

ORIGINAL ARTICLE

Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children

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Summary

Background The correct diagnosis of asthma in young children is often hard to achieve, resulting in undertreatment of asthmatic children and overtreatment in transient wheezers.

Objectives To develop a new diagnostic tool that better discriminates between asthma and transient wheezing and that leads to a more accurate diagnosis and hence less undertreatment and overtreatment. A first stage in the development of such a tool is the ability to discriminate between asthmatic children and healthy controls. The integrative analysis of large numbers of volatile organic compounds (VOC) in exhaled breath has the potential to discriminate between various inflammatory conditions of the respiratory tract.

Methods Breath samples were obtained and analysed for VOC by gas chromatography–mass spectrometry from asthmatic children ($n = 63$) and healthy controls ($n = 57$). A total of 945 determined compounds were subjected to discriminant analysis to find those that could discriminate diseased from healthy children. A set of samples from both asthmatic and healthy children was selected to construct a model that was subsequently used to predict the asthma or the healthy status of a test group. In this way, the predictive value of the model could be tested.

Measurements and main results The discriminant analyses demonstrated that asthma and healthy groups are distinct from one another. A total of eight components discriminated between asthmatic and healthy children with a 92% correct classification, achieving a sensitivity of 89% and a specificity of 95%.

Conclusion The results show that a limited number of VOC in exhaled air can well be used to distinguish children with asthma from healthy children.

Keywords diagnosis, discriminant analysis, predictive value

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Introduction

Asthma is a frequently occurring chronic inflammatory disease in children world-wide. Although effective therapies are available, asthma control is still not optimal in many patients [1, 2]. Until now, the criteria for the diagnosis and management of asthma have been based on clinical observations and lung function tests [3, 4]. However, symptoms may not always accurately represent the nature and extent of the underlying airway condition [5] and lung function tests are not definite in relation to

airway inflammation [6–9]. Young children showing symptoms like coughing, wheezing and dyspnoea may be suffering from various diseases of the respiratory tract, and it is therefore not always easy to achieve the proper diagnosis [10–14]. New diagnostic approaches with the potential to make an early diagnosis of asthma possible may not only considerably decrease underdiagnosis and undertreatment in true asthmatics but can also prevent overtreatment in transient wheezers.

In recent years, non-invasive techniques that may be useful for the assessment of airway inflammation have

been found in the analysis of exhaled breath. For instance, the collection and analysis of non-volatile compounds in exhaled breath condensate [10] and of volatile compounds such as nitric oxide (NO) [15–18] and carbon monoxide (CO) [19–21] in breath have been used to assess respiratory diseases. Fractional exhaled nitric oxide (FeNO) can be considered as a non-invasive marker of inflammation because its levels are elevated in children with asthma [22–24]. However, FeNO mainly represents eosinophilic inflammation, which may be present in different clinical conditions and therefore its discriminating power is limited [25, 26]. Also, ethane and pentane, which are detectable volatile organic compounds (VOC) in exhaled air, have been related to oxidative stress as a result of inflammatory conditions [21, 27, 28]. In all these approaches, single or small numbers of components are analysed and an attempt was made to use these as diagnostic tools.

Already in the early 1970s, Pauling *et al.* [29] demonstrated the presence of a large variety of VOC in exhaled breath. In 1985, Gordon *et al.* [30] showed that within this large number of VOC profiles could be found that differentiate between subjects with lung cancer and healthy controls, indicating the use of such profiles as possible biomarkers of disease. More recently, Phillips *et al.* [31] used discriminant analysis to select and measure 22 VOC from exhaled breath to classify subjects with and without lung cancer. In a recent follow-up study [32], they demonstrated that a VOC profile consisting of nine components is capable of classifying groups with and without lung cancer with a sensitivity of 85.1% and a specificity of 80.5%.

VOC can be determined by collection of exhaled breath and quantitative analysis by thermal desorption–gas chromatography *time-of-flight* mass spectrometry (GC–TOF–MS). Recently, we developed a highly accurate methodology to comprehensively analyse the entire range of VOC in exhaled air to search for unique profiles that can be used to identify certain patient groups [33]. The present study was set up to test the hypothesis that a variable method of sampling and a comprehensive chemical analysis technique in combination with adequate data processing and data analysis will provide a robust and easy-to-use tool for identifying asthma patients and healthy children based on their exhaled breath. We report the use of a totally non-invasive and easy-to-perform sampling method of exhaled air combined with an accurate measurement of a variety of VOC, which provides information concerning processes in the respiratory tract. Using discriminant analysis, we were able to select a limited number of compounds that discriminate asthmatic children from healthy controls and predict to which group children belong, thus confirming the hypothesis. In a consecutive prospective study, we will investigate the potency of this method to distinguish between healthy wheezers and asthmatic children.

Methods

Study population

A total of 120 children in the age range of 5–16 years were included in the study. Of these children, 63 were diagnosed with asthma and 57 were healthy controls. The asthmatic children were recruited from the outpatient clinic of the department of paediatric pulmonology of the University Hospital Maastricht. The asthma diagnosis was made on clinical grounds by an experienced children's lung specialist. Asthma was defined as a chronic inflammatory disorder with intermittent symptoms of cough, dyspnoea, wheezing and chest pain. The severity and control of asthma were assessed according to the international global initiative for asthma guidelines [3]. A subset of (42) atopic asthma patients was selected, based on an established positive Phadiatop and/or at least two RAST classes ≥ 2 . Appropriate therapy was prescribed by the patient's own physician. All 63 patients used an inhaled bronchodilator on demand and 55 of them used an inhaled corticosteroid daily. Sixty seven percent of this population had allergy as documented by RAST class 2 or higher for at least one common airborne allergen. The 57 control subjects were recruited from primary schools (36 out of 57) and from the outpatient paediatric clinic of the University Hospital Maastricht (21 out of 57). In this group, enuresis and constipation were the main reasons for consultation. The ISAAC questionnaire was used to exclude a history of respiratory problems in these children [34]. Patients with diseases that might interfere with the results of this study, mentally retarded children and active smokers were excluded from the study. The two populations were comparable in age, height and weight. The characteristics of the patients and controls are presented in Table 1. Informed consent was obtained from all children and their legal representatives. The study was approved by the Ethics Committee of Maastricht University, clinical trial registration number NCT 00413140.

Sampling

The subjects were asked to exhale their breath in a resistance-free plastic bag (Tedlar bag, SKC Ltd, Dorset, UK). Three to four exhalations were sufficient to fill the 5 L bag. No special provisions for the mode of exhalation, nor special directions concerning the diet of the subjects were given. The samples of both patients and controls were collected in the same room, to prevent the occurrence of a background bias. Therefore, and because of the data analysis methods used (i.e. discriminant analysis), no background samples were collected. Within 1 h of collection, the bag was emptied under pressure over a stainless-steel two-bed sorption tube, filled with carbograph 1TD/Carbopack X (Markes International, Llantrisant, Wales, UK).

Table 1. Characteristics of the study population

	Asthma (<i>n</i> = 63)	Controls (<i>n</i> = 57)
Age (mean±SD)	10.8±3.1	9.9±2.8
Height	144±17	141±17
Weight	38.2±14.9	34.8±10.9
Gender male/female	47/16	29/28 [†]
FEV ₁ (% predicted)	96.2±18.2	101.8±10.6
FEV ₁ /VC (%)	84.0±10.3	88.2±7.0
Atopy	42 (67%)	
Asthma severity*		
Intermittent	15 (24%)	
Mild persistent	12 (19%)	
Moderate persistent	17 (27%)	
Severe persistent	19 (30%)	
Allergic rhinitis	11 (17%)	
ICS use	55 (87%)	
ICS daily dose (µg)	576±357	

*Severity was classified according to the global initiative for asthma.

[†]Significantly different (*P* = 0.01). No confounding of gender on the discriminant functions for asthma vs. control and atopic vs. non-atopic asthma could be established.

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid.

Before and after loading the tubes were air-tight capped. The tubes were stored at room temperature until analysis.

Analysis

Our developed GC-TOF-MS methodology to accurately measure VOC and subsequent data handling was recently published and therefore the approach is summarized here briefly [33]. Analysis of the samples started with the release of the volatile compounds trapped on the sorption tubes by thermal desorption, using the Markes International Ultra-Unity automated thermal desorption equipment (Markes International). The gaseous mixture of released compounds was then divided; 90% of the sample was recollected on a second identical sample tube and stored for an optional second analysis. 10% of the sample was loaded onto a cold (5 °C) sorption trap, from which it was injected into a gas chromatograph (Trace GC, Thermo Fischer Scientific, Austin, TX, USA) and analysed by time-of-flight mass spectrometry (Tempus Plus, Thermo Fischer Scientific). For the GC-MS measurements, the following conditions were used: column: Restek RTX-5 ms, 30 m×0.25 mm ID, coated with 1.0 µm HP-5 phase; helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperature of the gas chromatograph was programmed as follows: 40 °C for 5 min, then increased by 10 °C/min until 270 °C, at which temperature it was maintained for 5 min. The mass spectrometer was set at a scan range of 35–350 AMU and scanned 5 times/s. The complete analytical procedure, including sampling, storage and instrumental analysis, was tested

for reproducibility. Instrumental reproducibility was determined by the analysis of identical exhaled air samples that were obtained by emptying a filled bag over a y-shaped connector onto two absorption tubes. The two absorption tubes were subsequently analysed by GC-TOF-MS. This experiment was repeated six times. The quantification of the similarity was performed by means of calculation of a distance measure (dot product rule). This distance measure is based on the similarity of the entire raw chromatogram. Distance measure calculation of all complementary files resulted in a distance measure ranging from 0.96 to 0.99. (A value of '1' denotes identical samples; the lower the value the lesser the degree of similarity.) Intra-individual variability was examined by repeated sampling of exhaled air from 10 subjects for 5 consecutive days and comparing the results per subject from day to day. Inter-individual variability was examined by sampling 10 subjects and comparing the data from subject to subject. Again, the similarity between several chromatograms was quantified using a distance measure as mentioned above. The intra-individual variability ranged from 0.80 to 0.99 and is far smaller than the inter-individual variability, ranging from 0.16 to 0.98.

The results of all tests support the methods used and were published elsewhere [33].

Data analysis

The GC-MS chromatograms of the breath samples of the 120 subjects involved in this research were recorded and retention times were normalized by calculating retention indices, relative to toluene and lining up using easily recognizable component peaks, to correct for chromatographic drifting. Parts of the chromatograms that occurred at a retention index <0.15 and at a retention index >2.8 were removed from the chromatograms, because of unreliable data from these parts, due to noisy mass spectra at the beginning of the chromatograms and column bleeding at the end of each run. The remaining data were carefully transformed to Excel files, containing almost 6000 different chromatographic peaks, determined by retention time and mass spectrum and combined with a relative intensity. In our approach, the measured mass spectra were compared with one another at the same retention time, rather than with library spectra. In this way, the resemblance of the original spectra determined the decision as to whether peaks at the same retention time represent the same component or not. Intensities below the detection limit were set at 0%. The detailed descriptions of the data handling procedures can be found elsewhere [33]. We applied no background subtraction, nor any corrections for the diet and the mode of exhaling in the sample bag of patients and controls. We realize that in this way, some noise on the results may be generated, but also that strict rules with regard to exhalation, diet and background subtraction would lead to less robust and less easy

sampling, and probably an increase of misclassifications as background corrections are not only hampered by the complex interdependence of excretion and uptake of VOC, it also increases the measuring fault. Furthermore, data analysis by discriminant analysis provides a tool to eliminate any randomly occurring variation, for instance due to diet (there is no reason to expect a difference in diet for patients and controls), breathing (when the subjects are entitled to exhale at their own choice, there is no reason to expect differences between the two groups as well) and background (the samples of patients and controls were taken divided over the sampling period and in the same room). It is our goal to select only those compounds that contain information regarding diseased vs. healthy status and are independent from other endogenous (gender, age, weight, genetic background) or exogenous factors (ambient air, diet, medication). Before data analysis was performed, all peaks that occurred in <8% of the samples (meaning <10 times in this study) were removed [33, 35]. This resulted in a final data matrix, containing 945 peaks and 120 subjects. This matrix was normalized by adjusting the sum of all peak intensities in each subject at 100%. In this way, the influence of the infrequently appearing peaks could be diminished. The resulting data were analysed by a stepwise discriminant analysis, using SPSS (SPSS 13.0 for windows, SPSS Inc. Chicago, IL, USA). The discriminant analyses were performed using a 20-fold cross-over approach. In this method all but 5% of the chromatograms (in this study, 5% equals six samples) are used to construct the discriminant function, which is subsequently used to predict to which group the ones left out belong. This is repeated 20 times, until all samples have been classified once. The discriminant functions obtained in this way are based on many (60–80) components and are optimal in terms of differentiation between both groups, but are not necessarily the best predictors for unknown samples, because of obvious overfitting. Therefore, the number of variables is gradually diminished, until a reasonable small number of components remains with sufficient prediction power. The reduction in components is accomplished by starting with the original large set of components from the first discriminant analyses, and then repeating the analysis with the least informative (the last) component of the results left out.

Results

The GC–MS analyses resulted in 120 chromatograms, representing the 63 asthmatic patients and the 57 controls. For the discriminant analysis, two groups were defined: group 0 consisted of the samples of the 57 controls, and group 1 consisted of the samples of the 63 asthma patients. This discriminant analysis resulted in a discriminant function comprising 72 components that

classified all samples correctly, i.e. 100% sensitivity and 100% specificity. In the discriminant analysis performed, all peaks that enhance the descriptive model were added, but for using the model as a reliably predictive tool the number of components is far too large, and leads to overfitting. Therefore, we gradually diminished the number of components to build the model, and registered the sensitivity and selectivity of the analyses as a function of the number of components involved. In this way using 22 components, a 100% correct classification was still obtained. A sensitivity of 95% at a specificity of 98% could be achieved with 14 components; with a total of eight components a 92% correct classification was still achieved, with a sensitivity of 89% and a specificity of 95%, whereas six components were sufficient to achieve a sensitivity and a specificity of both 84%.

Within this data set, validation was performed using 20-fold cross validation: six subjects (5%) were randomly selected of the 120 children included in this investigation. Subsequently, the remaining 114 chromatograms were used to build a model set and a discriminant analysis was performed. Next, the six removed chromatograms were added as a test set and their classification into healthy or diseased was determined to test the model based on 114 subjects. This analysis was repeated a total of 20 times, so that all 120 chromatograms were once part of a test set. Ultimately, this approach led to 20 (slightly) different models predicting the respective unknowns. A 100% sensitivity and specificity was attained when the number of compounds in the discriminant analysis was 25 or higher. Reducing the number of compounds involved in the analysis to eight compounds still resulted in 89% sensitivity at 95% specificity. The relation between the number of components used and the selectivity and specificity obtained using model sets of 114 chromatograms and test sets of six chromatograms is visualized in Fig. 1a. Based on GC retention time and mass spectrometric information, we were able to identify the most important compounds that discriminate the asthma patients from the healthy controls. These components are presented in Table 2.

The mean relative intensities of the first 10 components that were used to generate the discriminant function that discriminates between asthmatic and healthy children are presented in Fig. 2. Most of the 63 asthma patients used inhalation corticosteroids, but seven subjects were free of any medication. We used the 57 controls and all but the seven medication-free patients to build a new discriminant function. The discriminant function thus calculated was used to classify the seven medication-free subjects. All seven patients were correctly classified in this way as asthma patients, and not as controls, which would have been the case if the differentiation had been based on medication.

In a separate data analysis experiment, we compared the chromatograms of the 42 atopic asthma patients with

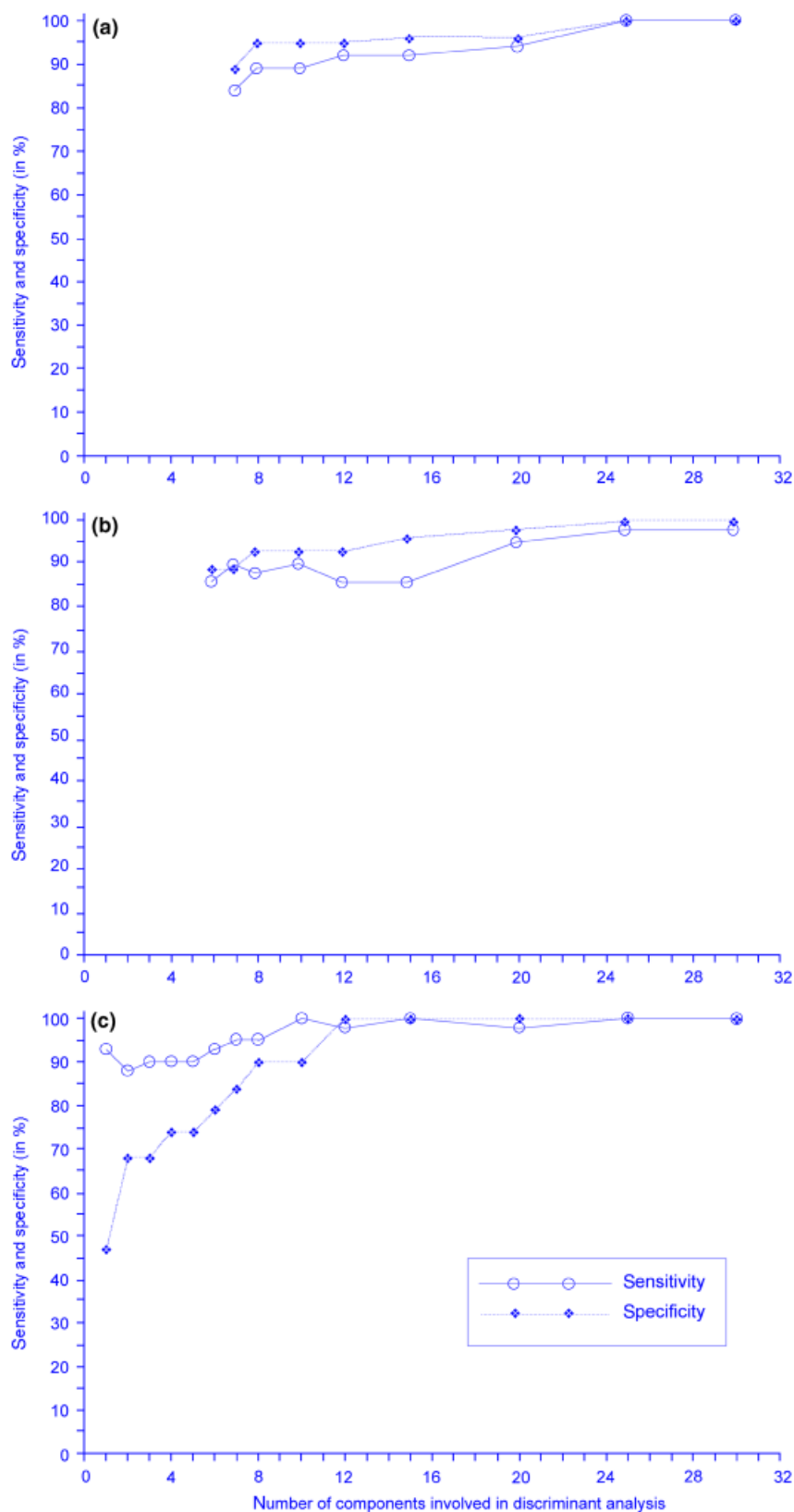


Fig. 1. Course of sensitivity and specificity as a function of the number of variables involved in the discriminant analyses. (a) Analysis of asthma vs. healthy controls, using 114 samples for the model and six for the test set, 20 times repeated. (b) Analysis of atopic asthma vs. healthy controls, using 95 samples for the model and five for the test set, 20 times repeated. (c) Analysis of atopic vs. non-atopic asthma, using 60 samples for the model and three for the test set, 20 times repeated.

those of the 57 controls. Discriminant analysis using the 20-fold cross-validation method showed 99% correct classification down to 25 components used. Using seven components still yielded a 90% sensitivity and an 89% specificity. The relationship between the number of components used in the analyses and the sensitivity and specificity is presented in Fig. 1b.

Finally, the chromatograms of the 42 atopic asthma patients and the 21 non-atopic asthma patients were

Table 2. Overview of the most important components used to discriminate asthma patients from healthy controls

Number*	Component
1	(Branched) hydrocarbon (C ₁₃ H ₂₈)
2	Carbon disulphide (CS ₂)
3	1-penten-2-on
4	butanoic acid
5	3-(1-methylethyl)-benzene
6	(Branched) hydrocarbon (C ₁₃ H ₂₈)
7	Unsaturated hydrocarbon (C ₁₅ H ₂₆)
8	Benzoic acid
9	p-xylene
10	(Branched) hydrocarbon (C ₁₁ H ₂₄)

*Numbers represent the order in which the components were included in the discriminant analysis.

compared using discriminant analyses. Our method, excluding 5% of the chromatograms from building the discriminant function and introducing these as unknowns, repeated 20 times, resulted in 100% sensitivity and specificity using 15 components. When seven components were used to determine the discriminant function, the specificity and selectivity were 95% and 84%, respectively, for the crossvalidation experiment. The relations between number of components involved in the analyses and the sensitivity and specificity are presented in Fig. 1c.

Discussion

Inflammation plays a critical role in many physiological changes of the body including inflammatory lung diseases like asthma. Inflammation is accompanied by oxidative stress and subsequently lipid peroxidation and during this process polyunsaturated fatty acids are converted into VOC that are secreted via the lungs. Hundreds of different VOC are present in human breath, and their relative concentrations may alter in the presence of the disease. Recently, we developed an approach to comprehensively analyse the entire range of VOC in exhaled air to search for unique profiles that can be used to identify certain

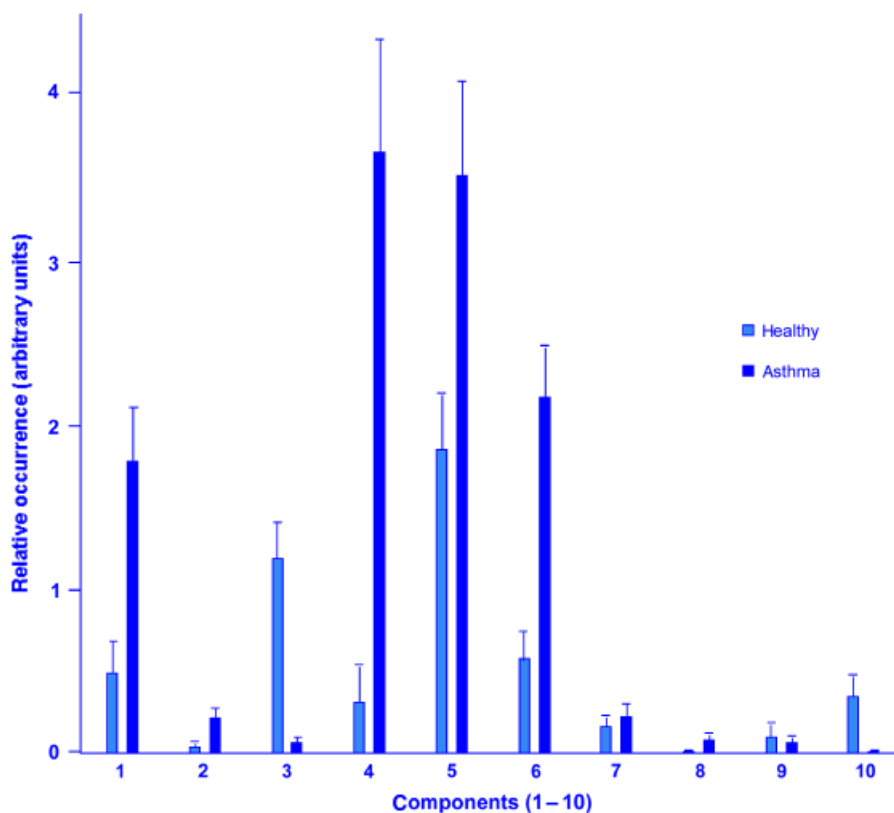


Fig. 2. Mean relative intensity (± standard error) of the 10 components that are used to generate the discriminant function that discriminates children with asthma from healthy controls. The number represents the order in which the components were included in the discriminant analysis (see also Table 2).

patient groups or exposed populations [33]. In the present study, we demonstrate the suitability of our highly accurate methodology tested on groups of asthmatic and non-asthmatic children. The results showed that discriminant analysis of exhaled breath chromatograms of 63 asthmatic children and 57 controls using 22 variables led to correct classification and cross validation of all subjects. Of course, it cannot be excluded that this number of components used for the classification might partly be based on statistical artefacts. However, reducing the number of components used to describe the discriminant function to six components clearly showed that the discriminating information present in the chromatograms was mainly based on real information with regard to the absence or presence of the disease, rather than on coincidence. This became even more evident, when the discriminant analyses were performed on 95% of the chromatograms (training set), leaving the remaining 5% (test set) as unknowns to be classified using the training model. In this case eight components were sufficient to reach 89% sensitivity at 95% specificity. As shown in Fig. 2, the majority of the discriminating compounds (nos 1, 2, 4, 5, 6, 7, 8) are enhanced but some compounds (nos 3, 9, 10) are also diminished in the exhaled breath of asthmatic children. Although a decreased amount in the patients is unexpected, the reason may be found in the complex equilibrium of formation and removal of VOC in the body. The hypothesis is that the inflammation-driven oxidative stress responsible for oxidizing polyunsaturated fatty acids abundantly present in cellular membranes may lead to a gamma of breakdown products excreted as VOC. Thus, it is obvious that the relative composition of VOC in exhaled breath can change as a result of the disease, and this change can be either an increase or a decrease of certain components. Indeed, we observed a considerable number of components that are significantly higher in patients compared with controls but these compounds are not included in the optimized discriminating model due to their limited discriminatory power. On the other hand, a decline in certain VOC may occur because especially the longer chain hydrocarbons are further oxidized into smaller compounds due to enhanced oxidative stress, and consequently their amounts are decreased in exhaled air of patients. It appeared that the absence of a number of these long-chained VOC is crucial for diagnostic ability. Apart from the oxidative stress hypothesis explaining changing VOC composition in patients, an alternative reason may be that asthma-affected lungs are remodelled, resulting in altered gas exchanges over the blood-lung barrier.

When the levels of sensitivity and specificity can be increased by increasing the number of samples in the data set, this method may be valuable for the diagnosis of asthma in children below 6 years of age, an age group in which a definitive diagnosis based on doctors' examina-

tion is still difficult. Because underdiagnosis and undertreatment in true asthmatics and overtreatment in transient wheezers are undesirable medically and in terms of public health, an earlier diagnosis would be valuable. The new diagnostic technique described in this study has the potential to make an early diagnosis of asthma possible and therefore a prospective study, aimed to find a relationship between the composition of exhaled air of children at a young age, and a definite diagnosis of the same children at an age of about 6 years, is worth performing. Furthermore, the experiments in which the exhaled breath samples of atopic asthma patients are compared with those of healthy controls showed the potential of this method to discriminate atopic asthma patients from healthy persons. The results of the analyses in which breath samples of atopic and non-atopic asthma patients were compared seem to indicate that not only can healthy individuals be differentiated from diseased patients in this way, but that it is also possible to differentiate one form of disease from another. At this stage, we did not identify the components involved in the discrimination between atopic asthma patients and healthy controls and non-atopic patients. Resolving the chemical identity of all relevant components will be enormously time consuming: some of the components can readily be identified, but for many there are various alternatives. To determine the identities, registration of GC retention times of (all) possible candidate compounds is necessary. The relevance of this identification largely depends on the probability to be able to link the components' structure to their respective biological origin.

The method used in this study and described above is designed to be easy and straightforward; it does not include background correction for ambient air, nor specific diet prescriptions. During the sampling period patients and controls were randomly sampled and therefore putative variations in background air are anticipated to affect both groups in the same manner and consequently have been eliminated in the discriminant analysis. Also, food intake and thus exhalation of food-related volatile compounds are expected to be free of systematic differences between both groups. Therefore, these components will have no discriminating power between these groups and have been eliminated in the data analysis process.

We are aware of a possible pitfall in the discrimination between the asthma patients and the healthy controls: because most patients are treated with inhalation steroids and the controls self-evidently not, the discrimination might (partly) be based on remains as for instance metabolites of the medication. However, all patients were instructed to not use their inhaler during 8 h before the sampling. We assume that compounds that are volatile enough to be exhaled, trapped on the sorption tubes and later on released from these tubes can be expected to be undetectable after this time interval. Besides, the

medication used was rather diverse and their chemical structures and metabolites were not reflected in the discriminating components. Furthermore, seven of the 63 asthmatic subjects included were not on treatment and were still well classified. In addition to this, the fact that the electronic nose does not discriminate treated asthma from non-treated asthma seems to indicate that treatment does not interfere with the discrimination [36]. In an additional experiment we used the 57 controls and 56 asthma patients (with medication) to construct a new discriminant function. In this new function, none of the medication-free patients' samples is involved. The seven medication-free patients' samples were then introduced as unknowns and all were correctly classified. This latter experiment shows that differentiation is not based on medication vs. no medication, but truly on asthma vs. non-asthma. On the other hand, the medication may have reduced the differences between the breath samples of patients and healthy controls, by suppressing the illness and the accompanying inflammation and thus inflammation products. Therefore, it can be speculated that comparing children with and without asthma, but all (still) without medication, may demonstrate even more difference in exhaled breath profiles than we found in this study. A completely new set of samples from both patients and controls may provide the ultimate test for our method. However, using 20-fold cross validation in our data-analysis experiments, we demonstrated that the model built on part of the data shows good performance in predicting the status of the left-out samples. By sampling breath from all children at age 3–4 with respiratory complaints, and comparing the VOC contents of these samples after a reliable diagnosis has been set (typically at age 6), we hope to be able to predict with sufficient sensitivity and specificity the outcome (i.e. asthma positive or negative) of young children with respiratory complaints. The results of the present study may provide a necessary, but of course not yet sufficient step towards this goal.

As indicated in the introduction, oxidative stress caused by inflammation and leading to lipid peroxidation may play an important role in the production of the deviant exhaled breath composition of diseased subjects. Products from this lipid peroxidation process, like ethane and pentane, are found in exhaled breath, but an extensive analysis of all components of exhaled breath in relation to the peroxidation reactions has not been performed before. In this study, we did not search for specific known components, but we measured all compounds (above C₃-size) in exhaled breath. The hydrocarbons we found to be discriminating might well be formed in the lipid peroxidation process, but other pathways cannot be excluded. Therefore, a more profound biological interpretation of the discriminating profile will be useful. For this, animal and *in vitro* cell culturing studies are warranted.

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