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Adam M. Peel, Maxim Wilkinson, Ashnish Sinha, Yoon K. Loke, Stephen J. Fowler, Andrew M. Wilson

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Volatile organic compounds associated with diagnosis and disease characteristics in asthma – a systematic review

[1]Adam M Peel, [2]Maxim Wilkinson, [1]Ashnish Sinha, [1]Yoon K Loke, [2]Stephen J Fowler, [1]Andrew M Wilson.

Author affiliation: [1]Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ. [2]Division of Infection, Immunity and Respiratory Medicine, School of Biological Sciences, The University of Manchester; Manchester Academic Health Science Centre and NIHR Manchester Biomedical Research Centre, Manchester University Hospitals NHS Foundation Trust, Manchester, UK.

Conflicts of interest: none **Corresponding author:** Adam M Peel; a.peel@uea.ac.uk

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Abstract

Background Metabolomics refers to study of the metabolome, the entire set of metabolites produced by a biological system. The application of metabolomics to exhaled breath samples - breathomics - is a rapidly growing field with potential application to asthma diagnosis and management.

Objectives We aimed to review the adult asthma breathomic literature and present a comprehensive list of volatile organic compounds identified by asthma breathomic models.

Methods We undertook a systematic search for literature on exhaled volatile organic compounds in adult asthma. We assessed the quality of studies and performed a qualitative synthesis.

Results We identified twenty studies; these were methodologically heterogenous with a variable risk of bias. Studies almost universally reported breathomics to be capable of differentiating - with moderate or greater accuracy - between samples from healthy controls and those with asthma; and to be capable of phenotyping disease. However, there was little concordance in the compounds upon which discriminatory models were based.

Conclusion Results to-date are promising but validation in independent prospective cohorts is needed. This may be challenging given the high levels of inter-individual variation. However, large-scale, multi-centre studies are underway and validation efforts have been aided by the publication of technical standards likely to increase inter-study comparability. Successful validation of breathomic models for diagnosis and phenotyping would constitute an important step towards personalised medicine in asthma.

1. Introduction

Asthma is a chronic disorder of the airways characterised by variable airflow obstruction commonly accompanied by inflammation. It is affects an estimated 339 million people worldwide (1), and generates a health service spending of approximately £1 billion *per annum* in the UK alone (2). Management of the condition is informed chiefly by symptoms and measures of airway calibre such as peak expiratory flow.

The identification and or quantification of metabolites offers an alternative route to diagnosis and disease management. Metabolites are low molecular weight (typically defined as <1500 amu[1]) organic and inorganic chemicals produced by cellular processes (including pathophysiological processes). The term 'metabolome' refers to the entire set of metabolites associated with a biological system(3). Change in the metabolome reflects change in underlying cellular activity(4) - disease pathophysiology can alter the relative concentrations of metabolites produced, or produce metabolites which are absent in health(5) - metabolomics is thus gaining traction as a means of biomarker discovery in disease(6).

Volatile organic compounds (VOCs) are carbon-based, low molecular weight compounds, volatile at room temperature. The study of endogenous VOCs generated by metabolic processes within the body and exhaled on the breath is commonly referred to as breathomics (7). Such studies produce data on a large number of compounds permitting inductive, hypothesis-generating approaches in which data are interrogated in order to identify disease-induced metabolomic permutations(8) without the prior identification of a candidate marker. This approach has been applied to many diseases including asthma.

Rufo et al (9) conducted a systematic review of the asthma breathomic literature in 2014, identifying 18 studies which reported on diagnostic accuracy. In a meta-analysis of six studies they calculated a pooled area-under-the-curve (AUC) value of 0.94. This figure needs to be interpreted with caution however as all but one of the included studies compared established-treated disease with non-disease (rather than testing diagnostic

[1] AMU = atomic mass unit

accuracy in those with a suspicion of disease) and the meta-analysis pooled diagnostic models which were comprised of differing VOCs. In addition, a mixture of adult and paediatric studies were included; age has since been identified as a factor which should be controlled for (10).

Interest in the field has continued to grow and a number of breathomic asthma studies have since been published. Neerincx et al (11) reviewed paediatric asthma breathomics, to which a systematic search has been appended (12); and recent reviews have provided an overview of metabolomics in exhaled breath (13) and across different biomediums (14, 15). In this study we aim to systematically review the literature on adult asthma breathomics - including studies of diagnosis and of disease characteristics - providing a comprehensive list of significant VOCs identified to-date.

2. Methods

A study protocol was developed in line with Prisma-P guidelines and registered with the International Prospective Register of Systematic Reviews (PROSPERO) (registration number CRD42017082727). The primary objective of the review was to ascertain the classification accuracy of VOC models for asthma diagnosis, phenotyping, and disease control. The secondary objectives were to identify the study methods used and to compile a list of those VOCs identified by studies as significant for use in future validation efforts.

In order to identify relevant literature the following strategy was used:

SEARCH TERMS

The following key words and MeSH terms were used - metabolomics, breathomics, exhaled breath, breath test, volatile organic compound* and asthma. The search string was optimised for each database; an example may be found in the appendix.

SEARCH STRATEGY

Inclusion criteria – Physician diagnosed asthma or asthma diagnosis according to recognised guidelines; clinical studies published in full; primary data; VOCs in exhaled breath studied (by any collection or analytical method).

Exclusion – Reviews; editorial; secondary data; studies of exhaled breath condensate; non-asthma studies; studies published in abstract form only.

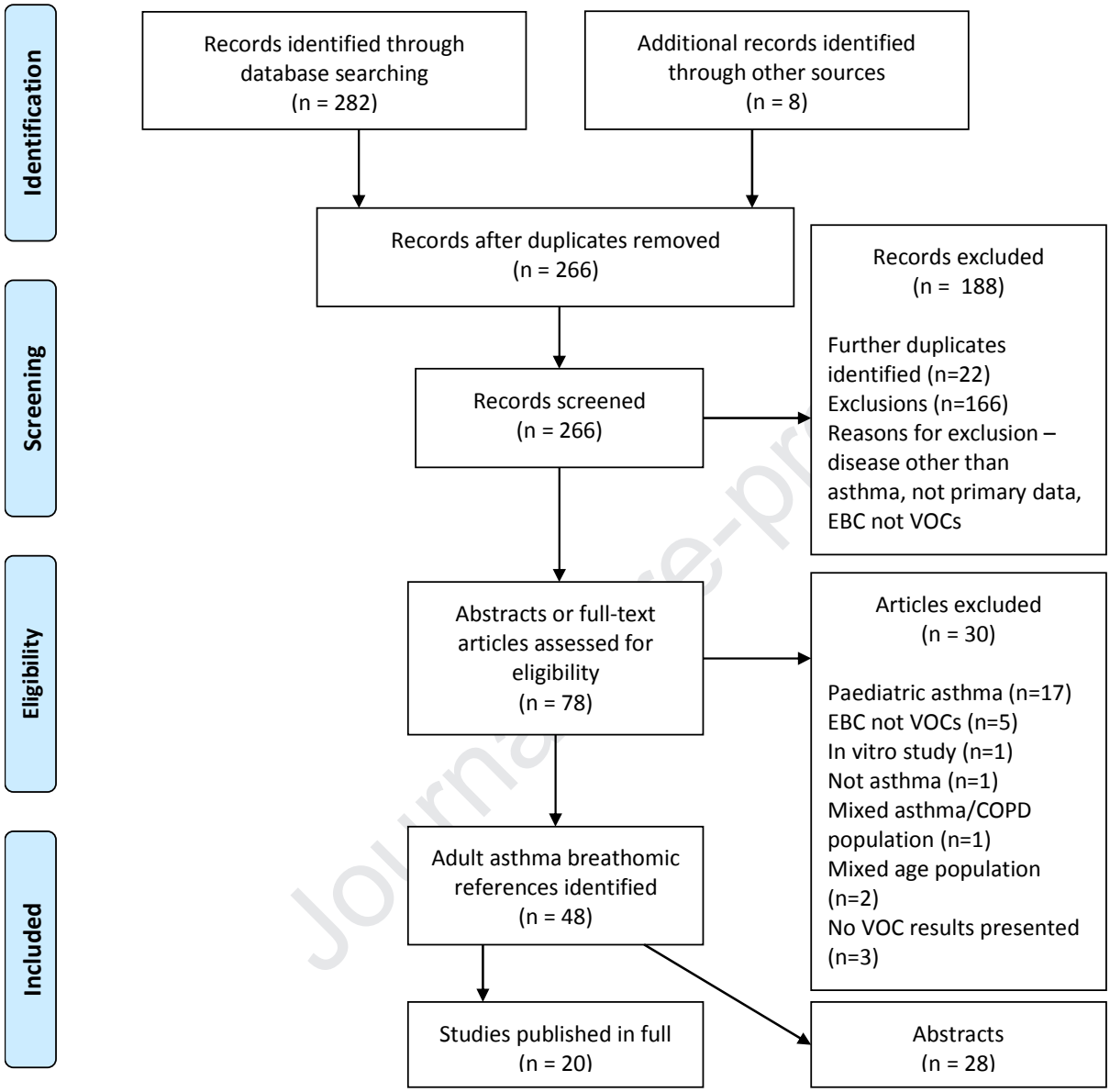
SEARCHES

PubMed; Medline (including Embase and OVID medline)

In addition, the references from Rufo et al's review; from the researchers' own reference libraries; and from the reference lists of included articles were searched. Researchers working in the field were asked to highlight papers they were aware of. The searches were conducted independently by two reviewers (AS and AP) on the 1st June 2017 and updated in November 2018.

Two reviewers (AP & AS) screened titles and abstracts for inclusion, resolving discrepancies through discussion with a third reviewer (MW). In total two hundred and ninety records were identified; this was reduced to two hundred and sixty six after removing duplicates. On screening abstracts and/or full texts, forty eight citations of adult asthma breathomic studies were identified, of which twenty eight were abstracts and twenty full journal articles. A PRISMA diagram (figure 1) describes the screening and selection process. Quality assessment was undertaken using the CASP diagnostic checklist (16) (see appendix table A4). Data extraction and quality assessment was independently conducted by two researchers (AP & MW).

Figure 1 – Prisma diagram



3. Results

Twenty journal articles met the criteria for inclusion (table 1). Fifteen of these compared VOCs in asthma and healthy controls (17-30), of which ten reported diagnostic accuracy (18-20, 22, 23, 26-30). Four studies reported on the ability to differentiate between asthma and COPD, one lung cancer and one allergic rhinitis (19, 28-31). Seven studies examined the ability to discriminate between phenotypes (18, 20, 26, 31-34) (two were cluster analyses), while three reported on levels of disease control or activity (20, 24, 32).

We included one paper (35) which failed to meet Rufo *et al*'s inclusion criteria (due to the absence of a comparator group) and excluded one (36) which used exhaled breath condensate as its sample medium. We included one paper (17) reporting on volatile organosulfides and excluded one study which recruited both adults and adolescents (37, 38).

Results were typically given as accuracy rates for the correct classification of samples - the area under the curve for receiver operator characteristics (AUROC), cross-validation values (CVV) or correlation coefficients. Table 1 displays the list of full publications along with results; Table A2 (appendix) summarises study design and breath sampling methods; while Table A3 (appendix) details the data processing and statistical methods used. There was heterogeneity in all aspects of study methodology, from sample collection through to statistical analysis. The majority of GC-MS studies used principal component analysis (PCA) in their statistical analysis however approaches to data pre-processing, discriminatory analysis and cross-validation varied. Given the methodological heterogeneity and variety of compounds upon which breathomic models were based, meta-analysis was deemed inappropriate; instead we present a narrative synthesis of study findings.

Table 1 – Included studies and results

Study	Country	Year	Population	Result
Awano **et al (17)**	Japan	2011	Asthma = 7 Non-asthma = 386 (both groups age range 60-65)	Asthma and the presence of dimethyl sulphide > 1.0nmol L^{-1} in mouth air Crude OR 7.4 (95%CI 1.4-39.0); Adjusted OR 6.9 (95% CI 1.1-44.2)
Brinkman et al (32)	Netherlands	2017	Asthma (partly controlled, mild-moderate) = 23	Baseline *vs* loss of control: eNose classification accuracy - 95%, GC-MS 68% Loss of control vs recovery: eNose classification accuracy - 86%, GC-MS 77% Significant association between exhaled metabolites and sputum eosinophils: Pearson r≥0.46, P<0.01
Dragonieri et al (18)	Netherlands	2007	Asthma (mild-severe) = 20 Controls = 20	Asthma vs controls: CVV 90-100%; M-distance 2.77-5.32. Mild vs severe asthma: CVV 65%; M-distance, 1.23.
Dragonieri et al (30)	Italy	2018	Asthma & allergic rhinitis (AAR): training set = 14; validation set = 7. Allergic rhinitis only (AR) and healthy controls (HC) as above	Training set AAR vs AR: CVA=86%, p<0.01; AUROC 0.93 AR vs HC: CVA=82%, p<0.01; AUROC 0.92 AAR vs HC: CVA=75% ,p<0.05; AUROC 0.87 Validation set AAR vs AR: CVA=83%, p<0.01; AUROC 0.92 AR vs HC: CVA=77%, p<0.01; AUROC 0.87 AAR vs HC: CVA=67 , p<0.05; AUROC 0.77
Fens et al (19)	Netherlands	2009	Asthma (mild-severe, persistent) = 20 COPD = 30 Controls = 40	Asthma vs COPD: accuracy 96%; p< 0.001 Asthma vs controls: accuracy 93 – 95%; p< 0.001
Fens et al (31)	Netherlands	2011	Asthma (stable) = 60 (21 w/ fixed airways) COPD = 40	Asthma vs COPD: accuracy 83-88%; p<0.001 AUROC 0.93-95 (95% CI 0.84–1.00); sensitivity 85-91%, specificity 90% Fixed asthma vs classic asthma: accuracy 58%; p=0.23; AUROC 0.68 (95% CI 0.50-0.85); sensitivity 60%, specificity 67%.
Ibrahim et al (20)	UK	2011	Asthma (mild-moderate) = 35 (sputum for phenotyping n=18) Controls = 23	Asthma vs controls: accuracy = 86% (PPV 0.85, NPV 0.89) Sputum eosinophilia: AUROC 0.98 (95% CI = 0.91-1.00; sensitivity = 0.75, specificity = 0.90). Sputum neutrophilia: AUROC 0.90 (95% CI = 0.76-1.00; sensitivity = 0.80, specificity = 0.75). Uncontrolled asthma: AUROC 0.97 (95% CI = 0.93-1.00; sensitivity = 0.89, specificity = 0.88).

Larstad et al (21)	Sweden	2007	Asthma (stable) = 13 Controls = 14	Baseline isoprene lower in asthmatic subjects (113 ppb vs 143; p = 0.03) No significant difference in baseline ethane, pentane, or nitric oxide.
Lazar et al (39)	Netherlands / Hungary	2010	Asthma (stable) = 10	Reduction in airway calibre was not associated with an altered eNose breath profile
Meyer et al (22)	Switzerland	2014	Asthma (mixed severity) = 195 Controls = 40	Asthma vs controls: accuracy 99% (sensitivity 100%, specificity 91%) Inter-cluster or cluster vs control accuracy: 82% – 95% Linear discriminant analysis for correct classification of all clusters and controls = 43%.
Montuschi et al (23)	Italy	2010	Asthma (mild, intermittent) = 27 Controls = 24	Asthma vs controls: diagnostic accuracy 88%
Olopade et al (24)	USA	1997	Asthma (acute exacerbation) = 12 Stable asthma = 11 Controls = 17	Significantly higher exhaled pentane levels during acute exacerbation ($p < 0.05$). No significant difference in exhaled pentane levels between stable/controlled asthma and healthy controls ($P>0.05$).
Paredi et al (25)	UK	2000	Asthma (steroid naive) = 14 Asthma (steroid treated) = 12 Controls = 14	Ethane in untreated asthmatics > healthy controls or ICS treated asthma ($p<0.05$) In untreated asthma, exhaled ethane correlated with levels of nitric oxide exhalation ($p<0.05$); those with FEV_1 <60% predicted had higher levels of ethane than those >60% ($p<0.05$).
Plaza et al (33)	Spain	2015	Asthma (persistent) = 52	Eosinophilic vs neutrophilic: accuracy 73%; P=0.008; AUROC 0.92 Eosinophilic vs paucigranulocytic: accuracy 74%; P=0.004; AUROC 0.79 Neutrophilic vs paucigranulocytic: accuracy 89%; P=0.001; AUROC 0.88
Reynolds et al (35)	UK	2014	Asthma & controls = 17	Discriminant analysis of asthma vs controls not reported
van der Schee et al (26)	New Zealand	2013	Asthma (mild-moderate) = 25 Controls = 20	Asthma vs controls: AUROC 0.77 (95%CI = $\pm$ 0.14; P = 0.002) Steroid responsiveness: AUROC = 0.88 (95% CI = $\pm$ 0.16; P = 0.008)
van der Schee et al (27)	Europe	2013	Asthma (U-BIOPRED, severity not specified) = 10 Controls = 10	Asthma vs control: eNose AUROC = 0.77 (95% CI = 0.22), p = 0.050 GM-MS AUROC = 0.84 (95% CI = $\pm$ 0.17), p = 0.011
Timms, Thomas & Yates (28)	Australia	2012	Asthma (GINA step 1-3) = 20 COPD = 17 Controls = 7	Asthma vs controls: eNose accuracy 70%, p=0.047 Asthma vs COPD: eNose accuracy 70%, p=0.019

de Vries et al (29)	Netherlands	2015	Asthma (mild to severe) = 37 Controls = 45 COPD = 31 Lung cancer = 31	Asthma vs COPD: accuracy 81%, AUROC 0.81 (95%CI ± 0.09), p=0.001 Asthma vs controls: accuracy 87%, AUROC 0.94 (95%CI ± 0.15), p<0.001 Asthma vs lung cancer: accuracy 68%, AUROC 0.71 (95%CI ± 0.09), p=0.045
de Vries et al (34)	Netherlands	2018	Asthma (mild to severe)= 278 COPD = 157. Training set=321; validation set 114.	Training set Clusters differing in ethnicity (p=0.01); systemic eosinophilia (p=0.02); neutrophilia(p=0.03); BMI (p=0.04); FeNO (p<0.01), atopy (p<0.01); exacerbation rate(p<0.01). Regression models predictive of eosinophilia (R^2=0.58); neutrophilia (R^2=0.41) Validation set Predictive models confirmed by validation set with the exception of BMI and neutrophilia

AUROC – area under the curve for receiver operator characteristics
CVA – cross-validation accuracy
CVV – cross-validation value
NPV – negative predictive value (percentage of true negatives)
PPV – positive predictive value (percentage of true positives)
SD – standard deviation

3.1 Quality assessment

We excluded studies published only in abstract form due to the inability to fully assess inclusion criteria, study quality and risk of bias. However, the exclusion of such publications creates a vulnerability to selective dissemination bias. Results from these abstracts can be found in table A4 (appendix).

Twenty studies were published in full and their quality assessed using the CASP checklist (see table A5, appendix).

Examining predictive models for their diagnostic test accuracy in asthma, there is no single valid and reliable test against which the new diagnostic can be measured. In a recent study of patients with a primary care diagnosis of asthma (40) the diagnosis could not be supported in 33% of cases; furthermore this is not a novel finding (41-43). The matter is further complicated by the heterogenous nature of the disease; inflammation is not an essential component of the disease, thereby limiting the use of existing inflammatory biomarkers. We included studies with diagnoses made by a physician or according to recognised clinical guidelines while accepting that, as a reference standard, this is likely to fall short of the assumed 100% accuracy. One study recruited from a severe asthma clinic with physician diagnosis inferred rather than explicitly stated (35).

Studies of diagnostic test accuracy should ideally examine the population in which the test would be employed - those with a clinical suspicion of disease or diagnostic uncertainty. The majority of studies compared healthy controls against participants with an existing asthma diagnosis (and commonly receiving treatment); such results are likely to over-estimate diagnostic accuracy and might perhaps better be characterised as hypothesis-generating or proof-of concept studies. In the majority of studies it was not clear that a random or continuous sample of patients had been used; where there is selection of participants there is risk of inclusion bias leading to over-estimation of test accuracy.

For those studies reporting on the occurrence of symptoms or loss of asthma control, the time between symptom reporting and VOC-sampling is important. The inherent variability of the disease and potential for symptoms to change means that any delay between reporting and measuring could lead to inaccuracy or obfuscate a relationship. Furthermore factors such as the time of day should be considered as asthma is a circadian disease and related VOCs display diurnal variation (44). Timing in studies was frequently implied rather than explicitly stated.

In the majority of studies it was not possible to say that index tests were conducted and interpreted without knowledge of the reference standard; blinding was rarely mentioned. Nonetheless the risk of bias is low; analytical methods such as gas chromatography-

mass spectrometry, and statistical methods such as PCA are hard to corrupt. Some risk nevertheless exists as storage time and conditions prior to processing have the potential to influence outcomes; in addition statistical methods for discriminant analysis are prone to over-fitting and require validation

Study participants were generally well described with the exception of body mass index (BMI) and ethnicity. BMI may affect markers of oxidative stress (45) and VOCs (10, 34); and evidence exists of ethnic differences in both pulmonary function (46, 47) and breath profiles (34, 48).

3.1.1 Technical validity

3.1.1.1 Breath sampling

There are two key methodological issues relating to sample collection a) that of how to best deal with ambient VOCs; and b) how to collect and store samples prior to analysis.

Ambient VOCs

A consensus method for dealing with ambient, environmental VOCs has been outlined by European Respiratory Society (ERS) technical standards (49). This recommends 1) parallel sampling of ambient air for background correction using alveolar concentration gradients, and 2) the use of VOC-filtered air. More detailed discussion of these issues can be found within the technical standards themselves (50). Of the included studies, ten measured ambient air VOCs; the way these data were utilised varied.

Exogenous VOCs in breath can be minimised through the use of filtered air but inhaled VOCs may be retained for some time and wash-out periods vary depending on the VOC in question. Wallace et al (51) estimate that some retention times may be as long as 3 days; and breathing synthetic air for 30 minutes was found to reduce but not eliminate ambient VOCs (52). If VOC analysis is to be clinically useful the period of time for which filtered air is breathed prior to assessment needs to be practicable; complete elimination of the 'exposome' is unlikely. Furthermore, ambient VOCs may be absorbed transdermally. In the case of some semi-volatile or aerosolised compounds, dermal uptake may be up to four times higher than inhalation (53, 54) however the relationship between dermally absorbed VOCs and their exhalation is largely unstudied. Current recommendations offer a pragmatic rather than a perfect solution; twelve of the included studies used filtered air.

Sampling methods

Two main approaches have been taken to the collection of samples prior to analysis – 1) the use of impermeable bags, 2) the use of sorbent materials.

Numerous studies have examined the properties of gas sampling bags (55-59). Beauchamp et al (58) summarise the drawbacks of this method which include material emissions, diffusion of VOCs (into or out of the bag), adsorption effects, reactive chemistry and the production of artefacts. While VOC losses have been described as within acceptable levels (57, 58) this could nonetheless result in those VOCs present at very low concentrations becoming undetectable; moreover the differential decay rates reported across VOCs could change relative concentrations over time.

Breath samples collected in impermeable bags can be concentrated using stainless steel tubes packed with adsorbent material. These may be stored (60, 61) before desorption and analysis; studies suggest storage at room temperature for fourteen days or less results in acceptable sample retention (27). Direct sampling onto sorbents is also possible. In both cases a decision has to be made as to which adsorbent(s) to use. Tenax – a porous polymer - is used in many of the studies; its hydrophobicity is suited to humid breath samples and it can adsorb a wide range of VOCs (62). Its ability to capture low mass VOCs is however limited and compound breakthrough may be an issue. Dual-bed sorbents are an attempt to combat these issues while also limiting the quantity of water adsorbed. If a deductive approach is used - looking for specific compounds - the appropriate sorbent(s) need to be selected. For inductive approaches there must be recognition that sorbent selection limits the range of VOCs collected; disease-related VOC permutations may go undetected if outside of this range. The stability of adsorbed samples is time and temperature dependant (63) ; of the six studies concentrating samples on sorbent tubes, two did not report the duration of storage, and three either did not report the temperature or stored samples at room temperature.

In addition to the storage of samples there is also variation in the nature of the sample. The majority of included studies using Tedlar bags collected mixed expiratory air by way of single or multiple exhalations. However, if the lung metabolome is the exclusive target of investigation there will be sample contamination from the upper respiratory tract. The importance of breath fraction to asthma breathomics is yet to be established. It is possible to quantify breath samples either by collection time or volume but a standard approach to this has yet to be established (49).

Ibrahim et al (20) used a novel device with a facemask and pressure sensor to selectively sample air from the lower respiratory tract directly onto sorbent tubes. This approach has since been commercialised in a device from Owlstone (Cambridge, UK). Fifteen of the studies used a collapsible reservoir (Tedlar or Nalophan bag) while one used a syringe.

3.1.1.2 Sample analysis

A range of methods have been applied to the analysis of breath samples including various forms of mass spectrometry; some offline - such as gas chromatography-mass spectrometry (GC-MS) - and others online - such as ion mobility spectrometry (IMS), proton transfer mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS) and field asymmetric ion mobility spectrometry (FAIMS). A full review of these methods may be found in Beale et al (62) and elsewhere.

Due to its sensitivity and selectivity, GC-MS has become the standard method by which to characterise the human metabolome (64), including that detectable via the breath (60) (although alternatives such as FAIMS may be equally efficacious(65)). GC-MS analysis requires a high level of technical expertise and the data produced needs extensive pre-processing prior to statistical analysis. This approach has been complemented by the electronic nose (eNose); chemical cross-reactive sensor arrays (66) over which breath samples may be passed inducing detectable changes in the sensor material, thereby characterising the relative concentrations of VOCs present (67). eNoses lack the ability of MS to identify VOCs - thereby precluding their use for biomarker discovery - but require less data pre-processing; less technical expertise; and their ability to produce real-time data holds promise in point-of-care diagnostics (see table 2).

Table 2 - eNose vs GC-MS

	eNose	**GC-MS**
Approach	Pattern recognition	Compound identification
Sensitivity and selectivity	Moderate	High
Insight into pathophysiological pathway	No	Yes
Real time use	Yes	No
Physical size	Small, portable	Large, immovable
Data pre-processing required	Some	Extensive
Technical expertise required for sample analysis	No	Yes

Of the studies in this review, nine used an eNose; seven mass spectrometry; and four a combination of the two.

3.1.2 Statistical validity

A range of statistical techniques may be used in the identification of disease-induced metabolomic permutations; these have been comprehensively reviewed elsewhere (7,

13, 62). Although strategies for avoiding false discoveries (68) and minimum reporting standards for data analysis in metabolomics (69) have been published, there is no standard statistical framework for analyses (70); the ERS technical standards are not prescriptive in this respect. As shown in table A5, approaches to data processing and pre-processing varied, both in the techniques used and the extent to which they were reported.

The majority of papers undertook inductive / untargeted analyses in which there was no *a priori* identification of compounds. Such analyses when applied to large data sets are prone to over-fitting and the resultant VOC models require validation; without this the performance of the model cannot purport to be accurate. Internal cross-validation is one of the methods commonly applied however the rigour this imparts may be limited by the small sample size of many of the included studies. Ten studies describe undertaking some form of internal validation such as leave one out cross-validation or boot strapping; only three studies used an external validation set (30, 31, 34).

Five studies (17, 21, 24, 25, 27) conducted targeted analyses based on compounds previous identified as associated with asthma or inflammation; a deductive approach not associated with the aforementioned statistical challenges. Although these findings provide support for the utility of certain VOCs in asthma breathomics, they were not an attempt to provide external validation to any one specific model. Furthermore, although Awano et al (17) specified compounds of interest *a priori*, their relationship with clinical variables (including asthma) was examined by way of post-hoc analyses and vulnerable to the risk of false discovery.

In studies other than those using an eNose, compound identification is possible. There are a number of databases which may be used including the Pacific Northwest National Laboratory (PNNL), the National Institute for Standards and Technology (NIST), Metlin, or in-house custom libraries constructed using reference standards. The extent to which use of different libraries might limit comparability is unclear; however, Sharpe et al (71) compared PNNL with NIST and reported that for all but one of the twelve compounds they compared, there was agreement between databases to within the level of experimental uncertainty. Few papers reported the libraries used for compound identification and none the match-percentages for compound identification. The chemical analysis working group metabolomics standards initiative (MSI) published proposed minimum reporting standards which include both data pre-processing and metabolite identification (72). Implementation of such reporting standards would allow identification of studies at risk of spurious candidate marker identification.

3.1.3 Clinical validity

Two potential confounders were common across studies – medication and study location. Participants with asthma were frequently taking medication such as inhaled corticosteroids (ICS) or β_2-agonists which healthy controls were not; any observed between-group difference in exhaled VOCs might be due to medication metabolites rather than disease-related changes in biochemical pathways. The extent to which this was addressed in studies varied, likely due to the emergent nature of this field of research and the inclusion of small-scale, proof-of-concept studies. Evidence regarding the extent to which medication might act as a confounder is unclear (18, 26, 45, 73) but exhaled VOCs have been reported to be capable of identifying those asthma patients in which oral corticosteroid and salbutamol urinary metabolites were present (74).

The second potential confounder was background bias. In many studies the site of recruitment differed between controls and those with asthma but it was unclear where breath sampling took place. de Vries et al (29) report no significant difference between samples from different medical sites (p=0.89); however the ambient VOC profile of hospitals may differ greatly from other locations (75) and a systematic difference in location could be the cause of sample differentiation, rather than disease metabolites. The application of background air subtraction and use of filtered air constitute an attempt to negate this but as discussed in section 3.1.2.1 there are limitations.

Other potential confounders such as smoking history, age, and gender (45) were not always well matched between groups (see table 2).

Asthma severity was frequently stated but where it was not, medication-use was rarely reported with sufficient detail to make an assessment of severity. Many studies contained a mixture of asthma severities; and while spirometry results were commonly presented measures of asthma control were not.

3.2 Qualitative synthesis

3.2.1 Asthma Diagnosis

The ability of breathomics to differentiate between those with asthma and healthy controls was examined by fifteen studies. These models reported moderate-to-excellent discriminative ability, citing CVVs of 90-100% (18), classification accuracies of 86%(20) to 99%(22), and AUROCs of between 0.70 (28) and 0.94 (29). It should be emphasised that these accuracy rates are based on populations with diagnosed disease; the studies were examining the difference in VOC profiles between healthy controls and those with established, treated, and frequently long-standing asthma. The diagnostic performance

of VOC models in a real clinical population with undiagnosed, untreated respiratory symptoms of relatively recent onset may be very different.

In many studies the risk of sampling bias was unclear; and in some studies there was a risk of confounding, for example large differences in the average age of groups (26, 29). While we included studies with physician diagnosed asthma the standard to which this was reported and conducted varied between studies. It is also worth noting that several studies used populations of mixed asthma severity; it is unlikely that breathomic models would be applied homogenously across such a population.

Five studies conducted a targeted analysis of compounds. In the case of pentane, Olopade et al (24) report significantly higher levels during acute asthma attack but both Olopade and Larstad et al (21) report no significant difference between controlled-asthma and healthy controls. Paredi et al (25) report significantly higher levels of ethane in untreated asthma compared with treated disease or healthy controls. They do not comment on treated-asthma versus healthy controls but Larstad et al (21) found no significant difference (in ethane levels) between a largely steroid-treated controlled asthma group and healthy controls. Larstad et al do however report a significantly lower level of isoprene in those with asthma. Awano et al cite an adjusted odds ratio of 6.9 (95% CI 1.1-44.2; $p<0.05$) for asthma in the presence of dimethyl sulphide; while van der Schee report AUCs of 0.79-0.84 ($p<0.05$) for the differentiation of asthma from controls using a five-compound model.

Ten studies performed untargeted analyses producing diagnostic models for the differentiation of asthma from healthy controls. Fewer studies aimed to differentiate between asthma and other respiratory diseases; four examined COPD and asthma reporting classification accuracies of between 70% and 96% (19, 28, 29, 31); one differentiated between asthma and allergic rhinitis reporting an AUROC of 93% (30); while another examined lung cancer and asthma, reporting a classification accuracy 68% (29). In all but the allergic rhinitis study there was a large difference in average age between the asthma and the other respiratory disease group.

3.2.2 Asthma Phenotypes

Eight studies examined asthma phenotypes including sputum cell type, steroid responsiveness, disease severity and airway reversibility.

Both Plaza et al (33) and Ibrahim et al (20) constructed models differentiating between eosinophilic, neutrophilic and paucigranulocytic phenotypes, with classification accuracies of 73% to 74% (33), and AUROCs of 0.79 (33) to 0.98 (20). Differentiation was likely not due to differences in ICS use (which were similar between groups in Plaza et al), but it was not reported whether there were other systematic between-group differences in

treatment regime. Brinkman et al report two VOCs significantly correlated with sputum eosinophilia (correlation coefficients of $r \geq 0.46$ & 0.47 ($P<0.01$)) but did not find any such correlations for sputum neutrophilia (32).

de Vries et al (34) examined a combined asthma and COPD population in a large multi-centre study. They identified clusters differing in eosinophilia ($p=0.02$), neutrophilia ($p=0.03$), atopy ($p<0.01$) and exacerbation rate ($p<0.01$). Further clusters based on ethnicity ($p=0.01$) and exhaled nitric oxide ($p<0.01$) were identified.

Van der Schee et al (26) examined eNose results for the prediction of steroid responsiveness, reporting an AUROC of 0.88 and greater accuracy than either sputum eosinophil count or FeNO. For the differentiation of mild from severe asthma Dragoneiri et al (18) report a CVV of only 65% (M-distance, 1.23). Similarly Fens et al (31) report an accuracy of just 58% (AUROC 0.68) for the differentiation of fixed and classic asthma.

Meyer et al (22) conducted a cluster analysis of both VOC data and clinical parameters. While VOC profiles were able to differentiate between some clinical clusters with good levels of accuracy, they also reported distinct clinical clusters with similar VOC profiles, and distinct VOC clusters with similar clinical characteristics.

3.2.3 Loss of asthma control

Four of the included studies examined some aspect of asthma control. Brinkman et al (32) conducted a prospective medication-withdrawal study. Classification accuracy for baseline versus loss of control - as measured by the asthma control questionnaire (ACQ) - was 95% using an eNose and 68% by GC-MS; loss of control versus recovery was 86% (eNose) and 77% (GC-MS). Ibrahim et al (20) using GC-MS report an AUROC of 0.96 for the identification of loss of control; and Olopade et al (24) report significantly higher levels of pentane during exacerbation compared to recovery. It is unlikely that the observed differences in breath profiles are due to changes in airway calibre - Lazar et al (39) undertook bronchial challenge testing on participants with stable asthma and reported no changes associated with bronchoconstriction.

3.2.4 Discriminant compounds

Nine of the included studies report on compound identities (presented in table 2). A total of seventy six compounds were cited as significant. Of these, nine were reported in more than one paper - 2,4-dimethylheptane; 2,6,10-trimethyldodecane; 2,6,11-trimethyldodecane; acetone; benzene; ethane; isoprene; phenol; and toluene - and two – acetone and isoprene - were reported by three studies. The models constructed by any given study were thus comprised of compounds largely or entirely absent from the models presented by other studies. Moreover, it was not always clear in which direction the compounds differed. In the case of isoprene, Dallinga et al and van der Schee (18,

27) found it to be elevated in asthma, while Larstad et al (21) report it to be lowered. Despite the lack of concordance between studies, where attempts have been made to validate previous models the results have been positive. van der Schee (27) used five compounds previously linked to asthma (acetone, isoprene, carbon disulphide, toluene and 1-propanol) and report an AUC of 0.79-0.84 ($p<0.05$).

Where compounds have not been identified but validation has been undertaken results have been similarly positive. Fens et al (31) report a phenotyping accuracy of 83-88% in an external validation exercise; Montuschi et al (23) validated their data in a distinct test set, reporting a diagnostic accuracy of 87.5%; de Vries et al (34) found the majority of clusters identified in their training set to be confirmed in an independent validation set; and Dragonieri (30) report diagnostic AUCs of 77- 92% in an external validation exercise.

Table 3 – Volatile organic compounds

Study	Discriminant compounds identified	Compound type	Direction of difference in asthma group (if appropriate)	Differentiated groups	Differences between case and control groups
Awano et al **(17)**	Dimethyl sulphide	Sulfur and nitrogen compounds	+	Asthma vs non-asthma	Not reported
Brinkman et al **(32)**	Acetonitrile	Sulfur and nitrogen	+	Control vs loss of control	NA – longitudinal study
	Methanol	Alcohol	+		
	Bicyclo[2.2.2]octan-1-ol.4-methyl	Alcohol	+		
	Acetonitrile	Sulfur and nitrogen	+	Sputum eosinophilia	Not reported
	Bicyclo[2.2.2]octan-1-ol.4-methyl	Alcohol	+		
Dragonieri et al **(18)**	Isopropanol	Alcohol	+	Asthma vs controls	Attempts to match age and gender and disease severity. Differences in FEV_1 % predicted and FVC % predicted.
	2,3-dimethylheptane	Alkane	+		
	2,4-dimethylheptane	Alkane	+		
	2,6,11-trimethyldodecane	Alkane	+		
	3,7-dimethylundecane	Alkane	+		
	4-methyloctane	Alkane	+		
	Alkane	Alkane	+		
	Toluene	Aromatic	+		
	Acetic acid	Acids & esters	+		
	Acetone	Ketone	+		
	Isoprene	Terpenoids	+		
Ibrahim et al **(20)**	2,6,10-trimethyldodecane	Alkane	+	Asthma vs controls	Closely matched in age, gender, and BMI. Differences in FEV_1, FVC & and FEV_1/FVC.
	2,6,11-trimethyldodecane	Alkane	+		
	Benzyl alcohol	Aromatic	+		
	3,4-Dihydroxybenzonitrile	Sulfur and nitrogen	+		
	2-methyldecane	Alkane	+		
	1-methyl-4-(1-methylethylidene)cyclohexene	Terpenoids	+		
	Butanoic acid,2,2-dimethyl-3-oxo-,ethyl ester	Acids & esters	+		
	2-butanone	Ketone	+		

	Allyl methyl sulphide	Sulfur and nitrogen	+		
	4-nitroso ethylester benzoic ac	Sulfur and nitrogen	-		
	2-butyl-cyclohexanol	Alcohol	-		
	5,5-Dibutylnonane	Alkane	-		
	4-ethenyl,1-,2-dimethyl benzene	Aromatic	-		
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)	Ketone	-		
	Pentadecanal	Aldehyde	-		
	Camphene	Terpenoids	-	Eosinophilic vs non-eosinophilic	Closely matched in age. Differences in FEV_1 % predicted, FVC % predicted, and in FEV_1/FVC
	1,1-Dimethylpropyl 2-Ethylhexanoate	Acids & esters	-		
	2,6,10-trimethyldodecane	Alkane	-		
	7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl) methanol	Alcohol	-		
	Cyclohexanone 3,7,7-trimethyl	Ketone	-		
	Bicyclo[4.1.0]hept-2-ene	Terpenoids	-		
	Cyclohexene-4-methylene	Alkene	-		
	Cyclopentene,1,3-dimethyl-2-(1-methylethyl)	Alkene	+	Neutrophilic vs non-neutrophilic	Differences in age, FEV_1 % predicted and FEV_1/FVC
	2,7-dimethyl naphthalene	Aromatic	+		
	3,5-dimethyl Cyclohexanol	Alcohol	+		
	Tetradecane, 4-methyl	Alkane	+		
	Decahydro-8a-ethyl-1,1,4a,6-tetramethylnaphthalene	Alkane	+		
	Benzene	Aromatic	-	Control vs loss of control	Differences in age, FEV_1 % predicted, FVC % predicted, and in FEV_1/FVC.
	Pentadecane, 1-methoxy-13-methyl	Ether	-		
	Heptanoic ac	Acids & esters	-		
	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)	Terpenoids	+		

	O-xylene 2-4-methylene, 3-methyl/butanal, 2-methyl	Aromatic	+		
	2,2,4,4-Tetramethyloctane	Alkane	-		
	(1E)-1-(methylsulphanyl)1-propene	Sulfur & nitrogen compounds	-		
	2,6-diisopropylnaphtalene	Aromatic	-		
Larstad et al **(21)**	Isoprene	Terpenoids	-	Asthma vs controls	Differences in gender, weight, FEV_1 % predicted and FVC % predicted.
	Ethane	Alkane	-		
Meyer et al **(22)**	1-Dodecanol, 3,7,11-trimethyl-	Alcohol	+	Asthma vs controls	Not reported
	Benzene	Aromatic	+		
	1,3-Dioxolane	Acids & esters	+		
	2-(phenylmethyl)-4-Cyclopentene-1,3-dione, 4-phenyl-	Acids & esters	+		
	Dodecane	Alkane	-		
	Phenol	Aromatic	-		
	Quinoline decahydro-	Sulfur & nitrogen	-		
	2-Propionyloxypentadecane	Acids & esters	-		
	Tetradecanoic acid	Acids & esters	-		
	Octanal	Aldehyde	-		
	2-Butyl-2,7-octadien-1-ol	Alcohol	?		
	2,4-dimethylheptane	Alkane	?		
	5-hexenoic acid	Acids & esters	?		
Olopade et al **(24)**	Pentane	Alkane	+	Controlled vs loss of control (acute)	NA – longitudinal study
Paredi et al **(25)**	Ethane	Alkane	+ (in untreated asthma)	Steroid treated vs non-steroid treated & healthy controls	Closely matched in age. Differences in gender, FEV_1 % predicted and RV/TLC % predicted
van der Schee et al **(27)**	Acetone	Ketone	+	Asthma vs controls	Differences in age, gender, and smoking
	Isoprene	Terpenoids	+		

	Carbon disulphide Toluene 1-propanol	Sulfur & nitrogen Aromatic Alcohol	+ + +		history.
		Abstracts			
Fens et al **(73)**	Acetone; 1,2-pentadiene; 2,4,4-trimethyl-1-pentene, phenol, D-limonene 4-tert-butylcyclohexyl-acetate	Ketone Alkene Alkene Alcohol Terpenoids Exyl-acetate	Not reported	Control vs loss of control	NA – longitudinal study
Brinkman et al **(76)**	Pantolactone,5 Methylacetate,32 Methylcyclohexane,22 Cyclohexane-D12,50 Pinene,22 Eucalyptol,74 2-methylfuran,70 Isopropyl alcohol	Acids & esters Acids & esters Alkane Alkane Terpenoids Terpenoids Aromatic Alcohol	Correlations	Sputum eosinophils Sputum neutrophil Blood eosinophils Blood neutrophils CRP FeNO ACQ FEV_1 % predicted	Not reported
Durrington et al **(77)**	2-Undecanal	Aldehyde	Diurnal variation in asthma which is not present in healthy controls		Closely matched in age. Difference in FEV_1
		Mixed age group			
Couto et al **(37, 78)**.	Nonane 2,2,4,6,6-pentamethylheptane Decane, Dodecane Tetradecane Nonanal Decanal Dodecanal	Alkane Alkane Alkane Alkane Alkane Aldehyde Aldehyde Aldehyde		Asthmatic vs non-asthmatic adolescent swimmers.	No significant differences reported.

The majority of abstracts did not publish details on compounds of interest, only Brinkman et al (76), Couto et al (38), Durrington et al (77) & Fens et al (73). The abstract by Fens et al was not subsequently published in full. The study by Couto et al was, however they included study participants under the age of eighteen. They report that samples from asthma and healthy controls could not be separated based on distinct metabolites. Brinkman et al present a list of compounds found to correlate with clinical variables; this was a univariate analysis without the more sophisticated methods such as Bonferonni correction which would normally be applied to a large dataset; moreover the validity of compound identification is hard to determine. While we present the compounds of interest for these four abstracts we draw attention to the inability to assess study quality, risk of bias, and full methodology.

4. Discussion

The accuracy of classification achieved by breathomic models suggests VOC-profiling in exhaled breath has potential for use in asthma diagnosis and management. The ability to discriminate between those with asthma and healthy controls has been consistently demonstrated but, to be of clinical use, these findings need to be validated in independent prospective cohort-studies undertaken in populations with only a clinical suspicion of asthma; this would enable the determination of diagnostic test accuracy. Given the high incidence of asthma misdiagnosis, development of such a test could be clinically significant and of benefit in the presence of diagnostic uncertainty.

Sputum eosinophil count has long been considered the definitive method for assessing lung inflammation, and when used to guide treatment has been shown to improve asthma outcomes (79). However, FeNO has been found to predict steroid responsiveness (80) and has now been integrated into national asthma guidelines for both management and diagnosis (81, 82). The ease of use and rapidity of results with FeNO measuring devices has led to more widespread clinical uptake than that achieved by sputum eosinophil count. However, VOC profiling has the potential for wider application than either including the identification of alternative sputum profiles (such as neutrophilic or paucigranulocytic); monitoring of control in non-eosinophilic phenotypes; identification of treatable traits; and the differentiation of transient pre-school wheeze and asthma.

A clinically-meaningful threshold has been determined for both sputum eosinophilia and FeNO and the reproducibility of measurements established. This is not yet the case for breathomic models. While VOC-measurements within-individuals may be reproducible and breath profiling may display good levels of accuracy, relatively few results have

been replicated or externally validated. It is important to note that, in a heterogenous disease such as asthma, findings based upon asthma populations defined by one 'gold standard' (such as sputum eosinophils) will not be accurately validated in a population based on an alternative diagnostic standard (e.g. physician diagnosed asthma) which may be composed of other or multiple phenotypes.

The inter-study variability reported in this review may in part be due to instrument variability. Between-laboratory comparisons for GC-MS data can be challenging due to the dynamic nature of the measuring equipment. However, this may be improved through the implementation of the MSI reporting standards coupled with comparative analysis of laboratory data quality. eNoses have demonstrated variability, both between manufacturers (83) and between devices of the same model (84), and sensor 'drift' can be difficult to detect. This may be, to some extent, a self-limiting problem; as potential markers are identified, study methodology may shift from inductive to deductive. With targeted studies it is possible to address calibration issues from the outset giving increased confidence in results.

Causes of inter-study variability do not lie exclusively with the instrumentation; metabolomics involves substantial inter-subject variation (62). This is not necessarily simply a result of comparing different asthma severities or phenotypes. A number of variables may have an effect on VOC profiles including the exposome (85), respiratory rate (86) and breathing route (87). In a study of healthy volunteers Philips et al (88) report the mean number of VOCs per breath sample to be <350 but the number of different compounds across their studies as a whole to be >3,400. Moreover, of the total compounds identified in their study only 27 were found in the samples of all participants. However, both Fens (19) and de Vries et al (29) report a high correlation coefficient for within-day repeatability and between-day repeatability for participants. It would seem then that breath prints are relatively stable within- but vary considerably between-individuals (50). Intra-individual variability secondary to asthma activity offers the opportunity for identification of disease biomarkers; and while inter-individual variability complicates the independent validation of results, Sterk argues this variation offers hope in terms of individual phenotyping (89) including the identification of treatable traits and implementation of personalized medicine.

Recent work in other diseases has shown that diet and lifestyle are important cofounders in breath VOC analysis (90). While this may apply to many of the smaller studies included within this review, with sufficiently large patient cohorts this may not be the case. In a study of 494 participants, variables thought to be highly confounding –

including age and smoking - appeared not to effect the ability of a diagnostic model to distinguish gastric cancer from healthy controls (91).

While studies have examined response to treatment in terms of identifying phenotypes such as 'steroid-responsive', there has been little published on the effect of therapy on exhaled VOC. Brinkman et al (92) report statistically significant correlations between exhaled VOC and medication metabolites as detected in urine, suggesting that exhaled VOC may offer a potential route for assessing therapeutic drug use. Further study of this area could have useful clinical application in the weaning of therapeutics.

Breathomic data sets are complex and the statistical approaches used in their analysis have developed over time as interest in the field has grown; their evolution is likely to continue as wider developments in metabolomics are applied to the field of breath research. The majority of studies published the results of principal component analyses with compound loadings or receiver operating characteristic curves with accuracy percentages; rarely were values such as the Cox & Snell R^2 published. The mean and standard deviation for individual compounds were also infrequently reported. Were such data to be made available, power calculations to determine sample sizes required to detect significant differences would be possible for attempts at the validation of individual biomarkers or prior models.

In common with other emergent fields of study (94) there is a conflict between innovation and standardisation. Due to its potential for both inductive and deductive approaches, and for both offline and online analysis, breathomics is likely to remain more heterogenous in its methodology than some other fields. However, the arrival of technical standards for exhaled biomarkers (49), minimum reporting standards (72) and CE-marked, production-line breath capture devices, goes a long way towards addressing some of the potential sources of confounding and variation. Despite the publication of such standards there is still considerable leeway in how samples may be processed and analysed; these decisions are crucial given that the clinical relevance and wider acceptance of results hinges on the correct selection and application of these techniques. The quality of analysis amongst the included papers is inconsistent and hampered by the low numbers of participants in many of the early studies. Internal validation of results does seem however to be becoming the norm, and as participant cohorts continue to grow the risk of overfitting diagnostic models will further reduce. Whilst the determination of which features within a dataset should be included in diagnostic models has improved, compound identification remains relatively poor with few of the studies checking the putative identifications against chemical standards. Better identification will

allow the biological origins of exhaled VOCs to be determined; the first step in linking breathomics to other 'omics in a systems biology approach.

5. Conclusion

Breathomics is well suited to the age of personalised medicine; the large data sets typically produced are highly individualised and reflect a multitude of metabolomic pathways; a feature which is particularly attractive for the study of complex heterogenous diseases such as asthma. The potential exists not only for diagnostics, phenotyping and the identification of treatable traits but – when coupled with other 'omics – the linking of phenotypes to endotypes. Results to-date are promising but validation in independent prospective cohorts is needed; this may be challenging given the high levels of inter-individual variation. However, addressing inter-study variation through the identification of important confounders, increasing study size, and methodological and analytical standardisation will facilitate these efforts. Identification of a limited number of compounds with strong discriminative ability may decrease processing time and aid the development of point of care testing; crucial if breathomics is to make the leap into clinical application.

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7. Appendix

Contents

Table A1 – Search String

Pubmed (http://www.ncbi.nlm.nih.gov/pubmed)

(("Breath Tests"[Mesh] OR "Exhalation"[Mesh] OR "exhaled"[All Fields] OR breath[All Fields]) AND ("Asthma"[Mesh] OR "asthma"[All Fields] OR "asthmatic"[All Fields]) AND ("Volatile Organic Compounds"[Mesh] OR "Volatile Organic Compound*"[All Fields])) OR (("asthma"[MeSH Terms] OR "asthma"[All Fields] OR "asthmatic"[All Fields]) AND (Breathomic*[All Fields] OR ("metabolomics"[MeSH Terms] AND ("exhalation"[MeSH Terms] OR "exhalation"[All Fields] OR "exhaled"[All Fields] OR breath[All Fields] OR "breath tests"[MeSH Terms]))))

Limits applied to results - 'humans'.

Table A2 – Research Methods: Breath Sampling

Study	Year	Title	Ambient air subtraction?	Filtered air used?	Storage method	Concentrated on sorbent tubes?	Internal validation?	External validation?	Analytical platform
Awano et al **(17)**	2011	Correlations between health status and OralChroma™-determined volatile sulfide levels in mouth air of the elderly	N	N	Syringe	N	X	X	GC (OralChroma™)
Brinkman et al **(32)**	2017	Exhaled Breath Profiles in the Monitoring of Loss of Control and Clinical Recovery in Asthma	N	Y	Tedlar bag	Y	X	X	eNose & GC-MS
Dragoneiri et al **(18)**	2007	An electronic nose in the discrimination of patients with asthma and controls	Y	Y	Tedlar bag	N	Y	X	eNose
Dragoneiri **(30)**	2018	Exhaled breath profiling by electronic nose enabled discrimination of allergic rhinitis and extrinsic asthma	N	Y	Tedlar bag	N	Y	Y	eNose
Fens et al **(19)**	2009	Exhaled breath profiling enables discrimination of chronic obstructive pulmonary disease and asthma	Y	Y	Tedlar bag	N	Y	X	eNose
Fens et al **(31)**	2011	External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease.	Y	Y	Tedlar bag	N	Y	Y	eNose
Ibrahim et al **(20)**	2011	Non-invasive phenotyping using exhaled volatile organic compounds in asthma	N	Y	X	Y	Y	X	GC-MS

Larstad et al **(21)**	2007	Determination of ethan, pentane and isporene in exhaled air – effects of breath-holding, flow rate and purified air.	Y	Y	Tedlar bag	Y	X	X	GC
Lazar et al **(39)**	2010	Electronic Nose Breathprints are independent of acute changes in airway caliber in asthma	Y	Y	Tedlar bag	N	X	X	eNose
Meyer et al **(22)**	2014	Defining adult asthma endotypes by clinical features and patterns of volatile organic compounds in exhaled air.	N	N	Tedlar bag	Y	X	X	GC-MS
Montuschi et al **(23)**	2010	Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma.	Y	N	Tedlar bag	Sorbent gauze	Y	N	eNose & GC-MS
Olopade et al **(24)**	1997	Exhaled pentane levels in acute asthma	Y	N	Tedlar bag	N	X	X	GC
Paredi et al **(25)**	2000	Elevation of Exhaled Ethane Concentration in Asthma	Y	N	Tedlar bag	N	X	X	GC
Plaza et al **(33)**	2015	Inflammatory asthma phenotype discrimination using an electronic nose breath analyzer	N	Y	Tedlar bag	N	Y	X	eNose
Reynolds et al **(35)**	2014	Analysis of human breath samples using a modified thermal desorption: gas chromatography electrospray ionization interface	?	Y	?	Y	X	X	TD-SESI-MS
van der Schee et al **(26)**	2013	Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. Clinical And Experimental Allergy	N	Y	Tedlar bag	N	Y	X	eNose
van der	2012	Effect of transportation and storage using sorbent tubes of exhaled	N	Y	Nalophan bag	Y	Y	X	eNose &

Schee et al **(27)**		breath samples on diagnostic accuracy of electronic nose analysis							GC-MS
Timms, Thomas & Yates **(28)**	2012	Detection of gastro-oesophageal reflux disease (GORD in patients with obstructive lung disease using exhaled breath profiling.	N	N	Tedlar bag	N	Y	X	eNose
de Vries et al **(29)**	2015	Integration of electronic nose technology with spirometry: validation of a new approach for exhaled breath analysis	Y	N	X	N	Y	X	eNose
de Vries et al **(34)**	2018	Clinical and inflammatory phenotyping by breathomics in chronic airway diseases irrespective of the diagnostic label	Y	N	X	N	Y	Y	eNose
		Summary:	Ambient air collected - 10 studies	VOC-filtered air -12 studies	Impermeable bags - 15 studies	Samples concentrated onto sorbent tubes - 6 studies	Internal validation conducted - 12 studies	External validation conducted - 3 studies	GC-MS / GC / TD-SESI-MS = 7 eNose = 9 Both = 4

GC-MS: Gas chromatography-mass spectrometry

TD-SESI-MS: Thermal desorption secondary electrospray ionisation mass spectrometry

Table A3 – Research methods: Statistical Analysis

Study	Data pre-processing	Compound identification	Data analysis
Awano **(17)**	Validation of gas chromatograms.	In-house	Univariate analysis chi-square and ANOVA; multivariate logistic regression.
Brinkman et al **(32)**	De-noising, peak detection & alignment, using XCMS. PCA, BoxCox power transformation, normalisation.	NIST	Univariate analysis; ANCOVA and Pearson correlation tests; FDR correction and standardised QR decomposition used. Multivariate analysis by PCA. T-test.
Dragonieri et al **(18)**	Savitzky-Golay filtering & baseline correction	NA	PCA & double cross-validatory implementation of linear canonical discriminant analysis. Pattern recognition algorithm & cross-validation estimate of error made.
Dragonieri et al **(30)**	?	NA	PCA, independent t-test, CDA, leave-one-out cross-validation, ROC-curve
Fens et al **(19)**	eNose sensor data reduced by PCA	NA	Linear canonical discriminant analysis & ROC. Cross-validation by leave one out method. Altman analysis with Bonferroni correction. Intra-class correlation coefficients.
Fens et al **(31)**	eNose sensor data reduced by PCA	NA	Linear canonical discriminant analysis; ROC curves, 10-fold boot strapping, combined with cross-validation.
Ibrahim et al **(20)**	**?**	**?**	Univariate logistic regression analysis, PCA, multivariate logistic regression. Discriminant function analysis with leave-one-out cross validation.
Larstad et al **(21)**	**?**	In-house	Kruskal-wallis, Wilcoxon signed rank test.
Lazar et al **(39)**	Savitzky-Golay filtering and baseline correction, sensor data reduced by PCA	NA	Mixed model analysis, paired T-tests.
Meyer et al **(22)**	Baseline correction, peak detection, normalisation of retention times, global normalisation.	**?**	Unsupervised hierarchical 2-step cluster analysis. Linear discriminant analysis.
Montuschi et al **(23)**	?	**?**	eNose sensor data reduced by PCA. Feed-forward neural network. Unpaired t test, Mann Whitney U test, Pearson coefficient.
Olopade et al **(24)**	**?**	**?**	Wilcoxon signed rank test.
Paredi et al **(25)**	**?**	**?**	One-way ANOVA with Bonferroni correction.

Plaza et al **(33)**	**?**	NA	PCA, univariate ANOVA, post-hoc least significant difference test. Linear canonical discriminant analysis. Leave-one-out validation. AUROC.
Reynolds et al **(35)**	Noise reduction, normalisation	**?**	Qualitative analysis of spectograms
Van der Schee et al **(26)**	eNose sensor data reduced by PCA	NA	Unpaired T-test, canonical discriminant analysis, cross validation by boot strapping. ROC and AUC. Pearson correlation coefficients.
Van der Schee et al **(27)**	Deconvolution, peak determination & peak alignment, background subtraction.	NIST	Principal component reduction, unpaired t-test, leave-one-out cross-validated linear canonical discriminant analysis. AUROC.
Timms Thomas & Dayle **(28)**	eNose sensor data reduced by PCA	NA	Canonical modelling. Cross-validation, interclass Mahalanobis distance.
de Vries et al **(29)**	Corrected for ambient VOCs; normalized	NA	PCA, univariate ANOVA, internal validation by bootstrapping, linear canonical discriminant analysis, AUROC.
De Vries et al **(34)**	Corrected for ambient VOCs (based on alveolar gradient), data normalised.	NA	PCA, unsupervised heirachical clustering using Euclidean distance and ward linkage. Similarity profile analysis. 10x algorithm repetition upon sub-sets. Between-cluster comparisons by ANOVA, Kruskal-Wallis or Chi-squared tests. Validated using independent data set. Supervised analysis by multiple linear regression, Regression model validated using independent data set.

? = not reported
AUC: area under curve
COW: Correlation optimized warping
HP-SPME/GC-qMS: Headspace solid-phase extraction, gas chromatography quadrupole mass spectrometry
NIST: National Institute of Standards and Technology
PLSDA: partial least squares discriminant analysis
SPLS: sparse partial least square discriminant analysis
TD-SESI-ToFMS: thermal desorption / secondary electrospray ionisation / time-of-flight mass spectrometry
ToFMS: Time-of-flight mass spectrometry

ANOVA: analysis of variance
AUROC: Area under a receiver operating characteristic curve
GC x GCMS: 2 dimensional gas chromatography mass spectrometry
MCCV – Monte Carlo Cross Validation
PCA: Principal component analysis
ROC: Receiver operator characteristics
SPME: Solid phase microextraction
TIC: Total Ion Chromatogram
WEKA: a suite of machine learning software / algorithms hosted by the University of Waika

Table A4 – Results: abstracts

Study	Year	Title	Journal or conference	Population	Results
Brinkman et al **(95)**	2014	Electronic noses capture severe asthma phenotypes by unbiased cluster analysis	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 77	Significant between-cluster differences in clinical characteristic reported but p values not cited.
Brinkman et al **(96)**	2013	Unbiased cluster analysis of severe asthma based on metabolomics by the U-BIOPRED electronic nose platform	European Respiratory Society Congress	U-BIOPRED Severe asthma n = 57	p-values for between-cluster differences in clinical characteristics 0.001 – 0.02
Brinkman et al **(97)**	2015	Unbiased clustering of severe asthma patients based on exhaled breath profiles	European Respiratory Journal Conference	U-BIOPRED Severe asthma n= 35	p-values for between-cluster differences in clinical characteristics and eNose profiles P = 0.02-0.04
Brinkman et al **(98)**	2015	Exhaled breath volatile organic compounds can classify asthma patients with high and low sputum eosinophils	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 27	Identifying sputum eosinophilia; AUROC 0.94 (95% CI, 0.85-1)
Brinkman et al **(99)**	2015	Longitudinal changes in exhaled breath GC/MS profiles during loss of asthma control by prospective steroid withdrawal	European Respiratory Society Congress	Subsequently published in full (32) (see table 1)	
Brinkman et al **(76)**	2016	Identification of exhaled volatile organic compounds (VOCs) associated with loss of asthma control	European Respiratory Society Congress		
Brinkman et al **(100)**	2016	Identifying biomarkers of loss of control/exacerbations in asthma from exhaled breath	European Respiratory Society Congress		
Brinkman et al **(74)**	2018	Exhaled volatile organic compounds as markers for medication use in asthma within the U-BIOPRED cohort	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 108	Identification of urinary oral corticosteroids (baseline, replication and validation) AUROCs 67 - 91; identification of urinary salbutamol AUROCs 70 – 82.
Capuano et	2012	Classification ability of two eletronic noses in asthma and COPD	European Respiratory Society	Severe asthma n = 10	Classification asthma vs COPD:

al **(101)**			Congress	COPD n = 9 Healthy controls n = 6	Cyranose 320 = 92%, Ten2010 = 86% Classification disease vs controls: Cyranose 320 = 88%, Ten2010 = 88%
Crespo et al **(102)**	2013	Discrimination of bronchial inflammatory phenotype of asthmatic patients by using the electronic nose	European Respiratory Society Congress	Asthma n = 44 (eosinophilic = 16 neutrophilic = 8 paucigranulocytic = 20)	Eosinophilic vs neutrophilic = 100% Eosinophilic vs paucigranulocytic = 100% Neutrophilic vs paucigranulocytic = 90%
Durrington et al**(77)**	2018	An 'omics' study to investigate the mechanisms underlying circadian rhythm in asthma.	American Thoracic Society Conference	Moderate atopic asthma = 10 Healthy control = 10	Significant diurnal variability in 7 VOCs including 2-undecanal (p=0.03) found in those with asthma but not controls.
Fens et al **(103)**	2011	Exhaled molecular patterns change after experimental rhinovirus 16 infection in asthma	European Respiratory Journal	Mild intermittent n = 9 Healthy controls = 14	Before and after RV16 inoculation Significant change in principal components in asthmatics P=0.1 p=0.15. No change in controls
Fens et al **(73)**	2015	Volatile organic compounds (VOCs) in exhaled breath of asthma patients differ between loss of control and stable phase	American Thoracic Society Conference	n = 23	Control vs loss of control: AUROC 0.98 (95% CI 0.96-1.00)
Greulich et al **(104)**	2013	An electronic nose can distinguish between different asthma phenotypes	European Respiratory Society Congress	Eosinophilic = 9 Non-eosinophilic = 11 Controls = 10	Eosinophilic vs non-eosinophilic p < 0.0001 AUROC 1.0 (95% CI – 0.96 – 1.0); CVV 59.1%
Ibrahim et al **(105)**	2010	Metabolomics of breath volatile organic compounds for the diagnosis and inflammatory phenotyping of adult asthma	American Thoracic Society Conference	Subsequently published in full (20) (see table 1)	
Meyer et al **(106)**	2012	Defining adult asthma endophenotypes by clinical features and patterns of volatile organic compounds in exhaled air	European Academy or Allergy & Clinical Immunology Congress	Subsequently published in full (22) (see table 1)	
Montuschi et al **(107)**	2010	Diagnostic performance of an electronic nose, fractional exhaled nitric oxide and lung function testing	American Thoracic Society Conference	Subsequently published in full (23) (see table 1)	
Pelit et al	2016	Breath print of severe allergic asthma	European	Severe allergic asthma = 27	Asthma vs controls: classification accuracy 88.6%

(108)		with SPME-GC-MS analysis of exhaled air volatile organic compounds	Respiratory Society Congress	Healthy controls = 42	(sensitivity 95.6%, specificity 95.8%)
Santini et al **(109)**	2014	Discrimination between oral corticosteroid-treated and oral corticosteroid-non-treated severe asthma patients by an electronic nose platform.	European Respiratory Society Congress	U-BIOPRED Severe asthma (adult) = 73	OCS vs no OCS: accuracy 71%
Santini et al **(110)**	2015	Breathomics can differentiate between anti IgE-treated and non-treated severe asthma adults	European Respiratory Society Congress	U-BIOPRED Severe = 39 Omalizumab vs non-use	eNose: accuracy 0.85 GCMS: accuracy 0.83
van der Schee et al **(111)**	2012	Predicting steroid responsiveness in patients with asthma using the electronic nose	American Thoracic Society Conference	Subsequently published in full (26) (see table 1)	
Schleich et al **(112)**	2015	Do volatile organic compounds (VOCs) discriminate between eosinophilic and neutrophilic asthma phenotype?	European Respiratory Society Congress	Asthma n= 276 (eosinophilic = 122 neutrophilic = 50 paucigranulocytic = 90)	Identification of good discriminatory VOCs reported. Identity of VOCs and accuracy results not reported.
de Vries et al **(113)**	2016	Exhaled breath analysis for identifying eosinophilic and neutrophilic inflammation in a mixed population of patients with asthma or COPD	European Respiratory Society Congress	Subsequently published in full (34) (see table 1)	
De Vries et al **(114)**	2017	Inflammatory phenotyping of chronic airway disease (including both Asthma and COPD) by breathomics	American Thoracic Society	Subsequently published in full (34) (see table 1)	
Wagener et al **(115)**	2012	Exhaled air volatile organic compounds and eosinophilic airway inflammation in asthma	European Respiratory Society Congress	U-BIOPRED? n = 36 Mod-to-severe	Correlation coefficients - VOCs & sputum eosinophilia (>3%): 0.42-0.47 VOCs & sputum eosinophilia (excl. participants on OCS): 0.49-0.62
Wagener et al **(116)**	2013	Exhaled breath profiling and eosinophilic airway inflammation in asthma – results of a pilot study	American Thoracic Society Conference	U-BIOPRED N = 27 (25 severe)	Eosinophilic vs non-eosinophilic Accuracy = 85%. AUROC 99% (95% CI 0.97-1.0).
Zanella et al **(117)**	2018	Breath print for asthma phenotyping	?	n = 245	Eosinophilic, neutrophilic, paucigranulocytic, mixed granulocytic. AUROC classification 0.68-0.71.

Mixed age population (adolescents)				
Couto et al Abstract **(37)** & published in full **(38)**	2015 & 2017	Oxidative stress in asthmatic and non-asthmatic adolescent swimmers - A breathomics approach	Paediatric Allergy & Immunology Congress of the European Academy of Allergy and Clinical Immunology	No separate clustering of groups on PCA analysis Controls demonstrated a more varied response to exercise; exhibiting a more pronounced decrease in the studied metabolites post-exercise.

It should be noted that nine of the abstracts and one full paper (60) were produced from a single large European programme of study - U-BIOPRED. These all analysed cohort sub-groups; it is not clear whether the same patients might feature as cases in more than one of these publications.

Table A5 – Quality Assessment

Study	1. Was	2. Was there	3. Did all	4. Could	5. Is the	6. Were the	7.	8. How sure	9. Can the	11. Were	12. What

	there a clear question for the study to address?	a comparison with an appropriate reference standard?	pt's get the diagnostic test and reference standard?	the results have been influenced by the results of the reference standard?	disease status of the tested pop. clearly described ?	methods for performing the test described in sufficient detail?	What are the results ?	are we about the results? Consequences and costs of alternatives performed?	results be applied to your patient or population of interest?	all outcomes important to the individual / population considered ?	would be the impact of using this test on your patients / population?
Awano (17)	✗	✗	✓	✓	✗	✓	✗	✗	✗	✗	✗
Brinkman (32)	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	✗
Dragonieri 2007 (18)	✓	✓	✓	✓	✓	✓	?	✓	✓	✗	✗
Dragonieri 2018 (30)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
Fens 2009 (19)	✓	✓	✓	✓	✓	✓	✓	?	✓	✗	✗
Fens 2011 (31)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
Ibrahim (20)	✓	✓	✓	✓	✓	✓	✓	✗	✓	✗	✗
Larstad (21)	✓	?	✓	✓	✗	✓	✗	?	?	✗	✗
Lazar (39)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
Meyer (22)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
Montuschi (23)	✓	✓	✓	✓	✓	✓	✓	?	✓	✗	✗
Olopade	✓	✓	✓	✓	?	✓	✓	?	?	✗	✗

(24)											
Paredi (25)	✔	✔	✔	✔	✔	✘	✔	✘	✔	✘	✘
Plaza (33)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✘	✘
Reynolds (35)	✘	?	✔	✔	✘	✔	✘	✘	✘	✘	✘
van der Schee 2013 (27)	✔	✔	✔	✔	✔	✔	✔	✔	?	✘	✘
van der Schee 2013 (26)	✔	✔	✔	✔	?	✔	✔	✔	?	✘	✘
Timms, Thomas & Yates (28)	✔	✔	✔	✔	✔	✔	✔	✘	?	✘	✘
de Vries (29)	✔	✔	✔	✔	✔	✔	✔	✔	?	✘	✘
de Vries 2018 (34)	✔	✔	✔	✔	✔	✔	✔	✘	✔	✘	✘

Note: CASP checklist question 10 “Can the test be applied to your patient or population of interest?” was omitted. This question refers to resource and opportunity costs for test implementation not appropriate to the field of research as it currently stands. Similarly, question 12 was answered in the negative due to the hypothesis-generating, proof-of-concept stage of the research.

HIGHLIGHTS

- The majority of published studies report breathomics capable of differentiating between samples from healthy controls, those with asthma, and those with other respiratory disease.
- Of the seventy six volatile organic compounds cited as significant in the literature, nine were reported in more than one paper.
- Validation of these findings in independent prospective cohorts is needed as the next step in developing disease biomarkers.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Dr Stephen J Fowler was co-author of one of the twenty studies included in this systematic review. As can be seen from the content of the review, the paper was not given any preferential treatment in the quality assessment process nor undue prominence in the text.