Exhaled volatile organic compounds predict exacerbations of childhood asthma in a 1-year prospective study

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ABSTRACT The hypothesis was that prediction of asthma exacerbations in children is possible by profiles of exhaled volatile organic compounds (VOCs), a noninvasive measure of airway inflammation. The aims of the present study were to determine: 1) whether VOCs in exhaled breath are able to predict asthma exacerbations; and 2) the time course and chemical background of the most predictive VOCs.

A prospective study was performed in 40 children with asthma over 1 year. At standard 2-month intervals, exhaled nitric oxide fraction (F_{ENO}), VOC profiles in exhaled breath samples, lung function and symptoms were determined in a standardised way. VOC profiles were analysed by gas chromatography–time-of-flight mass spectrometry.

16 out of 40 children experienced an exacerbation. With support vector machine analysis, the most optimal model of baseline measurements *versus* exacerbation within patients was based on six VOCs (correct classification 96%, sensitivity 100% and specificity 93%). The model of baseline values of patients with compared to those without an exacerbation consisted of seven VOCs (correct classification 91%, sensitivity 79% and specificity 100%). F_{ENO} and lung function were not predictive for exacerbations.

This study indicates that a combination of different exhaled VOCs is able to predict exacerbations of childhood asthma.

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Introduction

The purpose of asthma treatment is optimal control of the disease [1]. Several cross-sectional surveys have demonstrated that actual levels of asthma control fall far below the goals of national and international guidelines [2–4]. HAMMER *et al.* [3] reported poor control in 55% of children with asthma in the Netherlands. Poor asthma control and frequent exacerbations may lead to irreversible, pathological and functional airway changes [5]. Children with asthma often have a poor perception of their complaints relative to normal lung function, which results in under-reporting of symptoms and even underdiagnosis of asthma [1, 3, 6]. Another explanation for poor asthma control may be that although asthma is characterised by chronic airway inflammation, monitoring is not currently performed using measures of airway inflammation [7, 8]. Indeed, there are indications that exacerbations in asthma may be predicted by sputum eosinophils or by nitric oxide in exhaled breath [9, 10].

A relatively new noninvasive technique to assess airway inflammation/oxidative stress in the airways is profiling of volatile organic compounds (VOCs) in exhaled breath. In patients with asthma, production of reactive oxygen species (ROS) is increased [11], which causes lipid peroxidation of cell membranes and subsequent production of VOCs [12]. DALLINGA *et al.* [13] showed that VOCs in exhaled breath could discriminate between asthmatic children and controls with a high sensitivity and specificity. DRAGONIERI *et al.* [14] studied ''smellprints'' of VOCs by means of the ''electronic nose'' in 10 young adults with mild asthma, 10 older patients with severe asthma and 20 age-matched controls. They found that the electronic nose could discriminate patients with asthma from healthy controls, whereas the distinction between different asthma severities classes was more difficult.

The present longitudinal study investigated the hypothesis that specific VOCs in the exhaled breath of children with asthma are able to predict exacerbations before they are clinically manifest. The specific aims were to investigate: 1) whether VOCs in exhaled breath are able to reliably predict asthma exacerbations and to assess which combination of VOCs is most predictive; and 2) the time course of VOCs during an exacerbation.

Methods

Patients

Children with asthma aged 6–16 years were selected from the outpatient clinic of the Dept of Paediatric Pulmonology, Maastricht University Medical Centre (MUMC+), Maastricht, the Netherlands. All children were known to have had a diagnosis of asthma at our clinic for $\geqslant$6 months. Asthma was defined based on the following criteria of the Global Initiative for Asthma (GINA) and the Dutch Paediatric Pulmonology Society [1, 15]: 1) recurrent episodes with wheezing, breathlessness, chest tightness or coughing; and 2) one or more occasions with reversible airway obstruction (increase in forced expiratory volume in 1 s (FEV_1) after 400 μg salbutamol of $\geqslant$9% predicted [15]); and/or 3) bronchial hyperresponsiveness to histamine (defined as a provocative concentration of histamine required to induced a 20% fall in FEV_1 of <8 mg·mL^{-1}).

All children were treated according to the GINA guideline [1]. Treatment was only adapted on basis of symptoms and lung function. A child was considered atopic when a phadiatop test and/or radio-allergosorbent test (RAST) was positive (at least two RAST classes). Exclusion criteria were: 1) diseases that might have interfered with the results of the study (*e.g.* other chronic inflammatory diseases); 2) inability to properly perform measurements; or 3) active smoking.

Study design

The study design was a prospective longitudinal study over 1 year. In addition to fixed routine visits to the outpatient clinic every 2 months, patients were asked to visit the outpatient clinic an additional four times during an exacerbation. These extra visits were planned, at most, twice during the study. Extra measurements were planned at day 1, 3 and 5 of the exacerbation, and after return of FEV_1 to baseline values and improvement of symptoms (table 1).

Ethics

This study was approved by the Medical Ethics Committee, MUMC+. The www.clinicaltrials.gov registration number was NCT00404859. All parents gave written informed consent.

Exhaled breath sample collection

For at least 1 h before the experiments, no eating or physical exercise were permitted. Subjects were asked to inhale deeply and, subsequently, to exhale into resistance-free Tedlar bags (5 L) as previously described [13, 16, 17]. One to three exhalations were usually sufficient to inflate the bag. Details about exhalation pressure,

TABLE 1 Study design

	Standard				Exacerbation				Standard	
t	-3	-2	-1	0	e_1	e_2	e_3	e_4	+1	+2

The baseline measurement was defined as the routine measurement before the clinical onset of an asthma exacerbation, denoted by $t=0$. The routine measurements before $t=0$ were coded $t=$ -1, -2, -3, *etc.* The time interval between these measurements was 2 months. During the exacerbation, measurements were denoted by $t=e_1$, e_2, e_3 and e_4, referring to the additional measurements at day 1, 3 and 5, and at the end of the event, respectively. After the exacerbation, the standard measurements continued (denoted as t= +1, +2, *etc.*).

sampling time and reproducibility of the procedure are given elsewhere [16, 17]. The contents of the bag were transferred under standardised conditions onto stainless steel two-bed sorption tubes containing active carbon (Markes International, Llantrisant, UK). VOCs were trapped onto the carbon components until analysis of the sample. In order to analyse the sample, VOCs were released from the tubes by thermal desorption at 270°C (UNITY desorption unit; Markes International). VOCs were then separated by gas chromatography (GC) (ThermoFisher Scientific, Austin, TX, USA) and subsequently detected by time-of-flight mass spectrometry (TOF-MS) (Thermo Electron Tempus Plus; ThermoFisher Scientific) as previously reported [13, 16, 17].

Outcome measures

Primary outcome measure: asthma exacerbation

The occurrence of an asthma exacerbation was the primary outcome measure. The definition of an asthma exacerbation (moderate and severe) was based on the criteria of the American Thoracic Society and European Respiratory Society (ERS): 1) an increase in asthma symptoms (dyspnoea, cough and wheezing) and/or use of short acting β_2-agonists for $\geqslant$2 days; and/or 2) a need for treatment with oral corticosteroids; and/or 3) a need for hospital admission [18].

To recognise an exacerbation at an early stage, AM1 home monitors (CareFusion, Hoechberg, Germany) and modems (HC1; CareFusion) were used. FEV_1 measurements, use of rescue medication and the presence and severity of pulmonary symptoms were recorded daily at a fixed time. The intensity of symptoms was scored on a scale from 0 to 3. All patients were asked to perform the manoeuvres three times within 10 min and the highest FEV_1 (in litres) was stored. Data were sent digitally by telephone modem to a personal computer at the MUMC+ once per week. In the case of deterioration of FEV_1 values, and/or an increase in the presence and severity of pulmonary symptoms, patients were called to the hospital for additional measurements and a consultation of the responsible paediatric pulmonologist. At the outpatient clinic, asthma control scores and lung function tests were assessed and breath samples were collected.

Secondary outcome measures

Asthma control score

The asthma control score was assessed 2-monthly by using a validated questionnaire as described previously [3, 4]. The questionnaire contained questions about chronic airway symptoms, sleep disturbance, limitation of daily activity, asthma attacks, emergency or urgent care visits, and the need for short acting β_2-agonists [3].

Lung function tests

Short-acting bronchodilators were stopped $\geqslant$8 h and long-acting bronchodilators $\geqslant$36 h before testing. Dynamic spirometry was performed by means of the Flowscreen® (Carefusion) according to ERS standards [19]. The recorded parameters were: FEV_1, forced vital capacity (FVC) and maximum expiratory flow at 50% of FVC (MEF_{50}), all expressed as a percentage of the predicted normal value [19]. The reversibility to a β_2-agonist was determined 15 min after inhalation of 400 µg salbutamol *via* a spacer (Volumatic; GlaxoSmithKline, London, UK) at the first, fourth and seventh routine visit.

Analysis of data

Data acquisition and mining

Analysis of the data output files from GC–TOF-MS was performed in successive steps as previously described in detail [17]. In summary, the first step was to perform peak detection and baseline corrections on all analysis output files. Normalisation of the calculated peak areas was performed using an area scaling factor based on the cumulative area under the detected peaks. Retention times of all samples were assessed. Finally, the output files were merged by combining corresponding compounds based on the degree of

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similarity of the corresponding mass spectra, by determining the match factor values and similarity of retention time. The degree of mass spectrum similarity was calculated using a match factor based on the similarity index as described by STEIN and SCOTT [20].

Analysis

In order to analyse which VOCs were predictive of an asthma exacerbation, intra- and intersubject comparisons were made. Both comparisons were explored since they might provide different information with regards to the compounds that contribute to an early detection of exacerbations in asthma patients.

Intrasubject comparison

The first explored comparison was an intrasubject comparison between baseline measurements (t=0) and the first measurement of an exacerbation (t=e_1). The baseline measurement was defined as the sample taken before the start of the exacerbation (measurement 0), on average, 1 month prior to measurement e_1. The measurements during the exacerbation were chronologically denoted as e_1, e_2, e_3 and e_4. The baseline measurement before t=0 was denoted by t= -1, the one before that t= -2, *etc.*, as shown in table 1.

Intersubject comparison

The second analysis was an intersubject comparison. Baseline samples (t=0) of subjects suffering an exacerbation were compared to baseline measurements of subjects not suffering an exacerbation. Sampling dates of all incorporated samples were chosen as close to one another as possible.

Component selection

Subsequent component selection and determination of interesting compounds was performed in three ways. 1) Compounds detected in <8% of the samples were deleted from the dataset, according to PENN *et al.* [21]. 2) VOCs with significant different intensities between children with and without exacerbations were selected (t-tests with Bonferroni correction). Samples at measurement t=0 originating from the patients with an exacerbation were compared with samples of the group without an exacerbation (table 1). These samples matched the dates of the t=0 samples. 3) Support vector machine (SVM) classification models were used to analyse the ability of VOCs to predict an asthma exacerbation. A SVM is a method in statistics and computer science that analyses data and recognises patterns, and is used for classification and regression analysis. The standard SVM takes a set of input data and predicts, for each given input, which of two possible classes forms the input, making the SVM a nonprobabilistic binary linear classifier. Given a set of training examples, each marked as belonging to one of two categories, an SVM training algorithm builds a model that assigns new examples into one category or the other. An SVM model is a representation of the examples as points in space, mapped so that the examples of the separate categories are divided by a clear gap that is as wide as possible. New examples are then mapped into that same space and predicted to belong to a category based on which side of the gap they fall [22–24].

The SVM approach was chosen for its ability to construct predictive models with large generalisation power even in the case of large dimensionality of the data when the number of observations available for training is low [22]. SVMs are specifically useful since they seek a globally optimised solution and avoid overfitting, allowing for a large number of features or compounds. In order to obtain the best subset of compounds, the attribute selection option implemented in Weka (University of Waikato, Hamilton, New Zealand) was used [23]. Compounds were selected using an SVM attribute evaluator. The attribute evaluator evaluated the worth of a subset of compounds by considering the individual predictive ability of each compound along with the redundancy between them. Preferably, compounds were selected showing high correlations within the class and low intercorrelation. A subset of the highest ranking compounds was implemented into an SVM classifier trained with the sequential minimal optimisation algorithm of PLATT [24]. The SVM classifiers were validated and performance was tested using 10-fold cross-validation in which the entire dataset is split repeatedly into a test set (90% of samples) and a validation set (10% of samples).

Identification of VOCs

Identification of VOCs was performed by comparing the mass spectra with data from the NIST library (database of the National Institute of Standards and Technology) and by interpretation of the mass spectra by a specialised spectrometrist.

Power analysis

In order to find clinically meaningful correlations between independent predictors and the exacerbation rate of at least 0.6, 35 children are necessary to assess such a relationship with a two-sided alpha of 0.05 and a power of 98%. During the 12-month follow-up, a drop-out rate of 10% was assumed. 40 children with asthma were included in this study.

Results

Patient characteristics

Clinical characteristics at inclusion in the study are shown in table 2. The majority of the group was atopic: 29 (73%) out of 40 children. All but one patient was receiving maintenance treatment with inhaled corticosteroids. 38 of the 40 children completed the study, while two patients dropped out. One patient moved out of the region. Before he dropped out, he experienced one exacerbation. The data of this exacerbation were included in the analysis. The other child was not motivated to further participate. She had stable asthma.

Exacerbation rate

During the 1-year study period, 16 children developed an exacerbation of which 10 were moderate and six were severe. Only three patients developed a second exacerbation. The mean±SE time interval between the preceding standard measurement and the onset of an exacerbation was 39±4 days.

Volatile organic compounds

VOCs included in data analysis

In all 39 children with asthma, a total of 3,434 different VOCs were detected in the exhaled breath samples. On average, 343 VOCs were detected per individual. Each subject delivered seven to 15 samples depending on the occurrence of an exacerbation.

Significantly different VOCs

In total, independent t-tests showed 30 significantly different VOCs after Bonferroni correction ($p<0.001$). These VOCs were identified as markers associated with exacerbations.

Timecourse of VOCs

After selection of the significantly different compounds, these VOCs were tracked in all other samples (t= -2 to e_4). Their relative intensity was determined in all samples in order to provide more insight into the timecourse of intensities of these compounds prior to and during exacerbations. As an example, the course of the intensity of three VOCs during an exacerbation are shown in figure 1.

Ability to predict an asthma exacerbation

All VOCs that were present in ⩾8% of the samples were included in the SVM analyses in order to assess the ability of VOCs to predict an asthma exacerbation [16, 17]. Results of SVM models are based on VOCs considered together. The relationship between the number of different VOCs in the SVM model and the

TABLE 2 Clinical characteristics of the asthmatic children at inclusion of the study

Age years	10.7±0.4
Weight kg	38.8±2.0
Height cm	142.3±2.4
Males/females	29/11
ACQ score (range)	27.6±2.3 (9–64)
Lung function indices	
Reversibility, increase in FEV_1 % pred	5.9±1.1
FEV_1 % pred	99.6±2.2
FEV_1/VC %	83.9±1.5
FVC % pred	99.2±2.4
MEF_{50} % pred	82.6±3.8
Atopy yes/no	29/11
Total IgE $kU \cdot L^{-1}$	493.3±120.7
Active eczema	7 (18)
Allergic rhinitis	4 (10)
Treatment	
Dose of inhaled budesonide or equivalent μg	587±53
Long-acting β_2-agonist	21 (53)
Antihistamines	9 (23)
Leukotriene receptor antagonist	8 (20)

Data are presented as mean±SEM, n or n (%), unless otherwise stated. ACQ: Asthma Control Questionnaire; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; VC: vital capacity; FVC: forced vital capacity; MEF_{50}: mean expiratory flow at 50% of FVC; Ig: immunoglobulin.

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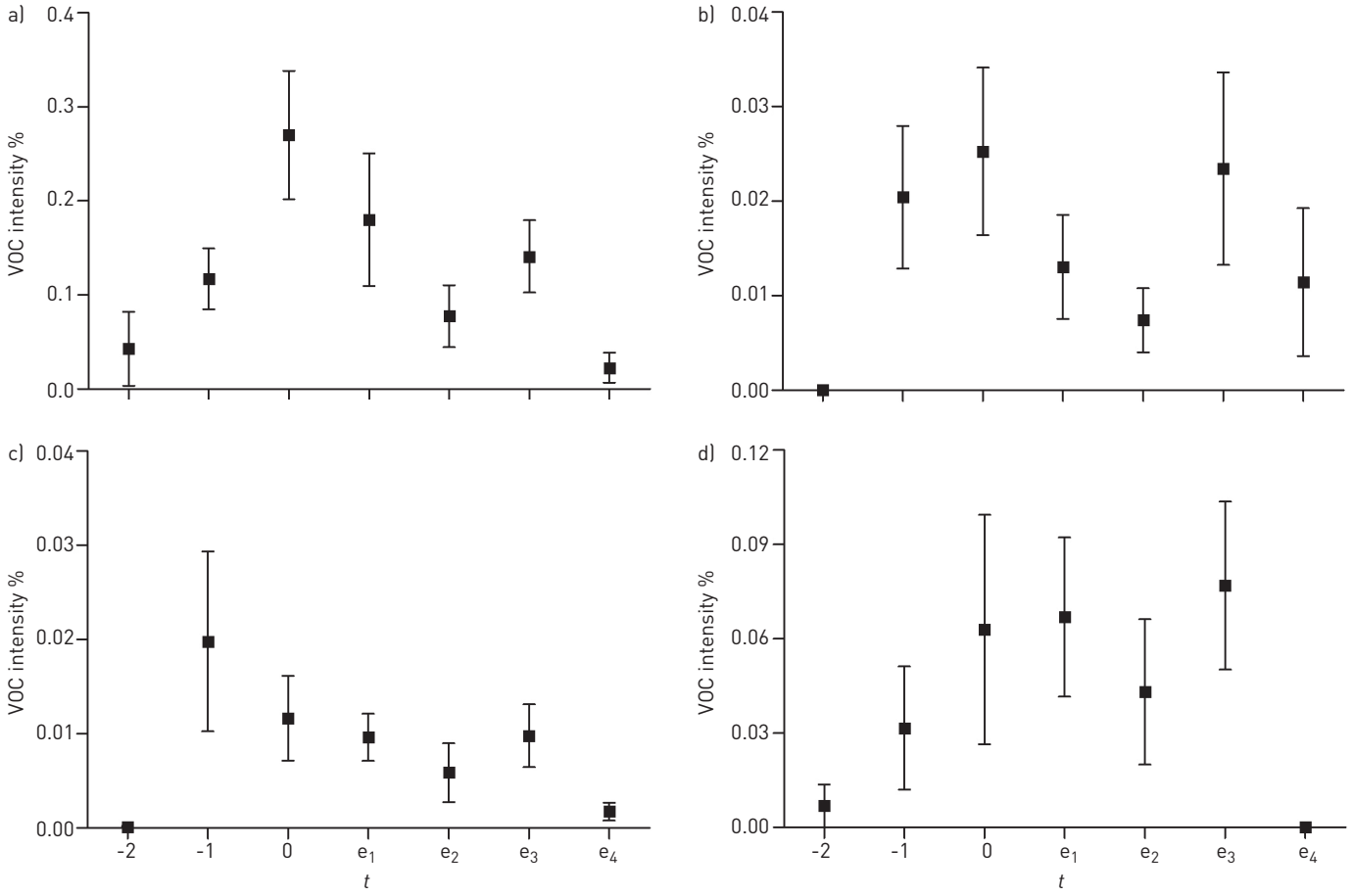

FIGURE 1 Intensity of four significantly different volatile organic compounds (VOCs) demonstrating their time course before and during an asthma exacerbation. a) *p*-xylene (Friedman test p=0.005); b) 8,11-octadecadiynoic acid methylester (Friedman test p=0.01); c) 3,6-octadecadiynoic acid methylester (Friedman test p=0.004); d) octadecane (Friedman test p=0.01). Data are presented as mean±SE. For definitions of the time points (*t*), see table 1.

corresponding sensitivity and specificity is shown in tables 3 and 4. In the intrasubject comparison (baseline *versus* exacerbation within patients), the optimally performing SVM was based on six VOCs. Exacerbations were correctly classified in 96% of samples (sensitivity 100%, specificity 93%, positive predicted value 89% and negative predicted value 100%) as shown in table 3. The optimally performing SVM of intersubject comparison (baseline resulting in exacerbation *versus* baseline not resulting in exacerbation, between patients) was based on seven VOCs. A correct classification rate of 91% was found with a corresponding sensitivity and specificity of 78% and 100% (positive predicted value 100% and negative predicted value

TABLE 3 The relationship between the number of volatile organic compounds (VOCs) in the support vector machine model and the prediction of exacerbations: intrasubject comparison

VOCs n	Sensitivity %	Specificity %	Correct classification %
9	100	100	100
8	100	100	100
7	100	93	96
6	**100**	**93**	**96**
5	100	79	89
4	100	71	86
3	100	64	82
2	100	50	75
1	100	29	64

An optimal model contained six VOCs and had a sensitivity of 100%, a specificity of 93%, a positive predicted value of 89% and a negative predicted value of 100% (bold).

TABLE 4 The relationship between the number of volatile organic compounds (VOCs) in the support vector machine model and the prediction of exacerbations: intersubject comparison

VOCs n	Sensitivity %	Specificity %	Correct classification %
9	79	95	88
8	71	95	85
7	**79**	**100**	**91**
6	64	100	85
5	71	100	88
4	64	100	85
3	43	100	76
2	36	100	73
1	21	100	67

An optimal model contained seven VOCs and had a sensitivity of 79%, a specificity of 100%, a positive predicted value of 100% and a negative predicted value of 86% (bold).

86%), respectively (table 4). Neither F_{ENO} nor lung function were no significant predictors of exacerbations. Univariate Cox regression analysis of the time until an exacerbation showed that neither F_{ENO} nor FEV_1 were significant predictors of an asthma exacerbation ($p=0.43$ and $p=0.60$, respectively).

Identification of VOCs

It was possible to identify five out of six VOCs of the optimally discriminating SVM model for intrasubject comparison and six out of seven VOCs for intersubject comparison (tables 5 and 6).

Discussion

This study indicates that VOCs in exhaled breath are able to predict asthma exacerbations in children. We found that a combination of six or seven VOCs was able to predict exacerbations both between and within patients with a high sensitivity and specificity. It was also possible to identify VOCs that significantly ''tracked'' the course of an exacerbation. The identified VOCs were mainly classified as hydrocarbons. Both F_{ENO} and lung function had no significant predictive value for exacerbations in this study.

To our knowledge, this is the first longitudinal study to analyse the ability of VOCs in exhaled breath to predict exacerbations of childhood asthma, and to study the course of VOCs during an exacerbation. In a recent study, IBRAHIM *et al.* [25] studied the potential of VOC profiling to assess asthma diagnosis, sputum inflammatory cell profile and asthma control in 35 adult asthmatics and 23 matched controls. They demonstrated that VOC profiles were able to accurately identify patients with sputum eosinophilia and poor disease control with cross-validated accuracies of 83% and 80%, respectively. In a cross-sectional study of 120 children (57 controls and 63 asthma patients), we found a good discrimination between asthma and controls based on an optimal combination of eight VOCs in exhaled breath [13]. In adults, DRAGONIERI *et al.* [14] assessed VOC patterns in exhaled breath by means of the electronic nose. They found that asthma could be differentiated from controls but the discrimination between different degrees of asthma severity was not satisfactory. In contrast, to GC–TOF-MS used in our study, the electronic nose in the study of DRAGONIERI *et al.* [14] was used to assess smellprints of different VOCs. These techniques are fundamentally different, since the smellprints are based on pattern recognition instead of assessing single mass spectra. The disadvantage of smellprints is that it may not measure specific VOCs that are important for the discrimination. The advantage of the approach used in the present study is that the most predictive VOCs are detected and that the chemical background of the compounds can be elucidated. This information can be used in the future to construct an electronic nose that is specifically useful for a certain disease (*e.g.* childhood asthma) and a specific clinical question (*e.g.* the early detection of exacerbations).

Regular 2-month assessments of F_{ENO} in this study were not predictive of exacerbations.

In a 6-month study by FRITSCH *et al.* [26] of 47 children with mild-to-moderate asthma, F_{ENO} at a cut-off point of 29 ppb was most predictive of an exacerbation with a sensitivity of 80% and a specificity of 60%. In the inhaled corticosteroid withdrawal study of PIJNENBURG *et al.* [27], F_{ENO} values >49 ppb 4 weeks after discontinuation had a sensitivity for an asthma relapse of 71% and a specificity of 93%. In nonatopic patients with asthma, F_{ENO} may not be elevated [28]. The advantage of VOC analysis in exhaled breath is that several inflammatory markers are measured in exhaled breath simultaneously and that the most predictive biomarkers can be selected, as indicated by the high sensitivity and specificity of the combination of VOCs in our study.

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TABLE 5 Identified compounds as implemented into the most optimal support vector machine classifiers of intrasubject comparison

1	*p*-xylene
2	3-methylpentane
3	2-ethyl-4-methyl-1-pentanol
4	Unknown
5	1-phenyl-1-butene
6	4,6,9-nonadecatriene

It is relevant to discuss several elements of our study. First, a group of 40 children with asthma was included, of which 16 children experienced an exacerbation during the year of follow-up. Although internal validation was performed in our study by 10-fold cross-validation and exacerbations could be predicted with good sensitivity and specificity, external validation of our study in larger study populations will be necessary to confirm the optimal combination of VOCs [29]. 29 of the 40 children were atopic but our number of children was too low to perform a *post hoc* analysis in this group.

Secondly, the presence of VOCs may be influenced by intrinsic (sex, age, weight, atopy and lung function) and extrinsic factors (*e.g.* ambient air or medication). Most of these intrinsic factors were well controlled in our study. Moreover, no relationship was found between these intrinsic factors, medication and exacerbations during the study. This almost rules out a possible influence of these factors on the results of this study. Currently, there is no consensus on the role of VOCs in ambient air on VOC profiling in exhaled breath. The children were all measured in the same room for each session and, therefore, were all influenced by ambient air in a comparable way. As reported previously by VAN BERKEL *et al.* [17] and DALLINGA *et al.* [13], we did not correct our measurements for chemical background appearing in the samples. This is because of the fact that it would not be possible to correct for the complex interdependencies between excretion and uptake of VOCs by easily subtracting the inhaled from the exhaled air. Moreover, background noise will be randomly distributed between subjects' samples and will, thus, neither exert any discriminatory power nor interfere with the outcome of the analyses. We are aiming with discriminative analysis to select only those compounds that are specific for the disease or condition and should, thus, principally not depend on background chemicals. An earlier report by our research group concluded that the procedure followed for sampling, chemical analysis, data handling and accurate data mining was highly reproducible [17]. In addition, chemical structures and metabolites of medication used were not reflected in the significantly predictive VOCs.

Thirdly, the origin of the identified VOCs remains in some instances uncertain. We were able to identify the majority of the predicting VOCs.

We hypothesise that discriminating VOCs reflect the degree of airway inflammation and oxidative stress. Saturated hydrocarbons (VOCs) in exhaled breath are formed during lipid peroxidation of fatty acid components of cell membranes. This process is triggered by ROS [17, 30–35].

In the future, it is relevant to study whether titration of anti-inflammatory treatment on the basis of VOCs is helpful in the prevention of exacerbations of asthma. In addition, studies are necessary to clarify the biochemical origin, pathophysiological function and optimal way of measuring predictive VOCs.

Breath analysis mirrors biochemical and immunological processes in the human lung. The present study indicates that VOC profiling is able to predict asthma exacerbations in children. Future studies are needed for external validation of the results in larger patient samples, to explore the nature of these VOCs and to investigate whether better asthma control can be achieved when exhaled breath analysis is included as a parameter in daily clinical practice.

TABLE 6 Identified compounds as implemented into the most optimal support vector machine classifiers of intersubject comparison

1	Unknown
2	2-ethyl-1,3-butadiene
3	Cyclohexane
4	2-octen-1-ol
5	1.2-methyl-4H-1,3-benzoxathiine
6	Benzene

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