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Exhaled biomarkers in lung cancer

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ABSTRACT: Lung cancer is the leading cause of cancer death. Results of therapeutic interventions are particularly discouraging when the disease is discovered in an advanced stage. Early diagnosis is limited by the fact that the disease usually develops asymptomatically and available screening methods do not fulfil the requirements for reliable discrimination between patients with lung cancer and subjects not suffering from the disease. Breath sampling is completely noninvasive and provides a potentially useful approach to screening lung cancer. Exhaled biomarkers contain both volatile and nonvolatile molecules. The profile of volatile organic compounds is different in patients with lung cancer than in control subjects. In exhaled breath condensate, the proteomic profile of breath from cancer patients differs from that of healthy smokers. We reviewed the scientific evidence demonstrating that a unique chemical signature can be detected in the breath of patients with lung cancer and that the exhaled breath biomarker profile could aid clinical decision making.

KEYWORDS: Biomarker, electronic nose, exhaled breath, exhaled breath condensate, lung cancer, smell

Lung cancer is the leading cause of global cancer death in both males and females. According to the most recent projection of global mortality, by 2030 it will emerge as the third and the fifth leading cause of death in high- and middle-income countries, respectively [1, 2]. Figures on disease outcome measures are very discouraging as even with the most advanced treatment strategies ~86% of lung cancer patients die within 5 yrs of diagnosis. With early detection and treatment, however, the 5-yr survival rate improves dramatically from 20% in patients with stage III lung cancer to 70% in patients with stage I disease [3]. Researchers, therefore, have sought out screening tests to detect lung cancer in the earliest stages and several promising new approaches have been proposed for this purpose, such as computer-assisted image analysis of chest radiographs, spiral computed tomography (CT) scanning, PCR-based assays of sputum and fluorescence bronchoscopy [4–7].

Breath chemical tests have a broad spectrum of applications ranging from the US Food and Drug Administration-approved exhaled nitric oxide fraction (F_{ENO}) measurement to monitor the effect of anti-inflammatory treatment in asthma, to volatile organic compound (VOC) determination and nonvolatile biomarker profiling in the cooled breath sample called exhaled breath condensate (EBC) [8–11]. Being completely noninvasive, sampling of the breath allows clinicians and researchers to assess different body functions in a flexible manner. Breath collection can be performed even in very severe patients and also repeated within short intervals. Therefore, breath testing is considered to be a potentially ideal candidate for screening purposes. Besides widely known constituents such as nitrogen, oxygen, carbon dioxide, inert gases and water vapour, exhaled breath also consists of thousands of volatile and nonvolatile components, mainly in trace amounts, making detection a challenging task. Application of highly sensitive cutting-edge technologies in sample analysis provides firm background for proper evaluation of this type of human sample. The use of innovative ''-omics'' technologies, including proteomics, metabolomics, mass spectromics, gas

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chromatography/mass spectrometry (GC-MS) and ion mobility spectrometries, offers great potential for the field of exhaled biomarker profiling [12]. Exhaled breath biomarkers have been assessed to understand disease pathomechanism and also to aid clinical decision making. For each purpose, completely different strategies have been implemented, including the determination of individual biomarkers and the recognition of signal patterns created by undefined compounds. Although pattern recognition is a challenging task from the statistical point of view, it is a powerful tool to analyse samples that comprise of a large number of different constituents.

BREATH TESTS TO DETECT LUNG CANCER

Exhaled VOCs and "smellprints"

Exhaled VOCs and their origin

VOCs in human breath were first described by PAULING *et al.* [13] in 1971. Now, it is known that exhaled breath contains thousands of different VOCs, most of them in picomolar (10^{-12} mol·L^{-1} or particles per trillion) concentrations. In normal subjects, more than 3,000 different VOCs can be detected; however, only 20–30 of these VOCs are present in all subjects. These are principally isoprene, alkanes, methylalkanes and benzene derivatives [14].

Exhaled organic compounds can originate from two main sources: exogenous volatiles that are inhaled (or absorbed through the skin) and then exhaled and those endogenously produced by different biochemical processes. Basic cellular functions including maintenance of cell membrane integrity, energy metabolism and especially oxidative stress are all known to be linked with VOC formation. Alkanes are generated during the lipid peroxidation of polyunsaturated fatty acids by reactive oxygen species and, although debated, the so-called breath alkane profile has been postulated as a new biomarker of oxidative stress [15, 16]. Aldehydes, ethane and penthane are all produced during lipid peroxidation and can be detected in exhaled breath [17]. Additionally, acetone formed *via* the decarboxylation of acetoacetate also arises from lipid peroxidation. Furthermore, the production of isoprene from acetyl-coenzyme A is associated with cholesterol biosynthesis [18]. These examples highlight that widely different biochemical pathways result in VOC formation and also imply that endogenously produced VOCs originate from several cell types.

Catabolism of many VOCs, including camphor, occurs through the cytochrome P450 (CYP) mixed oxidase enzymes [19]. Regardless of the distance of the organ where produced, VOCs can be transported by the blood to the lungs and exhaled during breathing. Therefore, the origin of exhaled VOCs is assumed to be mainly alveolar; however, direct comparison of VOC profiles from different parts of the lung and the airways is lacking. Changes either in the production or clearance of VOCs may result in alteration in their exhaled concentration, which can also be influenced by the gas-exchange properties of the lung. Several studies have assessed the changes in exhaled VOC profile in diseases of different organs. No detailed information is available, however, on the question of whether organ specific VOC profile exists and if functional changes in different organs are linked with altered exhaled VOC profile specific to a disease.

Collection of exhaled VOCs

Collection of exhaled breath for VOC detection is a simple procedure; however, there are several important methodological issues to consider (fig. 1). First, most of the components known to be present in exhaled breath can also be found in the environment. It is therefore necessary to distinguish the breath signal from an artefact of contamination with room air. One approach is to simultaneously collect VOCs in the breath and also in the air in order to determine the alveolar gradient (concentration in breath minus concentration in air) of each VOC as proposed by PHILLIPS *et al.* [9]. Although this method is easy to perform, it does not fully take into account the complexity of pulmonary absorption and exhalation of volatile substances. This approach has failed in $F_{E}NO$ studies and those studies may serve as examples for addressing the problem in another way. Another approach might be the use of a VOC filter at the inhalation port of the breath collecting apparatus which ensures the capture of environmental VOCs before the exhaled sample is taken. Nonetheless, no approach considers that exposure to environmental VOCs may have a sustained effect on exhaled VOC pattern since volatiles can readily be absorbed from the ambient air entering the blood, from where, along with endogenously produced molecules, they can be continuously released. This issue can be addressed by follow-up studies performing serial measurements with subjects inhaling VOC-free gases.

Secondly, due to the very low concentrations of volatiles in exhaled breath, optimised sample collection and very sensitive instruments are required for exhaled VOCs detection. Different research groups applied widely different approaches for breath collection (table 1). In the study of POLI *et al.* [20] a bag nonpermeable for VOCs was used for collection and VOCs were captured with a solid phase microextraction technique from the obtained sample. A portable breath collection apparatus has been developed by PHILLIPS and colleagues [9, 21], which collects breath VOCs onto sorbent traps for subsequent analysis by GC-MS. The potential influence of breathing pattern and the contribution of volatiles, from the upper airways and the mouth, to exhaled biomarkers might be of significance. Therefore, these aspects of sampling procedures have been

FIGURE 1. Sampling of exhaled breath for smellprint analysis by electronic nose. The subject inhales though a volatile organic compound filter and exhales with stable flow rate against resistance.

studied in detail in the case of measuring F_{ENO} but not in terms of exhaled VOCs, resulting in widely different sampling methods for collecting organic volatiles. Some researchers aimed to collect only alveolar air by asking the subjects to exhale from total lung capacity and sampled only the last fraction of the breath while others asked the participants to perform tidal breathing during sample collection. In a few studies, resistance was used against exhalation to close the soft palate in order to prevent nasal contamination of the sample. The importance of sampling procedures was comprehensively discussed in a recent study of MIEKISCH *et al.* [22].

Analysis of VOC patterns

Due to the feature that exhaled VOCs represent a complex mixture of entirely different molecules and that the usual aim of investigations is to discriminate between health and disease (or lung cancer and non-lung cancer) by an alteration in exhaled pattern, various statistical algorithms were applied in the reported studies (table 1) without direct comparison between these approaches. The number of VOCs selected for analysis was also variable. The lack of standardised sampling and statistical analytical procedures may be one of the main reasons for the differing results among research groups.

Selection of study groups, potential confounding factors

The last but not least important issue is the selection of the control group. Lung cancer is considered to be the endstage of multistep carcinogenesis. An unequivocal link between tobacco smoke and lung carcinogenesis has been established by molecular findings and also by epidemiological and

TABLE 1 Clinical utility of breath tests for detection of lung cancer[#]

First author [ref.]	Method used	Statistical approach	Cancer subjects n	Control subjects n	Sensitivity %	Specificity %
CHEN [23]	Solid-phase microextraction-GC (11 VOCs)	"breath diagnostic rule"[¶]	29	13 healthy 7 chronic bronchitis	86.2	69.2 71.4
MACHADO [24]	Electronic nose, filter used, pressure against exhalation, inhalation to total lung capacity	Principal components and canonic discriminant analysis	28: 7 SCLC, 21 NSCLC	109 Beryllium disease, asthma, COPD, healthy	71.4	91.9
MAZZONE [25]	Colorimetric sensor array, no filter, tidal breath	Random-forest method	49 NSCLC	94 COPD, IPF, sarcoidosis, PAH, healthy	73.3	72.4
MCCULLOCH [26]	Dog sniffing polypropylene organic vapour sampling tube, deep inhalation–exhalation, stored for 1–60 days	Double-blind, comparison of sitting and lying	55 NSCLC	83 healthy subjects	99	99
PHILLIPS [27]	GC-MS, 22 VOCs, sorbent trap	Forward stepwise discriminant analysis	60	48	71.7	66.7
PHILLIPS [28]	GC-MS, 9 VOCs, nose clip, tidal breathing, sorbent trap	Forward stepwise discriminant analysis	67	132 healthy subjects	85.2	80.5
PHILLIPS [29]	GC-MS, 16 VOCs, alveolar gradient of VOCs, sorbent trap	Multivariate analysis with fuzzy logic	193	211	84.6	80
PHILLIPS [30]	GC-MS, 30 VOCs, sorbent trap	Weighted digital analysis	193[+]	211[+]	84.5	81
POLI [20]	GC-MS, 13 VOCs, last portion of slow vital capacity, solid phase microextraction	Multinomial logistic-regression analysis	36 NSCLC	110 patients with COPD, healthy smokers and nonsmokers	72.2	93.6
WEHINGER [31]	Proton transfer reaction-MS 2 VOCs	Fisher's quadratic discriminant method	17	170	54	99

Total number of subjects is given. In some studies the training set and the validation set consisted of two different subject cohorts, while in others the same dataset was analysed twice. GC: gas chromatography; VOC: volatile organic compound; SCLC: small cell lung cancer; NSCLC: nonsmall cell lung cancer; COPD: chronic obstructive pulmonary disease; IPF: idiopathic pulmonary fibrosis; PAH: pulmonary arterial hypertension; MS: mass spectrometry. [#]: only studies providing values for sensitivity and specificity are listed; [¶]: if a subject's breath contained $\geqslant$1 out of 11 VOCs with a concentration that is higher than the diagnostic cutoff determined by the authors the patient is regarded as lung cancer patient, otherwise, the patient is regarded to have a noncancerous condition; [+]: data from [29] were analysed using a new statistical approach.

preclinical animal experimental data. In males and females, 90% and 78% of lung cancer cases, respectively, are estimated to be caused by tobacco smoking. Smoke contains many of the volatiles that are also present in the breath. Therefore, it is critical to assess the effect of tobacco smoking on the exhaled biomarker pattern, as it has already been approached from different aspects in reported investigations. In the majority of studies assessing exhaled biomarkers in lung cancer patients, a group of smokers without cancer was recruited for comparison. In this respect, the other major pulmonary disease associated with smoking, chronic obstructive pulmonary disease (COPD), deserves attention as well. COPD is characterised by typical lung function deterioration, chronic systemic and local airway inflammation and structural changes in lung parenchyma. The level of several exhaled biomarkers has been found to be altered in patients with COPD compared with healthy control subjects. Since the development of lung cancer is much more frequent in COPD patients than in controls, attention needs to be focused on the subtle differences in exhaled biomarker profiles between the two disease groups.

In spite of these uncertainties, there is, however, convincing evidence that the biochemical analysis of trace constituents in exhaled breath can provide valuable information when testing for lung cancer.

Differences in exhaled VOC profiles between lung cancer patients and control subjects

Different research groups have demonstrated that the exhaled VOC profile of patients with lung cancer differed from that of control groups. Using GC-MS, in 1985, GORDON *et al.* [32] showed that three VOCs had considerable difference in their presence in the 12 investigated patients with primary lung cancer compared with control subjects. Their model demonstrated 93% accuracy in separating the two groups. Three years later, two other groups confirmed their findings by using a different set of VOCs for discrimination of lung cancer patients from control subjects, but both studies had a relatively small sample size [33, 34]. The first report involving a larger number of subjects was published in 1999 by PHILLIPS *et al.* [27]. The authors chose 22 VOCs for analysis and determined their alveolar gradient. Their method showed 71.7% sensitivity and 66.7% specificity in selecting patients with lung cancer from control subjects. They demonstrated that some of the investigated alkanes were present in a lower concentration in the breath of patients with lung cancer than in samples obtained from controls. Their proposed explanation for these findings was that, during carcinogenesis, CYP enzymes are induced resulting in the enhanced catabolism of several VOCs. According to their views, CYP induction occurs in not only the tumour tissue but also other regions of the body, which might explain why the VOC profile is similar in all stages of lung cancer and does not change after tumour resection either. The same research group performed two larger scale multicentre studies applying exhaled VOCs profiling in lung cancer screening [28, 29]. The results of these studies showed that the test had a sensitivity of ~85% and a specificity of 80% in selecting patients with lung cancer (fig. 2). The data from the second study [29] were analysed using a different statistical approach, weighted digital analysis, that resulted in a similar sensitivity and specificity values (84.5% and 81%, respectively) [30]. The authors found that the exhaled VOC profile of patients with resected lung cancer was scored as cancer. They fitted this observation into their concept that CYP is induced not in cancer cells but in other cell types.

Other groups reported somewhat different results [20, 31]. In the study of WEHINGER *et al.* [31], a relatively low sensitivity (54%) with great specificity (99%) was found. POLI *et al.* [20], using a different method for sample collection, demonstrated that some VOCs are present in exhaled breath with elevated concentration in patients with lung cancer compared with control subjects, including asymptomatic smokers, nonsmokers and patients with COPD [20]. Exhaled breath of nonsmoker controls had higher levels of isoprene and heptane than in the ambient air, whereas patients with nonsmall cell lung cancer (NSCLC) or COPD patients and also control smokers showed higher exhaled levels of almost all substances compared with nonsmokers. None of the VOCs alone discriminated between the study groups; however, applying multinomial logistic regression analysis, the VOC profile was correctly classified in 80% of cases. Their method had comparable sensitivity and, even, better specificity for selection of lung cancer patients and controls. Furthermore, they showed that the concentration of isoprene and decane, but not other volatile compounds, decreased after tumour resection. Acccording to the authors' hypothesis, at least some of the exhaled VOCs are produced by tumour cells explaining why resection causes a decrease in volatile level. This theory is supported by GC-MS studies [23, 35] and also by findings obtained with electronic nose technology and canine smell [36, 37].

The study of CHEN *et al.* [23] determined VOC patterns in the culture medium of different cells, including lung cancer cells (squamous cell carcinoma, adenocarcinoma, bronchioloalveolar carcinoma and nonsmall cell carcinoma), normal airway epithelial cells, tastebud cells, osteogenic cells and lipocytes. Tumour cells were cultivated from human tissue obtained by surgical resection of lung cancer. The same patients also provided breath samples before the surgery. They found that 11 characteristic VOCs were present in higher amounts in lung cancer patients than in control subjects (chronic bronchitis patients and healthy subjects). There were some different, but also a few common, VOCs detected from the various types of lung cancer cell cultures. They proposed that VOCs found in the culture medium are the metabolic products of the cancer cells and can be used as disease biomarkers. Interestingly, they found a cancer-type VOC profile in lung tissue samples macroscopically appearing as normal tissue that was proved to contain tumour cells after 10 days of cell cultivation. They suggested that profound changes occur in the tumour microenvironment at the earliest stages of carcinogenesis, represented by altered VOC production/elimination; therefore, VOC profiling might be able to detect lung cancer in the earliest stage. In another study, the amount of acetaldehyde released from lung cancer cells *in vitro* was found to be increased; also supporting the theory that the VOC production/elimination profile is modified in cancer cells compared with controls [35].

Smoking status and tumour-node-metastasis (TNM) stage did not affect the results in any of the above studies. These

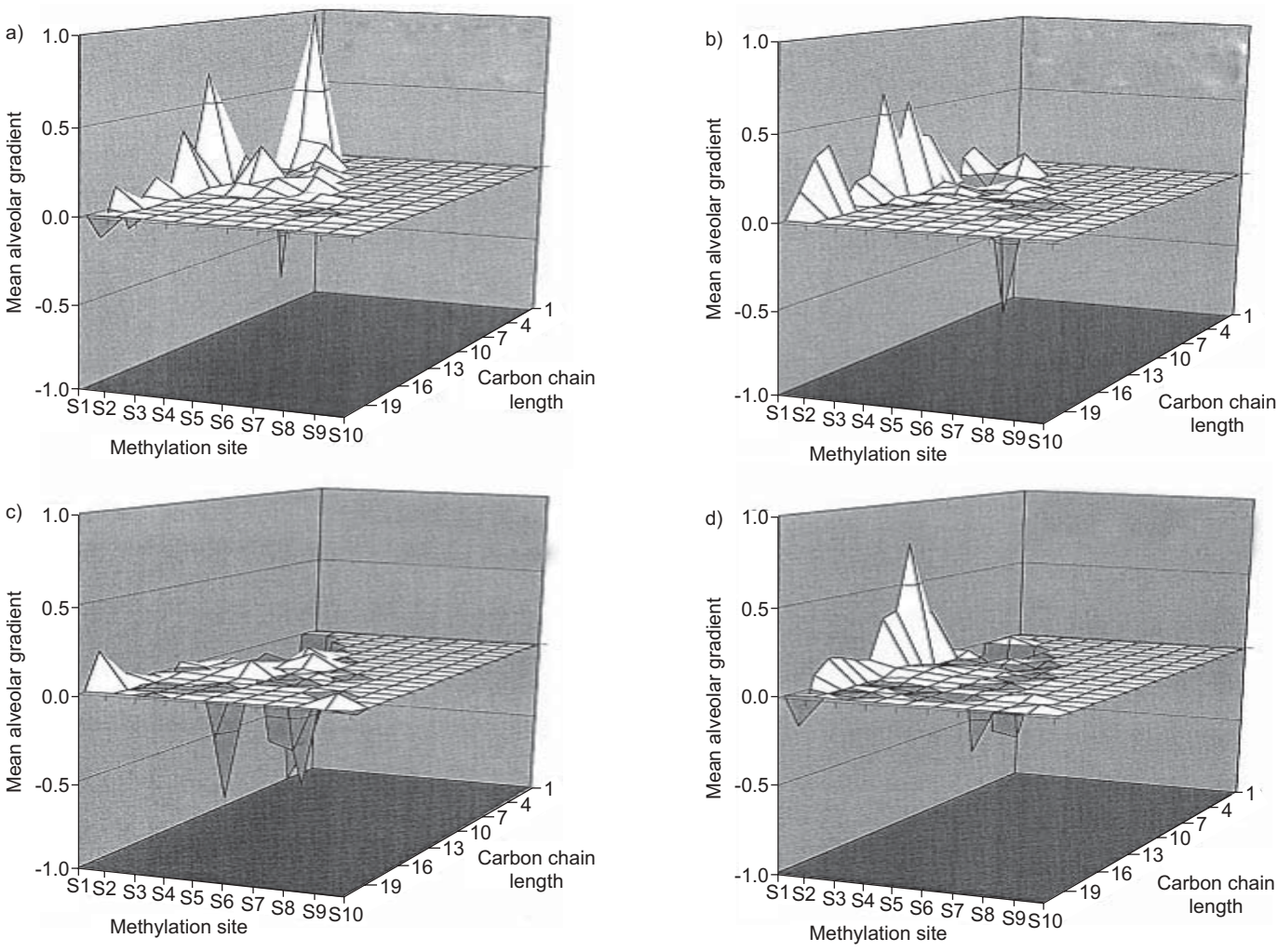

FIGURE 2. Analysis of exhaled volatile organic compound pattern by automated thermal desorption, gas chromatography, and mass spectroscopy. Surface plots of breath test results from a) healthy volunteers, b) cancer-negative bronchoscopy, c) primary lung cancer and d) metastatic lung cancer. Reproduced with permission from [28].

observations are highly important for a number of reasons. Smoking is associated with alterations in exhaled VOC pattern, as tobacco smoke contains abundant amounts of VOCs, and it also modifies cellular processes related to VOC production by increasing oxidative stress and lipid peroxidation. Variable smoking habit, therefore, may be expected as a confounding factor when using exhaled VOC profile in lung cancer detection. However, based upon published evidence, smoking did not change the discriminative power of VOC breath tests. In addition, TNM stage did not influence the outcome in any of the studies when the breath test was performed in stage I, as well as in more advanced stages of tumour growth, implying that exhaled profiling has the potential to evolve as a screening test for the disease.

GC-MS is a powerful tool for detecting small concentrations of VOCs but these systems are expensive and require highly skilled human resources that limit the applicability in everyday clinical practice. More simple and less expensive technologies have therefore also been studied.

Smellprint by electronic nose

Electronic noses rely on arrays of chemical vapour sensors that respond to specific characteristics of an odorant molecule including VOCs. Like the human olfactory system, the electronic system also generates a smellprint that can be compared with previously stored ones (fig. 3). Three recent publications demonstrated that breath samples from patients with lung cancer and those from healthy subjects can be distinguished by electronic nose technology [24, 38, 39]. In the first study, a quartz microbalance sensor system was used [38]. The quartz sensors were covered by metalloporphyrins to bind different VOCs. The authors found that the smellprint characterised the lung cancer and the control groups with 90% accuracy. A model developed from this system was reported to show 100% correct classification of lung cancer and 94% classification of controls. Another type of electronic nose, comprising of 32 separate carbon polymer sensors, was utilised in the study of MACHADO *et al.* [24]. In that study, the control group included both healthy nonsmokers and also patients with different lung diseases. The authors found a difference

between the smellprint of lung cancer patients and that of controls. In these studies, no signal difference was observed between patients with different clinical severity of the disease and no significant confounding effect of cigarette smoking and accompanying respiratory diseases, such as asthma and COPD, was detected. A recent study by DRAGONIERI *et al.* [39] demonstrated that the smellprint of lung cancer patients can be distinguished from that of COPD patients. These studies, however, were single-centre, cross-sectional recruiting a fairly limited number of lung cancer patients. In the study of MACHADO *et al.* [24], breath samples from a few patients after lung cancer resection were also included, which were classified into the noncancer group by the smellprint. These results are in line with those published by DI NATALE *et al.* [38] demonstrating that lung cancer patients after resection were grouped into the healthy or post-surgery groups but not to the cancer group. Based on these results, the authors concluded that altered smellprint is indeed related to the cancer tissue. Their hypothesis was confirmed by recent findings on cell cultures. In a recent study, cells from both tumour and normal cell lines were suspended in saline and a polymer composite electronic nose was used to evaluate the headspace gases [40]. The tumour cell lines, including adenocarcinoma, squamous cell carcinoma, and mesothelioma, were distinct from each other and from the normal fibroblast and smooth muscle cells as appeared on canonical discrimination plots.

The use of an electronic nose for detection of lung cancer offers several potential advantages but it has disadvantages as well. Strengths include high sensitivity, ease of administration of the test and portability of the detector. Limitations include loss of sensitivity in the presence of water vapour or high concentrations of a single component, sensor drift and the inability to provide absolute calibration, relatively short life of some sensors, necessity to do considerable method development work for each specific application, and the inability to obtain quantitative data.

Canine smell

Smellprint of humans is also recognised by dogs [41]. By the virtue of sensitivity of their smelling and their capacity to learn how to sign differences, dogs can be trained to discriminate exhaled breath samples from subjects with and without lung cancer [26]. In that double-blind study, dogs had an accuracy of 99% in discriminating between smells from exhaled breath of patients with lung cancer and controls. The stage of cancer, age of patients, smoking habit or most recently eaten meal did not influence the dog's diagnostic performance. Breath seems to serve as a better sample for canine discrimination than urine that was also proposed as a potentially good source of endogenously produced volatile compounds. In a recent study, GORDON *et al.* [42] could not train their dogs for proper discrimination of urine samples between patients with breast or prostate cancer from that of controls.

What makes smell: the cancer by itself or biochemical processes occurring at other sites in the body in response to the development of cancer? This question has recently been addressed using canine smell by HORVÁTH *et al.* [37] on ovarial cancer tissue. They were able to train dogs to discriminate ovarial cancer specimens from surgical ovarial tissue without cancer. Their results provide further evidence that metabolic pathways in cancer cells produce different volatile compounds (or at least a different pattern of them) than in noncancerous tissue.

According to a hypothesis, VOCs produced by tumours and detected by dogs are the products of major histocompatibility complex (MHC) genes [43]. These human leukocyte antigen (HLA) molecules have soluble isoforms that are present in blood, urine and sweat, and MHC-dependent odour components can be detected by an electronic nose [44]. There is an association between changes in HLA expression and cancer suggesting that the HLA-associated smellprint of human cancer could easily serve as olfactory cues. This hypothesis, although plausible, has not been tested.

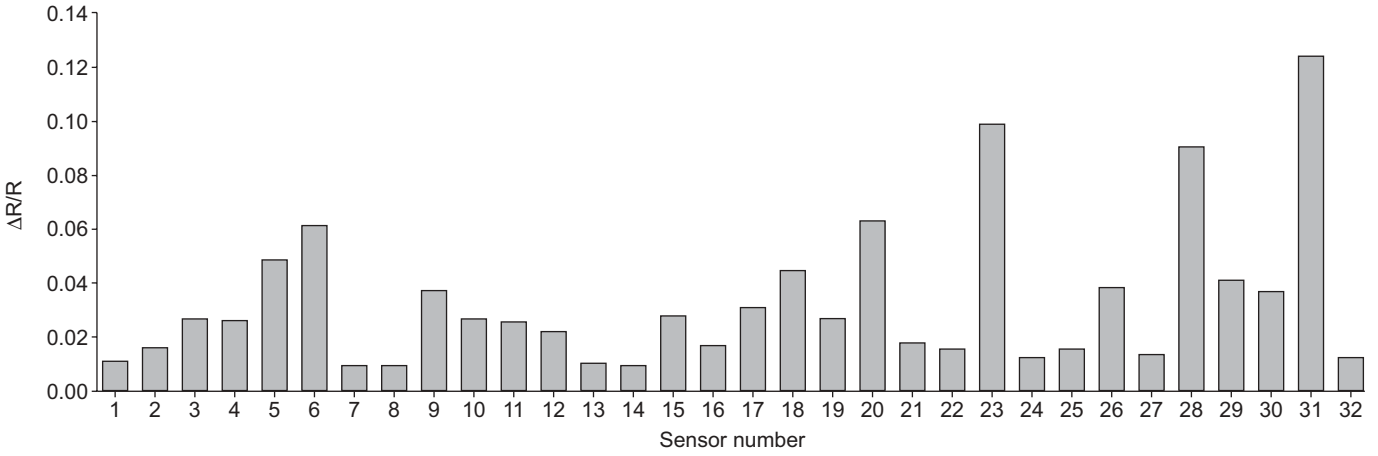

FIGURE 3. A representative smellprint of volatile organic compounds in exhaled breath recorded by an electronic nose with 32 polymer sensors as the change in sensor resistance (ΔR/R). The exhaled breath was collected from a lung cancer patient.

Colorimetric sensors

In the study of MAZZONE *et al*. [25] a colorimetric sensor array with 36 spots composed of different chemically sensitive compounds impregnated on a disposable cartridge was used to detect lung cancer from exhaled breath. The colour of these spots changes induced by the chemicals with which they come into contact. A total of 143 subjects were investigated (healthy subjects and patients with COPD, interstitial pulmonary disease, sarcoidosis and NSCLC). The model developed was able to predict the presence of lung cancer with a sensitivity of 73.3% and a specificity of 72.4%. Smoking, age and the stage of the disease did not affect the results in this study either.

Exhaled monoxides

Not only VOCs but also other gases have also been studied in the breath of lung cancer patients. A significant difference was observed in *F*eNO between lung cancer patients and control subjects, which was associated with altered expression of the inducible nitric oxide (NO) synthase [45]. Another study confirmed the observation of increased levels of *F*eNO and extended it demonstrating tumour-restricted tyrosine nitration in patients with cancer without increased NO synthase expression, suggesting an alteration of NO metabolism in the lungs of patients with cancer [46]. Other studies demonstrated a decrease in *F*eNO due to chemo- or radiotherapy in lung cancer [47, 48]. Considering the wide range of *F*eNO in healthy subjects and the confounding effect of tobacco smoking on *F*eNO level, the described mediator increase is not likely to be able to discriminate patients with lung cancer from those without.

Haem oxygenase (HO) is an antioxidant enzyme which catabolises haem to produce carbon monoxide (CO) and biliverdin. HO-1 is highly expressed in various tumour tissues and plays an important role in tumour cell growth through antioxidative and antiapoptotic effects. Although an exhaled CO level has been suggested to reflect HO activity and to be a marker of oxidative stress, this measurement is not likely to have a clinical utility for detection of lung cancer because smoking markedly affects exhaled CO levels [49, 50].

Gas sensor array technology is a rapidly growing field. Laser optic methods, arrays of nano- and mesowire sensors, and bioelectronic noses based on mammalian olfactory receptors in immobilised nanosomes represent the new generation of electronic noses for detection and discrimination of volatiles that may also be used in breath research [51–58].

EBC

Methodological issues of sample collection and biomarker measurement

Collection of EBC is a noninvasive method for obtaining nonvolatile compounds from the lungs. During EBC collection exhaled breath is directed through a cooling device, resulting in the accumulation of exhaled breath constituents in the cooling chamber. EBC contains numerous components. The principal component is condensed water vapour, representing nearly all of the volume (99%) of EBC samples. Only a small fraction of the condensate is derived from respiratory droplets containing nonvolatile molecules. EBC contains large number of ions, metabolites and other molecules including adenosine, ATP, ammonia, hydrogen peroxide, isoprostanes, lactate, leukotrienes, nitrogen oxides, peptides, prostaglandins, tromboxanes and various cytokines [10]. The mechanisms by which airway/ alveolar fluid substances are added to exhaled breath are not clear. The concentration of the different mediators is influenced by lung diseases and modulated by therapeutic interventions. Collecting devices, cooling temperature, type and length of storage, however, are all known to influence biomarker detectability in EBC. The European Respiratory Society/American Thoracic Society Task Force on EBC published recommendations for EBC sampling and biomarker detection discussing the most important methodological issues [10]. Several issues of concern were shared by studying exhaled volatiles and EBC. Oral/upper airway/salivary contamination, expiratory flow, surface and temperature of the collecting device, sample storage, and mode of mediator determination have all been shown to influence the results of EBC studies [59–76]. One specific consideration in EBC research is the dilution of droplets by water vapour. A so-called dilution factor has been suggested to counteract the potential variable dilution of biomarker molecules by water vapour [10, 77]. The use of mass spectrometry allows investigators to determine dilution factor together with the studied biomarker from the same sample [78]. Nevertheless, it is not yet proved that any of the proposed dilution factor measurements can properly serve to normalise data.

In EBC, mainly individual cytokines have been investigated in lung cancer using enzyme immunoassays (EIA). Commercially available immunoassays, however, are not validated for EBC, which is a very different matrix than that employed in many commercially available standards. Furthermore, cytokines are present in very low concentrations in raw EBC samples, frequently at or below the lower limit of detection of EIA assays. Assay validity is characterised by the lower limit of detection (LLD), reproducibility (intra- and interassay coefficient of variation (CV)), lower limit of quantification, range of linearity and specificity/selectivity of the test. Conventional validation of EIA reproducibility reported by the manufacturers is often undertaken only at concentrations on the linear section of the typical sigmoid curve for mediator concentration and, therefore, cannot be used for concentrations around the LLD. The variability of measurements increases greatly outside this linear portion; thus, in general, quantification only becomes acceptable at concentrations higher than the expected range of mediator level in EBC. BAYLEY *et al*. [76] validated some commonly used EIAs for EBC. They demonstrated that, using raw EBC samples, one has to face the fact that mediators including leukotriene B4, interleukin (IL)-8, secretory leukoprotease inhibitor and α_1-antitrypsin are present under or around the LLD, where quantification is not precise, both intra- and interassay CVs are high (>20–30%) and values fall into the flat part of the calibration curve [76]. They showed that the LLD for the IL-8 ELISA was consistent with the manufacturer's value of 10 pg·mL^{-1}. Quantification became acceptable, however, only at 40 pg·mL^{-1}, where the intra-assay CV was <12% and spike recovery was >88%. Below 40 pg·mL^{-1} the intra-assay CV ranged from 13.9% at 30 pg·mL^{-1} to 90.7% at 8 pg·mL^{-1}, with spike recoveries of 57.6% at 31 pg·mL^{-1} and 6.1% at 3.9 pg·mL^{-1}. These results suggest that EIA data on raw EBC samples should be cautiously interpreted. Detailed information on the performance of each assay is provided in

the kit manuals, such as a calibration curve for IL-6 EIA produced by Cayman Chemical (a frequent source of immunoassays used in EBC studies; Ann Arbor, MI, USA). LLD is given as 1.5 pg·mL^{-1} but the curve indicates that linearity is lost at ~8–10 pg·mL^{-1} with intra- and interassay CVs >20% under 25 pg·mL^{-1}. The manual marks the ''end of range'' and suggests to ''evaluate data cautiously'' under the range of linearity. This information can easily explain that the mean±SEM concentration of IL-6 in healthy control subjects varies between none detectable and 1.6±0.1 pg·mL^{-1} to 7.02 pg·mL^{-1}, depending on the study [79–81]. The same is relevant for the other cytokines measured by immonuassays in native EBC samples. A similarly wide range of tumour necrosis factor (TNF)-α for healthy control subjects has been determined by the numerous research groups (0–27 pg·mL^{-1}) [79, 82–84]. Most research groups studying TNF-α found this mediator in only a fraction of the samples (10–30%). Sample concentration (lyophilisation and resuspension) and the development of more sensitive techniques may assist in cytokine measurements, even if lyophilisation is known to be associated with some sample loss and mediator recovery can vary [85].

Although the number of studies using EBC is exponentially increasing only a few have investigated samples from lung cancer patients.

EBC biomarkers in lung cancer

Proteins

The total protein content of EBC has not been compared between patients with lung cancer and control subjects; different cytokines, however, have been assessed. There are two major approaches to studying cytokines in biological samples; one is the detection of individual molecules by different immunoassays, the other is the use of an array of sensors, called proteomics. The latter is thought to be a powerful tool for detecting lung cancer in blood. By the simultaneous evaluation of five selected serum cytokine markers, NSCLC patients could be separated from control persons with 90% specificity and sensitivity [86].

In raw EBC samples, IL-6 was measured in patients with NSCLC and healthy control subjects [87]. The reported mean±SEM concentration of IL-6 was 9.6±0.3 pg·mL^{-1} in patients with lung cancer and 3.5±0.2 pg·mL^{-1} in control subjects with a significant difference between the two groups. The same authors described that the concentration of IL-2 together with that of leptin and TNF-α is also significantly increased in raw EBC samples of patients with lung cancer compared with healthy control subjects [88].

The only study using the proteomic approach to study EBC in lung cancer was published by KULLMANN *et al.* [89] in 2008. To overcome the expected low concentration of cytokines in EBC, the authors concentrated the samples by lyophilising, resuspending and pooling them for antibody microarray analysis on 120 cytokines. Every cytokine on the array gave a signal in both groups. In total, 10 cytokines including eotaxin, fibroblast growth factors, IL-10 and macrophage inflammatory protein-3 were present with a >2-fold difference between the two groups. The results were not confirmed by other means of protein detection and sample pooling may have masked individual protein patterns. This pilot study, however, presents the first example that proteomics on EBC can be applied to study lung cancer but its potential for clinical practice remains unestablished.

DNA-related alterations

GESSNER *et al.* [90] found that p53 mutations can be detected in the majority of EBC samples in patients with NSCLC but not in samples form healthy controls. They also showed K-ras gene mutations in EBC samples from patients with lung cancer [91]. Similarly, microsatellite instability has been demonstrated in EBC from patients with NSCLC in a substantially higher proportion of patients than in control subjects [92, 93]. The microsatellite profile of DNA from EBC corresponded to that from lung cancer tissue of each patient with NSCLC.

Other markers

Oxidative stress has been implicated in the pathomechanism of lung cancer, and oxidative stress biomarkers, including hydrogen peroxide (H_2O_2) and 8-isoprostane, can be measured in EBC [10, 65]. No data are so far available comparing the level of EBC H_2O_2 between lung cancer patients and control subjects. In a pilot study, exhaled H_2O_2 was measured in lung cancer patients undergoing thoracotomy before and after surgery [94]. Samples from healthy control subjects were also collected but these data were not presented in the results. The conclusion of the study was that oxidative stress occurs during lung resection, as higher H_2O_2 concentration was found after lobectomy compared to baseline. In another study, the effect of chemotherapy was assessed on exhaled H_2O_2 concentration in lung cancer patients, showing a significant decrease in this biomarker level by treatment [48]. No difference was found in the concentration of EBC 8-isoprostane between patients with lung cancer and control subjects [95]. In the same study, concentration of vascular endothelial growth factor was found to be different between the two groups of subjects [95]. The concentration of endothelin-1, a molecule with known mitogenic activity, was increased in EBC of lung cancer patients compared to controls [96]. Furthermore, the level of chromium, a heavy metal usually associated with occupational lung diseases, was elevated in EBC from patients with lung cancer without known exposure to heavy metals [97].

All of the aforementioned detailed EBC studies were single-centre, cross-sectional observational studies usually including a relatively low number of patients. Moreover, none of them applied dilution factor for data interpretation and immunoassay studies did not present adequate signal validation. Prior to conducting large multi-centre studies on EBC biomarkers for lung cancer detection, full validation of the testing methods is undoubtedly required.

Future developments in breath testing

Refinement of breath sampling techniques and new developments in sensor array and nanotechnology are critically important in exhaled biomarker research. Regarding EBC, further studies improving the efficacy of breath condensers and aiming at appropriate standardisation of EBC collection and mediator determination are required before EBC biomarkers could be considered as clinically relevant biomarkers. Establishing reference values for the different biomarkers in

both smokers and nonsmokers, and presenting long-term data on intra-subject variability of EBC mediator concentration, are all essential elements of research [10, 98–100]. The use of metabolomics, proteomics and new modes of spectrometry further facilitate exhaled biomarker research [101–113]. Metabolomic pattern determined by using nuclear magnetic resonance measurements in EBC has been shown to discriminate between healthy and asthmatic children [111]. The contribution of extrapulmonary sources (tongue, periodontal tissues, nose, sinuses, *etc*.) to the volatile substances found in EBC samples, however, has not been addressed in the study [111, 114].

Lung cancer research is a rapidly growing field. Newly discovered pathways in carcinogenesis could provide potentially new biomarkers, some of which might be detectable in EBC or in the gas phase of the exhaled breath. Such biomarkers presumably include molecules that have been detected in breath samples in other conditions, for instance extracellular purines, including adenosine, ATP, AMP and hypoxanthine, together with oxidant-antioxidant balance and acidity [105–125].

Improving our understanding of metabolic pathways and their changes during carcinogenesis is also important in the evaluation of molecular breath patterns. Most likely, a complete pattern of different molecules (breathomics), rather than individual breath constituents, can provide a relevant ''exhaled biomarker fingerprint'' of lung cancer. New developments in statistical approaches [126–133] and data sharing between electronic noses [134] can facilitate this research area.

POTENTIAL PLACE OF BREATH FINGERPRINTS IN DETECTING LUNG CANCER

When trying to establish the potential place of exhaled biomarkers in screening and diagnostic algorithms fundamental questions need to be discussed including issues on diagnostic accuracy and the comparison of breath tests with the currently used reference techniques.

Diagnostic accuracy

The first step in the development of a new biomarker is the discovery phase. This is followed by rigorous evaluation of diagnostic accuracy and then by the evaluation of the impact of the biomarker on clinical outcomes. For quality control of studies assessing diagnostic procedures, an internationally accepted set of requirements was created by the participants of the Standards for Reporting of Diagnostic Accuracy (STARD) initiative [135]. The STARD guideline provides a framework to improve the accuracy and completeness of reporting of studies on diagnostic accuracy. It lists and explains 25 items as crucially important in the evaluation. These items consider the following points: definition of the biomarker, description of the study population (clinical and demographic characteristics, severity of the target disease, comorbidities and treatment), data collection, reference standard, technical details, adverse event reporting, cutoffs and/or categories of the results of the index tests, and the reference standard, blinding of the study, methods for calculating or comparing measures of diagnostic accuracy, methods for calculating test reproducibility, report on how indeterminate results, missing responses, and outliers of the index tests were handled, report on estimates of variability of diagnostic accuracy between subgroups of participants, readers or centres, if done, and the discussion the clinical applicability of the study findings. Numerous studies resulted from the STARD initiative and evaluated biomarker developments from different perspectives [136–145].

To what extent can studies on exhaled biomarkers for screening and diagnosing lung cancer meet the STARD criteria?

As previously mentioned, only a limited number of studies has investigated chemical breath tests as biomarkers of lung cancer. Numerous exhaled biomarkers are only in the discovery phase and only a few exhaled biomarkers have been evaluated, fulfilling the STARD criteria. Variability in sampling methods, study design, biomarker measurements, statistical means and lack of wide-scale multicentre comparison studies, however, limits the clinical applicability of these measurements. The most advanced evaluation has been carried out for the application of exhaled VOC profile as a biomarker of lung cancer [28–30]. The exhaled VOC profile has been carefully identified using different algorithms and its application has been compared with chest CT scan as a reference test for early detection of lung cancer in a multicentre lung cancer screening trial [29]. This study paves the way for further evaluation of breath tests for clinical use according to the STARD guidelines.

Lung cancer screening

The criteria for a screening test depend on both the disease and the method of screening. The disease must be sufficiently burdensome to the population, the disease must have a long preclinical latent period and the screening method must detect the disease at an earlier stage than would be possible by sign and symptoms. The screening test must have a good negative predictive value (NPV) to ensure that subjects with a negative screening test do not suffer from the disease. Tests with moderate specificity are inappropriate for population screening (with low probability of disease) because of the high risk of false-positive results. Furthermore, early detection must improve disease outcome, and the cost, feasibility and acceptability of screening, and early treatment should be available.

Lung cancer is the leading cause of cancer death today. Different screening programmes that can increase the rate of detection of early stage lung cancer have been the subject of considerable enthusiasm. To date, randomised controlled trials on chest radiograph and sputum cytology showed increase in survival time but have failed to demonstrate that screening with either modality decreases lung cancer mortality; therefore, none of these technologies is recommended [5, 6, 146, 147]. Studies on lung cancer screening with low-dose CT (LDCT) have appeared promising. In the International Early Lung Cancer Action Project (I-ELCAP) screening study, LDCT detected almost six-times as many stage I lung cancers as chest radiography and most of these tumours were no larger than 1 cm in diameter [148]. Numerous other publications resulted from the study demonstrating prolonged survival in the CT screened group with no difference in mortality [149–152]. Some authors, however, pointed out that the results should be interpreted cautiously because the study reported by the I-ELCAP [149] had no control group, lacked an unbiased outcome measure, did not consider what was already known about this topic from previous studies

and did not address the harms of screening [153]. In general, chest CT has a high NPV value (99.4–99.9%) with a moderate positive predictive value (PPV; 2.7–18%) as demonstrated by different study results [148, 154–157]. Based on the results of the currently available studies, CT-based lung cancer screening is not recommended by experts [158, 159].

Several other means for early detection of lung cancer have been explored, including fluorescent bronchoscopy, endobronchial ultrasound-guided transbronchial needle aspiration, induced sputum analysis by different means and blood biomarkers [160–178]. Although bronchoscopic approaches have been found to be sensitive and specific tools for staging lung cancer, they are invasive techniques that limit their applicability for screening. There is a great expectation towards these newer approaches to identify the early clonal phase of progression of lung cancer in high-risk populations, thus enabling cancers to be detected earlier than is possible with CT. Biomarkers may complement spiral CT, since tomography is not as sensitive for small central cancers as for peripheral nodules. This combined approach to lung cancer screening may improve the robustness of early lung cancer detection. Negative results using this approach, however, push experts to re-evaluate the interpretation of LDCT-screening results and have a closer look at the ''malignant potential'' of masses identified by tomography [179]. There are several distinct types of cancer biomarkers identified by different techniques (*i.e.* genetics, epigenetics, proteomics and metabolomics). A PubMed search using the key words ''blood biomarker lung cancer'' produced 4,001 findings and their detailed evaluation is beyond the scope of this series. In brief, a wide range of blood biomarkers has been investigated only in discovery studies. The studies on diagnostic accuracy of blood biomarkers for lung cancer demonstrate that their sensitivity varies between 58% and 86.9% with specificity between 75.5% and 85.7%. These values are comparable to those obtained by exhaled biomarkers (table 1).

How could breath fingerprinting fit into lung cancer screening?

When considering the potential role of breath testing in clinical practice, one needs to consider different aspects including efficacy, safety and cost. Based on the results of their study, PHILLIPS *et al.* [29] suggested that breath testing may have a place in a multimodality screening programme: high-risk patients could have primary screening with the breath test; if the results are positive, a chest spiral CT scan is to be performed and, if that has a positive result, cellular/histological confirmation should be completed from samples obtained by bronchoscopy and/or lung biopsy. This suggestion is based on the comparative performance of breath testing to other methods of screening. For example, a mammography study reported a PPV of 18% and NPV of 88% with 10% sensitivity and 94% specificity [180]. The available study on the application of smellprint in lung cancer screening programme serves as a feasibility study and shows proof of principle of potential usefulness for such a screening approach [29]. In the study of PHILLIPS *et al.* [29], the PPV and NPV of breath test were 10.8% and 99.5%, respectively, demonstrating that a breath test employed as a primary screening for lung cancer could potentially exhibit similar or greater accuracy than a mammogram, employed as a primary screen for breast cancer [29]. Recently, MAZZONE [52] presented an elegant calculation demonstrating the requirements for a clinically useful breath test for lung cancer diagnosis. The author not only discussed the requirements for breath testing in lung cancer screening settings but also addressed clinical decision making for indeterminate lung nodules. Although in that evaluation, the currently available breath testing approaches failed to demonstrate the expected high sensitivity and specificity values, the performance of dogs were within the limits suggesting that such a good discriminating potency can be achieved.

CONCLUSION

Lung cancer identification by breath test is a rapidly growing area of research that could provide groundbreaking improvements in molecule-oriented screening and monitoring of lung cancer. ''Breathography'' is not yet ready to play a similar role in lung cancer screening as that of mammography in screening for breast cancer. However, breath fingerprinting by innovative new means might complement other methods in the early detection of lung cancer. Increasing knowledge on individual smell fingerprint of humans may enable longitudinal studies aiming to describe changes in smellprint over time [181]. Wide-scale studies to characterise individual breath fingerprints and monitor environmental influence and the possible effect of nutrition and common diseases, such as hypertension and diabetes on smellprint are warranted before the potential place of breath fingerprint as a biomarker of lung cancer can be established in clinical practice.

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STATEMENT OF INTEREST

None declared.

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