

## Exhaled volatile organic compounds for diagnosis and monitoring of asthma

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### Abstract

The asthmatic inflammatory process results in the generation of volatile organic compounds (VOCs), which are subsequently secreted by the airways. The study of these elements through gas chromatography-mass spectrometry (GC-MS), which can identify individual molecules with a discriminatory capacity of over 85%, and electronic-Nose (e-NOSE), which is able to perform a quick onboard pattern-recognition analysis of VOCs, has allowed new prospects for non-invasive analysis of the disease in an "omics" approach. In this review, we aim to collect and compare the progress made in VOCs analysis using the two methods and their instrumental characteristics. Studies have described the potential of GC-MS and e-NOSE in a multitude of relevant aspects of the disease in both children and adults, as well as differential diagnosis between asthma and other conditions such as wheezing, cystic fibrosis, COPD, allergic rhinitis and last but not least, the accuracy of these methods compared to other diagnostic tools such as lung function, FeNO and eosinophil count. Due to significant limitations of both methods, it is still necessary to improve and standardize techniques. Currently, e-NOSE appears to be the most promising aid in clinical practice, whereas GC-MS, as the gold standard for the structural analysis of molecules, remains an essential

tool in terms of research for further studies on the pathophysiologic pathways of the asthmatic inflammatory process. In conclusion, the study of VOCs through GC-MS and e-NOSE appears to hold promise for the non-invasive diagnosis, assessment, and monitoring of asthma, as well as for further research studies on the disease.

**Key Words:** Asthma; Volatile organic compounds; Gas chromatography-mass spectrometry; Electronic-Nose; Breathomics; Non-invasive diagnosis

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**Core Tip:** The groundbreaking omics approach of non-invasive diagnosis of asthma by means of exhaled volatile organic compounds in several respiratory diseases, including asthma, is feasible and might revolutionize the diagnostic management of the aforementioned diseases.

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## INTRODUCTION

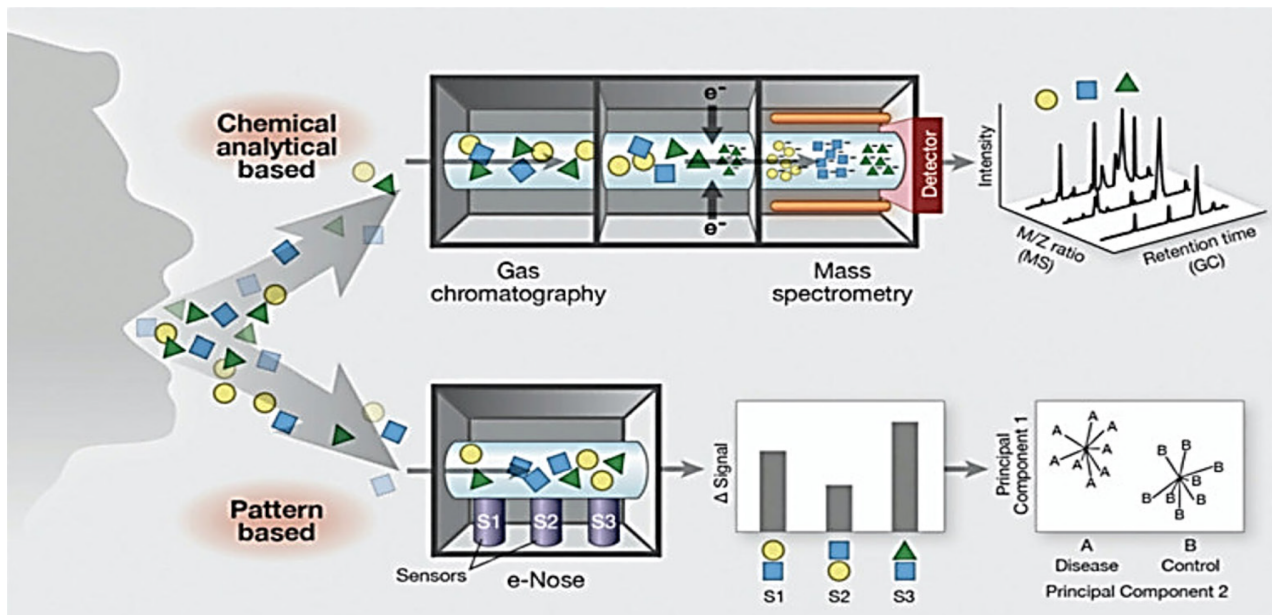
Asthma is a chronic disease of the airways that is characterized by reversible respiratory symptoms such as cough, breathlessness and chest tightness. The reason for these symptoms is the underlying chronic airway inflammation which results in bronchial hyperresponsiveness; therefore, quantifying airway inflammation in asthma has been in the focus of respiratory research for decades. Airway inflammation can be directly assessed *via* invasive (bronchoalveolar lavage, bronchial biopsy), and non-invasive techniques, such as spontaneous or induced sputum and exhaled breath analysis. However, neither bronchoscopy-based techniques nor sputum is feasible in routine clinical practice. Not-surprisingly, only the measurement of exhaled nitric oxide has become part of international guidelines[1,2]. Of note, exhaled nitric oxide reflects only on a distinct, albeit important, interleukin 4 and 13-driven asthmatic endotype and cannot fully encompass the whole burden of asthmatic inflammation[3].

Apart from nitric oxide, human breath contains thousands of molecules, including volatile organic compounds (VOCs). The origin of these substances is two-fold. Many of them are inhaled from the environment and exhaled into breath following or without modification in the human body. Other molecules are endogenously produced and reflect on the local or systemic metabolism, inflammation, and oxidative stress[4]. In line with this, exhaled VOC levels are significantly different in asthma and relate to disease activity and phenotypes. The analytical techniques to measure VOCs in breath have developed into two ways. Gas chromatography-mass spectrometry (GC-MS) is the gold standard method to quantify exhaled VOCs; however, it is costly, requires special analytical techniques and room capacity. An alternative for GC-MS is a sensor array which mimics the human olfaction, called electronic nose. The two techniques have very different purposes: GC-MS has the ability to perform precise structural analysis of individual molecules within the VOCs mixture present in the exhaled breath; on the other hand, electronic nose is not intended to identify individual molecules but rather to recognize patterns given by different fractions of the VOC mixture, which are then compared to a breath print database. Electronic nose is able to give quantitative response to a comprehensive VOCs profile, but in this case individual VOCs remain unidentified. This makes the two tools complementary and not interchangeable (Figure 1). In this review we will describe these techniques, the studies in asthma by focusing on the clinical aspects.

## GC-MS TECHNIQUE

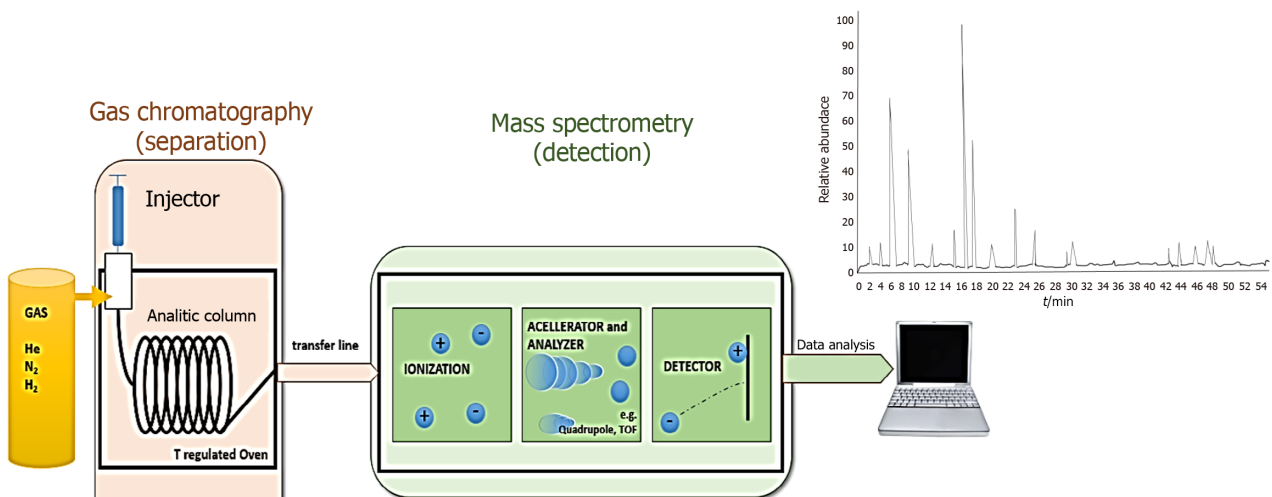
GC-MS is an analytical technique used to identify volatile compounds in a mixture, both qualitatively and quantitatively, with a high discriminative capacity of over 85%[5]. The exhaled sample is introduced into an injection chamber where it undergoes a thermal shock and is mixed with a transport gas contained in a high-pressure cylinder. Through a system that regulates pressure and flow, the transport gas is delivered continuously. The gas acts only as a carrier (mobile phase) and not as a solvent, entering the sample into a long analytical chromatographic column subjected to regulated temperatures. Within the chromatographic column, the so called “stationary phase” occurs, during which the molecules are separated. This phase represents the critical component of the system. The presence of gas allows the analyte to undergo absorption and desorption phenomena on the stationary phase. Each molecule will have its own absorption coefficient on the stationary phase, allowing for the separation of molecules and progressively determining their elution from the column based on a unique retention time for each compound, appearing as peaks on a detector as a function of time.

In GC-MS, the MS acts as the detector for GC (Figure 2). After selecting only volatile molecules with an absorption coefficient from the sample (excluding those in the dead volume), they are transferred to the mass spectrophotometer. MS



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**Figure 1 Differences in approach between the two techniques.** While gas chromatography-mass spectrometry utilizes an analytical chemical approach, electronic-Nose operates based on pattern recognition. GC: Gas chromatography; MS: Mass spectrometry.



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**Figure 2 Gas chromatography-mass spectrometry.** Combination of gas chromatography and mass spectrometry for the separation and identification of organic molecules, respectively: The gas serves as a medium for transporting the molecules present in the exhaled breath, which undergo absorption and desorption phenomena (Stationary Phase), passing sequentially along the transfer line. Here, the molecules are ionized and accelerated by electric fields. This allows for further separation and detection. Through data analysis, the detected molecules will appear as peaks, representing their relative abundance over the detection time. T: Temperature; TOF: Time of flight.

is an analytical technique used to identify individual particles in the sample based on their molecular weight. To do this, the volatile particles obtained from GC are initially ionized by means of an electron ionization and less frequently a chemical ionization (CI)[6]. The produced ions are then subjected to a gradient of electric fields (sometimes coupled with magnetic fields). Undergoing these forces, the particles are accelerated and separated based on their mass, charge, and velocity. The applied fields will deflect the trajectory of each individual particle, the less its mass, the greater the deflection: A particular electric field will be able to only allow ions with a specific mass/charge ratio to exit. These ions, if sorted based on their mass and charge, produce a pattern known as a "mass spectrum." The machine allows us to obtain not only the  $m/z$  ratio but also the intensity of each individual ion and its abundance in that particular sample[7]. To ensure accurate identification, the spectrum must be compared with a reference spectrum verified by a database such as the NIST/EPA/NIH Mass Spectral Library, inserting mass and charge values and the ionization conditions through which the databases identify the molecule that best matches those characteristics.

GC-MS allows for a more accurate study of the sample compared to the two techniques used separately since the retention time for GC and the chemical composition provided by MS allows us to determine the structure of the molecule under investigation. Also to be considered is that the use of different chromatographic columns and different detectors allows for the identification and quantification of a wide range of elements, making the entire method a very powerful tool (MS is commonly used to rapidly and accurately identify chemical compounds for a wide range of applications such as drug detection, pollution monitoring, petrochemical processing, and disease diagnosis through biomarkers)[8-12].

## GC-MS FOR VOCs' ANALYSIS IN ASTHMA

The rationale for studying VOCs in asthma is that chronic inflammation, which characterizes this disease, generates oxidative stress, as with any inflammatory state. As a result, polyunsaturated fatty acid membranes undergo a lipid peroxidation process that generates VOCs which are then secreted by the airways[13,14]. Alkanes (hydrocarbons) are found in exhaled breath as lipid peroxidation products and are released through respiration within seconds of their formation in tissues[15-17]. Aldehydes have also been linked to oxidative stress and inflammatory processes but their levels may vary depending on age and smoking status[18]. In individuals with asthma, the production of reactive oxygen species is increased, and the inflammatory process arises from various interactions between leukocytes, epithelial and stromal cells[19,20]. It has also been demonstrated that white blood cells in culture produce VOCs[21,22]. However, it is possible that pathways other than lipid peroxidation could form VOCs, such as hydrocarbons[23]. In fact, VOCs emitted through breath originate both locally in the airways and systemically throughout the body[24] and another possible explanation could be that lungs affected by asthma undergo remodeling, which leads to changes in gas exchange across the blood-lung barrier[23].

To date, GC-MS is the standard method used for characterizing the human metabolome, including VOCs present in human breath[25,26]. GC-MS can identify low-molecular-weight metabolites containing carbon and link VOCs to possible pathophysiological pathways helping us to better understand the asthmatic disease[27,28].

Consequently, several studies have investigated whether non-invasive diagnosis of asthma using GC-MS analysis of VOCs is feasible (Table 1). Dallinga *et al*[23] conducted a study on 120 children aged 5 to 16 years. Using time of flight (GC-TOF-MS), they detected eight VOCs in exhaled breath that were able to discriminate asthmatic children (63 out of 120, most of whom were using inhaled corticosteroids (ICS) and 42 out of 63 had atopic asthma) from controls (57 out of 120) with high sensitivity (up to 89%), specificity (up to 95%) and 92% correct classification, by repeating the experiment many times to test for reproducibility. The authors chose not to compare the obtained components with available library, but rather to compare their structural characteristics. Furthermore, the data analysis method used was discriminant analysis among the 945 initially obtained compounds, while admitting the possibility of statistical artifacts in the results. The most important components used to discriminate the two groups included alkanes and were: (Branched) hydrocarbon (C13H28), Carbon disulphide (CS2), butanoic acid, 3-(1-methylethyl)-benzene, (Branched) hydrocarbon (C13H28), Unsaturated hydrocarbon (C15H26), Benzoic acid, p-xylene, (Branched) hydrocarbon (C11H24) and 1-penten-2-on. The last three components were present in lower quantities in asthmatic subjects than in healthy ones. They also noted different VOCs between atopic asthmatic and non-atopic asthmatic patients but did not delve into their chemical identity. Sharma *et al*[29] conducted a pilot study to investigate the differences between asthmatic and non-asthmatic atopic subjects using a portable GC 30 min analysis (coupled to MS), making a statistical analysis using machine learning, linear discriminant analysis and principal component analysis (PCA) and identifying VOCs with NIST library. They collected VOCs from different subjects, including 30 asthmatics (both obese and non-obese, patients on ICS and non-ICS, with high and low eosinophil counts), 8 non-asthmatic but atopic individuals, and 35 neither asthmatic nor atopic individuals. They were able to distinguish three patient groups: The asthmatic/non-asthmatic group, the asthmatic/atopic non-asthmatic group, and the non-asthmatic/atopic non-asthmatic group, by analyzing the volatile compounds present in the exhaled air. The first group was identified by 9 VOCs: 2,4-dimethyl-heptane, 2,2,4-trimethyl-heptane, 3,3-dimethyl-octane, 2,3,5-trimethyl-heptane, 2,4,6-trimethyl-decane, 2,6,6-trimethyl-decane, 2-methyl-1-pentene and 2,8-dimethyl-undecane, with a classification accuracy of 94.4%. The second group was identified by 2 VOCs: 2-methyl-pentane and 2,5,9-trimethyldecane, with a classification accuracy of 90.5%. The third group was identified by 4 VOCs: 2-methyl-1-pentene, 2,4-dimethyl-heptane, 2,2,4-trimethyl-heptane, and 2,3,6-trimethyl-heptane, with a classification accuracy of 93.2%. Therefore, the VOCs were mainly alkanes, and compared to atopic patients, some overlapping compounds were noted (2-methyl-1-pentene, 2,4-dimethyl-heptane, 2,2,4-trimethyl-heptane). Furthermore, among asthmatic patients, the VOCs allowed the identification of subgroups: Obese asthmatic patients, asthmatics treated with ICS, asthmatics with high blood levels of immunoglobulin E (IgE), and those with upper respiratory illness. Therefore, it is very difficult to reconstruct a single profile for asthmatic patients, as their variables can be numerous. The importance of variability has also been emphasized by Gahleitner *et al*[30]. Their study was conducted on asthmatic children in United Kingdom, where there is the highest prevalence of pediatric asthma in the world and where GC-MS may be able to identify a pre-symptomatic stage of asthma in the future, making prevention possible.

They studied a small number of pediatric patients over the age of 8: A group of 11 children with asthma and another group of 12 healthy children. The authors paid close attention to the sampling phase, also evaluating the acceptability capacity of the children, which was found to be almost entirely good. Eight possible asthma markers (out of 25), mainly originating from the alveoli, were identified with a retention time between 2 min and 28 min: 1-(methylsulfanyl)propane, ethylbenzene, 1,4-dichlorobenzene, 4-isopropenyl-1-methylcyclohexene, 2-octenal, octadecyne, 1-isopropyl-3-methylbenzene, and 1,7-dimethylnaphthalene. The VOCs were collected on a Tenax/Carbotrap hydrophobic adsorbent trap, analyzed with thermal desorption GC-MS and various statistical techniques (including multivariate analysis,

**Table 1 Studies that have used gas chromatography-mass spectrometry<sup>4</sup> to detect volatile organic compounds in asthma**

Journal	Title	Main results	Ref.
<i>Metabolites</i>	Real Time Breath Analysis Using Portable Gas Chromatography for Adult Asthma Phenotypes	Pilot study, the first one to use a portable GC device (30 min analysis) that was coupled to a MS succeeded to distinguish by alkaline VOCs different subjects: 30 asthma, 8 atopic non-asthmatic and 35 non-asthma/non-atopic and their subgroups <sup>1</sup>	Sharma <i>et al</i> [29], 2021
<i>European Respiratory Journal</i>	Exhaled volatile organic compounds as markers for medication use in asthma	This is the inaugural investigation exploring exhaled VOCs in conjunction with drug utilization as detected by urinary metabolites in asthma. The study encompassed 78 adult patients with severe asthma, demonstrating the potential for detecting VOCs to monitor therapy, in this instance salbutamol and OCS <sup>2</sup>	Brinkman <i>et al</i> [43], 2020
<i>American Journal of Respiratory and Critical Care Medicine</i>	Exhaled Volatile Organic Compounds Are Able to Discriminate between Neutrophilic and Eosinophilic Asthma	The first study to provide surrogate markers for neutrophilic asthma. 521 patients divided into a discovery study group and a replication study group <sup>3</sup> . They found out that 2 VOCs for eosinophilic asthma and 3 VOCs for neutrophilic asthma had a classification performance comparable to that of blood eosinophilic count and FeNO	Schleich <i>et al</i> [14], 2019
<i>Clinical and Experimental Allergy Journal</i>	Exhaled breath profiles in the monitoring of loss of control and clinical recovery in asthma	This study demonstrates the superiority of e-NOSE over GC-MS in predicting exacerbations after ICS cessation (correct classification between 86% and 95% for e-NOSE and between 68% and 77% for GC-MS) in 22 patients with a mean age of 25 years. However, the VOCs analyzed with GC-MS were found to be correlated with eosinophilic sputum, which e-NOSE was not able to do <sup>4</sup>	Brinkman <i>et al</i> [49], 2017
<i>Journal of Breath Research</i>	Can exhaled volatile organic compounds predict asthma exacerbations in children?	7 VOCs detected in 32 children who experienced at least one exacerbation, were able to correctly predict the event 14 days earlier in 88% of cases. Sensitivity of the exam decreased in direct proportion to the temporal distance of the exacerbation <sup>5</sup>	Van Vliet <i>et al</i> [47], 2017
<i>Respiratory Research</i>	Defining adult asthma endotypes by clinical features and patterns of volatile organic compounds in exhaled air	16 VOCs able to distinguish asthmatic patients from healthy patients with a specificity of 91.1%, a sensitivity of 100%, and a correct classification of 98.7%. Moreover, 4 of these 16 VOCs were detected only in asthmatic subjects. The group was also able to identify 7 clusters of patients based on the clinical characteristics, the therapies carried out and the VOCs, demonstrating the hypothesis that a single asthma phenotype could be characterized by multiple inflammatory mechanisms, in fact they detected similar VOCs for clinical characteristics differentiate and vice versa <sup>6</sup>	Meyer <i>et al</i> [35], 2014
<i>PLoS One</i>	Profiling of volatile organic compounds in exhaled breath as a strategy to find early predictive signatures of asthma in children	The first study able to discriminate between asthmatic patients, transient wheezing patients and healthy controls using VOCs analysis. 252 children between 2 and 6 years and 17 VOCs identified with an accuracy of 80% open the door to early diagnosis and treatment of asthma in preschool children <sup>5</sup>	Smolinska <i>et al</i> [51], 2014
<i>American Journal of Respiratory and Critical Care Medicine</i>	Exhaled biomarkers and gene expression at preschool age improve asthma prediction at 6 yr of age	The association of VOCs, API and gene expression is able to discriminate asthma from preschool wheezing with an AUC 95%, PPV/NPV 90%/89% and <i>P</i> value < 0.0001 in this study of 198 children followed by 2 to 6 yr <sup>5</sup>	Klaassen <i>et al</i> [56], 2015
<i>Future Science</i>	Metabolomics pilot study to identify volatile organic compound markers of childhood asthma in exhaled breath	Although they found out eight distinguish asthma markers with a <i>P</i> value < 0.05, the group highlights the exiguous number of patients (23 children of which 12 healthy for control). The authors stress the importance of the variability of conditions <sup>7</sup>	Gahleitner <i>et al</i> [30], 2013
<i>European Respiratory Journal</i>	Exhaled volatile organic compounds predict exacerbations of childhood asthma in a 1-yr prospective study	Six or seven VOCs had been identified to be able to predict exacerbation (SVM) with a correct classification rate of 96%, a sensitivity of 100% and specificity of 93%. On the opposite, FeNO and lung function had not been able to give the same result <sup>5</sup>	Robroeks <i>et al</i> [45], 2013
<i>Thorax</i>	Non-invasive phenotyping using exhaled volatile organic compounds in asthma	A total of 12 VOCs were used to discriminate between asthmatic subjects, subjects with eosinophilic sputum, those with neutrophilic sputum, and those with uncontrolled asthma. The discriminatory accuracy of the VOC groups for the 4 patient groups ranged from 79% (neutrophilic asthma) to 89% (for loss of control asthma) <sup>5</sup>	Ibrahim <i>et al</i> [39], 2011
<i>Clinical et Experimental Allergy</i>	Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children	The group identified eight discriminating compounds between an asthmatic patients children group (63 people) and a control healthy group (57 people), with a sensitivity of 89% and a specificity of 95% and a claim of 92% correct classification. They also tested the reproducibility and intra/inter-individual variability <sup>8</sup>	Dallinga <i>et al</i> [23], 2010

<sup>1</sup>Thermo Scientific Single Quadrupole Mass Spectrometry (ISQTM series). Volatile organic compounds (VOCs) were analyzed with ChromeleonTM and identified with NIST 2014 library.

<sup>2</sup>The group used gas chromatography (GC)-time of flight (TOF)-mass spectrometry (MS) for breath and liquid chromatography coupled with high resolution-MS (LC-HR-MS) for urine.

<sup>3</sup>The discovery study's VOCs were analyzed by GC-TOF-MS. The replication study's VOCs were analyzed by a comprehensive two-dimensional Gas Chromatography (two columns with two different stationary phases) coupled to HR-TOF-MS, GCxGC HRTOFMS. VOCs have been identified with NIST 2014 library.

<sup>4</sup>The only study of the list that made double analysis, by electronic-Nose (four different platforms) and GC coupled to Quadrupole MS; identification by NIST library.

<sup>5</sup>VOCs were analyzed by GC-TOF-MS and identified with NIST library.

<sup>6</sup>VOCs were analyzed by GC-TOF-MS and compared between the two groups of the study. No library were used.

<sup>7</sup>VOCs were analyzed by GC-MS (after being collected into a Tenax/Carbotrap hydrophobic adsorbent trap) and identified with NIST except two of them.

<sup>8</sup>They used their developed GC-TOF-MS methodology[48], with two absorption tubes and a GC-TOF-MS. The group did not use a library to compare mass spectra in their approach. Instead, they compared the measured mass spectra with each other at the same retention time. This allowed them to determine whether peaks at the same retention time represented the same component or not based on the similarity of the original spectra.

ICS: Inhaled corticosteroids; GC: Gas chromatography; TOF: Time of flight; MS: Mass spectrometry; VOCs: Volatile organic compounds.

discriminant analysis, 2D-PCA, leave-one-out cross-validation algorithm and the two-tailed *t*-test), and identified with NIST. While most of these VOCs likely originated from the environment, the only component presumably of endogenous origin and therefore a potential marker of asthma was 2-octenal, which had already been identified in studies on innate immunity and oxidation[31]. These VOCs were present in significantly higher quantities in patients with asthma compared to healthy subjects, especially for 1,4-dichlorobenzene. This element has already been previously linked to asthma and compromised ventilatory function[32,33]. Additionally, 4-isopropenyl-1-methylcyclohexene had also been correlated with asthma symptoms[34]. It is important to note that while there were similar profiles detected among the controls, those among the asthmatic subjects were found to be very different from each other, emphasizing, as said above, the variability of the disease and how difficult it is to reconstruct a unique profile for the asthmatic patient. Meyer *et al*[35] found that among 945 VOCs detected by GC-TOF-MS and analyzed by discriminant analysis, 16 VOCs were able to distinguish adult patients with asthmatic (195 subjects) from 45 adult healthy controls with a specificity of 91.1%, a sensitivity of 100% and an overall correct classification rate of 98.7%. Furthermore, four of these 16 VOCs were detected only in patients with asthma. They did not use libraries but compared the VOCs between the two groups. The study was also able to identify seven endotypes of asthma using an unsupervised hierarchical two-step cluster analysis, based on clinical characteristics, therapies administered and the four VOCs [1-Dodecanol, 3,7,11-trimethyl-, 1,3-Dioxolane, 2-(phenylmethyl)- and 2 unknowns]. Although some of these clusters presented similar clinical characteristics and treatments, they produced different VOCs. Similarly, clusters with similar VOCs had different clinical characteristics and were under different treatment regimens. This may indicate that VOCs reflect different inflammatory mechanisms even within the same phenotype, as previously proposed by Lötvall *et al*[36], suggesting a future subdivision of phenotypes based on different molecular patterns represented by VOCs. Currently, the phenotypic subdivision of asthma is mainly based on clinical and demographic characteristics, although with significant differences between adults and children[37, 38]. Unlike Ibrahim *et al*[39], this study was not able to identify specific VOCs that could correlate with specific inflammatory mechanisms. However, in further clustering analysis, patients with similar clinical characteristics presented different VOCs, different levels of IgE and eosinophilia, suggesting that VOCs may reflect the presence of this type of inflammation (*i.e.*, IgE and eosinophil-mediated), bringing it in line with Ibrahim *et al*[39].

Ibrahim's group[39] conducted a study on adult subjects with no history of smoking, consisting of 35 asthma patients and 23 healthy controls. GC-TOF-MS and various statistical analysis tools (including logistic regression, PCA, multivariate logistic regression, discriminant function analysis, and leave-one-out cross-validation) were used to detect significant VOCs differentiating between 4 types of patient groups: Asthma patients *vs* healthy controls, asthma patients with sputum eosinophilia ( $\geq 2\%$ ), asthma patients with sputum neutrophilia ( $\geq 40\%$ ), and patients with uncontrolled asthma [asthma control questionnaire (ACQ)  $\geq 1$ ]. Asthma patients was diagnosed based on clinical assessment rather than bronchial hyperresponsiveness. For the first group, 4 VOCs were identified with a discriminatory accuracy of 86%. For the second group, 3 VOCs were identified with a discriminatory accuracy of 83%, a sensitivity of 75%, a specificity of 90%, and an area under the curve of the ROC curve (AUROC) of 0.98 (higher than that of FeNO, which had an AUROC of 0.69). Only one VOC was able to distinguish patients with neutrophilic asthma from those with non-neutrophilic asthma with an accuracy of 79% and an AUROC of 0.90. For the fourth group, 4 VOCs were identified with a discriminatory accuracy of 89% and an AUROC of 0.97. Despite the fact that patients with uncontrolled asthma almost all had sputum eosinophilia, the discriminative VOCs detected in the two groups were still different. Several of the found VOCs were alkanes and methylated alkanes. The longer chain alkanes (such as 2,6,11-trimethyl-dodecane, previously detected in the electronic-Nose (e-NOSE) study by Dragonieri *et al*[40]) appear to be more specific to asthma. They also detected 2-Methyldecane, an isomer of undecane found by Schleich *et al*[14]. In this regard, the ability of Ibrahim's group to correlate VOCs with sputum was confirmed by Schleich's group as well[14]. This group, having previously worked to detect VOCs associated with asthma subtypes *in vitro*[41], was the first to investigate whether GC-MS could represent an alternative to induced sputum for identifying inflammatory phenotypes (eosinophilic asthma if  $\geq 3\%$  eosinophils, neutrophilic asthma if  $\geq 76\%$  neutrophils, and paucigranulocytic asthma if  $< 3\%$  eosinophils and  $< 76\%$  neutrophils)[42]. They compared the two diagnostic techniques in a prospective study that involved 521 adult asthma patients diagnosed according to guidelines, divided into a discovery study group (276 patients) and a replication study group (245 patients), with overlapping numbers of patients in each group with eosinophilic, paucigranulocytic, and neutrophilic asthma. While a classic GC-TOF-MS was used for the first study, a 2D-GC-high resolution (HR)-TOF-MS was used for the second, and all VOCs obtained were analyzed using various statistical techniques, including conditional inference trees and conditional random forests (RFs), and compared with the NIST library. Comparing the eosinophilic group with the paucigranulocytic group, they identified two VOCs that were abundant in patients with paucigranulocytic asthma but much less so in eosinophilic asthma (Hexane and 2-hexanone, AUC = 0.99 for the discovery study. AUROC = 0.68 for the replication study, where there were hexane, 2-hexanone, 1-propanol). This allowed them to understand how low concentrations of hexane and 2-hexanone could identify eosinophilic asthma. Comparing paucigranulocytic asthma with neutrophilic asthma, they found that in the discovery study, neutrophilic asthma presented abundant amounts of 3-tetradecane followed by pentadecane (AUC = 0.85), whereas in the replication study, nonane and undecane (isomer of 3,7-dimethyl-

nonane) were more abundant in neutrophilic asthma and paucigranulocytic asthma, respectively (AUROC = 0.70). Comparing neutrophilic asthma with eosinophilic asthma, the discovery study showed high amounts of 3,7-dimethylnonane, followed by nonanol and then 1-propanol in the first asthma subtype compared to the second (AUC = 0.92). The replication study instead detected an increase in nonanol, hexane (AUROC = 0.71), and 1-propanol in neutrophilic asthma compared to eosinophilic asthma. Finally, comparing neutrophilic asthma with other phenotypes, the replication test showed that nonanal, propanol, and hexane gave an AUC of 0.73% (AUC of 0.97% in subjects with high neutrophil counts in induced sputum) with an accuracy of 76%, a sensitivity of 81%, and a specificity of 43%, appearing as the first biomarkers of neutrophilic asthma. Adding undecane to these three markers did not improve the results. The accuracy in diagnosing eosinophilic asthma through VOCs decreased to 60%. Therefore, VOCs can identify both eosinophilic and neutrophilic asthma with accuracy similar to that of FeNO and eosinophil count for eosinophilic asthma. Moreover, combining the study of VOCs with that of FeNO and eosinophil count increases accuracy, specificity and sensitivity of the result compared to tests performed individually (AUROC = 0.87). The diagnosis of eosinophilic asthma through VOCs was still superior in terms of specificity and sensitivity compared to that performed through eosinophil count and FeNO.

This study is particularly significant because markers of neutrophilic asthma are deficient. Moreover, the markers predominantly used to identify neutrophilic asthma (1-propanol, nonanol, and hexano) appear to be unaffected by age, smoking, or ICS use in diagnosing the subtype through VOCs.

Similarly to one of Ibrahim's attempts, such as trying to find VOCs capable of identifying uncontrolled asthma, other studies have also been conducted to explore non-invasive therapeutic monitoring of asthma. The Brinkman *et al*'s investigation[43], in particular, represents the initial and promising step towards the development of future rapid and noninvasive adherence tests for inhaled therapies[44]. They recruited 78 adults with severe asthma from the U-BIOPRED cohort and explored the association between exhaled VOCs using GC-TOF-MS and levels of salbutamol in urine, as well as oral steroid therapy using liquid chromatography coupled with HR-MS. The biomarkers were analyzed using various statistical techniques and then compared with the NIST library. Four VOCs (lysine, glycolic acid, 4-carene and octanal) were found to be associated with traces of asthma medication in urine samples from severe asthmatics. The baseline AUC was 82.1 for salbutamol and 78.8 for oral corticosteroids. The group was able to correlate exhaled VOCs with the detection of salbutamol and OCS in urine, suggesting the possibility of an analytical approach to monitoring therapy adherence through exhaled VOCs, thus providing a noninvasive analysis. Therefore, VOCs are indeed simple to measure in clinical practice and may serve as a reflection of compliance[44].

The usefulness of GC-MS in asthma is also evident in studies assessing its ability to predict exacerbations, which is a crucial aspect of treatment[2,45]. The ability of VOCs to predict an exacerbation has great potential due to the fact that airway inflammation is already pronounced prior to the onset of symptoms, enabling treatment at an early stage, before the onset of clinical symptoms.

Robroeks *et al*[45] conducted the first longitudinal study aimed at evaluating the ability of VOCs to predict asthma exacerbations. The study included 40 children aged between 6 and 16 years, non-smokers, without other chronic pathologies that could interfere with the results and of whom 73% were atopic. The diagnosis of asthma and its treatment were carried out according to the GINA guidelines[2]. During the 12-mo study, 16 out of 40 children developed an exacerbation [diagnosed according to the indications provided by the American Thoracic Society (ATS) and European Respiratory Society (ERS)][46], with 10 of them experiencing moderate exacerbations and the remaining experiencing severe exacerbations. In total, GC-TOF-MS detected 3434 VOCs, with an average of 343 VOCs per patient. The obtained VOCs were subjected to both inter-individual comparison (patients who experienced exacerbation *vs* patients who did not) and intra-individual comparison (VOCs at baseline and VOCs during exacerbation). To select significant VOCs, identified using the NIST library, statistical analyses such as independent t-tests and Bonferroni correction were applied (which allowed the identification of 30 significant VOCs). The VOCs that were present in at least 8% of the samples were included in the support vector machine classification model, which was chosen for its great ability to construct predictive models. Six VOCs (p-xylene, 3-methylpentane, 2-ethyl-4-methyl-1-pentanol, 1-phenyl-1-butene, 4,6,9-nonadecatriene, and one Unknown VOC) predicted asthma exacerbations in children with an advance notice of  $39 \pm 4$  d, with a classification rate of 96%, 100% sensitivity, 93% specificity, a positive predictive value of 89% and a negative predictive value of 100% in the intra-subject comparison, while 7 VOCs (2-ethyl-1,3-butadiene, cyclohexane, 2-octen-1-ol, 1,2-methyl-4H-1,3-benzoxathiine, benzene, and one unknown VOC) predicted exacerbations with a classification rate of 91%, 78% sensitivity, 100% specificity, a positive predictive value of 100% and a negative predictive value of 86% in the inter-subject comparison. The group also demonstrated, through Univariate Cox regression analysis, that FeNO ( $P = 0.43$ ) and lung function (FEV1) ( $P = 0.60$ ) were unable to predict exacerbations. This study shows that specific VOCs, mostly hydrocarbons, can predict with a good accuracy asthma patients who will experience exacerbations in the future compared to those who will remain stable.

van Vliet *et al*[47] and Robroeks *et al*[45]' work attempted to study the phenomenon in a larger population. They conducted a one-year prospective observational cohort study[47] involving a sample of 94 children aged between 6 and 18 years, 76% of whom were atopic and 65% had controlled asthma at the beginning of the study. The participants were non-smokers and were not affected by specific pathological conditions or undergoing specific therapies. All patients had undergone ICS therapy in the year prior to the study. Although the design and method of this study were comparable to that of Robroeks *et al*[45], the results sometimes differed. In this case, too, the participants were enrolled after diagnosis and treatment based on the GINA guidelines (along with the Dutch Society of Pediatrics guidelines and its Pediatric Pneumology section) and exacerbation identification using ATS/ERS criteria. 48% of the participants experienced at least one exacerbation. GC-TOF-MS detected 2416 VOCs. Statistical analyses such as RF classification modeling, PCA and ROC-curves, were used to select specific VOCs identified through the NIST library. 7 VOCs, including 3 aldehydes, 1 cyclic alkane hydrocarbon, 1 ketone and 1 aromatic compound, plus 1 unidentified VOC (1,2-dimethylcyclohexane; 2-

ethylhexanal; 2(or 3)-methylfuran; 6,10-dimethyl-5,9-undecadien-2-one; nonanal; octanal, one Unknown) were characterized by an AUROC of 90%, sensitivity of 88% and specificity of 75%. However, the accuracy decreased if the sample was collected more than two weeks (14 d) prior to exacerbation, as statistical analyses for the VOCs detected 21 d prior to exacerbation had a sensitivity of 63%. The accuracy model performed 14 d before exacerbation was studied only in 32 patients, which is why new studies will need to be conducted to generalize the model. Nonetheless, it can be affirmed that these 7 VOCs are correlated with the presence of asthmatic airway inflammation. Considering that 2/3-methylfuran had previously been detected[48] as a compound correlated with smoking, it may in this case be a detector of passive smoke exposure.

It is necessary to note that some studies, such as Brinkman *et al*[49], have observed the superiority of e-NOSE over GC-MS in predicting exacerbations. The group conducted a prospective intervention study on 23 partially controlled mild-to-moderate asthma treated with ICS patients who were non-smokers for at least 12 mo, with a mean age of 25 years, 95% of whom were atopic and 75% were women. The study measured VOCs at three time points: Baseline, during loss of control, and at recovery using composite e-NOSE platforms (four different brands) and GC with a Quadrupole MS. The group then studied the association between VOCs and induced sputum, as inflammatory cells tend to increase during exacerbations[49]. The study of airway inflammation *via* sputum or VOCs is even more important considering that it does not always have a clinical correlate[50]. To induce exacerbation, patients stopped ICS for eight weeks (while continuing other asthma therapies except LABA) or until loss of control, then took oral corticosteroids (OCS) for one week and ICS to reach recovery. Four weeks after the exacerbation event, VOC analysis was performed again. The VOCs obtained from the three measurements were subjected to various statistical analyses, including univariate analysis of covariance (ANCOVA) and PCA, false discovery rate correction, paired *t*-tests, Friedman test and QR decomposition, and then identified using the NIST library. This allowed for the identification of three VOCs from GC-MS (methanol, acetonitrile, bicyclo[2.2.2]octan-1-ol, 4-methyl and one PC1) and two components from e-NOSE (PC1 and PC2) as discriminative substances. In particular, the classification accuracy for baseline versus loss of control-as measured by the ACQ-was 95% using an e-NOSE and 68% by GC-MS; loss of control versus recovery was 86% (e-NOSE) and 77% (GC-MS). Furthermore, the VOCs produced during exacerbation were different from those produced during steady-state and recovery, which also explains the findings of Robroeks *et al*[45]. The study suggests that exhaled breathprints can be considered useful composite markers for identifying loss of asthma control following cessation of ICS, with e-NOSE technology demonstrating higher accuracy in distinguishing between baseline, loss of control, and recovery conditions compared to GC/MS. Additionally, the study analyzed the relationship between exhaled breath components and airway inflammation and lung function. Significant correlations (Pearson  $r \geq 0.46$  and  $P < 0.01$ ) were found between certain chemical compounds identified by GC-MS (acetonitrile, bicyclo[2.2.2]octan-1-ol, 4-methyl and PC1) and the presence of eosinophils (but not of neutrophils) in airway sputum. In contrast, none were observed for e-NOSE. It is noteworthy that 4-methyl-bicyclo[2.2.2]octan-1-ol contains a characteristic bicyclic ring, which matches the compound described by Ibrahim *et al*[39] as 3,7,7-trimethyl-bicyclo[4.1.0]hept-2-ene [known as: (+)-3-Carene], reported to be correlated with sputum eosinophils [39]. Finally, both GC-MS and e-NOSE showed correlations between VOCs, pulmonary function and FeNO.

Pediatric asthma has another major problem, namely that of entering into the differential diagnosis with transient wheezing. The inability to accurately discriminate the two pathologies leads to an undertreatment of asthma and an overtreatment of wheezing[23]. Indeed, no tests are available that predict at early stage who will develop asthma and who will be a transient wheezer. Some authors wanted to observe if GC-TOF-MS can help us to solve the problem, differentiating between healthy, asthmatic and wheezing patients, thus making early diagnosis and treatment possible.

Smolinska *et al*[51] conducted for the first time a large and well-designed study that demonstrated how VOCs analysis, using GC-TOF-MS can help distinguish asthmatic from transient wheezing children. Using the ISAAC questionnaire, 252 children aged approximately 2-3 years, both symptomatic for wheezing and healthy controls, were recruited. The patients were followed up until the age of 6. During this period, 3256 VOCs were detected, analyzed, selected, and ultimately used at the end of the observation period to distinguish three patient groups: Healthy patients (49 patients), patients with wheezing (121 patients), and those who had or developed asthma (76 patients). The group performed a complex statistical analysis using two multivariate methods, exploratory analysis with r-PCA and PCA, RFs was used, corroborated and validated with out-of-bag error, dissimilarity Partial Least Squares Discriminant Analysis (d-PLS-DA), more effective than PLS-DA, and the Duplex algorithm; the outcomes were then validated with an independent test set. As a result, out of the 3256 different compounds detected, 17 VOCs (acetone, 2,4-dimethylpentane, 2,4-dimethylheptane, 2,2,4-trimethylheptane, 1-methyl-4-(1-methylethenyl) cyclohexene, 2,3,6-trimethyloctane, 2-undecenal, biphenyl, 2-ethenyl-naphthalene, 2,6,10-trimethyldodecane, octane, 2-methylpentane, 2,4-dimethylheptane, 2-methylhexane and 3 unknown VOCs), some of which had been identified previously[23,39,52-54], were deemed significant for their potential discriminatory power with an accuracy of 80% in the independent test set. Among these, mostly hydrocarbons and long-chain alkanes, the following VOCs were present in higher concentrations in asthmatic patients compared to those with wheezing, by the age of 6: 2,4-dimethylpentane, 2,4-dimethylheptane, 2-undecenal, octane, 2-methylpentane, 2,4-dimethylheptane, and 2-methylhexane. Octane and 2,3,6-trimethyloctane, in particular, showed greater variability compared to other compounds. Over the 4-year period, patients with wheezing exhibited greater heterogeneity of results than patients with asthma. While this study demonstrated that VOCs in exhaled breath can predict the subsequent development of asthma (which may guide early treatment), it also highlighted the non-invasive capacity of GC-MS, applied to children as young as 2 years old to diagnose asthma, which is currently only possible after the age of 6 with current techniques[55].

The same and other authors aimed to investigate in a new study[56] the usefulness of these VOCs in enhancing the discriminative accuracy of the Asthma Predictive Index (API) by associating it not only with VOCs in exhaled breath but also with gene expression, exhaled breath condensate (EBC), and airway resistance. To this end, 198 patients aged between 2 and 4 years with wheezing symptoms but without specific comorbidities were enrolled and followed up until

the age of 6. The Duplex algorithm divided the original data set into a discovery set and a validation set. Logistic regression models, quantifying discrimination, chi-square test, Hosmer-Lemeshow goodness-of-fit test were used for data analysis. After six years, 76 patients were diagnosed with asthma, while 122 patients were diagnosed with preschool wheezing. The API alone had a discriminative power expressed in an AUC of 61%. The combination of API and VOCs in the discovery set increased the AUC by 28%, bringing it to 89%, with a sensitivity of 84% (from 66% of the API alone), a specificity of 82% (from 56% of the API alone), with positive predictive value (PPV)/negative predictive value (NPV) of 82%/83%, a 95% CI between 83 and 95, and a  $P$  value  $< 0.0001$ . The result was confirmed in the validation set, with an AUC of 83% and a  $P$  value  $< 0.01$ . The further association of gene expression study (in particular TLR4, Catalase, and tumour necrosis factor- $\alpha$ ) with API and VOCs further improved the discriminative power, giving an AUC of 95%, PPV/NPV of 90%/89%, and a 92%-99% CI in the discovery set, which was confirmed in the validation set with an AUC of 86% and a CI of 77%-96%. However, after backward logistic regression, only 9 of the 17 discriminative VOCs were included in the model. Of these nine VOCs, four compounds were positively associated (acetone, octane, 2,4-dimethylheptane, 2-methylpentane) and five compounds were negatively associated with the development of asthma (2-methylhexane, 2,3,6-trimethyloctane, 2,6,10-trimethyldodecane, 2,4-dimethylpentane, 2-undecenal). The results obtained from the association of API with EBC biomarkers or airway resistance studies were disappointing, as there was no increase in the AUC in either case. Similarly, the association between API and gene expression alone increased the AUC by 17% in the discovery set and 12% in the validation set, where it was not confirmed. From this study, it can be inferred that when VOCs were added to the API status, they significantly improved an accurate asthma diagnosis at preschool age. The main VOCs of these studies are reported in Tables 2-7.

## ELECTRONIC NOSE TECHNOLOGY

Technological progressions during latest years have resulted in the elaboration of chemical sensor arrays which can fingerprint VOC mixtures. These are known as “electronic noses”, due to their resemblance of mammalian olfactory system for smells[57]. Rather than recognizing and quantifying single molecular elements of VOC mixtures, e-NOSES are able to discriminate different VOC-patterns by comparing the incoming odor with formerly learnt patterns[58] which are also known as breathprints (Figure 3). When VOCs impact on the e-NOSE sensors, a change in their conductivity occurs, followed by the generation of electrical signals which result in exclusive VOC-spectrums[58]. To date, several e-NOSE technologies are available, which have been tested in every plausible field dealing with volatiles and gases, predominantly in army, environmental monitoring, food and beverage industry, and more recently for diagnosis diseases[59].

Currently available e-NOSES are the Cyranose 320, based on a carbon black-polymer sensor array[40], the Aeonose, using micro hotplate metal-oxide sensors[60], the Tor Vergata e-NOSE, operating with quartz crystal microbalances (QCM) covered with metalloporphyrins[61] the BIONOTE e-NOSE, which has QCM sensors utilizing anthocyanin-coated gold electrodes[62], the Common Invent e-NOSE, working with metal oxide semiconductor sensors[63], the SpiroNose, based on cross-reactive metal-oxide semiconductor sensors[64] and the Owlstone Lonestar e-NOSE, developed on field asymmetric ion mobility spectrometry[65], e-NOSES detect mixtures of VOCs to create breathprints-they do not generally identify individual molecular compounds (Figure 4).

After the introduction of e-NOSES in the biomedical setting, VOCs pattern analysis of the exhaled breath has become an achievable option, due to the ability to perform a quick on-board pattern-recognition analysis, without delivering information about the individual molecular components[66,67]. This high-throughput analysis is fundamentally an ‘omics’ approach, similar to genomics, transcriptomics, and metabolomics[68].

## E-NOSE APPLICATIONS IN ASTHMA

The first proof of concept study investigating the role of an e-NOSE in the field of asthma was performed in 2007 by Dragonieri *et al*[40], who analyzed a population of patients with an established diagnosis of mild and severe asthma, as well as a group of healthy controls. All participants inspired VOC-filtered air by tidal breathing for 5 min, and a single expiratory vital capacity was collected into a Tedlar bag, which was successively sampled by a Cyranose 320[40]. According to exhaled breathprints, the Cyranose was able to distinguish mild and severe asthma from controls [cross-validation value (CVV) 95% and 90%, respectively]. Moreover, individuals with mild asthma could also be discriminated from those with severe asthma, even though less sharply (CVV = 65%)[40]. The same group, by using the same e-NOSE, showed that breathprints of patients with Allergic Rhinitis clustered distinctly from those with Allergic Asthma and Rhinitis [cross validated accuracy (CVA) = 85.7%], as well as from healthy controls (CVA = 82.1%). As expected, breathprints from Allergic Asthma and Rhinitis were also separated from those of healthy controls (CVA = 75.0%)[69]. Asthmatics were also discriminated from those with COPD (accuracy 96%)[70] and patients with asthma with persistent airway obstruction from COPD, regardless from smoking (accuracy 88%, AUROC curve 0.95)[71]. Remarkably, van der Schee *et al*[72] used a Cyranose 320 to predict the response to corticosteroid therapy in a population of 25 patients with asthma, showing that the e-NOSE was more accurate than sputum eosinophil counts (AUROC 0.883,  $P = 0.008$  vs AUROC 0.610,  $P = 0.441$  respectively) and exhaled Nitric Oxide (0.545,  $P = 0.751$ )[72]. Regarding asthma sub-phenotyping, Plaza *et al*[73], compared VOC breathprints in three asthma subtypes (eosinophilic, neutrophilic, and paucigranulocytic) assessed by inflammatory cell counts in induced sputum[73]. Exhaled breath from 52 individuals with persistent asthma was analyzed by a Cyranose 320. Breathprints were significantly different in eosinophilic compared to neutrophilic (accuracy 73%,  $P = 0.008$ , AUROC = 0.92), as well as paucigranulocytic asthma (accuracy 74%,  $P = 0.004$ , AUROC = 0.79), and

**Table 2 Discriminative volatile organic compounds between asthmatic and non-asthmatic subjects**

Identified compound	Ref.
Acetic acid	[40]
Acetone	[40]
Alkane	[40]
1,2-Dichlorobenzene	[30]
3-(1-methylethyl)-benzene	[23]
Ethyl benzene	[30]
1-isopropyl-3-methylbenzene	[30]
Benzoic acid	[23]
butanoic acid	[23]
(Branched) hydrocarbon (C11H24)	[23]
(Branched) hydrocarbon (C13H28)	[23]
(Branched) hydrocarbon (C13H28)	[23]
Unsaturated hydrocarbon (C15H26)	[23]
2,4,6-Trimethyldecane	[29]
2,6,6-Trimethyldecane	[29]
Octadecyne	[30]
1,3-Dioxolane, 2-(phenylmethyl)	[35]
2,6,11-trimethyl dodecane	[40]
1-Dodecanol 3,7,11-trimethyl	[35]
2,3-dimethyl heptane	[40]
2,4-Dimethylheptane	[29]
2,3,5-Trimethylheptane	[29]
2,2,4-Trimethylheptane	[29]
4-isopropenyl-1-methylcyclohexene	[30]
isoprene	[40]
Isopropanol	[40]
1,7-Dimethylnaphtalene	[30]
4-Methyloctane	[40]
3,3-Dimethyloctane	[29]
2-Octenal	[30]
2-methyl-1-pentene	[29]
1-penten-2-on	[23]
1-(Methylsulfonyl)propane	[30]
Carbon disulphide (CS <sub>2</sub> )	[23]
toluene	[40]
2,8-Dimethylundecane	[29]
3,7-Dimethyl undecane	[40]
p-Xylene	[23]

neutrophilic clustered distinctly from the paucigranulocytic phenotype (accuracy 90%,  $P = 0.001$ , AUROC = 0.88), suggesting the potential of using an e-NOSE as a less-invasive alternative to sputum cytology. Similarly, Wagener *et al* [74], by means of a platform with four different e-NOSEs (Tor Vergata, Cyranose 320, Owlstone Lonestar, and Common Invent) separated eosinophilic from non-eosinophilic asthma breathprints in 27 patients with an accuracy of 85% and

**Table 3 Discriminative volatile organic compounds of different asthma subtypes and inflammatory disease characteristics**

Inflammatory characteristics	Identified compound	Ref.
Eosinophilic asthma <i>vs</i> paucigranulocytic asthma	Hexane 2-hexanone	[14]
Neutrophilic asthma <i>vs</i> eosinophilic asthma	3,7-dimethylnonane; Nonanol 1-propanol	[14]
Neutrophilic asthma <i>vs</i> paucigranulocytic asthma	3-tetradecane pentadecane nonane undecane (isomer of 3,7-dimethylnonane)	[14]
Neutrophilic asthma <i>vs</i> paucigranulocytic and eosinophilic asthma	Nonanal propanol hexane	[14]
Neutrophilic sputum cell $\geq$ 40%	Cyclopentene, 1,3-dimethyl-2-(1-methylethyl) C <sub>10</sub> H <sub>18</sub> Naphthalene, 2,7-dimethyl	[39]
Eosinophilic sputum cell $\geq$ 2%	Camphene (7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl) methanol Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl Cyclohexene-4-methylene	[39]
Blood eosinophils level	2-octene; 3,3-Dimethyloctane; 1-Fluorododecane; 2,6,6-Trimethyldecane	[29]

**Table 4 Discriminating volatile organic compounds in asthmatic exacerbations and uncontrolled asthma**

Identified compound	Ref.
Acetonitrile	[49]
Benzene	[39,45]
1,2-methyl-4H-1,3-benzoxathiine	[45]
2-ethyl-1,3-butadiene	[45]
2-Butanone, 3-methyl/butanal, 2-methyl	[39]
1-phenyl-1-butene	[45]
6,10-dimethyl-5,9-undecadien-2-one	[47]
2(or 3)-methylfuran	[47]
2-ethylhexanal	[47]
Cyclohexane	[45]
1,2-dimethylcyclohexane	[47]
Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)	[39]
Methanol	[49]
Nonanal	[47]
Pentadecane, 1-methoxy-13-methyl	[39]
3-methylpentane	[45]
2-ethyl-4-methyl-1-pentanol	[45]
(1E)-1-(methylsulphanyl)1-propene	[39]
Octanal	[47]
2-octen-1-ol	[45]
4-methyl-Bicyclo[2.2.2]octan-1-ol	[49]
2,2,4,4-Tetramethyloctane	[39]
4,6,9-nonadecatriene	[45]
p-xylene	[45]
O-xylene	[39]

AUROC of 99% [74]. In addition, Brinkman *et al* [43] used the aforementioned platform with four different e-NOSEs in a population of 78 patients with severe asthma, revealing exhaled molecular phenotypes which were associated with changing inflammatory profile and oral steroid use. Thus, breath analysis might contribute to the management of severe asthma [75]. The same group used the four e-NOSEs panel in combination with GC-MS to differentiate between stable and

**Table 5 Discriminatory volatile organic compounds in asthma therapies**

	Salbutamol	OCS	ICS
Methyl-acetate	[43]		
Butanal	[43]		
3-Methyl-butanal	[43]		
Butyrolactone	[43]		
Carene		[43]	
Carvone	[43]		
Chloroacetic acid odecyl ester			[29]
1-Butyl-1-methyl-2-propyl- cyclopropane			[29]
2,6,6-Trimethyldecane			[29]
Glycolic acid		[43]	
Lysine		[43]	
Octanal		[43]	
1-Propanol	[43]		
Methyl propionate	[43]		
3,6-Dimethylundecane			[29]

OCS: Oral corticosteroids; ICS: Inhaled corticosteroids.

**Table 6 Discriminatory volatile organic compounds between asthmatic subjects and subjects with transient wheezing**

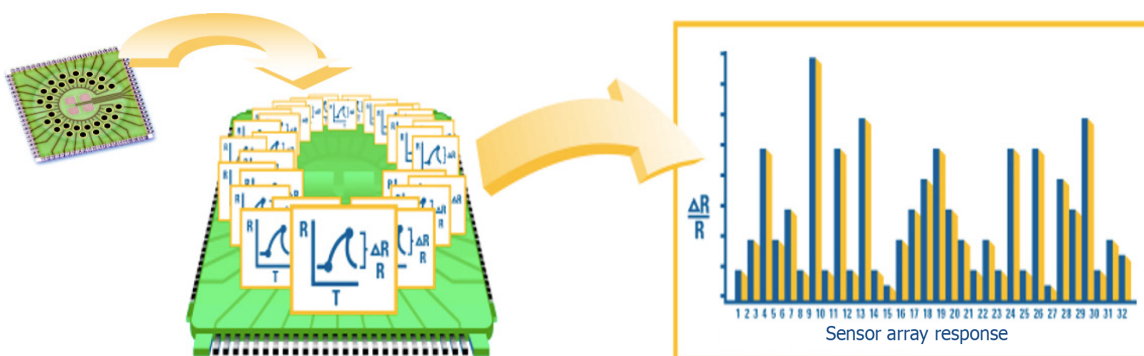
Identified compound	Ref.
Acetone	[51,56]
Biphenyl	[51]
1-methyl-4-(1-methylethenyl) cyclohexene	[51]
2,6,10-trimethyldodecane	[51,56]
2,4-dimethylheptane	[51,56]
2,2,4-trimethylheptane	[51]
2-methylhexane	[51,56]
2-ethylnaphthalene	[51]
2-methylpentane	[51,56]
2,4-dimethylpentane	[51,56]
octane	[51,56]
2,3,6-trimethyloctane	[51,56]
2-undecenal	[51,56]

unstable episodes of asthma[49]. PCA of e-NOSEs merged data showed 95% distinction between stable asthma and during loss of control, and 86% between loss of control and recovery[49]. Interestingly, GC-MS data revealed lower classification accuracies of 68% for stable *vs* loss of control, and 77% for loss of control *vs* recovery[49]. However, GC-MS detected exhaled metabolites that were significantly related with sputum eosinophils, whereas e-NOSE data did not correlate with sputum neutrophils and eosinophils[49], thus indicating a potential advantage of using both techniques together.

Concerning atopy, Abdel-Aziz *et al*[76] analyzed exhaled breath profiles by using either an integrated e-NOSE platform or the SpiroNose in four independent cohorts of pediatric and adult patients with asthma. Breath profiling discriminated between atopic and nonatopic participants with AUROC of 0.84 and 0.72 in the training and validation sets, respectively [76].

**Table 7 Discriminative volatile organic compounds in asthmatic/non asthmatic subjects with comorbidities**

	Asthmatics with obesity	Asthmatics with upper respiratory illness	Asthmatics vs non-asthmatics/atopics	Non-asthmatics/non-atopics vs non-asthmatics/atopics
2-Methylbutane		[29]		
1-Cyclopropane ethanol		[29]		
2,5,9-Trimethyldecane			[29]	
2,4-Dimethylheptane				[29]
2,3,6-Trimethylheptane	[29]			[29]
2,2,4-Trimethylheptane				[29]
n-Hexane	[29]	[29]		
Isoprene		[29]		
2-Methyloctane	[29]			
2,6-Dimethyl (S,E)-4-octene	[29]			
2-Methylpentane			[29]	
2,3,4-Trimethylpentane	[29]			
2-methyl-1-pentene				[29]
4-Methyl-1-pentene		[29]		



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**Figure 3 Working principle of electronic-Noses.** The combination of the signal from all sensors generates the so-called “breathprint”.

Furthermore, Tenero *et al*[77] showed that an e-NOSE could discriminate pediatric patients based on their current level of asthma control. In detail, a Cyranose 320 distinguished between a group of healthy controls associated with children with non-symptomatic controlled asthma and individuals with partially-controlled and symptomatic uncontrolled asthma with an AUROC of 0.85, and a sensitivity and specificity of 0.79 and 0.84, respectively[77].

Using the Tor Vergata e-NOSE, Montuschi *et al*[61] compared its diagnostic accuracy with conventional methods, such as F<sub>E</sub>NO, and lung function testing in a population of intermittent or mild persistent asthma and healthy controls were studied[61]. The e-NOSE discriminated between asthma and healthy controls in 87.5% of cases, overtaking F<sub>E</sub>NO (79.2%), lung function (70.8%), and F<sub>E</sub>NO coupled with lung function (83.3%). When combining e-NOSE analysis of exhaled breath with F<sub>E</sub>NO the diagnostic accuracy for asthma reached 95.8%[61].

Nevertheless, Banner *et al*[60] investigated the potential of an Aeonose e-NOSE in discerning among exhaled breath of pediatric patients with asthma, Cystic Fibrosis, and healthy controls[60]. The Aeonose showed high accuracy in differentiating asthma from Cystic Fibrosis (AUROC = 0.90, sensitivity 89%, specificity 91%) and Cystic Fibrosis from controls (AUROC = 0.87, sensitivity 85%, specificity 77%), whereas the accuracy was lower when comparing asthma and healthy controls (AUROC = 0.79, sensitivity 74%, specificity 91%)[60].

Using a Spironose, de Vries *et al*[78] adequately discriminated asthmatics and controls with an accuracy of 87% and a AUROC: 0.94 ± 0.15[78]. With the same e-NOSE, Lammers *et al*[79] obtained an adequate discrimination between pre- and post- rhinovirus challenge with an AUROC = 0.82 (95%CI = 0.65-0.99) in healthy and 0.97 (95%CI = 0.91-1.00) in asthmatic



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**Figure 4** Example of a commercially available electronic-Nose. Cyranose 320 is a small and easily portable device.

adults, suggesting that this technology might be useful in monitoring virus-driven flare-ups in asthma[79].

Additionally, van Bragt *et al*[80] showed that a Spirognose could accurately identify asthmatics who had a recent exacerbation (AUROC = 0.76 for both training and validation sets, indicating that asthma flare-ups have a specific exhaled breath pattern which is detectable by the e-NOSE[80]. Finally, in the study by Bikov *et al*[81]. Cyranose 320 could not differentiate asthmatic patients with and without exercise-induced bronchoconstriction, exercise challenge induced alterations in breathprints in both groups[81].

## LIMITS AND FUTURE DIRECTIONS

Although GC-MS is a highly sensitive and specific method, it cannot currently be used in clinical point-of-care settings due to various issues. Equipment limitations: Include high cost (EUR: 60000-150000), large size, requirement for highly trained personnel[80], offline pre-concentration and chromatographic separation, and lack of portability (prior to Sharm's work)[5]. Moreover, results cannot be obtained in real-time and can take anywhere from minutes to hours to generate[82] (so far).

Regarding e-NOSE technique, it is much cheaper than GC-MS, portable and easy to perform with quicker availability of results. However, there is no VOC identification, which is essential for investigation the pathophysiologic pathways underneath the disease, although it seems less important for the clinician's point of view.

Additional issues include sample extraction and data processing techniques, as well as statistical analyses used to interpret results: The type of GC-MS and e-NOSE technique used[23,30], as well as its various parameters (such as the type of gas used, stationary phase temperature, column type, sampling, storage, and instrumental analysis) can all impact the results. For example, GCxGC-HR-TOF-MS appears to be an ideal instrument for the analysis of VOCs in complex samples, such as breath[83]. Furthermore, not all VOC molecules detected are found in libraries, which can result in a loss of important information[14]. Additionally, Caldeira and his research team attempted to identify the characteristics of allergic asthma by using a combination of GC-MS and headspace solid-phase microextraction. This method was used to improve the extraction of components in the sample by pretreating it[53]. Regarding sample collection, a number of issues still need to be clarified, including the appropriate time of day to collect samples relative to the circadian rhythm [47,84], the setting of the examination, foods or drinks consumed before the examination, the breathing time necessary to collect the sample, the breathing pattern (that affect the origin of the VOCs, with differences in the concentration of VOCs from the upper or lower airways; for instance, it is expected that alveolar breath has higher concentrations of VOCs[82]), the use of a full face mask or free plastic bag to collect the sample (among others) and The patient acceptability which affect the level of stress, impacting the quality of their breath sample and the VOCs detected. Environmental factors can also make it difficult to distinguish between asthmatic and non-asthmatic VOCs[52], such as inter- and intra-individual factors: Chronic exposure to the environment, age, sex, physiological metabolic activities, exercise, drug intake, comorbidities, resident microbiome, smoking (van Berkel *et al*[48], identified four VOCs as biomarkers of recent exposure to smoke 2,5-dimethylhexane, dodecane, 2,5-dimethylfuran and 2-methylfuran. It is also a complicating factor that patients have difficulty in declaring their actual smoking habit[82]) and other exogenous elements like diet, food and water[14,29,85]. For example, the concentration of methylated alkanes appears to increase with age and oxidative stress. This increase in oxidative stress may not only be attributed to asthma, but also to comorbidities such as obesity or hypertriglyceridemia[86,87]. Pentane is a marker of oxidation and increases in response to an increase in oxygen free radicals resulting from various inflammatory conditions[88], along with ethane[89]. Moreover, these same inflammatory molecules can also be found in other diseases, which alter the relative concentrations of VOCs normally produced by the body, thus becoming potential contaminants beyond environmental sources[23]. These factors contribute to significant variability in results and explain the presence of various irrelevant peaks in asthma. In fact, healthy individuals are capable of exhaling 3481 different VOCs, identified by GC-MS, and over 200 of these compounds were detected in most breath samples. Although there are many interindividual variations, these variations do not appear to be significant

within the same person[88].

## CONCLUSION

In conclusion, the study of VOCs through GC-MS and e-NOSE appears to hold promise, based on the experiments conducted so far, for differential diagnosis between non-asthmatic and asthmatic subjects, identification of subtypes, therapy monitoring, exacerbation prevention, distinguishing from comorbidities (such as obesity), aiding in the differential diagnosis with preschool wheezing, and perhaps distinguishing asthmatic patients from those with atopy. To achieve these goals, however, standardization of the technique is necessary. It is clear that accuracy, specificity, sensitivity, and reproducibility vary significantly depending on the GC-MS and e-NOSE techniques used. Therefore, further studies will be necessary to standardize methods with a good cost-benefit balance, making modifications to the technique to make it more accessible. It will also be necessary to refine and standardize the patient's external conditions to the asthmatic disease, the sampling techniques, and the VOCs processing itself[30]. Finally, it will be fundamental to find stable biomarker VOCs that are less sensitive to the environment and more disease-specific, so that they can be quantified and identified in a reproducible manner, regardless of comorbidities or other factors[24]. However, it is important to consider that since VOCs are a result of oxidative stress from numerous inflammatory processes, a deeper understanding of the inflammatory processes underlying the diseases is necessary to identify them correctly through VOCs.

After about 70 years of perfecting techniques and 2000 years of observing the asthma disease, there is still a long way to go to finally make the study of VOCs through GC-MS and e-NOSE clinically available.

## FOOTNOTES

**Author contributions:** Savito L wrote the first draft; Scarlata S performed the supervision; Bikov A contributed to the conceptualization, english editing; Carratù P performed the data collection; Carpagnano GE conceptualization, data collection; Dragonieri S wrote the first draft and performed the supervision.

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