may have had reduced or altered bacterial growth in the oral cavity due to loss of vital teeth. However, results of Barker et al. who described reduced concentrations of dimethyl sulfide in exhaled breath of patients with cystic fibrosis may suggest correlations of dimethyl sulfide concentrations with metabolic effects other than bacterial growth [27].

Depending on the kind of normalization or data processing different statistical information was obtained in our study. After normalization of results onto end tidal PCO_2 differences of exhaled 2-methyl-1,3-butadiene and toluene concentrations gained significance. Since end tidal PCO_2 was not equally distributed among the study groups but showed significant differences between cancer patients, smokers and non-smoking controls, statistical differences appearing after normalization to PCO_2 were most likely due to the unequal distribution of PCO_2 rather than to cancer related effects that might have been revealed through normalization. In addition we found dependency of 2-methyl-1,3-butadiene exhalation from age and gender which has already been described [30,31].

Table 6
Serum biomarker concentrations and statistically significant differences with respect to lung cancer classifications.

	NSCLC	SCLC	NSCLC vs. SCLC	p-value
NSE (μg/L)	16.2 (10.9–17.5)	34.2 (25.7–48.6)	s.	0.001
CEA (μg/L)	7.4 (2.0–21.5)	7.8 (2.8–24.2)	n.s.	0.867
Cyfra 21-1 (μg/L)	4.4 (2.0–20.8)	2.9 (1.8–3.9)	n.s.	0.221

Concentrations expressed as median values (25th–75th percentile). Non parametric test for independent measures (Mann–Whitney Rank test followed by Dunn's method) was used to compare lung cancer classifications.

Normalization onto BSA did not change any statistical results. Cope et al. [32] have already pointed out, that BSA might not be a good parameter to normalize as the relationship between substance exhalation and BSA is principally not clear. In order to address the influence of inter-individual variation physiological parameters like body weight, body height, blood pressure, heart rate and expired PCO_2 must be recorded and taken into account. However, up to now, in VOC breath analysis no standard exists for normalization. The problem consists in deciding which kind of normalization makes sense and is most likely to yield "true" results. The method of normalization has to be chosen in the way that:

- normalization parameters are equally distributed among the study groups, and
- relationships between normalization parameters and normalized data make sense in terms of physiology and biochemistry.

In contrast to cancer recognition differentiation between smokers and non-smokers was reliably possible by means of smoking related exposure markers. This was true for univariant and multivariate data processing. Compounds occurring in cigarette smoke, like acetonitrile, benzene, 2,5-dimethylfuran and toluene have been described as typical breath biomarkers for recognition of active and passive smoking [9,33–35]. Accordingly, acetonitrile, 2,5-dimethylfuran, toluene and o-yxlene, were predominantly detected in exhalation samples of smokers but not in the breath of non-smokers or ex-smokers. These substances represent markers of exposure and have to be clearly distinguished from any endogenous compounds. Differences between smokers and non-smokers in exhaled acetone concentrations were most probably due to physiological effects of nicotine deprivation as smokers had to stop smoking at least one hour before giving breath samples [36].

Results clearly demonstrate that exhaled substance concentrations may depend on a variety of parameters, such as environmental conditions or patients' medical history, other than the disease under investigation. Even the smartest statistical algorithm applied to results of breath testing will fail if these confounding variables are not taken into account. Due to high inter-individual variation normalization of data is necessary. However, normalization parameters have to be chosen with care as compensating effects of normalization may be different from one substance to the other. Well-founded biomarker identification, intelligent normalization and data

Table 5
Serum biomarker concentrations and statistically significant differences between study groups.

	Lung cancer	Smoker	Controls	LC vs. S	LC vs. C	C vs. S	p-value
NSE (μg/L)	17.2 (12.8–27.8)	12.8 (9.3–14.8)	15.0 (11.6–17.1)	s.	n.s.	n.s.	0.002
CEA (μg/L)	7.4 (2.0–22.9)	1.9 (1.2–3.1)	1.2 (0.7–1.8)	s.	s.	s.	<0.001
Cyfra 21-1 (μg/L)	3.2 (1.9–5.9)	0.6 (0.5–0.9)	0.8 (0.6–1.3)	s.	s.	n.s.	<0.001

Concentrations expressed as median values (25th–75th percentile). Non parametric test for independent measures (Kruskal–Wallis test followed by Dunn's method) was used to compare lung cancer patient, healthy smoker and non-smoking controls.
Abbreviations: NSE: neuron-specific enolase; CEA: carcino-embryonic antigen; Cyfra 21-1: cytokeratine fragment 21-1.

processing will help to obtain clinically relevant information from breath analysis.

List of abbreviations

BSA	body surface area
C	non-smoking controls
CEA	carcino-embryonic antigen
Cyfra 21-1	cytokeratine fragment 21-1
GC	gas chromatography
d.	detected
LC	lung cancer patients
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
n.d.	not detected
n.s.	not significant
NSCLC	non-small cell lung cancer
NSE	neuron-specific enolase
PCA	principal component analysis
PDMS	polydimethylsiloxane
S	smoker
s.	significant
SCLC	small cell lung cancer
SEM	standard error of the mean
SPME	solid-phase micro extraction
TNM	classification of malignant tumours
VOC	volatile organic compound

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