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ScienceDirect
International Journal of Mass Spectrometry 265 (2007) 49–59
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Lung cancer detection by proton transfer reaction mass-spectrometric analysis of human breath gas

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Received 8 May 2007; received in revised form 25 May 2007; accepted 25 May 2007
Available online 6 June 2007

Abstract

Background: Determination of the diagnostic usefulness of proton transfer reaction mass spectrometry (PTR-MS) for detecting primary lung cancer through analysis of volatile organic compounds (VOCs) in exhaled human breath was demonstrated in this investigation. Unlike, for example, gas-chromatographic analyses, PTR-MS can be used without time-consuming preconcentration of the gas samples.
Methods: By means of PTR-MS, exhaled breath samples from primary lung cancer patients ($n = 17$) were analyzed and compared with both an overall control collective (controls total, $n = 170$) and three sub-collectives: hospital personnel (controls hospital, $n = 35$), age-matched persons (controls age, $n = 25$), and smokers (controls s, $n = 60$), respectively.
Results: Among the VOCs present at reasonably high concentrations, the ones leading to the product ion at $m/z = 31$ (VOC-31, tentatively protonated formaldehyde) and $m/z = 43$ (VOC-43, tentatively a fragment of protonated *iso*-propanol), were found at significantly higher concentrations in the breath gas of the primary lung cancer patients as compared to the healthy controls at the following median concentrations (with interquartile distance, iqr): For VOC-31 the median concentrations were 7.0 ppb (iqr, 15.5 ppb) versus 3.0 ppb (iqr, 1.9 ppb) with $P < 10^{-4}$. For VOC-43 the median concentrations were 244.1 ppb (iqr, 236.2 ppb) versus 94.1 ppb (iqr, 55.2 ppb) with $P < 10^{-6}$. The discriminative power between the two collectives was further assessed by ROC-curves obtained upon variation of the chosen threshold concentration and by Fisher's Quadratic Discriminant Method.
Conclusions: Within the limits of pilot study, VOC-31 and -43 were found to best discriminate between exhaled breath of primary lung cancer cases and healthy controls. Simple and time-saving breath gas analysis by PTR-MS makes this method attractive for a larger clinical evaluation. It may become a new valuable tool for diagnosing primary lung cancer.

Keywords: Primary lung cancer; Proton transfer reaction mass spectrometry; Exhaled breath; Volatile organic compound; Receiver operating characteristic curve

Abbreviations: GC, gas chromatography; I-ROC, integral of the ROC-curve; *m/z*, mass-to-charge ratio; PTR-MS, proton transfer reaction mass spectrometry; ROC-curve, receiver operating characteristic curve; SPME, solid-phase micro-extraction; VOCs, volatile organic compounds

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doi:10.1016/j.ijms.2007.05.012

1. Introduction

In developed countries, particularly in North America and Europe, lung cancer is a leading cause of cancer death [1]. In Europe, lung cancer is the most common form of cancer diagnosed (13.2%) and of cancer death (20%) [2]. Therefore, many efforts have been made to develop proper screening methods for an early diagnosis of this disease including chest-X-ray, sputum cytology, and low-dose (spiral) computer tomography (LDCT) [3]. Observational studies suggest that out of these, LDCT appears to be the most promising screening method [4]. However, up to date, effectiveness of LDCT is still unclear and results from clinical trials have to be awaited to judge whether or not this method would be able to reduce lung cancer mortality rate [5]. Apart from this unclear clinical effectiveness, the low cost-effectiveness of LDCT is another aspect that would favour alternative screening methods [6].

The detection of volatile organic compounds (VOCs) in human breath gas by Pauling et al. [7] opened new insights into the human body, which consequently led to a new challenging scientific field: analysis of VOCs for clinical diagnosis and therapeutic monitoring [8–14]. VOCs enter the body from air as emissions from traffic, industry products or natural sources [15,16] and some originate from the body itself [17]. Most of the VOC-concentrations are in the nanomolar (10^{-9}) and picomolar (10^{-12}) range, requiring highly sensitive devices and sophisticated analytical methods for their proper detection [16,18–20]. By means of gas chromatography (GC) and mass spectrometry (MS), 3481 different VOCs have been described in the breath gas of healthy controls with an average number of about 200 different VOCs being detectable in one individuals' breath gas [21]. Thereby, a 'common core' of 27 different VOCs was found in all individuals [21] and among these, isoprene, acetone and different alcohols belong to those with the highest concentrations [22].

Besides being non-invasive and therefore painless to patients, collection of breath gas is easy to handle and time-saving [23]. On the contrary, definition of normal ranges of single VOCs in human breath gas is tricky, because VOCs have been described to be influenced by different factors such as age [24], gender, smoking [25], circadian rhythm and heart rate [8], respiration rate [26], nutritional status [27–29], and composition of environmental air [30,31]. Therefore, to minimize the influence of these factors, comparable guidelines for breath gas collection and analysis are required [14,32].

Concerning lung cancer, Phillips et al. described 22 VOCs – most of them alkanes, alkane derivatives and benzene derivatives – that showed significant differences between patients with and without this disease [33]. Within another cross-sectional study employing GC–MS, they identified nine VOCs – again alkanes and alkane derivatives – as the best set of markers of lung cancer [23]. Other study groups, employing sensor array analyses ('electronic nose') [34] and GC–MS combined with solid-phase micro-extraction [35] also showed that rather a combination of VOCs than single VOCs alone successfully discriminate between individuals with and without lung cancer.

In the following, we report the results of an investigation based on breath gas samples from 17 primary lung cancer patients and 170 healthy individuals analyzed by means of proton transfer reaction mass spectrometry (PTR-MS), from which two new potential biomarkers for primary lung cancer emerge. Compared to GC–MS, PTR-MS delivers relative VOC concentrations with high sensitivity (down to parts per trillion) *without* sample preconcentration. Such preconcentration steps are time-consuming. Adsorption and desorption of breath gas furthermore may depend on the particular adsorption medium used such as Tenax, Carbopack, Carboxen, and on its capacity to adsorb organic substances in the presence of carbon dioxide and water which are present in exhaled breath at relatively high concentrations. In addition, PTR-MS allows on-line measurements (over a full night, for example). The drawback of PTR-MS, on the other hand, is its inability to distinguish between substances having the same molecular weight. All substances identified by product ions with particular mass-to-charge ratios (m/z) must therefore be considered as possible contributors.

The much earlier seminal work of Španěl et al. [29] sampling the headspace of urine from prostate and bladder cancer patients showed that formaldehyde was present in the urine, and hence the blood stream, of these cancer patients but not at measurable concentrations in the urine of healthy controls. Thus, it was suggested that formaldehyde would be present in the exhaled breath of these cancer patients.

2. Methods and materials

2.1. Study subjects

We have investigated 17 patients suffering from histologically confirmed primary lung cancer, as indicated in Table 1 who were selected from the haematologic-oncological care division of the Innsbruck Internal Medicine Department. In addition, 170 healthy persons recruited among the hospital staff and elsewhere were united into a total control collective which provided the

Table 1
Histological findings and TNM-stage in primary lung cancer patients

Carcinoma type	Number of patients
Small cellular	4
Non-small cellular	13
Epidermoid	3
Adenocarcinoma	5
Large cell	3
Neuroendocrine	2
Total	17
TNM-stage	**Number of patients**
I	9
II	3
III	3
IV	1
V	1
Total	17

Table 2
Demographics of the investigated subjects

	Controls (total)	Controls (hospital)	Controls (age)	Primary lung cancer (total)
Total number (female/male)	87/83	17/18	17/8	4/13
Smokers (unknown/non-smokers/previous smokers/smokers)	4/95/11/60	0/15/4/16	2/19/1/3	0/4/5/9
Age: mean ± S.D. (a)	41.0 ± 13.4	32.8 ± 6.8	65.6 ± 7.9	62.4 ± 11.2

reference breath gas samples and served as a super-collective for the following three sub-collectives:

- a hospital control collective composed of 35 members of the medical and nursing staff matching the patients with respect to exposure to the hospital indoor air,
- an age-matched control collective composed of 25 individuals matched with respect to age,
- a smoking control collective composed of 60 smoking individuals.

The demographics of all 187 subjects involved are summarized in Table 2. Informed consent to the study approved by the local ethics board was obtained from all patients and controls. All procedures used were in accordance with the recommendations found in the Helsinki Declaration of 1975.

2.2. Study design

Within this retrospective pilot study, we analyzed breath gas samples from untreated primary lung cancer patients and controls by means of PTR-MS. With respect to age, environmental air, gender, smoking behaviour and tumour stage, we scanned human breath gas samples to detect possible marker molecules (ions with a mass-to-charge ratio, m/z, in the range of 21–230) that characterize primary lung cancer.

2.3. Methods

At least two breath gas samples per patient were collected the same day into 3-L Tedlar® (polyvinylfluoride) bags (SKC 232 Series; Eighty Four, PA, USA), the patients providing all their samples prior to the first chemotherapeutic cycle in order to prevent possible difficulties caused by interfering drug metabolites. Mixed expiratory breath samples were taken, with no restriction on a particular part of breath. While donating breath, all patients and controls were sitting at rest inflating a bag through a disposable mouthpiece. To obtain reasonably constant fractions of alveolar and dead space breath in one bag, all individuals were advised to *breath normally* during sample collection. Breathing rate was *not* recorded. In addition to samples of exhaled breath, inhaled ambient air was collected for reference. After each use, bags were prepared for re-use by a thorough cleaning procedure: first, they were rinsed with pure nitrogen two to three times, then heated over night at 95 °C filled with flushing gas, and finally re-rinsed two to three times before being depleted and stored. Before storage, selected bags were re-analyzed by PTR-MS to check that no residual VOCs persisted after the cleaning procedure.

All samples were analyzed using PTR-MS (Ionicon FDTs; Innsbruck, Austria) scanning over the mass-to-charge ratio, m/z (relative ionic mass m to charge number z), range 21–230. Analysis was done on the day of breath sampling (with intermediate storage at room temperature). In the PTR-MS technique, VOCs from a gas sample are chemically ionized by proton transfer from the primary ion H_3O^+. Here, the rate constant for the proton transfer,

$$M + H_3O^+ \rightarrow MH^+ + H_2O$$

has been taken to be $k = 2 \times 10^{-9}\,cm^3\,s^{-1}$. The rate constant may differ for different VOCs in the range $1.5 \times 10^{-9}\,cm^3\,s^{-1} < k < 4 \times 10^{-9}\,cm^3\,s^{-1}$. Our determined concentrations are thus uncalibrated. For calibration, it would be necessary to confirm the substance identifications at chosen mass-to-charge ratios m/z and use the rate constants for these particular chosen compounds.

For quality control, atmospheric (outdoor) air, the mass spectrum of which was clearly distinct from that of all other samples, was conveyed to the instrument after each sample to enable unambiguous selection of those PTR-MS record sections that render pure single samples. To prevent condensation on the walls, the sample containers were tempered in an oven at 42 °C, i.e. 5 °C above the temperature of exhaled breath gas; likewise, the complete PTR-MS inlet line and reaction chamber were heated to 42–45 °C by means of a heating coil. For further technical information on the PTR-MS the reader is referred to the literature [16]. In short, it is a mass spectrometer capable of analysing samples that resulted in ions following proton transfer in the mass range 21–512 u *without* previous separation of the analytes.

2.4. Analysis of PTR-MS spectra

The raw PTR-MS spectra (count rates for all measured mass-to-charge ratios m/z) were processed using MATLAB software version 7.0.4.365 (R14) (The MathWorks Inc.; Natick, MA, USA). They were interpreted as spectra reflecting unique product ions generated from the respective molecular reactant VOCs in the sample via proton transfer from the precursor ions H_3O^+. Correspondingly, the product ions are assumed to be protonated molecular ions (ionic mass = molecular mass + proton mass) or protonated molecular fragment ions (ionic mass = molecular mass + proton mass − mass of lost fragment) as detected by the PTR-MS. Thus, the mole fractions (here simply referred to as concentrations) of the VOCs themselves, here denoted by the m/z ratio of their presumed product ion, have been computed from the ratios of the product ion to the precursor ion count rate using an approximate formula proposed by the

instrument manufacturer [36]. The absolute VOC concentration values computed are uncalibrated and may be inaccurate. This is, in particular, the case for formaldehyde (product ion at $m/z = 31$), with its low proton affinity exceeding only somewhat that of water.[1] The concentrations for ions at m/z 31 have been corrected for isotope effects from m/z 30 (=NO^+ which contributes $^{15}NO + N^{17}O = 0.37 + 0.04\% = 0.41\%$ to m/z 31). Accurate absolute values for formaldehyde concentrations can only be achieved with PTR-MS by appropriate calibration measurements.

2.5. Tentative identification of ions

We tentatively identified VOC-31 as formaldehyde and VOC-43 as isopropanol. The identification of VOC-31 is based on the fact that PTR-MS does not detect ethane and nitric oxide (which would also appear at m/z 31). In PTR-MS, isopropanol (with a molecular mass of 60) fragments to m/z 43 by loss of water. We would like to stress that we presume that the ion at m/z 43 is also a fragment from another compound than isopropanol. This would also explain the relatively high concentrations of VOC-43 in our control group, when compared with the much lower concentrations found by Turner in a study of healthy volunteers. The fragment m/z 43 can be both $C_3H_7^+$ (as from propanol) or CH_3CO^+ as sometimes occurs from the reactions of aldehydes, ketones and carboxylic acids.

2.6. Selection of data

In certain situations, the inhaled air shows a higher concentration of some compound than the exhaled air. In such a case, the corresponding concentrations of the respective compound in exhaled air do certainly *not* reflect the blood concentrations of this compound (if consideration of blood concentrations is applicable at all, which is not the case for a compound like, e.g. nitric oxide, which is produced in the lungs and the sinuses). A similar caveat holds if the concentration of a compound in inhaled air is just below the concentration in exhaled air. We therefore applied the following *filter* to our data of concentrations in exhaled air. A value for the expiratory concentration is taken into account if and only if

$$(\text{inspiratory concentration})_i \leq 0.6(\text{expiratory concentration})_i. \quad (1)$$

Hence, this filter discards all those expiratory concentrations that are only marginally higher or even smaller than the respective inspiratory concentration.

There have to be *exceptions* to this filter condition (1) for low expiratory concentrations: If we compare two groups, say, non-smokers with smokers, the expiratory concentrations of compounds in non-smokers are often so small, that the indoor air concentrations (inhaled) and the expiratory concentrations are in the same range. If these expiratory concentrations are filtered out, almost all data are "lost" due to some background level of indoor air concentrations. We therefore do *not* filter out these low expiratory concentrations, even though we have to concede that these expiratory concentrations are only *upper bounds* for the "real" expiratory concentrations (which would appear if the indoor air would be absolutely clean and free of any contamination).

For VOC-31 and -43 the thresholds were taken as 5 and 150 ppb, respectively. Therefore, all exhaled concentrations of VOC-31 smaller than 5 ppb and all exhaled concentrations of VOC-43 smaller than 150 ppb were taken into account. The thresholds were chosen in a way not to exclude the many low expiratory concentration values (in particular, those of healthy volunteers), which are in the same range as inspiratory concentrations. Otherwise, many of the low concentrations of compounds would have been discarded, consequently giving rise to a wrong statistical distribution of concentration values (with no low concentrations appearing at all). Even though there is some arbitrariness in the choice of these thresholds, consideration of some threshold value (or possibly weighted thresholds) seems to be unavoidable. We are aware of the fact that expiratory concentrations lower than inspiratory concentrations have to be used with great care: we consider such expiratory concentrations just as an upper bound for the blood concentrations (if the latter make sense at all, which would *not* be the case, e.g. for nitric oxide, which is formed in the airways and the sinuses).

An alternative approach (not used here) would be to consider the differences

$$\text{expiratory concentration} - \text{inspiratory concentration}. \quad (2)$$

We consider our approach (1) to be physiologically more informative than just taking differences in concentrations whenever a VOC behaves like carbon dioxide, the concentration of which in exhaled air (about 4%) is, within the normal inspiratory range, independent of the CO_2 concentration in inhaled air (0.03–2% in indoor air) [37]. In particular, "negative concentrations" do not arise in our approach (1).

2.7. Statistical data analysis

The significance of differences between the various collectives and sub-collectives was decided by virtue of P-values obtained from Wilcoxon rank sum tests. $P < 0.05$ indicate significant differences in accordance with common standards. As the confidence interval of a P-value strongly depends on the element (subject) number of the collectives involved, assessing the discriminating efficiency of a quantity (VOC concentration) purely based on a P-value may be very misleading. The corresponding receiver operator characteristic (ROC) curve is more useful for this purpose because it depends much less on the subject numbers. ROC-curves are graphical representations of sensitivity versus (1 − specificity) upon variation of the discriminating threshold quantity (concentration) over its domain. The resulting integral of the ROC-curve serves as an indicator for good separation of patients from healthy volunteers. This integral (I-ROC) may at best reach a value of 1. We computed not only ROC-curves comparing all patients with all healthy volunteers, but also ROC-curves for the comparison of persons in

[1] A. Jordan, private communication (2004).

these two groups being older than 30 (or older than 40, or older than 50).

As an additional method, we split the patient and volunteer groups randomly into a respective learning set (60% of persons in respective groups) and a test set (40% of persons in respective groups). We applied Fisher's Quadratic Discriminant Method [38] to the combined information given by logarithmic concentrations of VOC-31 and -43, as well as age. The MATLAB command classify.m was used, with quadratic boundaries between groups, taking a prior probability of lung cancer of 5%. The prediction of class-affiliation of the 40% test persons was determined (after training using the persons in the learning set). The corresponding accuracy, sensitivity, specificity, positive predictive value and negative predictive value were determined in 1000 independent simulations (with 1000 different choices of learning and test set). Here the accuracy is defined as:

$$\text{accuracy} = \frac{\text{number of true positives} + \text{number of true negatives}}{\text{total number of persons}}$$

The extraction of many different datasets from one set of experimental values is related to the bootstrap technique [39,40].

3. Results

A selection of results obtained from statistical evaluation taking into account only exhaled air samples (fulfilling our filter condition (1)) is summarized in Table 3 and depicted in Figs. 1–3. Among the tentatively assigned trace compounds being found in the breath gas of cases and controls at reasonably high concentration, $x > 10$ ppb, there are three that according to criterion (3) occur at significantly different concentrations in the breath gas of the primary lung cancer patients and control subjects. These are the VOCs ionized to the product ions detected at $m/z = 31$ (VOC-31), 43 (VOC-43) and 69 (VOC-69), respectively. In fact, as demonstrated by the following concentration ratios, the mean concentrations $\mu[x]$ of both VOC-31 (tentatively assigned to formaldehyde; ethane, which has the same molecular weight as formaldehyde, is undetectable by PTR-MS, because its low proton affinity is smaller than that of water) and VOC-43 (tentatively assigned to *iso*-propanol, observed product ion interpreted as fragment ion resulting from VOC protonation followed by H_2O loss [36]) are higher in the patient breath gas collective than in the control breath gas collectives, whereas the opposite is true for VOC-69 (tentatively assigned to isoprene), though to a lesser extent:

$$\mu[x_{31}](\text{patients})/\mu[x_{31}](\text{controls}) \approx 2.3,$$
$$\mu[x_{43}](\text{patients})/\mu[x_{43}](\text{controls}) \approx 2.6,$$
$$\mu[x_{69}](\text{patients})/\mu[x_{69}](\text{controls}) \approx 0.6.$$

The ratios obtained for VOC-31 and -43 are supposed to be large enough to qualify these VOCs as potentially useful discriminators. Thus, VOC-31 and -43 are the promising candidates that emerge from the present study as potential biomarkers for primary lung cancer. Another species of the selection is the VOC ionized to the ion at $m/z = 59$ (VOC-59), tentatively assigned

Table 3
Concentration (median and iqr) of various VOCs in the breath gas of controls *n* and primary lung cancer cases

VOC	*n*	Controls *n* (ppb)		Primary lung cancer cases (ppb)		*P*
		Median	Iqr	Median	Iqr	
31[a]	Total	3.0	1.9	7.0	15.5	9.3E−05
	Hospital	2.8	2.0			4.0E−04
	Smokers	2.9	1.7			2.1E−04
	Age	3.9	0.9			2.5E−02
43[a]	Total	94.1	55.2	244.1	236.2	2.1E−07
	Hospital	122.4	39.6			1.4E−04
	Smokers	119.2	73.9			1.4E−05
	Age	69.2	55.0			1.2E−05
59	Total	759.8	582.5	741.7	808.6	n.s.
	Hospital	793.3	575.5			n.s.
	Smokers	757.0	578.1			n.s.
	Age	1179.0	863.8			n.s.
69	Total	81.8	56.1	52.1	26.7	3.0E−03
	Hospital	88.0	59.3			6.2E−03
	Smokers	81.0	46.0			4.4E−03
	Age	78.6	59.5			2.9E−02
108	Total	0.5	0.4	0.7	0.5	n.s.
	Hospital	0.6	0.4			n.s.
	Smokers	0.7	0.5			n.s.
	Age	0.5	0.3			n.s.

Tentative assignments: 31, formaldehyde; 43, propanol; 59, acetone; 69, isoprene; 108, *o*-toluidine. Collectives: Overall controls (total); hospital controls (hospital); age-matched controls (age); *P*-values obtained from Wilcoxon rank sum tests; if $P < 0.05$, difference significant by definition. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb (VOC-31) and 150 ppb (VOC-43), respectively. Acronyms: iqr, interquartile range; VOC(s), volatile organic compound(s); ppb, part per billion (1E−09); n.s., non-significant.

[a] Best single discriminators.

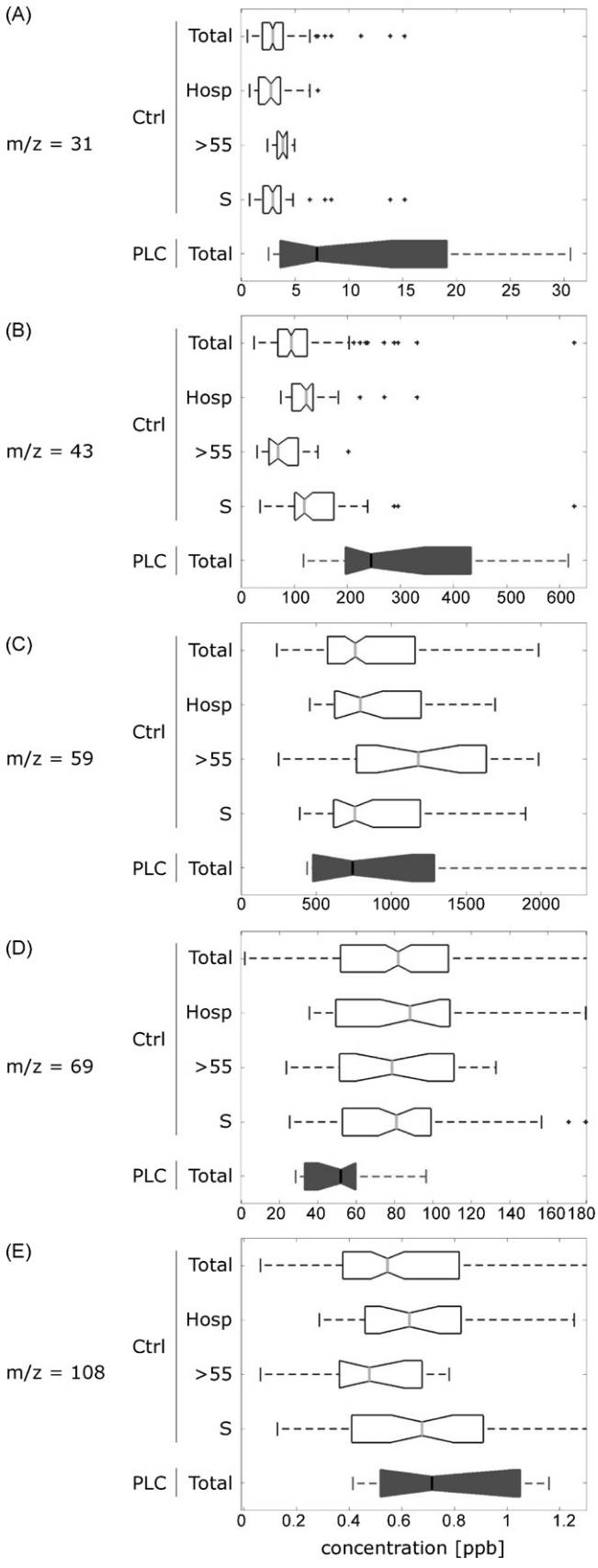

Fig. 1. Concentration distributions of various VOCs in the breath gas of three different control collectives (white boxes) and primary lung cancer patients (black boxes); *m/z*, tentative assignment: (A) 31, formaldehyde; (B) 43, propanol; (C) 59, acetone; (D) 69, isoprene; (E) 108, *o*-toluidine. Boxes mark the lower, median and upper quartiles; whiskers conform to MATLAB convention; outliers (+) are values out of the whisker range. A few outliers are outside the range of concentration shown. Note differences in scale on *x*-axis. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb (VOC-31) and 150 ppb (VOC-43), respectively. Acronyms: VOC, volatile organic compound; ppb, parts per billion.

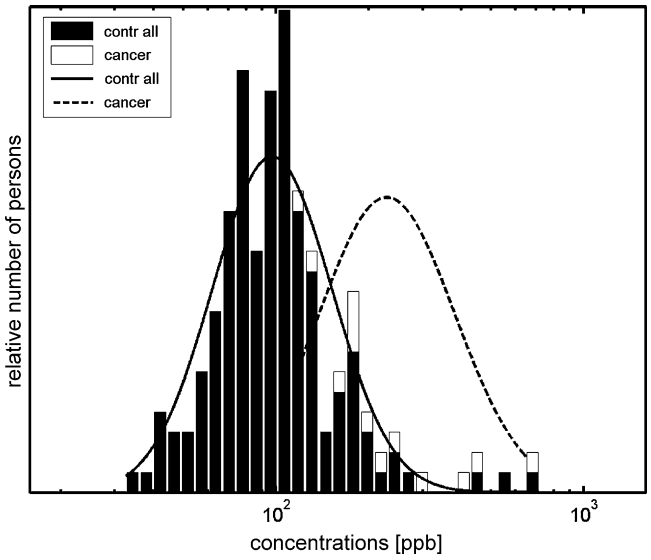

Fig. 2. Histogram showing the distributions of concentrations for VOC-43 on a logarithmic scale for the overall control group compared with the group of primary lung cancer patients.

to acetone. It has been included as a prime example of a non-discriminating substance present at equally high concentrations in the exhaled air of both patients and controls. The VOC ionized to the ion at $m/z = 108$ (VOC-108), tentatively assigned to 2-methyl-benzenamine (*o*-toluidine), is another example of a non-discriminating compound being found at equal concentrations in the breath gas of both patients and controls, but unlike VOC-59 its concentrations are vanishingly small. Fig. 3 shows box plots of the concentration distributions of the compound ionized to the ion at $m/z = 42$ (VOC-42) as observed in the breath gas of smoking and non-smoking primary lung cancer cases and controls. From these plots it clearly appears that the breath gas concentration of VOC-42, tentatively assigned to acetonitrile, discriminates well between smokers and non-smokers, but not between patients and controls.

The primary lung cancer marker candidates VOC-31 and -43 underwent a closer statistical analysis. Additional sub-collectives of interest were generated starting from the super-collectives of patients and controls, respectively (see Tables 4 and Fig. 4, bottom). Based on ROC-curves, Fig. 5 finally addresses the question of efficiency of the breath gas concentration of VOC-31 (Fig. 5, top) and VOC-43 (Fig. 5, bottom) in discriminating between the primary lung cancer patients and the total control collective. No particularly chosen threshold for these VOC concentrations, however, gives rise to a complete

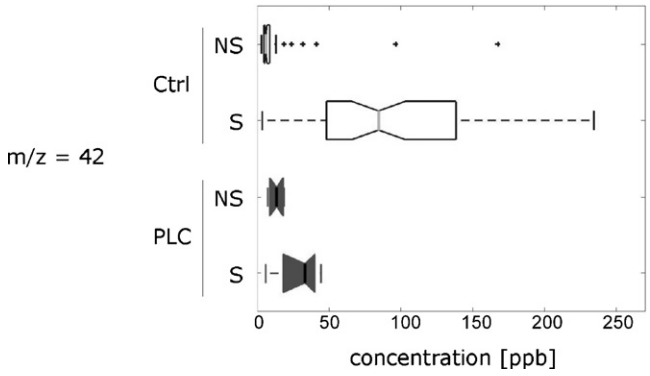

Fig. 3. Concentration distributions of VOC-42 (tentatively acetonitrile) in the breath gas of smoking (S) and non-smoking (NS) primary lung cancer patients (black boxes) and controls (white boxes). Boxes mark the lower, median and upper quartiles; whiskers conform to MATLAB convention; outliers (+) are values out of the whisker range. A few outliers are outside the range of concentration shown. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb (VOC-31) and 150 ppb (VOC-43), respectively. Acronyms: VOC, volatile organic compound; ppb, parts per billion.

Table 4a
Concentration (median and iqr) of VOC-31 in the breath gas of control and primary lung cancer sub-collectives

Sub-collective	Control (ppb)		Primary lung cancer patients (ppb)		*P*
	Median	Iqr	Median	Iqr	
Not older than 55 a	2.9	1.9	5.2	16.2	5.5E−02
Older than 55 a	3.9	0.9	8.0	14.0	9.8E−05
Smokers	2.9	1.7	7.4	9.5	5.9E−04
Non-smokers	3.1	1.9	6.7	20.1	7.0E−03
Females	3.0	1.8	3.1	1.1	n.s.
Males	2.9	2.0	8.3	16.1	1.2E−05
Total	3.0	1.9	7.0	15.5	5.1E−06

Tentative assignments: VOC-31, formaldehyde. *P*-values obtained from Wilcoxon rank sum tests; if $P < 0.05$, difference significant by definition. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb. Acronyms: iqr, interquartile range; VOC, volatile organic compound; ppb, part per billion (1E−09); n.s., non-significant.

Table 4b
Concentration (median and iqr) of VOC-43 in the breath gas of control and primary lung cancer sub-collectives

Sub-collective	Control (ppb)		Primary lung cancer patients (ppb)		*P*
	Median	Iqr	Median	Iqr	
Not older than 55 a	95.2	53.9	236.2	63.8	2.4E−03
Older than 55 a	69.2	55.0	244.1	448.3	1.6E−04
Smokers	119.2	73.9	257.4	311.8	2.4E−04
Non-smokers	76.8	42.2	194.2	235.0	2.7E−04
Females	89.7	51.5	413.5	397.0	2.1E−02
Males	99.5	60.2	244.1	170.6	2.3E−05
Total	94.1	55.2	244.1	236.2	3.1E−07

Tentative assignments: VOC-43, propanol. *P*-values obtained from Wilcoxon rank sum tests; if $P < 0.05$, difference significant by definition. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 150 ppb. Acronyms: iqr, interquartile range; VOC, volatile organic compound; ppb, part per billion (1E−09).

Table 5
Results from Fisher's Quadratic Discriminant Method for the combination of the parameters VOC-31, VOC-43, and age

Parameter	Mean ± S.D.
Accuracy	0.96 ± 0.02
Sensitivity	0.54 ± 0.20
Specificity	0.99 ± 0.01
Positive predictive value	0.90 ± 0.16
Negative predictive value	0.96 ± 0.02

The mean ± S.D. was computed based on 1000 different values for the indicated parameters. Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb (VOC-31) and 150 ppb (VOC-43), respectively. Acronym: VOC, volatile organic compound.

discrimination. We computed not only ROC-curves comparing all patients with all healthy volunteers, but also ROC-curves for the comparison of persons in the two groups being older than 30 (or older than 40, or older than 50) (Fig. 5). Corresponding integral values that serve as indicators for good separation of patients from healthy volunteers are summarized in Fig. 5.

Finally, together for VOC-31, -43, and age, we split the patient and volunteer groups randomly into a respective learning set (60% of persons in respective groups) and a test set (40% of persons in respective groups). Fisher's Quadratic Discriminant Method was then used to compute the accuracy, sensitivity, specificity, positive predictive value and negative predictive value for correct class-affiliation in the test set. With 1000 simulations (and corresponding 1000 different learning and test sets) we arrived at the results summarized in Table 5. The sensitivity is low with 0.54 ± 0.20 for the combination of the parameters VOC-31, -43 and age. The accuracy is 0.96 ± 0.02, the positive predictive value is 0.90 ± 0.16, the negative predictive value 0.96 ± 0.02, the specificity is 0.99 ± 0.01. Hence, relatively few healthy volunteers in our cohort were wrongly categorized as cancer patients. Note that these statistical results are based on a prior probability of 5% for lung cancer in the overall population and that different prior probabilities lead to different results, i.e. change specificity and sensitivity (whereas the accuracy remains roughly the same).

4. Discussion

The VOC composition of the air inhaled just before and during breath gas sampling certainly influences the VOC composition of the exhaled breath. Hence, organic trace gases present in the ambient air must be taken into account. For instance, all compounds tentatively identified with the selection of Fig. 1 are found in environmental air. Formaldehyde is emitted from wood products and textiles [41] and it occurs as a central metabolic intermediate in methylotrophic bacteria [42]. Isoprene is also produced by plants in large amounts. Isopropanol is a frequently used solvent utilized in the production of or contained in many cosmetics and sanitary products. Acetone is another widely used solvent and furthermore is a product of photo-oxidation of some alkanes and alkenes being found in urban air [43]. Similarly, the probability of exposure to *o*-toluidine is high in view of the

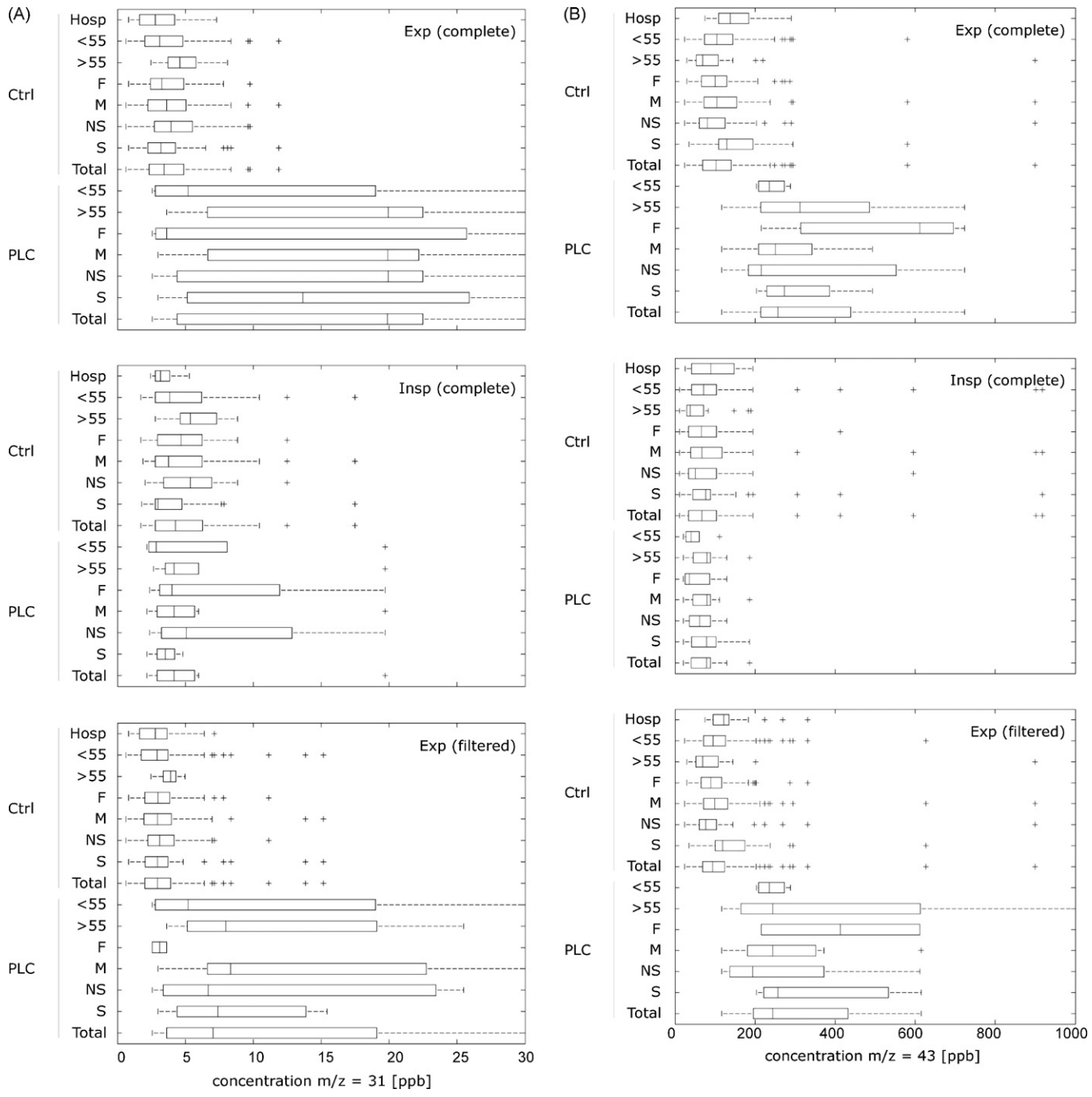

Fig. 4. (A) Concentration distributions of VOC-31 (tentatively formaldehyde) are shown: exhaled (top), inhaled (middle), and exhaled after applying our data selection by the filter condition (1) and an additional threshold of 5 ppb (bottom). Primary lung cancer patients and control sub-collectives (white boxes) and the corresponding sub-collectives are shown. Sub-collectives: Hosp, hospital controls; <55, not older than 55 a; >55, older than 55 a; F, women; M, men; S, smokers; NS, non-smokers. Boxes mark the lower, median and upper quartiles; whiskers conform to MATLAB convention; outliers (+) are values out of the whisker range. A few outliers are outside the range of concentration shown. Acronyms: VOC, volatile organic compound; ppb, parts per billion. (B) Concentration distributions of VOC-43 (tentatively isopropanol) are shown: exhaled (top), inhaled (middle), and exhaled after applying our data selection by the filter condition (1) and an additional threshold of 150 ppb (bottom). Primary lung cancer patients and control sub-collectives and corresponding sub-collectives are shown. Sub-collectives: Hosp, hospital controls; <55, not older than 55 a; >55, older than 55 a; F, women; M, men; S, smokers; NS, non-smokers. Boxes mark the lower, median and upper quartiles; whiskers show the 10 and 90 percentiles; outliers (+) are values out of the whisker range. A few outliers are outside the range of concentration shown. Acronyms: VOC, volatile organic compound; ppb, parts per billion.

fact that the chemical is a constituent of more than 90 different dyes and many other industrial products [44]. Yet, since in the present pilot study the concentrations of all VOCs examined more closely are found to be roughly constant throughout all collected samples of inhaled ambient air (concentration of VOC-31 and -43 shown in Fig. 4, middle; others not shown), we attribute observed differences in the breath gas concentration of these VOCs to individual physiological alterations rather than to dif-

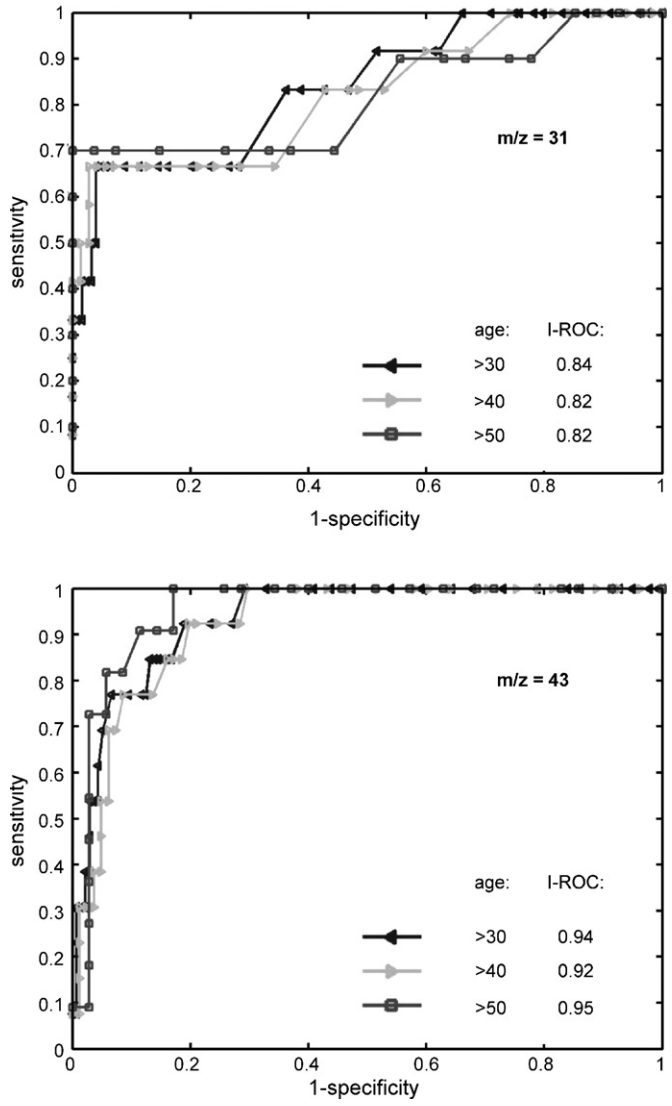

Fig. 5. ROC-curves for the two potential primary lung cancer markers VOC-31 and VOC-43 in the study population: sensitivity vs. (1 − specificity) upon variation of the threshold breath gas concentration discriminating between patients and control collective. Best achievable discrimination is indicated by the integrals of the ROC-curves (I-ROC; see Section 2 for further details). Note that only those patients and healthy volunteers have been considered, which fulfill the filter condition (1), or are below the threshold of 5 ppb (VOC-31) and 150 ppb (VOC-43), respectively.

ferences in short-term exposure to the respective VOCs. Future work should certainly investigate carefully the rate of uptake of compounds from indoor air, the metabolization of such inhaled compounds and exhalation of metabolites, as well as the washout times for these compounds (after a person leaves a room with high concentrations of these compounds in indoor air).

Formaldehyde is one of the potential primary lung cancer biomarkers emerged from this study if the tentative assignment of VOC-31 is correct. Not yet proposed as a single biomarker for primary lung cancer, it is also found in the breath gas of healthy subjects at low concentrations of a few ppb. It is an intermediate of some toxic and mutating impact [45] occurring in oxidative conversions of the hepatic metabolism. It arises in the cytochrome P_{450}-dependent methanol catabolism governed by the microsomal alcohol-oxidizing system and in the methyl ether *O*-dealkylation catalyzed by the microsomal mono-oxygenase [46]. Methylotrophic bacterial strains naturally occurring in the physiological human (oral and gastrointestinal) microflora [47] have also been described to generate and consume formaldehyde [42]. In addition, expression of immunomodulatory enyzme indoleamine (2,3)-dioxygenase (IDO) could be involved in the accumulation of formaldehyde [48]. IDO is the rate-limiting enzyme in the degradation of the essential amino acid tryptophan via the kynurenine pathway and forms *N*-formyl-kynurenine, which is subsequently converted to kynurenine. During this latter biochemical reaction, formaldehyde is released. Accelerated degradation of tryptophan due to enhanced IDO activity has been found in patients suffering from various types of cancer, e.g. including colorectal carcinoma and malignant melanoma, and it was associated with shorter survival of patients [49,50]. Genotyping the cytochrome P_{450}-polymorphisms suspected of being responsible for increased primary lung cancer incidence [51] as well as measurement of *O*-dealkylation activity [52] may shed more light on breath formaldehyde related to primary lung cancer. Isopropanol is the other potential primary lung cancer biomarker emerged from this study if the tentative identification of VOC-43 as isopropanol is correct. It is known to be abundant in the exhaled breath of healthy individuals [53]. In addition, isopropanol has been already found in the breath gas of lung cancer patients without being explicitly proposed as single marker [34]. Isoprene, tentatively identified with VOC-69,[2] is one of the most abundant VOCs in exhaled human breath [10] besides acetone. Its concentration in blood may be related to the rate of blood cholesterol formation. Its concentration in breath depends on heart rate, breathing rate and breathing volume [54]. The fact that the breath gas concentration of VOC-69 is slightly smaller in primary lung cancer patients than in healthy volunteers suggests that the opposite sign of the respective differences in VOC-31 and -43 is not just an artefact due to different content of dead space volume in the exhaled breath samples. Finally, no primary lung cancer marker potential is apparent from our records on VOC-108 tentatively assigned to *o*-toluidine as suggested in an early investigation [55].

Phillips et al. proposed methylated alkanes detected in exhaled human breath as biomarkers for primary lung cancer and found their concentration to depend on age [23,24,56]. Unfortunately, most alkanes cannot be detected by PTR-MS. Our primary lung cancer marker candidates VOC-31 and -43 do not correlate with age as shown by the described sub-collectives ≤55 and >55 years in Fig. 4 and Tables 4a and 4b. Furthermore, they exhibit no gender- or smoking-specific effects as the marker alkanes suggested by Michael Phillips (Fig. 4 and Tables 4a and 4b). Hence, our mismatch with respect to smoking does not seem to be significant (Table 2). Nevertheless, because of our low number of patients, this interpretation should be considered preliminary.

[2] Isoprene partly fragments to *m/z* 41 in PTR-MS.

There is evidence for acetaldehyde (molecular mass 44 u) to be a possible marker for lung cancer. *In vitro*, two non-small cellular cancer cell lines (SK-MES and CALU-1) were found to emit acetaldehyde in proportion to the overall cell number independently of the glucose levels in the cell culture medium [12,29]. This suggests that tumour size, i.e. tumour stage, could be related to breath gas concentrations of acetaldehyde *in vivo*, as found for several cancer markers in blood. Acetaldehyde is detected at $m/z = 45$ by means of PTR-MS, where unfortunately a fraction of protonated CO_2 is also detected (exhaled breath contains $4\% = 4 \times 10^7$ ppb CO_2). Consequently, the fraction of acetaldehyde has not been determined in our experiments, even though VOC-45 seems to be increased in the breath of primary lung cancer patients. However, regarding the concentrations of our suggested marker molecules VOC-31 and -43, no differences related to tumour stage were observed (data not shown).

With the PTR-MS and the SIFT-MS [57] technique, time-consuming preconcentration and separation procedures of breath samples are not needed, and breath samples can readily be analyzed. Both techniques would allow on-line measurements, which would be a further improvement in comparison with bag-collection of exhaled breath samples. Nevertheless, in our setting, we cannot bring patients to the PTR-MS or the PTR-MS to patients.

An advantage of PTR-MS is that abundant molecules in air such as nitrogen, oxygen and water do not interfere with measured VOCs. Sensitivity in the range of a few ppbv and even ppt [36,57] allows absolute quantification also of VOCs. By PTR-MS and SIFT-MS, one important criterion for daily clinical screening, namely simplification of sample collection and rapid as well as online (real time) analysis would be fulfilled. Besides, it is inexpensive (no laboratory consumables such as columns, carrier gases or molecular biological kits are necessary) and convenient for patients.

The severe disadvantage of PTR-MS is its inability to distinguish between substances which have the same molecular mass. Formaldehyde, for example, has the same molecular mass as nitric oxide and ethane: only the fact that nitric oxide and ethane are not protonated by the primary ion H_3O^+ (due to their low proton affinity) leads us to conjecture that $m/z = 31$ concentrations should be attributed to formaldehyde. From this point of view, the use of GC–MS with library identification is superior to PTR-MS investigation of breath gas samples.

5. Conclusions

To the best of our knowledge this is the first pilot investigation using PTR-MS for the analysis of breath samples from primary lung cancer patients compared to a collective of healthy controls including healthy volunteers working in a hospital area. Although the total number of patients is small, PTR-MS investigations revealed significant differences for distinct masses between primary lung cancer patients and healthy volunteers: VOC-31 and -43 served as best single discriminators between cases and controls.

Our study is a pilot study: the interquartile distances in the concentrations of VOC-31 and -43 are huge (in the same range as the median values). This indicates that various different effects contribute to the increase in concentration of VOC-31 and -43. The small *P*-values (see Table 3) partly result from the relatively large number of healthy volunteers in our study and therefore should not be overestimated. Even for m/z 42 (tentatively assigned to acetonitrile from cigarette smoking), there is no perfect separation between the groups of smokers and non-smokers in Fig. 3.

'Contaminated' room air, age and smoking do not seem to be responsible for the observed increases in concentration for VOC-31 and -43 in primary lung cancer patients. Nevertheless, future investigations should validate such effects in more detail, and determine, in particular, uptake of compounds (from indoor air in a particular contaminated room) and washout times (after leaving this room). Due to our small study population, other influencing factors (i.e. gender, tumour histology and tumour stage) have to be further evaluated.

Beyond doubt, GC analysis and peak identification with computer-based library of mass spectra will be necessary to confirm and further discussions of a plausible role for formaldehyde and (*iso*)propanol in lung cancer pathophysiology are required. Due to the small study population, further clinical evaluation needs to be done to strengthen our findings that ions with VOC-31 and -43 are useful markers for correct diagnosis of lung cancer.

Acknowledgments

This project has been supported in part by the European Commission (project BAMOD, project no. LSHC-CT-2005-019031). We appreciate very much the support by the University Clinics of Innsbruck (TILAK) and its Director Dr. Herbert Weissenböck. Anton Amann thanks Wolfram Miekisch, Jochen Schubert, David Smith, Patrik Španěl, Svorad Stolc and Viktor Witkovsky for improving the manuscript by many very valuable suggestions.

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