

P R A C E O R Y G I N A L N E
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Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting

Lotne związki organiczne (VOCs) w wydychanym powietrzu pacjentek z rakiem piersi w warunkach klinicznych

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Abstract

Background: Carcinogenic products in the exhaled breath of cancer patients are of growing medical interest as they can serve as noninvasive disease markers. Breath analysis can be used as an alternative or complementary diagnostic tool in breast cancer patients who have a different pattern of chemical composition in their breath. This study aims to verify the existence of specific volatile organic compounds (VOCs) in the breath of breast cancer patients.

Methods: This prospective study included ten patients suffering from breast cancer and ten healthy pair-matched women. Breath samples of each member of the two respective groups were taken and scanned by gas chromatography/mass spectrometry for the presence of volatile organic compounds such as alkanes, ketones, halogenated hydrocarbon, aldehydes, and esters.

Results: The spectrum of VOCs differed significantly within the two groups. Five specific VOCs could be identified as typical discriminatory markers in the breath samples. Four VOCs were elevated in the healthy controls, one specific VOC was found to be elevated in women affected by breast cancer.

Conclusions: This pilot study revealed a specific VOC pattern using gas chromatography in the breath of breast cancer patients. Five specific breast cancer-VOCs were identified. At relatively low cost the identification of VOCs may be used to detect breast cancer.

Key words: **breast / mass spectrometry (GC/MS) / early diagnosis of breast cancer
cancer / volatile organic compounds / breath analysis / gas chromatography /**

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Mangler M, et al. *Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting.*

Streszczenie

Cel: Coraz bardziej rośnie zainteresowanie medycyny produktami karcinogenezy w wydychanym powietrzu pacjentów chorych na raka jako możliwych nieinwazyjnych markerów choroby. Analiza powietrza wydychanego może być wykorzystana jako alternatywne lub pomocnicze narzędzie w raku piersi u pacjentek, które mają odmienny skład chemiczny oddechu. Celem tego badania była weryfikacja obecności lotnych związków organicznych (VOCs) w wydychanym powietrzu pacjentek z rakiem piersi.

Metoda: Przeprowadzono prospektywne badanie, do którego włączono 10 pacjentek cierpiących na raka piersi i 10 zdrowych dobranej do pary kobiet. Pobrano próbki wydychanego powietrza od każdego uczestnika badania i poddano gazowej chromatografii/spektrometrii masowej na obecność następujących lotnych związków organicznych: alkanów, ketonów, halogenowanych wodorowęglanów, aldehydów i estrów.

Wyniki: Spektrum VOCs różniło się istotnie w obu grupach. Pięć specyficznych VOCs zdefiniowano jako typowe markery w wydychanych próbkach. Cztery VOCs były podwyższone w grupie kontrolnej, natomiast jeden specyficzny VOC był podwyższony w grupie kobiet z rakiem piersi.

Wnioski: To badanie pilotażowe, przy pomocy gazowej chromatografii, wykazało specyficzny wzór VOC w wydychanym powietrzu u pacjentek chorych na raka piersi. Zidentyfikowano pięć specyficznych dla raka piersi VOCs. Przy relatywnie niskich kosztach identyfikacja VOCs może być wykorzystana do wykrywania raka piersi.

Słowa kluczowe: **rak piersi / lotne związki organiczne / analiza oddechu /
/ gazowa chromatografia / spektrometria masowa (GC/MS) /
/ wczesna diagnostyka raka piersi /**

Introduction

In a society where breast cancer is a leading cause of cancer death, early detection of the disease in women at risk of breast cancer or in the general population is highly desirable. Annually, 46,000 women in Germany are affected and 17,000 to 18,000 die from breast cancer. Breast cancer is the most frequent kind of malignancy in women, causing 4% of deaths in females [1].

Early detection of the disease minimizes therapeutic intervention and reduces mortality [2,3]. General mammography screening is undoubtedly profoundly effective for secondary prevention of breast cancer, regardless, it generates high expenses. The costs are estimated at 70-130€ per patient, amounting to the annual sum of over 100 million € in Germany [1].

Alternative or complementary strategies are needed to increase the diagnostic accuracy and/or to lower the expenses of breast cancer detection.

A recent study showed that 'a man's best friend', the dog, may in fact contribute to the process of early cancer detection by sniffing patient breath [4]. The authors reported that ordinary trained household dogs achieved the sensitivity of 0.88 (95% CI, 0.75, 1.00) and the specificity of 0.98 (95% CI, 0.90, 0.99) in the identification of patients with breast cancer [4]. The authors postulate the identification of chemical compounds in an exhaled breath that are associated with the presence of cancer. The compounds are most likely cancer-induced metabolites. These metabolites, including volatile organic compounds (VOCs), are important biomarkers of inflammatory and oxidative processes of anthropogenic and biogenous origin [5-8].

According to the definition of the WHO, VOCs are volatile organics classified according to their retention time (C6 -C16) and boiling point (50°C – 250°C). Thus, subsuming a group of substances including molecules with different physical, chemical and toxicological attributes such as alkanes, alkenes, ketones, halogenated hydrocarbons, aldehydes, esters, unsaturated hydrocarbons, terpenes, siloxanes, alcohols and aromates. Exogenous VOCs can be found in indoor air, building materials

or electrical equipment [20]. Endogenous VOCs are produced by people or animals and released through exhalation. There is a wide inter-individual variation in their composition. Still, certain core VOCs that can be reproduced in the exhaled air of individuals can be found [19].

Thus, the difference in the concentration of VOC between individuals with and without cancer is the assumed explanation why dogs can sniff out cancer [4]. There have been attempts to copy the success of the dogs by establishing artificial noses [8]. Artificial noses are subtle machines using a nanocomposite array of different organic polymer sensors. Exposing the sensors to VOCs, they change their electrical resistance as they swell ("smelling by swelling"- mechanism) [7,8]. This change in resistance is then transcribed in an algorithmic analysis. A perfect artificial olfactory replacement which could be compared to the human or canine olfactory system has, however, not yet been invented [8].

Breath analysis is a powerful tool to detect various diseases [7,9-18]. Breath is a complex aerosol containing more than a thousand of different nonvolatile (dissolved in microdroplets) and volatile components [7,19]. This organic composition can be used as a reliable diagnostic source.

Breath sample tests have been implemented in the diagnosis and management of asthma by measurement of the exhaled nitric oxide (NO) or identification of lactose intolerance by breath hydrogen (H₂) analysis using gas chromatography [9]. Helicobacter pylori infection is detected by using the urea breath test. In patients with heart transplants, breath samples are used to predict tissue rejections and may replace myocardial muscle biopsies [10]. Furthermore, promising breath screening tests are evaluated in patients suffering from heart diseases, schizophrenia, rheumatoid arthritis, or preeclampsia and asthma and COPD maybe recognized early [5, 28, 29, 30].

Breath samples are obtained non-invasively and the analysis is inexpensive.

Identification of VOCs is technically rather challenging as VOCs are produced in the range of nano- and picomolars. The methods of collecting the air samples described in literature are not easily transferable to an everyday clinical setting.

Clinical management, however, requires an optimized and easy technique to collect the air samples, preferably in a standardized setting.

Our aim was to find certain specific ‘smellprints’ in the breath of breast cancer patients by identifying a group of specific biomarkers (VOCs) using a simple and approved method of the gas chromatography/mass spectrometry (GC-MS). We also wanted to verify the practicability of the test using an everyday clinical setting for our analysis.

Methods

The procedures of the study received ethical approval from the institutional ethics committee responsible for human experimentation.

This prospective study included two groups of women: 10 successive patients with breast cancer and 10 healthy pair-matched control subjects. All patients and test persons gave their written informed consent to participate in this study. The controls had a mammography without pathological findings within the last six months before the testing. Women with concomitant medical problems were excluded from the study.

The method used in this study was based on three steps (Figure 1):

- sampling a representative amount of breath,
- purification and cleansing of the sample,
- measurement of VOCs concentrations.

Breath sampling

Preliminary tests were carried out in order to choose the most appropriate experimental setup using material with zero emission rates.

We took breath samples of the patients in the morning before the operation in a standardized setting: drinking, eating, and brushing teeth were not allowed within the last eight hours before sampling. The temperature in the examination room was kept constantly at 24°C.

We collected an amount of 1000 milliliters of the exhaled alveolar breath from each study member, as well as compartment air samples during the same time (Figure 2).

A chemically inert glass container with a capacity of 1000 ml was used as a storage medium for the breath samples (Figure 2&3).

The glass container was connected to a Tenax test tube containing adsorption filter polymers to collect the VOCs (Fig. 2&3). Conjoined with the tube was an automatic pump (Fig. 2&3). The patients were asked to exhale through a custom-built device connected to the glass container five times after discharging the air from the anatomical dead space (approx. 300ml). After collecting the breath in the glass container the connection between test tube and glass container was opened and the pump was switched on to draw the air into the test tube using a flow rate of 100ml/min. Then the test tube was sealed at both ends and transferred for laboratory analysis. Compartment air samples were taken in an identical fashion.

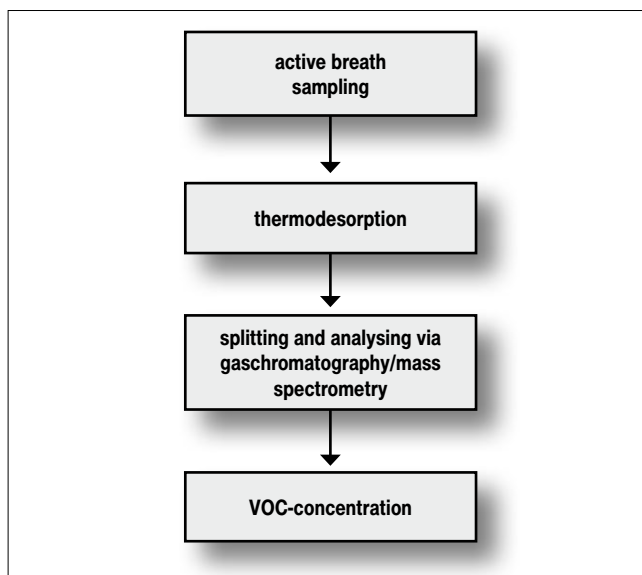


Figure 1. Scheme of taking and analyzing the probes.

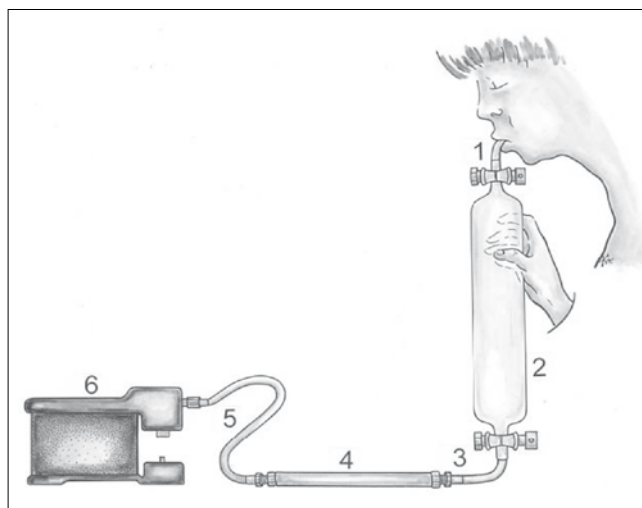


Figure 2. Collection of breath during 5 exhalations discharging the air from the anatomical dead space (approx. 300ml). 1: exhalation tube, 2: glass container, 3 and 5 inert connection tubes, 4: absorbent, 6: pump.



Figure 3. Test person during breath sample.

VOC analysis

An internal standard is added to the samples to guarantee an exact analysis. The probes are cleansed with helium. The VOCs are separated from the samples by thermal desorption. During thermodesorption, the loaded tube is heated up in a thermodesorption unit starting at 30°C, heating up to 280°C by 60°/min, maintaining this temperature for eight minutes. The VOCs are now dissolved within the inert carrier gas helium, this being the 'mobile phase' of the system. They are then flushed into a cold trap and frozen at -80°C. After heating up the cold trap to 280°C by 12°C/min, the substances are transferred from the carrier gas to the gas chromatograph (GC) and adhere to its capillary column, the so-called 'stationary phase', which separates the molecules from each other at different temperatures and allows them to be quantified and qualified via GC-MS.

The interactions of dissolved VOCs with the stationary phase causes different compounds to elute at different times, respectively, called the retention time. The analysis of the differing boiling points and the comparison of the retention times helps to identify the substances. The dispersed substances pass a detector in the gas chromatograph which transfers certain changes of the physical composition of the carrier gas into electrical signals. These signals are transferred to a computer or recorder interpreting the changes.

By evaluating the peaks and 'area under the curve', the quality and the quantity can be described, respectively.

By adding the method of mass spectrometry one can not only increase the accuracy of the detection but also find minute amounts of substances. Mass spectrometry does not analyze the mass of particles alone but the mass-to-charge ratio by generating a mass spectrum. The dispersed substances of the stationary phase in the GC are transferred to the MS. There they are ionized by the ion source via collisions. Electrons are colliding with the molecules to be analyzed producing ions. These fragile ions collapse building smaller fragments. The fragments can be analyzed by their mass-to-charge ratio. The detection limit of the method is 1 µg/m³ for the substance under investigation.

Values of compartment air samples were subtracted from breath sample values. The data was recorded with a relational database management system and analyzed via SPSS (Version

15.0) using the Kolmogorov-Smirnov test, Mann-Whitney U test, t-test, and ROC curve analysis.

Results

The mean age of patients was 64.5 (41-73) years, the mean body-mass-index was 25.50 kg/m² (19 – 36), 9/10 (90%) patients were postmenopausal. The mean age of the healthy women in the control group was 58.5 (51-70) years, the mean body-mass-index was 26.30 kg/m² (22 – 31), 10/10 (100%) women were postmenopausal. In all patients breast cancer was confirmed histologically: invasive ductal carcinoma (n=7), invasive lobular carcinoma (n=2) and tubular cancer (n=1). Cancer stages were: pT1a (n=3), pt1c (n=4), pT2 (n=3). Two patients had one affected lymph node, respectively. In all patients the disease was limited to the breast.

Unspecific VOCs

Using the gas chromatography in combination with mass spectrometry, we found a total of 109 different VOCs and a variation of 83 to 100 different volatile organic compounds in the breath of patients and controls. We identified various VOCs belonging to different classes including 26 alkanes, 7 alkenes, 4 ketones, 11 halogenated hydrocarbons, 11 aldehydes and alcohols, 9 esters, 4 unsaturated hydrocarbons, 13 terpenes, 3 siloxanes, and 21 aromates. The identified VOCs had either a negative (amount < 0 µg/m³) or positive alveolar gradient (amount > 0 µg/m³). Variances differed in the patients.

Regarding the analyses of the quantity of VOCs, the patients exhaled more alkanes and alkenes when compared to controls. Regarding other substances such as ketones, halogenated hydrocarbons, aldehydes, esters, unsaturated hydrocarbons, terpenes, siloxanes, alcohols, and aromates, the alveolar gradients did not differ.

We found a broad distribution concerning range, median, and standard deviation in most of the VOCs (examples are given in Figure 4).

Specific VOCs

Among all the substances tested, five VOCs belonging to the alkanes, alkenes, halogenated hydrocarbons, aromates, or

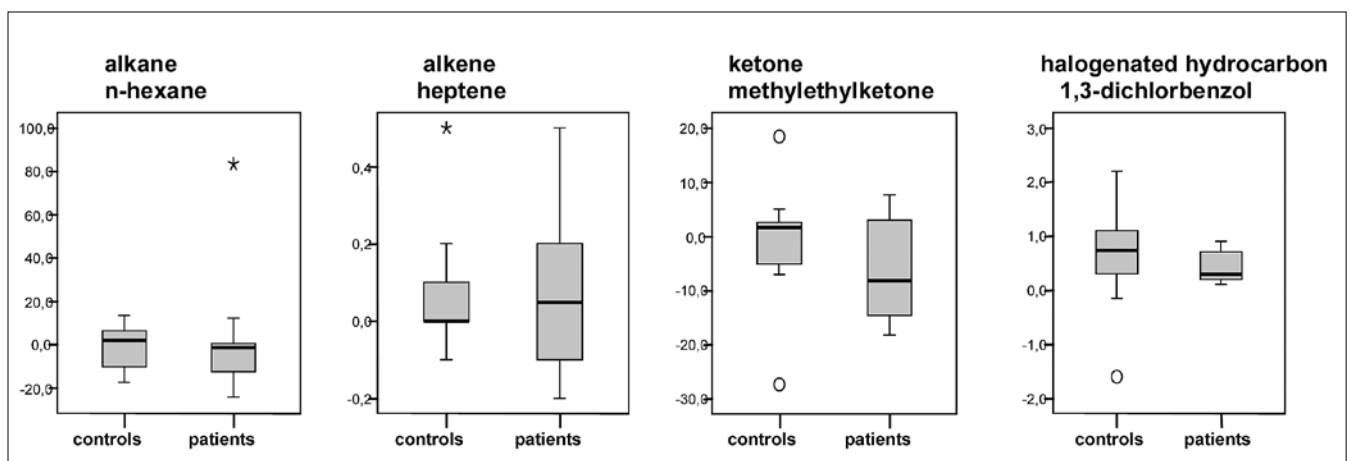


Figure 4. Boxplots of exemplary unspecific VOCs in µg/m³, ranges do not differ significantly within the two groups.

Mangler M, et al. *Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting.*

terpenes were found to be significantly different in the group of breast cancer patients using the t-test and Mann-Whitney U test. The five identified substances were: 3-methylhexane, decene, caryophyllene, naphthalene, and trichlorethylene. Trichlorethylene alone was significantly elevated in the group of breast cancer patients and showed a positive alveolar gradient. The values of 3-Methylhexane, decene, caryophyllene, and naphthalene were significantly decreased in the patient group and, except for naphthalene, all presented a negative alveolar gradient. (Table I).

Discussion

There is increasing evidence that searching for biomarkers by analyzing breath has an important place, not only in the clinical management of patients with pulmonary diseases [5, 26]. Dogs achieved an impressive accuracy when exposed to the smell of patients with lung or breast cancer [4].

Inspired by these recent findings, breath sample analysis for detection of women with early breast cancer has become an important goal. A non-invasive parameter with adequate accuracy could replace invasive diagnostic methods. However, until now a perfect artificial olfactory system replacing the human or canine olfactory system has not yet been found [8].

Thus, our study aimed at copying the canine nose and identifying certain specific 'smellprints' in the breath of breast cancer patients by using a simple and approved method such as the GC-MS.

The questions what kind of mechanism underlies the method of breath analysis and why VOC concentrations differ between healthy individuals and individuals with diseases remain. Some VOCs have a positive alveolar gradient, others a negative one. Phillips et al., defined the expression 'alveolar gradient' as abundance of VOC in breath minus abundance in air [10,16,19, 21]. The polarity of the alveolar gradient appears to depend mainly on whether a VOC originates from inside or outside the body [23]. When the alveolar gradient is negative the rate of clearance is increased. Most volatile substances have a negative alveolar gradient in lung cancer patients and a positive alveolar gradient in healthy subjects because of the enhanced activity of cytochrome P 450 due to oxidative stress. Oxidative stress is induced by an overbalance of reactive oxygen species and oxygen radicals in the mitochondria and peroxisomes of tumor cells subsequently eluding into cytoplasm [24, 25, 27]. There they oxygenate and damage not only the DNA and important proteins, but also polyunsaturated fatty acids, that are being converted into alkanes and methyl alkanes, e.g. these substances are mainly eliminated via the lung itself or metabolized via the enhanced activity of the cytochrome P450 of the lung [16, 21, 27]. As their quantity changes with the amount of oxidative stress, they can be traced in the breath. Oxidative stress, however, can be caused by other mechanisms such as inflammation or infection which may bias the results.

Studies to distinguish between early and late stage of lung cancer were unsuccessful [16, 21]. This finding could also serve as evidence that the different pattern of VOCs is mainly induced indirectly by the enhanced activity of cytochrome P 450 in cancer patients and not by the tumor metabolites themselves. Detecting the enhancement of cytochrome P450 as an early consequence of tumor metabolism, could be an adequate method to diagnose

cancer. Nevertheless, the enhancement of cytochrome P450 is not the only explanation why an alteration of certain VOCs can be reproduced in affected patients. Direct interaction of tumor metabolites in cells also results in altered (higher or lower) levels of certain VOCs. Tumor cells produce different metabolites leading to increased levels of benzol and alkaline derivates in the affected tissues, the latter being mainly eliminated via the exhalation or via the enhanced activity of cytochrome P 450 in the lung [21, 22, 26].

The change of VOC pattern may already be traceable in early stages of breast cancer when histological detection fails [21]. Phillips et al., identified nine substances to trace lung cancer and eight substances to detect breast cancer, Diana Poli et al., proclaimed 13 substances with significantly changed concentrations in patients with lung cancer differing from Phillips' results [21, 15]. Various other studies showed different significant patterns of VOCs and only singular matches were shown [13, 15, 17, 18, 21]. We scanned our breath samples for 109 substances in total including the substances identified in literature.

We did find a significant alteration of the concentration of five specific VOCs: 3-methylhexane, decene, caryophyllene, naphthalene, and trichlorethylene. The decrease in the concentration of 3-methylhexane, decene, caryophyllene, and naphthalene in our breast cancer patients can result from the altered activity of cytochrome P 450, whereas the increase of trichlorethylene might derive directly from tumor cell metabolites. Our findings do support the possible role of application for breath analysis in cancer patients. In a clinical everyday setting the relevance and reproducibility of VOC analysis could be confirmed.

In our study we could neither reproduce the specific alterations of biomarkers found in Phillips' breast cancer group nor those of other cancer studies [21]. These results point out the fragility of VOC analysis. Exact pathogenesis of VOCs is unknown, nutrition might have an impact on the exhaled pattern of VOC and age does increase the quantity of VOCs [22].

Different methods to analyze the breath samples may be found [6, 13]. In recent investigations technical problems of sampling and analysis occurred and, due to lack of normalization and standardization, great variations were found between the results of different studies [11].

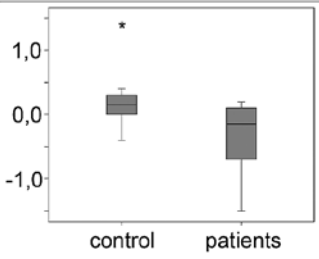
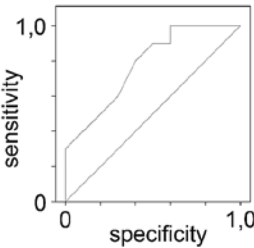
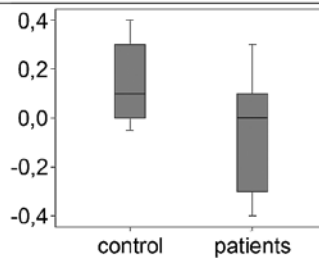
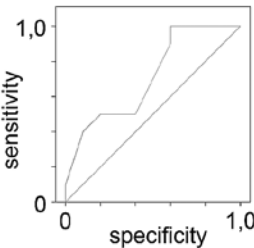
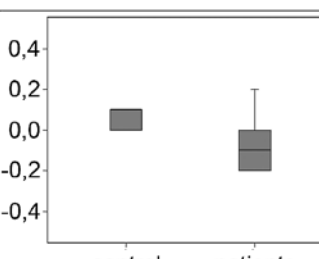
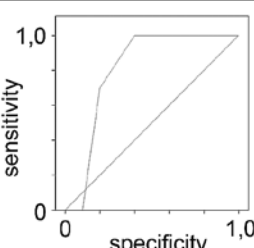
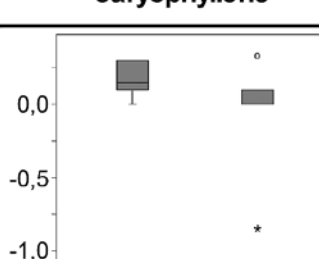
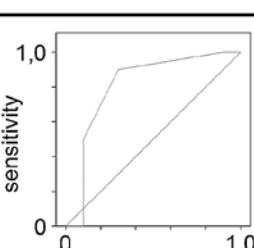
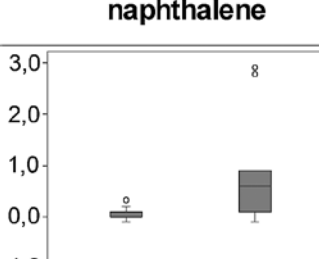
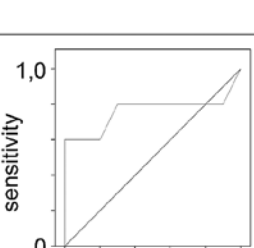
Out of the described techniques GC/MS linking fulfils the best qualifications to identify and quantify the smallest traces of volatile compounds [6, 13]. Therefore, we chose GC/MS as it is the most approved method with the best results.

We scanned our breath probes for almost all VOCs identified in cancer patients so far, but we found no consistency in the existing data. Looking at a set of volatile markers may enable recognition and diagnosis of patients with breast cancer in the near future but nevertheless, extensive research has to be done.

Conclusion

In an everyday clinical setting breath analysis can be implemented as an extra tool in breast cancer diagnosis. We found a significantly different pattern of VOCs in the breath of breast cancer patients when compared to healthy controls. Still, further and multi-center studies need to be performed to verify the reproducibility of breath analysis in breast cancer patients.

Table 1. Cut off values after ROC curveanalysis.

substance in $\mu\text{g}/\text{m}_3$	cut off value in $\mu\text{g}/\text{m}_3$	sensitivity	specificity
 <p>3-methylhexan e</p>	 <p>-0,55</p>	100%	40%
 <p>decene</p>	 <p>-0,125</p>	100%	40%
 <p>caryophyllene</p>	 <p>-0,05</p>	100%	60%
 <p>naphthalene</p>	 <p>0,05</p>	90%	70%
 <p>trichlorethylen e</p>	 <p>0,05</p>	80%	70%

Mangler M, et al. Volatile organic compounds (VOCs) in exhaled breath of patients with breast cancer in a clinical setting.

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List of abbreviations

GC/MS	– gas chromatography/mass spectrometry
VOC	– volatile organic compounds
ROC curve	– receiver operating characteristic curve

Competing interests

The authors declare that they have no competing interests.

Contributions of the authors

MM – participated in the design of the study and helped with the acquisition of data, patients, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and participated in the final approval of the version to be published.

CF – participated in the design of the study and helped with the acquisition of data, patients, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content; and participated in the final approval of the version to be published.

ML – added substantial contributions to concept and design, revised it critically for important intellectual content; and participated in the final approval of the version to be published.

OS – added substantial contributions to concept and design, revised it critically for important intellectual content; and participated in the final approval of the version to be published.

AS – added substantial contributions to concept and design, revised it critically for important intellectual content; and participated in the final approval of the version to be published.

DS – added substantial contributions to concept and design, revised it critically for important intellectual content; and participated in the final approval of the version to be published.

All authors read and approved the final manuscript.

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