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Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis

João Cavaleiro Rufo¹, Joana Madureira¹, Eduardo Oliveira Fernandes¹, André Moreira²

¹ GEAC, INEGI - Institute of Science and Innovation in Mechanical Engineering and Industrial Management, Porto, Portugal

Rua Dr. Roberto Frias, 4200-465, Porto, Portugal

² Faculty of Medicine of the University of Porto, Porto, Portugal & Centro Hospitalar São João E.P.E., Porto, Portugal

Al. Prof. Hernâni Monteiro, 4200 - 319 Porto, Portugal

Corresponding author:

João Cavaleiro Rufo

INEGI

Rua Dr. Roberto Frias - 4200-465 – Porto, Portugal.

Tel: +351 229578710 Email: jcruf@gmail.com

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Abstract

We aimed to assess the value and classification rate of exhaled volatile organic compounds (VOCs) in asthma diagnosis.

A PRISMA oriented systematic search for published studies regarding exhaled VOCs in asthma diagnosis was conducted based on pre-defined criteria. Studies presenting sensitivity and specificity values for the test were included in the meta-analysis. Pooled diagnosis odds ratios (DOR), area under the curve (AUC) and positive and negative likelihood ratios (LR) for exhaled VOC profiles were calculated; and publication bias, threshold effect and heterogeneity were estimated.

Eighteen studies were selected for the qualitative analysis and 6 met the criteria for inclusion in the quantitative analysis. Mean (95%CI) pooled DOR, positive and negative LR were 49.3 (15.9 to 153.3), 5.86 (3.07 to 11.21) and 0.16 (0.10 to 0.26), respectively. The AUC value was 0.94. Only 3 of the 18 reviewed studies performed an external validation of the model using a different dataset.

The results from the revised studies suggest that exhaled VOCs are promising biomarkers for asthma diagnosis and that several compounds, mainly alkanes, may be significantly associated with asthma inflammation. However, there are still various constraints associated with standardization and externally validated studies are needed to introduce exhaled VOCs profiling in a clinical scenario.

Keywords: asthma; exhaled biomarkers; diagnosis; volatile organic compounds; VOCs.

Abbreviations used:

ATS: American thoracic society

COPD: Chronic obstructive pulmonary disease

DOR: Diagnostic odds ratio

eNose: Electronic nose

ERS: European respiratory society

GC-DMS: Gas chromatography coupled with a differential mobility spectrometer
sensor

GC-FID: Gas chromatography coupled with flame ionization detector

GC-MS: Gas chromatography coupled with mass spectrometry

GINA: Global initiative for asthma

ISAAC: International study of asthma and allergies in childhood

NLR: Negative likelihood ratio

PCA: Principal component analysis

PLR: Positive likelihood ratio

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

QUADAS: Quality Assessment of Diagnostic Accuracy Studies

ROC: Receiver operating characteristics

ROS: Reactive oxygen species

SROC: Summary receiver operator characteristic

VOC: Volatile organic compound

Introduction

The currently used clinical approaches for asthma diagnosis have multiple downsides, either considering specificity, sensitivity, invasiveness and/or expensiveness (1). Moreover, certain age groups, such as young children or the elderly, have a higher probability to suffer from distinct phenotypes of asthma with different metabolomics and immune responses, as well as different responses to pharmacological treatment (2-4). Concomitant respiratory diseases are very common in the aforementioned age groups and their symptoms may origin extra confounding factors for the diagnosis of asthma, such as transient virus-induced symptoms in children or chronic obstructive pulmonary disease in the elderly (5, 6). Therefore, new methodologies for an early and improved diagnosis of asthma are constantly being researched. One of these promising and non-invasive methodologies is the measurement of volatile organic compounds (VOCs) in the exhaled breath (7, 8).

To comprehend the potential of exhaled VOCs as biomarkers of asthma, it is important to understand their origin and how they relate to the pathology. Chronic airway inflammation and oxidative stress are normally associated with asthma (9). In general, the oxidative stress is caused by reactive oxygen species (ROS) produced by inflammatory cells activation resultant from the inflammation process. These ROS will promote the degradation of polyunsaturated fatty acids present in lipidic structures, such

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as the bronchial epithelium cells membrane, originating the formation of volatile hydrocarbons. Either formed by physiological or by pathophysiological events, these VOCs enter the bloodstream and are subsequently excreted in the exhaled breath in different configurations according to their origin (10-12). These configurations may also include VOCs that are locally released in the airways due to bronchial epithelial cell inflammation, forming a mixture with those produced by systemic metabolism.

VOCs can be measured by several techniques such as gas-chromatography coupled with mass spectrometry (GC-MS) or the electronic nose (eNose) technology. While GC-MS is a largely recognized and validated methodology for measuring VOCs, the efficiency of the eNose technologies in certain applications is still under discussion by the scientific community. The eNose often consist in an array of sensors that respond electronically when exposed to a mixture of volatile compounds, leading to the identification of VOC patterns or profiles, also known as breath-prints or smell prints (12-15). Although Röck *et al.* (2008) described more than 50 eNose models, the Cyranose[®] 320 (Sensigent, California, USA) is one of the currently most used models in the collection of exhaled VOCs, having showed good within-day and between-day reproducibility (16).

Since collecting breath is a simple, inexpensive and non-invasive process, this methodology for profiling airway diseases looks particularly promising. However, one should have in mind that VOCs in exhaled breath may also be originated from exogenous sources, such as the ambient air pollution, and may enter the organism via skin, ingestion or inhalation, being also excreted in the exhaled breath (17). Therefore, it should be necessary to assess the composition and levels of VOCs in the indoor/outdoor environment where the patient spends more time of exposure in order to discriminate if the exhaled VOCs are from endogenous or exogenous sources. In addition, the

metabolism of resident or pathogenic bacteria may be a potential source of VOCs (18). Therefore, determining the specific origin of these compounds in exhaled breath may prove to be a challenge.

The clinical uses of exhaled VOCs as biomarkers for pulmonary diseases have already been properly reviewed by *van de Kant et al.* (2012). However, presumably due to the wide range of pulmonary diseases included in the analysis, further information regarding asthma diagnosis was left to be reviewed. Moreover, several additional studies have been published concerning the clinical use of VOCs for asthma diagnosis. In this review, all the currently published methodologies regarding VOCs measurement and/or profiling for the diagnosis of asthma and its phenotypes were systematically analysed and the sensitivity and specificity data retrieved from those studies were included in a meta-analysis whenever possible.

Methods

Search strategy, inclusion criteria and data extraction

A PRISMA oriented systematic search (19) was performed until 31 October 2014 in PubMed, Scopus, Compendex, Inspec, ScienceDirect, Academic Search Complete, Web of Science and the Cochrane library. The search was conducted through the combination of the keywords “asthma”, “exhaled”, and “VOCs” or “volatile organic compounds”. Published peer-reviewed full-text articles in English concerning clinical studies of asthma diagnosis through VOCs monitoring were assessed for eligibility. The inclusion criteria for qualitative synthesis were (a) asthma defined by a trained physician or according to official guidelines, such as those validated by the Global

Initiative for Asthma or the American Thoracic Society/European Respiratory Society; (b) VOCs measured in exhaled breath; and (c) clinical studies.

The exclusion criteria consisted in (a) less than two defined groups for comparing VOC levels or VOC profiles; (b) studies focused on diagnosing symptoms of asthma, not the disease itself. Moreover, presenting the sensitivity and specificity values for the asthma diagnosis test was an additional inclusion criterion for meta-analysis. Two reviewers independently applied the inclusion criteria and any differences were resolved by consensus.

Information regarding study design, settings, population, methodologies (including sample collection, VOC analysis techniques, targeted biomarkers, environmental air VOCs assessment) and outcomes was gathered. The diagnosis tests' sensitivity and specificity data was retrieved whenever possible for meta-analysis.

Quality assessment

The quality assessment of the selected studies was conducted according to the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool (20). To better represent the quality assessment, QUADAS quality scores were defined: items classified as “Yes” added 1 point to the score; items classified as “No” and as “Unclear” added 0 points to the score.

Statistical analysis

The statistical analysis was performed using the Stata IC software (version 13, StataCorp LP, College Station, TX, USA) and the Meta-DiSc software (version 1.4) (21). The sensitivity and specificity of the included studies were used to construct a contingency table. The bivariate meta-analysis model was employed to obtain the

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pooled value of sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) and generate the bivariate summary receiver operator characteristic (SROC) curve. Heterogeneity between studies was evaluated using the Chi-square and I^2 tests. A value of $p < 0.05$ was considered as being statistically significant and $I^2 \geq 50\%$ indicated the existence of significant heterogeneity (22, 23). To detect the threshold effect, the Spearman's test of correlation was used to evaluate the relationship between sensitivity and specificity. The publication bias of the selected studies was assessed using Begg's funnel plot and Egger's test (24).

Results

Study selection, characterization and quality assessment

A total of 288 studies were found using the aforementioned search methodology. With the removal of duplicates, 248 studies were accepted for screening. This number was later increased to 256 after the inclusion of studies found by reference list searching. During the screening, 236 studies were excluded for not fulfilling the inclusion requirements, thus 20 studies were selected for full revision. Two studies were later excepted according to the exclusion criteria: one study was focused on asthma symptoms diagnosis (25) and the other did not comprise two defined groups for comparisons (26). Therefore, 18 studies were included in the qualitative synthesis (27-44). However, of these 18 studies, only 6 presented the required data for the quantitative synthesis (**Figure 1**).

Data from the 18 studies concerning asthma diagnosis by exhaled breath VOCs assessment was reviewed thoroughly. In total, 560 patients with asthma diagnosed by a physician and/or according to official guidelines (including ATS, ERS, GINA and ISAAC) were investigated in 7 different countries. The most recurrent methodology for sampling collection consisted in Tedlar[®] bags for trapping the exhaled breath (45) although the sampled volume varied between 2 mL to 10 L. The most used technique for analysing VOCs was gas chromatography coupled with mass spectrometry (GC-MS), which was used in 10 studies, followed by the Cyranose[®] 320 technology (5 studies), whereas only 3 studies used gas chromatography coupled with flame ionization detector (GC-FID). One study performed exhaled breath analysis with gas chromatography coupled to differential mobility spectrometer and a mass spectrometer (GC-DMS-MS). Only 3 of the 18 reviewed studies performed an external validation of the model using a different dataset, while the majority of the studies only used a cross-validation approach due to population size limitations, which does not provide as much certainty of the findings as external validation does. While **Table 1** shows a summarized overview of the collected information, the most important outcomes are presented in the discussion. Moreover, the 18 studies were scored according to QUADAS (**Table 1S**).

Data analysis

Six of the 18 selected studies allowed data extraction to be included in the meta-analysis (28, 29, 35, 37, 42, 44). Sensitivity and specificity of exhaled VOC profiles in asthma diagnosis tests were retrieved from these studies and grouped according to the methodology used. For the purpose of the meta-analysis, the methodology of analysis used (eNose or GC-MS) was not discriminated since it only represents different means to produce similar outcomes for diagnosing asthma (VOC profiles). The data retrieved from the aforementioned studies is presented in **Table 2**.

The sensitivity and specificity values of the exhaled VOC profiles test in asthma diagnosis were analysed. Heterogeneity in specificity was observed among the 6 studies ($I^2=65.1\%$; $p=0.014$), which did not occur with sensitivity ($I^2=46.6\%$; $p=0.095$) (**Figure 2**). Therefore, the random effects model was employed. The pooled results reported a mean (95% CI) sensitivity of 87% (82% to 91%) and specificity of 86% (80% to 90%) (**Figure 2**). The mean (95% CI) pooled PLR was 5.86 (3.07 to 11.21), indicating that individuals with asthma had approximately 6 times higher chance of presenting asthma-associated exhaled VOC profiles when compared with individuals without asthma, and the mean (95% CI) pooled NLR was 0.16 (0.10 to 0.26). The mean (95% CI) pooled DOR was 49.3 (15.9 to 153.3) and the area under the SROC curve (AUC) was 0.94 (**Figure 2 and 1S, respectively**).

Publication bias

To assess publication bias, the Begg's funnel plot and the Egger's test were used (**Figure 2S**). Both the funnel plot and the Egger's test p-value ($p=0.100$) suggested no publication bias, although this observation is limited by the reduced number of studies included in the meta-analysis.

Diagnostic threshold and heterogeneity

To evaluate the diagnostic threshold, the Spearman's correlation coefficient between sensitivity and 1-specificity was calculated. The correlation coefficient was -0.429 ($p=0.397$), suggesting that there was no heterogeneity from the threshold effect. Moreover, heterogeneity was also explored by meta-regression to assess the contribution by sample size; type of methodology used and the country where the study was performed. However, evidence of heterogeneity was not found for none of the aforementioned characteristics ($p>0.05$).

Discussion

Our systematic review and meta-analysis showed: first, exhaled VOC profiles have high sensitivity and specificity values, respectively 87% and 86%, for diagnosing asthma; secondly, individuals with asthma had 6 times higher chance of being diagnosed through exhaled VOC profiles than healthy controls; and thirdly, diagnosis odds ratios and area under the curve values are respectively, 49.3 and 0.94. Taken together these observations suggest that exhaled VOC profile analysis may be an accurate test for asthma diagnosis. Nevertheless, these results should be interpreted with caution since the sensitivity and specificity values were retrieved from studies using not only different analysis methods but also different algorithms for VOC profiling. Therefore, despite how much promising the meta-analysis results may seem to be, there is still much to be done before introducing VOC biomarkers in a real clinical context.

In common with all meta-analyses, this systematic review may have included studies in which the characteristics of the subjects were too dissimilar for comparison or the spectrum of patients used was not completely representative of the one that would receive the test in practice. Nevertheless, this was considered and reported on the quality rating of the included studies, and apparently no publication bias existed. Secondly, included studies used different methodologies for assessing VOCs (mostly Cyranose[®] 320 and GC-MS), and most importantly used distinct VOC profiles for diagnosing individuals with asthma, making it impossible to further investigate how the diagnostic potential correlates with these characteristics due to the reduced number of trials available. Nevertheless, as shown by the overall collected information, exhaled VOC assessment appears to be a tool with significant accuracy for asthma diagnosis.

Studies with larger populations may help to better understand and validate the correct accuracy, sensitivity and specificity of exhaled VOC profiles in asthma diagnosis.

These findings are particularly important when compared with the sensitivity and specificity values of the currently used clinical tools in asthma diagnosis. For instance, the pooled sensitivity values of VOC profiles (87%) are significantly higher when compared with those obtained by spirometry considering both $FEV_1 < 80\%$ and $< 90\%$ (29% and 35%, respectively) (46, 47). Bronchodilator reversibility $> 12\%$ also showed a poor sensitivity value (36%) when compared with VOC profiles (48). On the other hand, the GINA self-reported symptom questionnaires showed highly skewed sensitivity/specificity ratios (10.9 for ≥ 1 reported symptoms and 0.2 for ≥ 5 reported symptoms) while VOC profiles presented a ratio approximated to 1.0 (49). **Table 2S** shows the sensitivity and specificity values of other asthma diagnostic tools (46).

Exhaled VOCs for asthma diagnosis

The first reported attempt to use exhaled VOCs for asthma diagnosis was performed by *Olopade et al.* (1997), who measured the pentane levels in the exhaled breath of 12 subjects with acute asthma, 11 with stable asthma and 17 healthy controls. Although pentane levels were shown to be significantly higher in more severe forms of asthma (see next section), they were not significantly different between individuals with stable asthma and healthy controls, suggesting that this VOC may not be adequate for the diagnosis of the disease itself. Similarly, *Lärstad et al.* (2007) found no significant differences in pentane levels between patients with asthma and healthy controls.

Paredi et al. (2000) had more success with exhaled breath ethane assessment, showing that individuals with asthma presented significantly higher levels of ethane in their breath when compared to healthy controls ($p<0.05$). However, similarly to pentane (38), exhaled ethane was more associated as a biomarker of severity being able to significantly distinguish between steroid-naïve and steroid-treated patients with asthma ($p<0.01$) (39). These results were later supported by *Lärstad* and co-workers, who showed that asthma pathophysiology may be slightly associated with exhaled ethane (36). For unknown reasons, *Lärstad et al.* also found that individuals with asthma presented significantly decreased concentrations of isoprene in exhaled breath when compared to healthy controls (36). These results suggest that isoprene may be negatively associated with asthma. Nevertheless, since the concentrations of exhaled isoprene may be highly affected by cholesterol synthesis, diet, exercise and age, its potential as a biomarker of asthma may be limited (50).

In a clinical study comprising a population of 20 individuals with asthma and 20 controls, *Dragonieri et al.* (2007) analysed exhaled VOC profiles with both Cyranose[®] 320 and GC-MS techniques. The smell prints (VOC profiles detected by electronic nose) of the patients with asthma were fully separated from healthy controls showing a significant diagnosis potential. However, explorative GC-MS analysis showed the presence of similar VOCs in asthma when compared with the control group, suggesting that further studies are required in order to determine specific discriminative VOC concentrations between individuals with and without the disease. *Dallinga et al.* (2010) also analysed by GC-MS the VOC profiles in the exhaled breath collected from 63 children with asthma and 57 controls and the outcomes of the study revealed 8 promising VOCs, mostly hydrocarbons, that were able to discriminate between asthmatic and healthy children achieving sensitivity of 89% and a specificity of 95%.

Similar promising results were shown by *Montuschi* and co-workers (2010) using a metalloporphyrins coated QMB sensor (prototype model, University of Rome Tor Vergata) was used to discriminate between 27 patients with asthma and 24 healthy subjects with high diagnostic performance. Classification rates were based on neural networks. Mass-spectrometry fingerprinting was used in a subset of samples to confirm the eNose classification. In this case, principal component analysis (PCA) was used for classification, showing clear between-group distinction. The specific compounds responsible for the discrimination were not identified. This was one of the few included studies that performed external validation of the VOC profiles used in the analysis, achieved through an alternative dataset to test the classification model. Diagnostic performance of the metalloporphyrins coated QMB sensor was also compared with FeNO and spirometry and their combination using two different procedures for breath sampling. When *Caldeira et al.* (2011) measured 44 VOCs in the exhaled breath of 35 children with asthma and 15 healthy controls by GC-MS, they showed that it was possible to discriminate children with allergic asthma and healthy controls with 88% classification rate and that alkanes and aldehydes were the compounds with more discriminative power. *Caldeira* and co-workers did specify the most promising alkanes in a later study, which consisted on nonane, (2,2,4,6,6)-pentamethylheptane, decane, (3,6)-dimethyldecane, dodecane and tetradecane (28).

A 15 VOC profile obtained through GC-MS and assembled by PCA was able to differentiate between 35 individuals with asthma and 23 healthy controls, in a study performed by *Ibrahim et al.* (2011). Also in UK, *Gahleitner* and co-workers (2013) identified through GC-MS a panel of 8 compounds that were able to discriminate between 11 subjects with asthma and 12 healthy controls. However, none of these

VOCs matched the promising compounds found on the aforementioned study by *Caldeira et al.* (2012).

Using gas chromatography coupled with a differential mobility spectrometer sensor (GC-DMS) to analyse VOCs in the exhaled breath samples of 13 individuals with asthma, 5 subjects with COPD and 13 healthy controls, *Schivo et al.* (2013) demonstrated that it was possible to differentiate patients with asthma from healthy individuals. However, the most discriminant compounds in this analysis could not be identified since there was no analyte diagnostic library for the GC-DMS methodology at the time of the study in spite of the parameters which could be used for species identification.

A study involving asthma diagnosis by Cyranose[®] 320 VOC profiling, conducted by *van der Schee* and co-workers (2013), showed that this technique is able to significantly discriminate between patients with asthma and healthy controls with greater accuracy than FeNO. Unfortunately, the eNose systems are able to identify VOC breath-prints but cannot specify the most discriminant compounds (15).

In a study with 252 participants comprising a random sample of children with asthma and healthy controls, *Smolinska et al.* (2014) were able to correctly predict 80% of early asthma cases by analysing exhaled breath VOCs through GC-MS. The authors identified 14 compounds from total of 17 that showed promising diagnosis potential. Similarly to several aforementioned studies, alkanes were the VOCs with the highest discriminant value, again suggesting that some alkanes may be linked to pathological processes associated with asthma. It is important to mention that the model used in this study was validated using an external dataset.

Table 3 shows a list of the most discriminant VOCs for asthma diagnosis identified in the aforementioned studies. The results suggest that alkanes are indeed associated with asthma pathophysiology although the specific identity of the compounds appears to remain undetermined (51). Moreover, in the case of few compounds the results appear to be contradictory in different studies. For instance, the results from *Dragonieri et al.* (2007) suggest that acetone is positively associated with asthma, while in the study performed by *Caldeira et al.* (2011) acetone is negatively associated with asthma. These inconsistencies may be associated with each individual intervariability, but may also be caused by exposure to different environments and, consequently, exhalation of different exogenous VOCs (52, 53). They might also reflect differences in breath sampling procedures.

Exhaled VOCs in differential diagnosis

Comorbidity is frequent in children and elderly individuals. In these circumstances, asthma diagnosis may prove to be even more difficult since several respiratory diseases share common symptoms. Such is the case of COPD, which is often misdiagnosed as asthma. Four of the 18 selected studies were focused on differentiating COPD from asthma using exhaled breath VOCs analysis.

In a study conducted in the Netherlands, by *Fens* and co-workers (2009), the authors aimed to correctly distinguish a population comprised by 30 subjects with COPD, 20 subjects with asthma and 40 healthy controls, analysing their exhaled breath by Cyranose[®] 320. The results showed that the breath-prints of individuals with asthma are significantly different from those with COPD or with no respiratory diseases (controls) with an accuracy of respectively 96 and 95%. Although repeatable and reproducible, the

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results were only expressed in Cyranose[®] 320 breath-prints thus no specific compounds were identified. The external validation of the mentioned results was demonstrated in a later study by *Fens et al.* (2011) where they showed that the accuracy for discriminating asthma from COPD was not confounded by “current smoking” status. This may be a further advantage over current diagnostic methodologies, such as FeNO assessment, that are known to be influenced by smoking (54).

Timms and co-workers (2012) performed a study aiming to distinguish patients suffering from common obstructive lung diseases (including asthma) with concomitant gastro-oesophageal reflux disease from those without gastro-oesophageal reflux disease, using the Cyranose[®] 320 technology. The population included 20 individuals with asthma and the results showed that the breath-prints from individuals with gastro-oesophageal reflux disease were highly distinguishable from those without reflux, in the asthma population.

In the aforementioned study performed by *Schivoet al.* (2013), the authors hypothesised that GC-DMS could be used to differentiate between individuals with asthma and individuals with COPD, using VOCs profiles. However, they observed that after analysing the VOCs profiles it was not possible to distinguish between COPD and asthma. The small size of the sampled population (13 individuals with asthma and 5 with COPD) was pointed by the authors as the main reason behind these results.

Despite GC-MS being the preferential technique for assessing VOCs in exhaled breath in the 18 selected studies, the Cyranose[®] 320 was the most used technology in differential asthma diagnosis. Consequently, no discriminant VOCs have been identified in these type of diagnosis, although the obtained results appeared to be very promising with breath-prints from individuals with COPD being significantly different from those

of individuals with asthma, as well as the breath-prints from individuals with comorbidities, which were significantly different from those without comorbidities. However, there is not much published information concerning exhaled VOCs analysis to distinguish different phenotypes of asthma (such as allergic vs non-allergic asthma).

Exhaled VOCs in asthma severity monitoring

Several studies showed a potential to differentiate asthma severity using exhaled VOCs assessment (30, 38-40, 44). This could be useful to monitor steroid treatment and to perceive exacerbations in patients with asthma.

In the aforementioned work performed by *Olopade et al.* (1997), the results suggested that exhaled pentane levels were increased in acute asthma and dropped significantly once acute asthma subsided. Similarly, *Paredi et al.* (2000) showed that exhaled ethane levels were also significantly increased in steroid-naïve when compared with steroid-treated patients with asthma.

Delfino and co-workers (2003) sampled the breath of 21 individuals with asthma by GC-MS and quantified 8 VOCs including benzene, methylene chloride, styrene, tetrachloroethylene, toluene, m,p-xylene, o-xylene, and p-dichlorobenzene. The results showed no significant associations between exhaled VOCs concentrations and symptoms of asthma. However, there were no alkanes on the sampled set of VOCs in this study, which supports the concept that alkanes may be the VOCs more commonly associated with asthma pathophysiology. This may also explain the distinct results obtained in the two previously mentioned studies, by *Olopade et al.* (1997) and *Paredi et al.* (2000), since pentane and ethane are both alkanes.

Intra and intersubject comparisons regarding asthma exacerbations were performed by Robroeks and co-workers (2013) in a prospective study comprising 40 children with asthma. All samples were analysed by GC-MS. Exhaled breath was collected before and after an exacerbation in 16 children. The authors showed that, using support vector machine analysis, 6 VOCs were able to differentiate stable from exacerbated asthma measurements within patients with a classification rate of 96%. Similarly, a model of 7 VOCs were able to differentiate the whole exhaled breath samples of individuals with baseline measurements of stable asthma from those with baseline measurements of exacerbated asthma with a classification rate of 91%. These results suggest that not only are exhaled VOCs capable of differentiating exacerbated asthma from stable asthma measurements, they may also predict individual-specific exacerbations in children, supporting the usefulness of exhaled VOCs analysis as a monitoring tool for asthma treatment.

The Cyranose[®] 320 technology also produced promising results in the study performed by van der Schee *et al.* (2013). Exhaled VOC profiles were shown to predict steroid responsiveness in patients with asthma with greater accuracy than FeNO and sputum eosinophils count. These findings suggest that exhaled VOC profile analysis may be an important tool for assisting asthma treatment administration.

Exogenous VOCs in exhaled breath

When measuring exhaled VOCs as biomarkers of physiological processes one should consider that a large part of the compounds in the exhaled breath may have exogenous origins. Potential misinterpretations may have occurred in the revised studies since the origins of certain VOCs in breath were assumed as endogenous and thus considered

useful as biomarkers of asthma despite the possible contribution of food, bacteria or environmental contaminants (53, 55, 56).

The human body can absorb and exhale VOCs originated in several food products or cooking practices (57, 58). In the revised studies, the patients were usually instructed to refrain from eating or drinking in the 2 hours preceding breath collection to limit the influence of exogenous VOCs on the exhaled breath. However, the kinetics of most VOCs is largely unknown and further research is needed in order to achieve a standardized approach for reducing the influence of food VOCs in the exhaled breath.

Eliminating the VOCs originated in the inhaled air from the breath collection requires a more challenging and complex approach since the participants cannot refrain from breathing in large periods of time preceding the breath collection. The more commonly used method for reducing the error originated by inhaled VOCs is the alveolar gradient approach. It is based on subtracting the inhaled concentration of a compound from the exhaled concentration of the same compound in order to determine the endogenous concentrations of each compound. If the result is positive, the compound is defined as endogenous (53, 59). Although several of the revised studies considered the alveolar gradient, there were some studies that did not perform this step. Even in the circumstances where alveolar gradient was taken into consideration there is a possibility that other factors, such as the smoking history, may significantly hamper asthma diagnosis (17).

To avoid misinterpretations associated with exogenous VOCs in exhaled breath analysis for asthma diagnosis, all the aforementioned sources of exposure should be considered in future studies. Moreover, the standardization of proceedings to reduce the impact of

exogenous VOCs in breath analysis will be an important improvement to allow reliable comparisons between studies.

Conclusions

There has been an increasing interest in non-invasive tools for asthma diagnosis. Exhaled VOCs are among the most promising biomarkers and several compounds, mainly alkanes, have been identified as significantly associated with asthma. In this review, the more discriminant compounds identified by GC-MS in previous studies were listed and sorted as positively or negatively associated with asthma. The eNose studies also produced interesting results although it was not possible to identify the most discriminant VOCs. However, there are still various constraints associated with exhaled breath collection standardization and analysis validation. The exogenous factors that may influence exhaled VOCs concentrations, such as food, bacteria and environmental contaminants, must be taken into consideration in future studies to avoid misinterpretation on the results. Moreover, pharmacological treatment must be taken into consideration since it may influence the type and quantity of VOCs in the exhaled breath. Nevertheless, if these constraints are disentangled, exhaled breath VOCs profiling may be clinically used as an additional tool for asthma diagnosis or combined with other diagnostic tools, such as nuclear magnetic resonance spectroscopy of exhaled breath condensate, for a more comprehensive breathomics approach to asthma diagnosis (60).

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Authors' contribution

JCR and AM were responsible for the initial draft, systematic search and statistical analysis. JM and EOF critically revised the manuscript making important contributions to the final draft. All authors provided substantial contributions to the conception or the analysis of the work, revised the manuscript for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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Table 1 – Characteristics of included studies on exhaled VOCs for asthma diagnosis.

Reference	Year	Country	Objective	Participants	Breath collection	Technique	Targeted markers	Short conclusions
(31)	1997	USA	Asthma diagnosis; Asthma severity monitoring	12 subjects with acute asthma, 11 subjects with stable asthma and 17 healthy controls	Tedlar bag (50 mL sampled)	GC-FID	1 VOC (Pentane)	Exhaled pentane levels are increased in patients with acute asthma and decrease significantly once acute asthma subsides.
(32)	2000	United Kingdom	Asthma diagnosis; Asthma severity monitoring	26 subjects with asthma and 14 healthy controls	Tedlar bag (2 mL sampled)	GC-FID	1 VOC (Ethane)	Exhaled ethane is elevated in asthma when compared to steroid-treated patients and controls.
(23)	2003	USA	Asthma severity monitoring	21 subjects with asthma	Evacuated stainless steel canister	GC-MS	8 VOCs	No significant associations have been found. Benzene was the most promising marker.
(29)	2007	Sweden	Asthma diagnosis	13 subjects with asthma and 14 healthy controls	Tedlar bag (3 L sampled)	GC-FID	Ethane, Pentane and Isoprene	There might be a minor contribution of ethane from the airways in subjects with asthma. The concentrations of isoprene are low in subjects with asthma for unknown reasons.
(24)	2007	Netherlands	Asthma diagnosis; Asthma severity monitoring	10 subjects with severe asthma, 10 subjects with mild asthma and 20 controls	Tedlar bag (1 L sampled)	Cyranose [®] 320, GC-MS	VOCs profile	Smellprints of patients with mild asthma were fully separated from young controls, and patients with severe asthma could be distinguished from controls. Patients with mild and severe asthma could be less well discriminated.
(26)	2009	Netherlands	Differential diagnosis (Asthma vs. COPD)	30 subjects with COPD, 20 subjects with asthma and 40 controls	Tedlar bag (10 L sampled)	Cyranose [®] 320	VOCs profile	VOC profiling of exhaled air can distinguish patients with asthma from those with COPD or control subjects.
(22)	2010	Netherlands	Asthma diagnosis	63 children with asthma and 57 healthy controls	Tedlar bag (5 L sampled)	GC-MS	VOCs profile	A total of eight components discriminated with high accuracy between asthmatic and healthy children.
(30)	2010	Italy	Asthma diagnosis	27 subjects with asthma and 24 controls	Tedlar bag (2 L sampled)	Metalloporphyrins coated QMB sensor, GC-MS	VOCs profile	VOCs breath-prints could discriminate between patients with asthma and healthy subjects with a high diagnostic performance.
(20)	2011	Portugal	Asthma diagnosis	35 children with asthma and 15 healthy controls	Tedlar bag (1 L sampled)	GC-MS	44 VOCs	Discrimination between allergic asthma and control children was attained with a high classification rate mainly by the compounds linked to oxidative stress, such as alkanes and aldehydes.

(25)	2011	Netherlands	Differential diagnosis (Asthma vs. COPD)	40 subjects with COPD and 60 subjects with asthma	Tedlar bag (10 L sampled)	Cyranose® 320	VOCs profile	External validity of breath-prints significantly distinguishing fixed asthma from COPD and classic asthma. Discriminative accuracy was not confounded by current smoking.
(28)	2011	United Kingdom	Asthma diagnosis	35 subjects with asthma and 23 healthy controls	3 L collected directly in adsorbent pipes (Tenax)	GC-MS	VOCs profile	A model derived from 15 VOCs classified patients with asthma with high accuracy.
(21)	2012	Portugal	Asthma diagnosis	32 children with allergic asthma and 27 healthy controls	Tedlar bag (1 L sampled)	GC-MS	VOCs profile	A pattern of six compounds belonging to the alkanes characterized the asthmatic population.
(36)	2012	Australia	Differential diagnosis (Asthma with GORD vs. Asthma without GORD)	17 subjects with COPD, 20 subjects with asthma and 7 healthy controls	Tedlar bag (no volume specified)	Cyranose® 320	VOCs profile	The Cyranose® 320 distinguished exhaled breath profiles of asthmatic patients with GORD from asthmatics without GORD.
(27)	2013	United Kingdom	Asthma diagnosis	11 subjects with asthma and 12 healthy controls	2.5 L collected directly in adsorbent pipes (Tenax)	GC-MS	VOCs profile	A panel of eight candidate markers were found to differentiate between the asthmatic and healthy children in the test cohort.
(33)	2013	Netherlands	Asthma severity monitoring (prospective study)	40 children with asthma	Tedlar bag (5 L sampled)	GC-MS	VOCs profile	With support vector machine analysis, the most optimal model of intravariation baseline measurements versus exacerbation was based on six VOCs. The model of intervariable baseline values consisted on seven VOCs.
(34)	2013	USA	Asthma diagnosis; Differential diagnosis (Asthma vs. COPD)	13 subjects with asthma, 5 subjects with COPD and 13 healthy controls	Exhaled breath condensate collector (10 to 15 minutes)	GC-DMS-MS	VOCs profile	VOC profiles distinguished asthma from healthy controls. It was not possible to distinguish between COPD and asthma.
(37)	2013	Australia	Asthma diagnosis	25 subjects with asthma and 20 controls	Inert bag (not specified)	Cyranose® 320	VOCs profile	The Cyranose® 320 significantly discriminated between asthma and controls and may be used to predict the patient's response to steroids.
(35)	2014	Netherlands	Asthma diagnosis (Randomized trial)	76 children with asthma, 121 children with transient wheezing and 50 controls	Tedlar bag (1 L sampled)	GC-MS	VOCs profile	A set of 17 VOCs discriminated preschool asthmatic children from transient wheezing children.

GORD – Gastro-oesophageal reflux disease; GC-MS – Gas chromatography coupled with mass spectrometry; GC-FID – Gas chromatography coupled with flame ionization detector; GC-DMS – Gas chromatography coupled to a differential mobility spectrometer and a mass spectrometer.

Table 2 – Studies included in the meta-analysis.

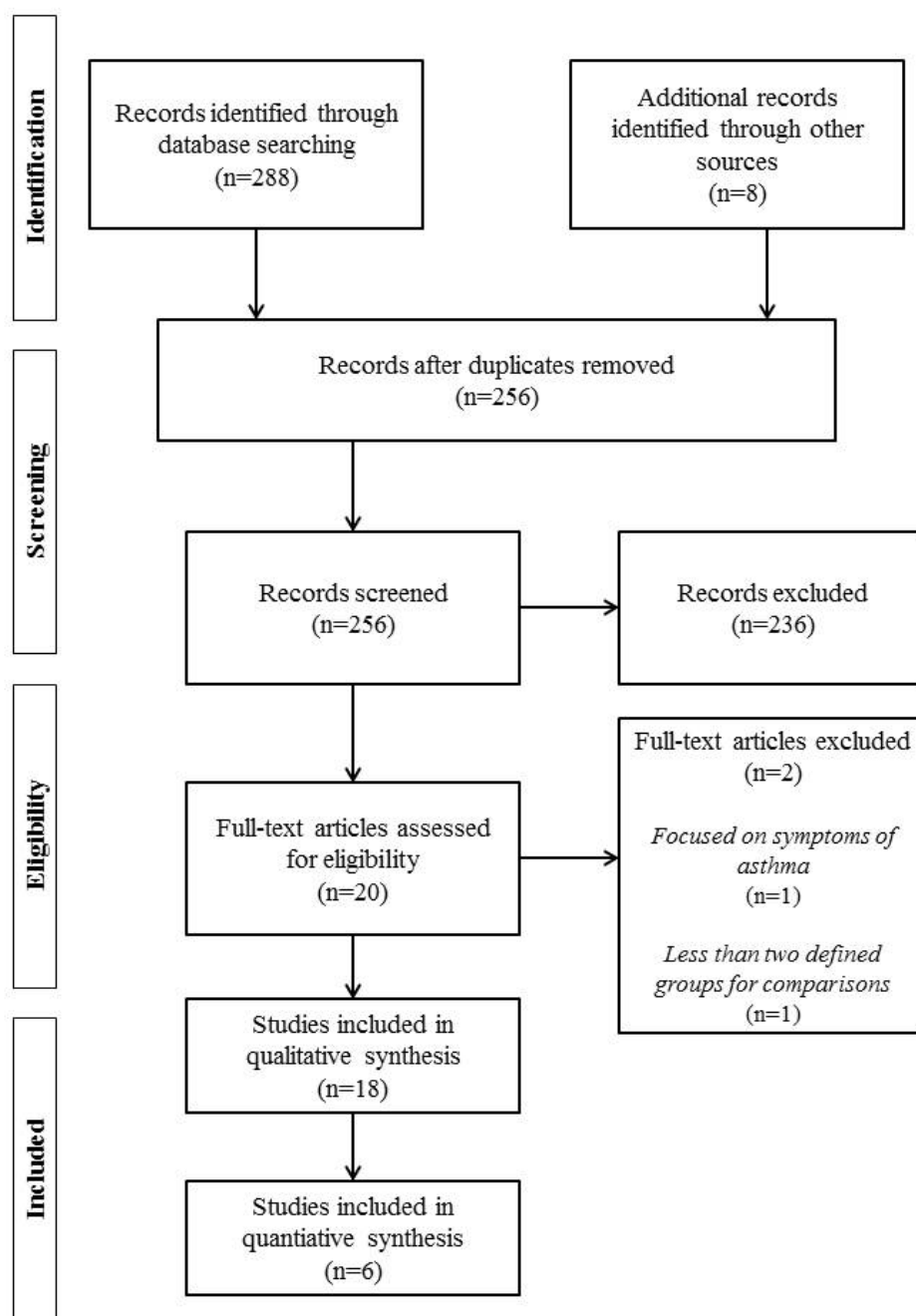
Reference	Technique	Sample size	Accuracy	Sensitivity	Specificity
(36)	Metalloporphyrins coated QMB sensor	12 subjects with asthma vs 12 controls (analysis in alveolar exhaled breath)	88%	100%	75%
(43)	Cyranose® 320	25 subjects with asthma and 20 controls (post-steroid comparison)	82%	84%	80%
(28)	GC-MS	63 children with asthma vs 57 healthy controls	92%	89%	95%
(34)	GC-MS	35 subjects with asthma vs 23 healthy controls	86%	85%	89%
(27)	GC-MS	32 children with allergic asthma vs 27 healthy controls	98%	96%	95%
(41)	GC-MS	76 children with asthma vs 50 healthy controls	77%	82%	74%

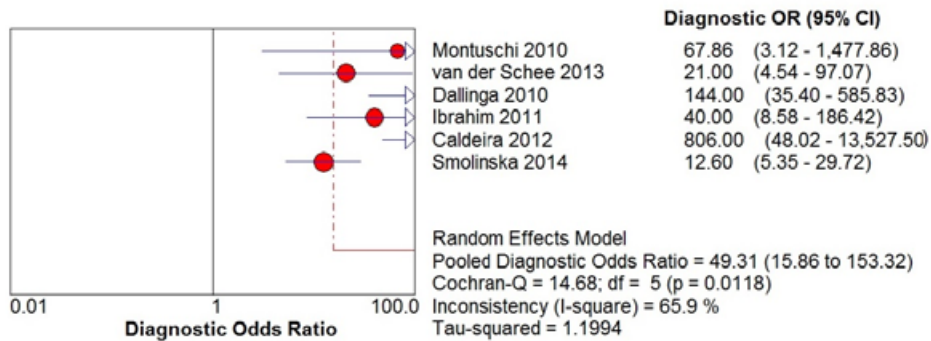
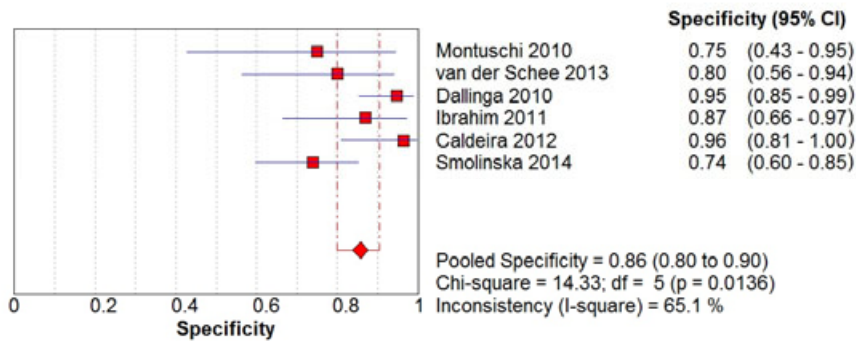
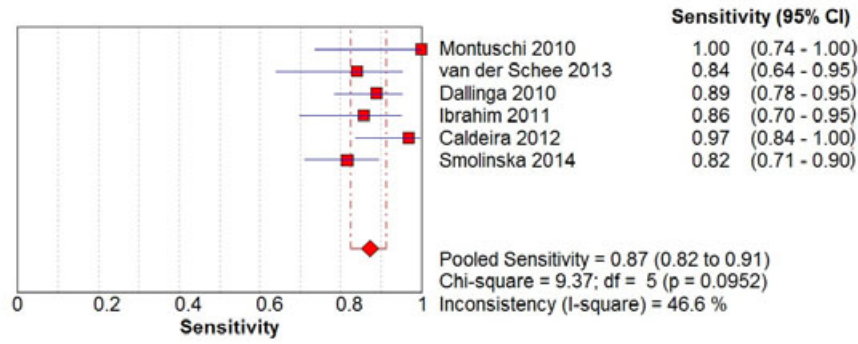
Table 3 – Most discriminant VOCs for asthma diagnosis identified in the revised studies.

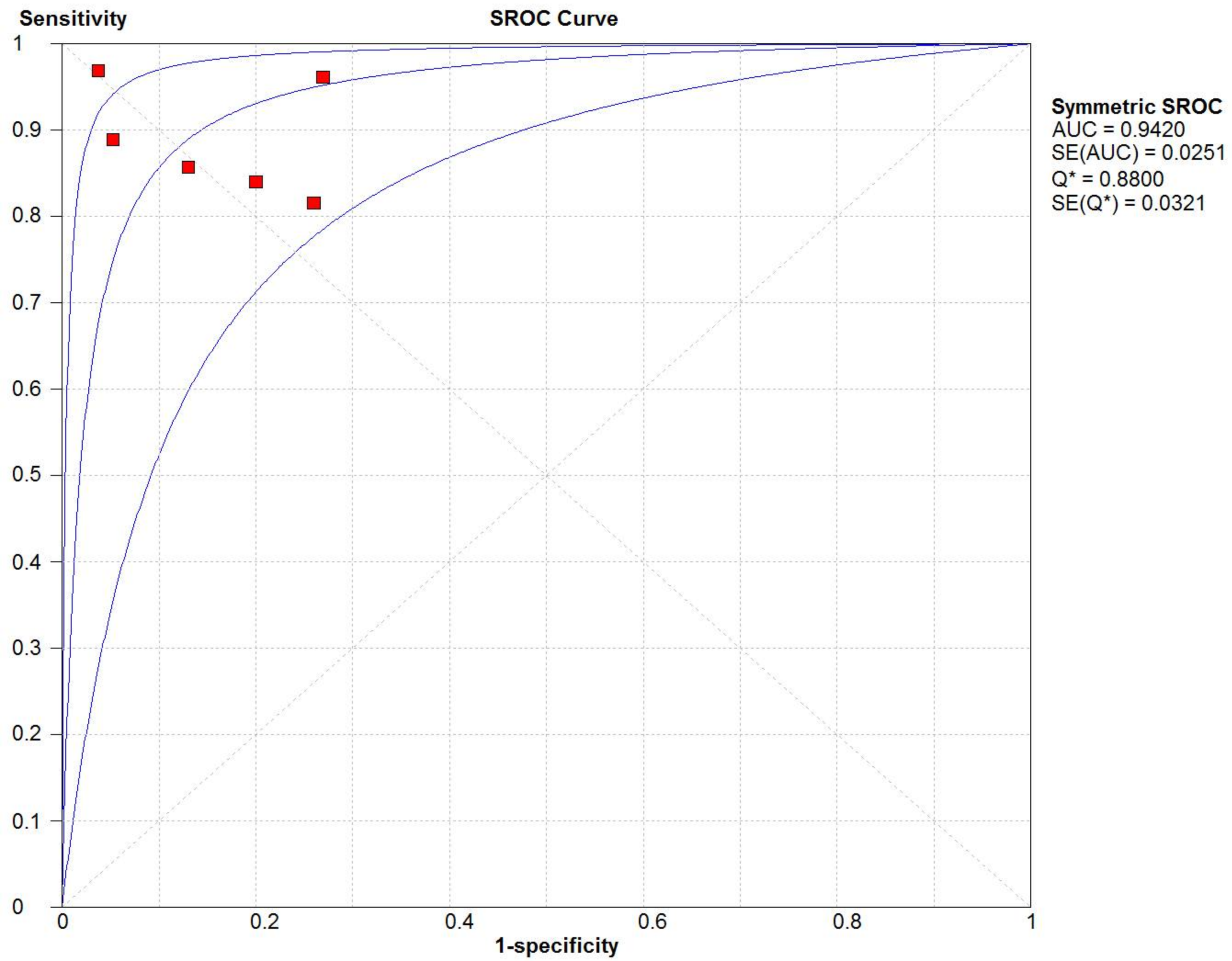
VOCs	Group	Reference	Identification
1,2-propanediol	Alcohol	(van de Kant., 2013)	(-)
2-butylcyclohexanol		(Ibrahim et al., 2011)	(-)
2-propen-1-ol		(van de Kant., 2013)	(-)
Hexadecan -2-ol		(van de Kant., 2013)	(+)
Isopropanol		(Dragonieri et al., 2007)	(+)
2-octenal	Aldehyde	(Gahleitner et al., 2013)	(+)
2-undecenal		(Smolinska., 2014)	(+)
4-methyl-2-pentenal		(van de Kant., 2013)	(+)
Decanal		(Caldeira et al., 2012)	(-)
Dodecanal		(Caldeira et al., 2012)	(-)
Nonanal		(Caldeira et al., 2012)	(-)
Pentadecanal		(Ibrahim et al., 2011)	(-)
1-(methylsulfanyl)propane	Alkane	(Gahleitner et al., 2013)	(+)
2,2,4-trimethylheptane		(Smolinska., 2014)	(-)
2,2-dimethylhexane		(Caldeira et al., 2011)	(+)
2,3,6-trimethyldecane		(Caldeira et al., 2011)	(+)
2,3,6-trimethyloctane		(Smolinska., 2014)	(-)
2,3-dimethyl heptane		(Dragonieri et al., 2007)	(+)
2,4,6-trimethyl-decane		(van de Kant., 2013)	(-)
2,4-dimethylheptane*		(Dragonieri et al., 2007)	(+)
2,4-dimethylheptane*		(Caldeira et al., 2011)	(-)
2,4-dimethyloctane		(Caldeira et al., 2011)	(+)
2,4-dimethylheptane		(Smolinska., 2014)	(+)
2,4-dimethylpentane		(Smolinska., 2014)	(+)
2,6,10-trimethyldodecane		(Smolinska., 2014)	(-)
2,6,10-trimethyl-dodecane		(Ibrahim et al., 2011)	(+)
2,6,11-trimethyl dodecane		(Dragonieri et al., 2007)	(+)
2,6,11-trimethyl-dodecane		(Ibrahim et al., 2011)	(+)
2-methyl-1-butene		(van de Kant., 2013)	(-)
2-methyl-decane		(Ibrahim et al., 2011)	(+)
2-methylhexane		(Smolinska., 2014)	(+)
2-methylpentane		(Smolinska., 2014)	(+)
3,6-dimethyldecane		(Caldeira et al., 2012)	(+)
3,7-dimethyl undecane		(Dragonieri et al., 2007)	(+)

4-methyloctane*		(Dragonieri et al., 2007)	(+)
4-methyloctane*		(Caldeira et al., 2011)	(-)
5,5-Dibutylnonane		(Ibrahim et al., 2011)	(-)
Alkane		(Dragonieri et al., 2007)	(+)
Decane		(Caldeira et al., 2011)	(+)
Decane		(Caldeira et al., 2012)	(+)
Dodecane		(Caldeira et al., 2011)	(+)
Dodecane		(Caldeira et al., 2012)	(+)
Ethane		(Paredi et al., 2000)	(+)
Ethane		(Larst��d et al., 2007)	(+)
Isododecane		(Caldeira et al., 2012)	(+)
Nonane		(Caldeira et al., 2012)	(+)
Octadecyne		(Gahleitner et al., 2013)	(+)
Octane		(Smolinska., 2014)	(+)
Pentane		(Olopode et al., 1997)	(+)
Tetradecane		(Caldeira et al., 2011)	(+)
Tetradecane		(Caldeira et al., 2012)	(+)
Tridecane		(Dallinga et al., 2010)	(+)
Undecane		(Dallinga et al., 2010)	(-)
1-dodecene		(Caldeira et al., 2012)	(-)
2,4,4-trimethyl-1-pentene		(van de Kant., 2013)	(-)
2,4-octadiene		(van de Kant., 2013)	(-)
2-ethyl-1,3-butadiene		(van de Kant., 2013)	(+)
3-methyl-1-butene		(van de Kant., 2013)	(+)
4-methyl-1-decene		(van de Kant., 2013)	(+)
Benzyl alcohol	Aromatic alcohol	(Ibrahim et al., 2011)	(+)
Benzoic acid	Aromatic carboxylic acid	(Dallinga et al., 2010)	(+)
1,2,3-trimethylbenzene	Aromatic hydrocarbon	(van de Kant., 2013)	(+)
1,4-dichlorobenzene		(Gahleitner et al., 2013)	(+)
1,4-dichloro-benzene		(van de Kant., 2013)	(-)
1,7-dimethylnaphthalene		(Gahleitner et al., 2013)	(+)
1-methylethynylbenzene		(van de Kant., 2013)	(+)
2-ethenylnaphthalene		(Smolinska., 2014)	(-)
3-(1-methylethyl)-benzene		(Dallinga et al., 2010)	(+)
4-ethyl-o-xylene		(Ibrahim et al., 2011)	(-)
b-Cymene		(Gahleitner et al., 2013)	(+)
Biphenyl		(Smolinska., 2014)	(-)
Ethylbenzene		(Gahleitner et al., 2013)	(+)
Limonene*		(Gahleitner et al., 2013)	(+)
Limonene*		(Smolinska., 2014)	(-)
Naphthalene		(van de Kant., 2013)	(+)
p-xylene		(Dallinga et al., 2010)	(-)
Toluene		(Dragonieri et al., 2007)	(+)
Acetic acid	Carboxylic acid	(Dragonieri et al., 2007)	(+)
Butanoic acid		(Dallinga et al., 2010)	(+)
Pentanoic acid		(van de Kant., 2013)	(+)
1,1',3',1''-Ter(cyclopentane)	Cycloalkane	(Dallinga et al., 2010)	(+)
1-pent-2-one	Ketone	(Dallinga et al., 2010)	(-)
2-butanone		(Ibrahim et al., 2011)	(+)
6-methyl-2-heptanone		(van de Kant., 2013)	(-)
6-methyl-5-hepten-2-one		(Caldeira et al., 2012)	(-)
Acetone*		(Dragonieri et al., 2007)	(+)
Acetone*		(Caldeira et al., 2011)	(-)
Acetone*		(Smolinska., 2014)	(-)
Acetophenone		(van de Kant., 2013)	(-)
2,6-Di-tert-butylquinone	Miscellaneous	(Ibrahim et al., 2011)	(-)
3,4-Dihydroxybenzonitrile		(Ibrahim et al., 2011)	(+)
Allyl methyl sulfide		(Ibrahim et al., 2011)	(+)
Ethyl 2,2-dimethylacetoacetate		(Ibrahim et al., 2011)	(+)
Ethyl 4-nitrosobenzoate		(Ibrahim et al., 2011)	(-)
Isoprene*		(Larst��d et al., 2007)	(-)
Isoprene*		(Dragonieri et al., 2007)	(+)
Isoprene*		(Caldeira et al., 2011)	(+)
Nitrocyclohexane		(van de Kant., 2013)	(+)
Terpinolene		(Ibrahim et al., 2011)	(+)

(+) indicates a positive association with asthma, whereas (-) indicates a negative association with asthma; * indicates conflicting outcome associations between different studies.







Begg's funnel plot with 95% confidence limits

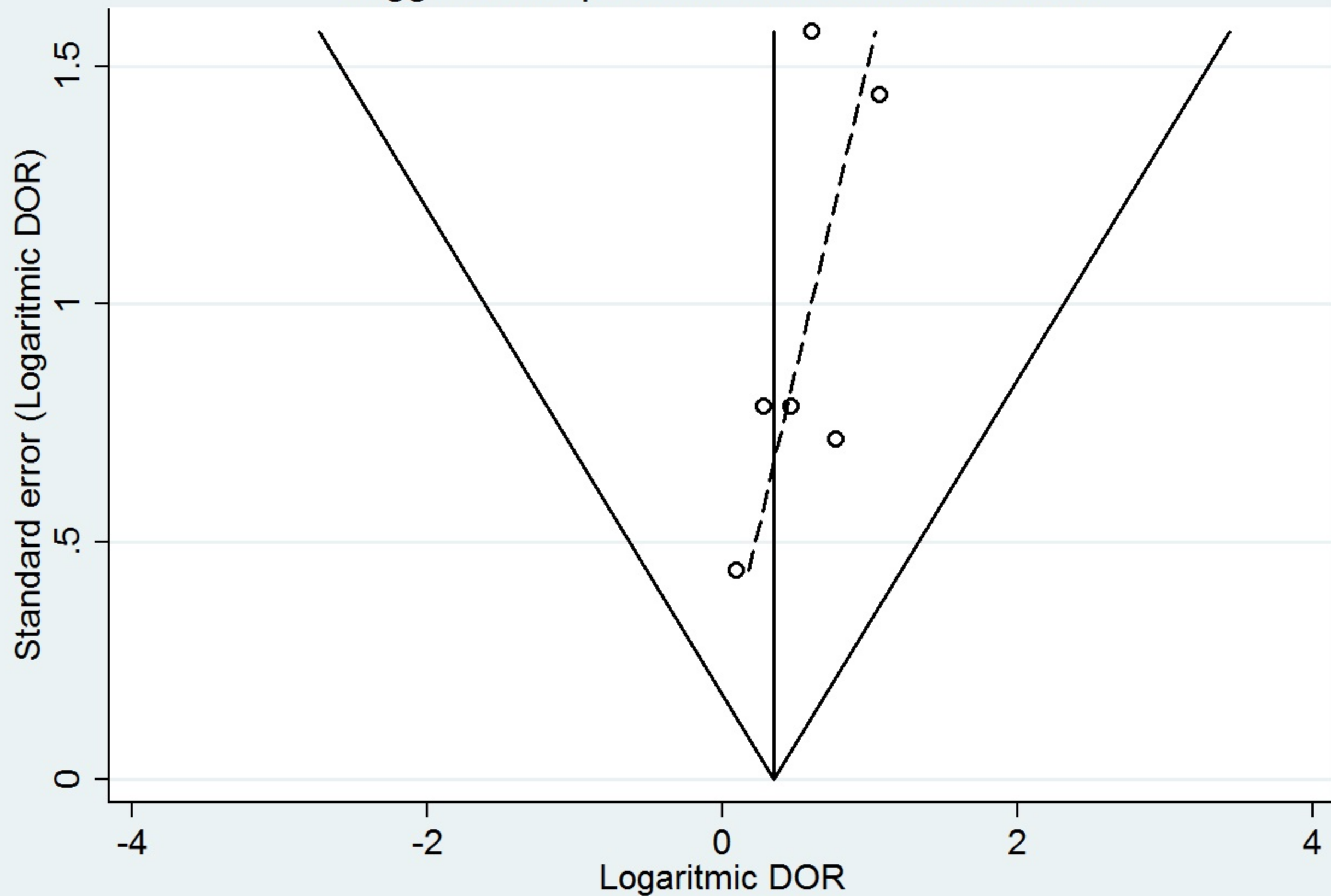


Table 1S - Quality of studies using QUADAS (Quality assessment of studies of diagnostic accuracy included in systematic reviews) [13]

Study	QUADAS items														Quality Score
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Olopade et al. [31]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Unclear	10
Paredi et al. [32]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Unclear	Yes	Yes	Unclear	9
Delfino et al. [23]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Larstad et al. [29]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Dragonieri et al. [24]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Fens et al. [26]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Dallinga et al. [22]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Montuschi et al. [30]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Unclear	10
Caldeira et al. [20]	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	9
Fens et al. [25]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
Ibrahim et al. [28]	No	Unclear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	10
Caldeira et al. [21]	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	9
Timms et al. [36]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Yes	10
Gahleitner et al. [27]	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Unclear	Unclear	Yes	Yes	Unclear	8
Robroeks et al. [33]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	13
Schivo et al. [34]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear	Yes	Yes	Yes	11
van der Schee et al. [37]	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Unclear	Yes	Yes	Yes	Yes	12
Smolinska et al. [35]	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Unclear	Unclear	Unclear	Yes	Yes	9

Table 2S – Sensitivity and specificity for each diagnostic test for asthma.

Diagnosis Method	Reference	Population		Sensitivity (%)	Specificity (%)
		Asthma	Non-asthma		
Spirometry	Schneider [39]	90	129	16	100
Fev1 < 80% predicted	Smith [40]	17	30	29	100
Fev1 < 90% predicted	Smith [40]	17	30	35	93
Bronchodilator reversibility > 12%	Tse [41]	1041	250	36	90
Sputum Eosinophils	Smith [40]	14	26	86	88
FeNO > 20 ppb	Smith [40]	16	28	88	79
FeNO >46 ppb	Schneider [43]	83	77	32	93
GINA questionnaire ≥ 1 symptoms	Lim [42]	164	516	98	9
GINA questionnaire ≥ 5 symptoms	Lim [42]	164	516	19	92
VOC profiles	Current study			87	86