Discovery and Validation of a Volatile Signature of Eosinophilic Airway Inflammation in Asthma

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Volatile biomarkers of airway eosinophilia

1.24 Methods on Non-Invasive Assessment of the Airways: Exhaled Breath and Condensates

At a Glance Commentary

Current Scientific Knowledge on the Subject: Indirect biomarkers of eosinophilic airway inflammation such as FeNO and blood eosinophils are routinely used in practice but are not directly related to sputum eosinophils.

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What This Study Adds to the Field: This study has developed and provided initial clinical validation for a direct biomarker of sputum eosinophilia in severe asthma using headspace and exhaled VOC analysis and could offer the opportunity for future point of care testing to directly monitor eosinophils in the airway.

This article has an online data supplement, which is accessible at the Supplements tab.

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Abstract

Rationale: Volatile organic compounds (VOCs) in asthmatic breath may be associated with sputum eosinophilia. We developed a volatile biomarker-signature to predict sputum eosinophilia in asthma.

Methods: VOCs emitted into the space above sputum samples (headspace) from severe asthmatics (n=36) were collected onto sorbent tubes and analysed using thermal desorption gas chromatography-mass spectrometry (TD-GC-MS). Elastic net regression identified stable VOCs associated with sputum eosinophilia ≥3% and generated a volatile biomarker signature. This VOC signature was validated in breath samples from: (I) acute asthmatics according to blood eosinophilia $\geq 0.3 \times 10^9$cells/L or sputum eosinophilia of ≥ 3% in the UK EMBER consortium (n=65) and U-BIOPRED-IMI consortium (n=42). Breath samples were collected onto sorbent tubes (EMBER) or Tedlar bags (U-BIOPRED) and analysed by gas-chromatography-mass spectrometry (GC×GC-MS -EMBER or GC-MS -U-BIOPRED).

Main Results: The *in vitro* headspace identified 19 VOCs associated with sputum eosinophilia and the derived VOC signature yielded good diagnostic accuracy for sputum eosinophilia ≥ 3% in headspace (AUROC (95% CI) 0.90 (0.80-0.99), p<0.0001), correlated inversely with sputum eosinophil % (r_s= -0.71, p<0.0001) and outperformed FeNO (AUROC (95% CI) 0.61 (0.35-0.86). Analysis of exhaled breath in replication cohorts yielded a VOC signature AUROC (95% CI) for acute asthma exacerbations of 0.89 (0.76-1.0) (EMBER cohort) with sputum eosinophilia and 0.90 (0.75-1.0) in U-BIOPRED - again outperforming FeNO in U-BIOPRED 0.62 (0.33-0.90).

Conclusions: We have discovered and provided early-stage clinical validation of a volatile biomarker signature associated with eosinophilic airway inflammation. Further work is needed to translate our discovery using point of care clinical sensors.

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Introduction

Asthma is a complex chronic disease characterised by type-2 inflammation in 40-60% of patients, associated with cognate elevation of relevant airway immune cells (1). According to international guidelines, approximately 4% of patients with asthma have severe disease, characterised by treatment failure to high dose inhaled steroids and current add on therapies, and require high cost targeted biologic therapies- for example, eosinophil depleting therapies targeting IL-5 and its receptor (2). Furthermore, several orally active biologic agents are now in development for type-2 asthma in patients that have a high unmet need but are 'pre-biologic' care.

Concomitant with the development of high-cost therapies in moderate-severe asthma is the need for biomarkers that can be readily translated for disease stratification, monitoring, and deeper understanding of disease mechanisms. Whilst sputum phenotyping has proven to be invaluable in the characterisation of airway inflammation in severe asthma (3), sputum sampling is time consuming, biased towards patients that can generate a sample at any given time and costly - limiting its application across centres. FeNO has emerged as useful biomarker in asthma and can be used to identify steroid responsive airways disease (4), as well as to stratify biologic therapy (5). However, FeNO levels are largely driven by iNOS activation as a consequence of IL-13 induction (6) and are modified by corticosteroids, as well as other confounders such as diet, smoking and co-existing rhinitis (7). Consequently, the development of a non-invasive exhaled breath biomarker of eosinophilic airway inflammation in patients with severe asthma, would offer the potential for non-invasive and potential future near patient phenotyping of airway inflammation in severe asthma and may identify novel mechanisms of disease beyond T2 activation.

We recently systematically reviewed the evidence for the association of VOC biomarkers with type-2 inflammatory clinical biomarkers (including sputum eosinophilia) across 44 studies, in asthma and COPD (8). Notably, Schleich *et al*, recently identified hexane and 2-hexanone as exhaled breath VOCs able to discriminate between eosinophilic and pauci-granulocytic airway inflammatory phenotypes in asthma (9) and Ibrahim *et al*, identified several exhaled VOC able to discriminate eosinophilic from non-eosinophilic phenotypes with a high classification accuracy (10). In addition, several studies have

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sought to determine the utility of VOC biomarkers for airway inflammation by examining the headspace volatilome of cultured primary cells (11-13) to identify VOCs potentially associated with a putative cellular source.

However, no study to date has sought to validate *in vitro* detected volatile biomarkers in exhaled breath samples acquired from relevant clinical target asthma populations - with a view to maximising their potential for translation into the clinic.

This study aimed to: **(I)** discover a panel of *in vitro* volatile biomarkers associated with sputum eosinophilia (≥3%) in headspace (region directly above the sputum samples entraining emitted VOCs) of sputum from severe asthmatics and develop a VOC biomarker signature from the VOCs, and **(II)** validate the VOC biomarker signature in exhaled breath samples (*in vivo*) within two independent clinical cohorts in the context of acute asthma exacerbations (EMBER consortium) (14) and stable severe asthma (U-BIOPRED consortium) (15), characterised according to blood or sputum eosinophilia.

2 Material and methods

2.1 Patient cohorts

All patient cohorts for biomarker discovery and exhaled breath validation are summarised in **Figure 1** and their clinical, breath sampling and biomarker discovery/replication is summarised in **Table 1** .

2.1.1 Discovery cohort

Thirty-six patients were recruited from the adult severe asthma service at the Glenfield Hospital (UK) between 2018 and 2019. All patients had a diagnosis of severe asthma according to the ATS/ERS 2014 consensus criteria (16), were recruited at least six weeks post exacerbation and were required to have been on stable treatment for at least 4 weeks prior to study entry. Clinical characteristics of the discovery cohort are provided in **Table 2**. Participants were asked to provide a spontaneous sputum sample, which

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was immediately transferred to the lab for the collection of 1L of headspace onto sorbent tubes (Carbograph 1TD and Tenax TA 60/40 - Markes International, Llantrisant), using a previously validated and reported protocol (**supplement section 1.3**)(17).

Subsequently, within 1 hour of sputum collection, it was processed for the differential cell counting using the protocol described in **supplement section 4.3.2** in order to group sputum headspace samples as eosinophil enriched (n=22) and non-eosinophil enriched (n=14) according to the sputum eosinophil threshold of 3% (**supplement Table S5**).

Both control and sputum headspace samples were then analysed by thermal desorption gas chromatography coupled to mass spectrometry (TD-GC-MS) as previously reported(17). Summarised in **Table 1.**

2.1.2 Exhaled breath clinical validation cohorts

Separate exhaled breath clinical validation studies were performed in order to evaluate the biomarker signature accuracy to predict eosinophilic asthma exacerbations: **E**ast **M**idlands **Bre**athomics Pathology Node **-EMBER** cohort (14), and severe eosinophilic asthma: **U**nbiased **BIO**markers in **PRED**iction of respiratory disease outcomes – **U-BIOPRED** cohort (15). Summarised in **Table 1.**

EMBER cohort (n=65): Participants from an acute breath phenotyping cohort (14) were admitted to hospital for severe asthma exacerbations (Glenfield Hospital, UK). Breath samples were acquired within 24 hours of hospital admission and were collected onto sorbent tubes using a ReCIVA sampler (Owlstone Medical, Cambridge, UK) **as described in supplement section 1.4 and Figure S2**, and analysed by two-dimensional gas chromatography with a flame ionisation detector and mass spectrometry (GC×GC-FID-MS) as previously reported in the core EMBER study (18) and analytical validation study (19). Participants were then characterised according to blood or sputum eosinophila [(blood eosinophils $\geq 0.3 \times 10^9$/L; n=20 and blood eosinophils $< 0.3 \times 10^9$/L; n=45) or spontaneous sputum eosinophils ≥ 3% (n=9) and sputum eosinophils < 3% (n=15) - in a subgroup of participants]. FeNO data was not available in EMBER – as patients had by in large presented with severe exacerbations

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(**supplementary tables S6-S7**), with airflow obstruction and elevated respiratory rates and consequently were unable to perform FeNO tests acutely.

U-BIOPRED cohort (n=42): The U-BIOPRED study design has been previously reported (20). Breath sampling in U-BIOPRED was as previously reported and is described in **supplementary section 1.5**, into Tedlar bags followed by trapping of volatiles onto Tenax sorbent tubes (15). For this secondary analysis **(n=42),** patient data with non-smoking- severe asthma only were selected from the U-BIOPRED cohort. Participants had accessible raw data acquired using GC-MS for mass spectral analysis and generation of peak tables for the pre specified VOCs in the biomarker signature. Of these 42 participants, 12/42 had a concurrent blood eosinophilia $\geq$ 0.30 $x10^9$/L and a subgroup (22/42) underwent sputum induction at the same visit as breath sampling, within this subgroup 14/22 participants had a concurrent sputum eosinophilia $\geq$ 3%. 40/42 participants in U-BIOPRED had concurrent FeNO measurements.

2.2 Sample Size

Sample size calculations for the biomarker discovery cohort are provided in the **online supplementary material, section 3.1**.

2.3 Volatile biomarker data acquisition and chemometric analyses

Detailed methods for volatile data acquisition and chemometric analyses are provided in the **online supplementary material (sections 2.1-2.3)** and are reported in prior publications for EMBER(17, 18). respectively and U-BIOPRED (15). A summary of the **(I)** exhaled breath capture and storage and **(II)** analytical methods for analysis and quantitation of VOCs is provided in **Table 1**.

2.4 Statistical methods for discovery and validation studies

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Detailed statistical methods are summarised in **Table 1** and provided in the **online supplementary material (section 3.2-3.5)**. We adopted a supervised machine learning method as unsupervised principal components analysis of all 393 headspace features (**Figure S4 Supplement**) did not separate clinically relevant groups. In brief elastic net (e-NET) regression models with cross validation were used to identify a stable set of canonical VOCs (n=19) in the discovery cohort associated with sputum eosinophilia ≥ 3%. These VOCs were then aggregated into a non-weighted biomarker signature using the stable non-zero, e-NET regression coefficients and z- transformed VOC concentrations of the biomarkers (**Figure S5 Supplement**). The biomarker signature was then validated in EMBER and U-BIOPRED via identification of the same VOCs and the use of ROC curves to examine the diagnostic accuracy for both blood (≥ 0.30 x10^9/L) and sputum (≥ 3%) eosinophilia within these respective cohorts. eNET regression models were used across all three cohorts to confirm the validity of biomarker diagnostic accuracy**.** eNET allows for grouped feature selection i.e. the selection of features that form natural groups will be preserved, the eNET also performs variable selection (shrinkage to zero). We thus viewed the elastic net as a reasonable approach given the correlation structure within the chromatographic feature matrix. For all e-NETS models were run 100 times with 10-fold cross-validation. eNETs were fitted to shuffled diagnostic labels to confirm that diagnostic predictions were not simply due to random noise and overfitting (**Figure S5 Supplement**).

In the discovery cohort, eNET models were also run on the room air acquired VOCs to establish the validity of the VOC models sputum headspace. In U-BIOPRED sensitivity analyses were run with and without the VOC Phenol - due to the potential for contamination of phenol from the Tedlar bag sampling. In both the discovery and U-BIOPRED cohort breath biomarker signatures were compared to FeNO to establish the comparative diagnostic accuracy of FeNO and breath biomarker signatures. In U-BIOPRED logistic regression analysis followed by ROC regression was used to evaluate the combined diagnostic accuracy of FeNO and the breath biomarker signature.

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3 Results

3.1 Sputum headspace discovery study

Figure 1 summarises both the design of the sputum headspace volatile biomarker-based model discovery study to predict airway eosinophilia, and the clinical validation studies in exhaled breath samples from EMBER and U-BIOPRED. **Table 1** reports the clinical, breath sampling and analytical procedures in all three cohorts, whilst **Table 2A** summarises the clinical characteristics in the discovery cohort and **Table 2B** the sputum differential cell count results in the discovery cohort. Sputum sample classification according to sputum eosinophil count threshold of 3% revealed 22 eosinophil-enriched sputa (≥3%) and 14 non-eosinophil-enriched sputa (<3%). The comparison of the differential and total sputum eosinophil count (median, IQR) between the two groups showed, as expected, a significant higher number of eosinophils in eosinophil-enriched sputa compared to the other groups ($p<0.0001$; **Table 2B**).

TD-GC-MS analysis of 36 sputum headspace (volatiles emitted directly above the sputa) samples detected 393 features after thresholding based on frequency of observation and removal siloxane artifacts. The eNET regression selected 19 features (VOCs). The selected VOCs are listed and grouped according to their chemical class in **Table 3**, which also reports the results of a literature search to check whether the selected VOCs had previously been identified as biomarkers of asthma or airway eosinophilia. The chemical structures of the 19 VOCs within the biomarker score are highlighted in **Figure 2** and were compared to VOCs associated with sputum eosinophilia in previous GC-MS based human exhaled breath volatile studies in asthma.

The normalised peak area value of each VOCs was compared between eosinophil-enriched and non-eosinophil-enriched sputa, and significant differences between the groups were observed for 1-hexanol ($p= 0.02$), styrene ($p= 0.017$), phenol ($p= 0.005$), decane ($p= 0.019$), benzothiazole ($p= 0.03$) (**Figure 3A and supplementary Figure S7**). The majority of the other VOCs demonstrated higher normalised

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peak area values in the headspace of non-eosinophil-enriched sputa compared to headspace of eosinophil-enriched sputa.

Biomarker signature generation is summarised with some worked examples in **Figure S6 and section 3.2 of the supplementary methods.**

In **Figure 3B** the biomarker signature values generated for each sputum headspace samples were compared between eosinophil-enriched and non-eosinophil-enriched sputa, showing a statistically significant difference ($p<0.01$) with higher values for non-eosinophil-enriched sputum headspace samples compared to eosinophil-enriched ones. Receiver operating characteristic (ROC) curve analysis to estimate the ability of the VOC signature to discriminate between eosinophil-enriched and non-eosinophil-enriched sputa, showed an AUC of 0.90 [0.80-0.99] ($p<0.0001$) and was substantially greater than either blood eosinophils (AUC: 0.61 [0.42-0.80]) or FeNO (AUC:0.61 [0.35-0.86]) (**Figure 3C**). We observed moderate correlations between the VOC signature and sputum eosinophilia % (r_s= -0.71; $p<0.0001$) **Figure 3D**, as well as absolute sputum eosinophil counts (r_s= -0.54; p= 0.0006).

TD-GC-MS analysis of control background room air headspace samples was also performed, in this context the AUROC 95% CI was 0.5 (0.5-0.5). Further details are outlined in the **online supplementary material Figure S11a**.

3.2 Clinical validation study for the developed volatile biomarker score

Demographics and clinical characteristics for the acute asthma EMBER and stable severe asthma U-BIOPRED cohorts are summarised **Table 1** and detailed in in **Tables S6-S9** of the **online supplementary material.**

In the EMBER cohort, 16/19 VOCs within the biomarker signature were detected in exhaled breath samples GCxGC spectra; the missing VOCs were phenol, 2-butoxyethanol and benzothiazole. In the

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U-BIOPRED cohort, 17/19 VOCs within the biomarker signature were detected in exhaled breath sample GC-MS spectra, the missing VOCs were 1-hexanol and 2-butoxyethanol.

The VOC signature demonstrated good diagnostic accuracies for sputum eosinophilia in both EMBER and U-BIOPRED exhaled breath cohorts (AUROCs: ~0.90), with narrow confidence intervals for the given sample size, in addition to combined sensitivities and specificities of >0.80 **(Figure 4 & Table 4)**. Models run on shuffled diagnostic labels in both EMBER and U-BIOPRED demonstrated significantly lower ($p<0.01$) mean AUROCs (~0.59), indicating that the models did not suffer from significant overfitting (**Figure S11, supplement**). In contrast and as expected for a VOC signature developed from sputum headspace, diagnostic accuracies for blood eosinophilia were modest and had wider confidence intervals **Figure 4 & Table 4**.

We did not find any correlation between blood CRP level (n= 6/63 with an available CRP had a CRP > 50 mg/L) or individual VOC concentration/eNET signature values in EMBER. Furthermore analysis of rhinovirus-19 using qPCR (**Supplementary methods section 4.7**) in the sputum of the EMBER cohort (n=17/24 with available sputum for PCR analyses), identified only weak but statistically significant unadjusted inverse correlations between tridecane (r_s= -0.55, p=0.02) & 1-hexanol (rs=-0.52, p=0.02).

Further analyses of the U-BIOPRED cohort **(Figure 5)** identified that two VOCS (toluene and benzaldehyde) within the multi marker signature were numerically and significantly lower in exhaled breath in the eosinophilic patients compared to the non-eosinophilic patients ($p<0.05$, unadjusted) **(Figure 5a)**. In addition, the concentrations of several VOCs, in particular the reactive aldehyde species (RASP) (nonanal, decanal, hexanal and benzaldehyde) correlated significantly with the eNET score values for the multi marker VOC signature (**Figure 5b**), indicative of dysregulated RASP in eosinophilic asthma. VOC biomarker score values were significantly higher in the eosinophilic patients compared to the non-eosinophilic patients (**Figure 5c**). Comparative analyses using ROC logistic

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regression evaluating the diagnostic accuracy FeNO alone or FeNO in combination with the breath biomarker signature, indicated that FeNO did not provide any added accuracy to the exhaled VOC signature and had modest diagnostic accuracy alone AUROC 95% CI (0.62 (0.33-0.90)) (**Figure 5D**). Diagnostic accuracy for the VOC score in predicting sputum eosinophilia when phenol (a potential tedlar bag contaminant) was removed from the multi-marker data was unaffected, AUROC 95% CI (0.90 (0.75-1.0)).

4 Discussion

This is the first report to identify a non-invasive breath biomarker signature of eosinophilic airways disease in asthma using exhaled breath volatilomics. Our study used robust and previously validated discovery and replication methods for headspace volatile detection and quantification (17, 18). Breath biomarker signature values were directly correlated with both the percentage and total eosinophil counts in sputum. In addition, we have identified that the breath biomarker signatures discovered in sputum headspace were predictive of eosinophilic asthma exacerbations identified by sputum in the EMBER cohort (14, 18) and of severe eosinophilic asthma in the U-BIOPRED cohort (20).

Our findings are important and indicate that the VOC biomarkers are associated with sputum eosinophilia via robust discovery and external validation in two independent disease cohorts - underscoring their validity as biomarkers of eosinophilic inflammation. Additionally, discovery of the biomarker score in a severe asthma cohort with eosinophilia in both the presence and absence of anti-IL-5 therapy, raises the possibility that future studies could evaluate the utility of the VOC biomarker signature for monitoring mucosal eosinophilia in patient receiving biologics or potentially for stratifying biologic response - however these assertions would need to be tested prospectively, in appropriately designed studies.

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Diagnostic accuracies for sputum eosinophilia and combined diagnostic sensitivity and specificity were high, much more so than for blood eosinophilia, as would be expected for a biomarker developed from sputum headspace. Furthermore in the discovery cohort and U-BIOPRED, the VOC signature substantially outperformed FeNO. Furthermore, in the EMBER cohort, we found only small but statistically significant correlations between rhinovirus-16 viral load in sputum and two of the VOCs in our marker score (tridecane and 1-hexanol) and no correlations between the VOCs and CRP as a marker of bacterial exacerbations. These observations would suggest that our biomarker score is specific for eosinophilic inflammation, rather than any specific etiotype of asthma exacerbation and may reflect the level of tissue eosinophilia - however further research is warranted in this area to confirm and follow up on these findings.

Given the challenges of sputum induction and processing, and obtaining FeNO in severe exacerbations of asthma - the identification of a canonical set of VOCs associated with sputum eosinophilia in both headspace and breath could pave the way for rapid point of care triage of eosinophilic disease in the future using point of care ion mobility, or potentially printed and disposable colorimetric VOC detection arrays (21), as well as other types of sensors such as eNOSEs (22) - specifically trained to identify the VOCs in this study.

The eosinophil associated VOCs identified in sputum headspace samples were mostly represented by aromatics hydrocarbons, aldehydes, and alkanes, and a small number of alcohols and ketones. Our previous systematic review of volatile biomarkers of type-2 inflammation in asthma and COPD (8) - highlighted the potential importance of aldehydes and hydrocarbons, however it also highlighted the variability of associated biomarkers across the literature and in addition methodological reporting of VOC biomarkers. Intriguingly, we identified several biomarkers that were derivatives of previously reported VOC biomarkers of eosinophilic inflammation. Specifically oxygenation, hydroxylation or hydrogenation of several of our reported biomarkers yielded biomarkers previously associated with sputum eosinophilia in adults with moderate-severe asthma (9, 10). For example, the eosinophil associated VOC - hexane reported by Schleich *et al* (9), is rapidly transformed to 1-hexanol by hydroxylation and subsequently Hexanal through oxidation via alcohol dehydrogenase enzymes - both

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of these VOCs were reported as eosinophil associated VOCs in our discovery analyses. These observations potentially highlight the importance of alcohol dehydrogenase-based oxidation (i.e. of 1-hexanol to hexanal) in the context of eosinophilic airways diseases.

RASP were the second most represented category among the detected volatile biomarkers of airway eosinophilia. Oxidative stress generates reactive oxygen species (ROS), which trigger lipid peroxidation (23), with the consequent production of several RASP. Exhaled levels of hexanal have been reported as predictive of asthma exacerbations in childhood (24). In keeping with these prior observations, we identified several reactive aldehydes species in U-BIOPRED that correlated highly with the exhaled VOC signature. Given the potential for RASP inhibition (25) using oral small molecule inhibitors, it is plausible that exhaled VOCs could be utilised a biomarker of target engagement with RASP inhibitors.

In keeping with previous observations in cell culture studies of metabolically active cells (26), we observed that several VOCs were depleted in the headspace of eosinophilic sputa. For example, the aromatic hydrocarbon -styrene, was depleted in the headspace of eosinophilic sputa, relative to non-eosinophilic sputum, whilst also being enriched in the headspace of sputum relative to background. Styrene is a volatile monomer widely used in the production of polymers and reinforced plastics, however the relative depletion in eosinophilic sputum and enrichment in headspace compared to background -would auger against external contamination as a causative factor in our data. Microbial and fungal degradation of plastic compounds containing styrene is well reported across a host of pathogens that could plausibly be resident within eosinophilic sputum samples (27).

Our research has several limitations, firstly the derived biomarker signature of eosinophilic inflammation in sputum headspace - was derived from spontaneous sputum sample and not samples obtained by induction. Consequently, our discoveries may be more applicable to the upper and larger airways, as opposed to lower airway eosinophils acquired by sputum induction of broncho alveolar lavage analysis. Although a previous report, failed to identify relevant differences in sputum cellularity between induced and spontaneous samples with the exception of a better cell viability in induced

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sputa (28). Despite this limitation, the biomarker signature that we derived was validated in exhaled breath against the designated target, in two independent populations characterised by eosinophilic exacerbations and stable severe eosinophilic asthma – underscoring the potential validity for future research. Additionally, our replication cohorts, were of small sample sizes with diagnostic accuracy studies in subgroups of patients characterised by either sputum eosinophilia, indicating the need for further research validating the VOCs identified as eosinophilic airway biomarkers.

In conclusion, we have discovered in tissue headspace and replicated in exhaled breath – a biomarker signature of tissue eosinophil associated VOCs, several of which could be plausibly linked to eosinophilic airway inflammation and have been reported in previous discovery VOC studies as derivatives of the biomarkers reported here. Future studies should target quantification and detection of the VOCs described in our report using point of care sensors such as printed colorimetric arrays or eNOSEs, in well powered studies designed to develop point of care tests for eosinophilic airway inflammation in asthma.

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Tables

Table 1: Summary of the methodological aspects of the three datasets (discovery cohort, EMBER cohort and U-BIOPRED cohort)

	Discovery cohort	**EMBER Cohort**	**U-BIOPRED cohort**
Sample type	**Headspace**	**Exhaled breath**	**Exhaled breath**
Number of subject/samples	N=36	N=65 – (cohort with sputum eosinophils -24/65)	N=42 – (cohort with a sputum eosinophils - 22/42)
Asthma conditions GINA 2023 treatment step in the sputum cohorts	Stable severe asthma *GINA 5 (‡anti-IL-5±†mOCS): 14/36 GINA 5 (mOCS): 6/36 GINA 4-5 (&ICS/$LABA only): 16/36	Acute asthma exacerbation GINA 1: 2 GINA 2-3: 9 GINA 4-5 (none on mOCS): 13 Asthma Biologic: 0	Stable severe asthma GINA 4-5: 8 (not on mOCS) GINA 5: 14 (all on mOCS) Asthma Biologic: 0
¥Asthma phenotype according to sputum eosinophils ≥ 3%	Eosinophilic: 22/36 Non-Eosinophilic: 14/36	Eosinophilic: 9/25 Non-Eosinophilic: 16/25	Eosinophilic: 14/22 Non-Eosinophilic: 8/22
Fraction of exhaled breath collected	Not applicable – headspace of native sputum eosinophils	Mixed expiratory breath	Mixed expiratory breath
Exhaled breath container	Dedicated headspace collection systems, reported in [17]	ReCIVA® Breath Sampler, 1L of breath sampled from the phase II/III slope of the CO_2 sensor trace	10 L Tedlar bag (SKC, Eighty-Four, PA, USA)
Pre-concentration method	Carbograph 1TD and Tenax® TA 60/40 (Markes International, Llantrisant)	Carbograph 1TD and Tenax® TA 60/40 (Markes International, Llantrisant)	Tenax (Tenax GR SS 6 mm×7 inch; Gerstel, Mülheim an der Ruhr, Germany)
Sample storage	Into sorbent tubes, at 4°C for no longer than 15 days	Into sorbent tubes, at 4°C after dry purge, for no longer than 72 hours	Up to 39 days on sorbent tubes at 4°C, breath samples were purged onto to sorbent tubes within 10 minutes of acquisition.
Analytical platform	TD-GC-MS	TD-GCxGC-FID-MS	TD-GC-MS
Data processing platform	AnalyzerPro (Spectral Works, Runcorn, UK-v 5.7)	- MassHunter GC–MS Acquisition B.07.04.2260 (Agilent Technologies Ltd, Stockport, UK) - GC Image™ v2.6 - GC Project and Image Investigator (JSB Ltd, Horsham, UK)	- Masshunter Quantitative Analysis (Agilent Technologies)
Multivariate statistical method	Elastic net regression fitted using the cv.glmnet function from the glmnet package in R.3.6.1, (R Core Team, https://www.R-project.org).	Elastic net regression fitted using the cv.glmnet function from the glmnet package in R.3.6.1, (R Core Team, https://www.R-project.org).	Elastic net regression fitted using the cv.glmnet function from the glmnet package in R.3.6.1, (R Core Team, https://www.R-project.org).

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Legend: * Global Initiative for Asthma 2023, treatment intensity step. (17) Peltrini R et al, headspace (mOCS), Inhaled corticosteroids (ICS, Long-acting beta antagonist (LABA).

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Table 2A Clinical characteristics of severe asthmatic donors of sputum samples in the discovery cohort.

	[*]Patients with eosinophil-enriched sputa (n=22)	[†]Patients with non-eosinophil enriched sputa (n=14)	*p-value*
Age (years)	67 (58-71)	58 (56-66)	0.23
BMI (Kg/m^2)	29.6 (26.4-37.6)	31.1 (29.1-35.2)	0.49
Sex (% females)	45	28	0.31
Age of asthma onset	39 (7-53)	35 (29-49)	0.72
Asthma duration (years)	24 (8-33)	23 (12-38)	0.95
Smoking status (Current-never-ex)	1/20 – 13/20 –8/20	0/12 – 7/12 – 7/12	0.56
Post bronchodilator FEV_1 (% predicted)	75 (53.8-92.4)	53 (49.5-81)	0.17
Post bronchodilator FEV_1/FVC (% predicted)	89.1 (76.3-99.3)	73.9 (66.7-99.1)	0.47
FeNO (ppb)	37 (23-52)	26 (14-68.5)	0.40
Blood eosinophils (x10^9/L)	0.21 (0.08-0.46)	0.10 (0.05-0.3)	0.30
ACQ 6-score	1.67 (1 – 3.17)	2.67 (0.67-4.08)	0.85
[‡]Atopy (% Yes)	53	60	0.70
Number of patients on daily dose of maintenance OCS	13/22	7/14	0.31
Maintenance OCS dose (mg or prednisolone/24 hours)	5(5-10)	7.5 (5-15)	0.31
[$]Number of patients on high dose of ICS	20/22	11/14	> 0.99
Number of patients on long-acting beta antagonist (LABA)	21/22	12/14	> 0.99
Number of patients on long-acting muscarinic antagonist (LAMA)	11/22	5/14	0.72
Anti-leukotrienes (Montelukast)	5/22	5/14	0,24
Number of patients on concurrent anti-IL5 therapy (Mepolizumab)	8/22	5/14	0.71
Anti-IL-5 (Mepolizumab) treatment duration (weeks)	8(6-12)	12(4-16)	0.98

Legend: Data are summarised as median (Q1-Q3), and a non-parametric test (Mann-Whitney) was performed for continuous variables.

[*] *Sputum eosinophils ≥ 3%*
[†] *Sputum eosinophils < 3%*
[‡] *Positive skin prick test*
$ Beclomethasone Dipropionate (pMDI, HFA) > 1000 mcg per day

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Table 2B Total and differential cell count of severe asthmatics sputum samples in the discovery cohort.

	*Patients with eosinophil-enriched sputa (n=22)	†Patients with non-eosinophil enriched sputa (n=14)	*p-value*
Total cells (x10^6/g)	3.02 (1.03-5.51)	2.86 (1.46-3.84)	0,88
Eosinophils (%)	5.37 (3.00-8.75)	0.37 (0.25-0.93)	<0.0001
Eosinophils (x10^6/g)	0.09 (0.08-0.20)	0.012 (0.007-0.03)	<0.0001
Neutrophils (%)	74.62 (65.01-83.37)	77.52 (64.43-91.95)	0.39
Neutrophils (x10^6/g)	1.82 (0.63-3.72)	1.84 (0.97-2.85)	0.88
Macrophages (%)	14.33 (6.87-23.12)	11.37 (5.5-30.25)	0.73
Macrophages (x10^6/g)	0.40 (0.13-1.10)	0.27 (0.14-0.82)	0.66
Lymphocytes (%)	0.39 (0.00-0.75)	0.87 (0.31-1.75)	0.14
Columnar epithelial cells (%)	3.75 (0.76-5.50)	3.25 (1.31-6.68)	0.57
Squamous cells (%)	1.91 (0.03-5.35)	5.35 (2.43-8.51)	0.08
Viability (%)	69.86 (61.45-83.50)	68.55 (55.28-74.85)	0.76
Plug weight (mg)	189.5 (106.5-264.25)	141.5 (57.75-213.75)	0.51

Data are summarised as median (Q1-Q3), and a non-parametric test (Mann-Whitney) was performed for continuous variables.
* *Sputum eosinophils ≥ 3%*
† *Sputum eosinophils < 3%*

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Table 3 Chemical classification and prior reports from biomarker studies of discovery VOCs.

Chemical class	[a] NIST library match	[b] MSI levels	[c] CAS registry number	Summary of literature findings	Citation
Ketones	Acetone	1	67-64-1	-Predictive of pre-clinical asthma in pre-school wheezers in children - -Discriminated asthma with other atopic diseases in women of childbearing age	(29) (30)
Aromatic hydrocarbon	Benzene	1	71-43-2	-Predictive of asthma diagnosis in children	(31)
	Toluene	1	108-88-3	-Associated with asthma symptoms in children with mild asthma	(32)
	Phenol	1	108-95-2	-Prenatal exposure to phenols can promote asthma development -May be a contaminant from Tedlar bag sampling of VOCs	(33)
	p-Xylene	1	106-42-3	-Predictive of asthma diagnosis in children with asthma -Predictive of asthma exacerbations in school age children with asthma	(31) (34)
	Styrene	1	100-42-5	-Occupational exposure has been shown to elicit airway hyper responsiveness and eosinophilic inflammation in the context of occupational asthma -Observed in the headspace of epithelial cells in the context of hypoxia	(35, 36)
	α-Methyl styrene	1	98-83-9	-Not reported in the context of asthma. Biomarker of pre-cancerous gastric lesions and gastric cancer.	(37)
	Benzothiazole	1	95-16-9	-Not reported in the context of asthma. However potential for development of benzothiazole derived anti-viral therapeutics based upon robust *in-silico* virtual and high throughput screening data.	(38)
Aldehydes	Benzaldehyde	1	100-52-7	-No clear association in the context of asthma in exhale breath. -----Oral ingestion in murine allergic asthma models demonstrated elevated number of eosinophils and neutrophils and Th2 cytokines in BAL fluid significantly decreased after the treatment	(39)
	Decanal	1	112-31-2	-Reactive aldehyde species, including decanal, were dysregulated in in exhaled breath of adults with asthma exacerbations	(18)
	Nonanal	1	124-19-6	-Detected in breath and discriminated neutrophilic from eosinophilic asthma, when classified by sputum cell counts. -Predictive of asthma exacerbation risk in children with asthma.	(9) (40)
	Hexanal	1	66-25-1	-Reactive aldehyde species, including hexanal, were dysregulated in exhaled breath of adults with severe asthma exacerbations	(9)
	2-Ethylhexanal	1	123-05-7	-Predictive of asthma exacerbation risk in children with asthma.	(40)
Alkanes	Decane	1	124-18-5	-Together with other VOCs discriminated exhaled breath of atopic asthmatics compare to healthy controls & patients with allergic rhinitis	(41)

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	Isothiocyanato-cyclohexane	1	1122-82-3	-Predictive of respiratory disease in neonates	(42)
	Tridecane	1	629-50-5	-Detected in the breath and associated with lung disease including asthma and lung cancer but is considered as a lung cancer biomarker	(43) (44)
Alcohols	1-Hexanol	1	111-27-3	-Associated with obstructive lung function and Cladosporium exposure in a general population home dwelling study	(45)
	2-Butoxy-ethanol	1	111-76-2	-Discriminated headspace of activated eosinophil vs activated neutrophils in *ex vivo* cell cultures of healthy non asthmatic, non-atopic donors	(11)
Others	Methylene chloride	1	75-09-2	-Found ubiquitously in exhaled breath of children with mild asthma	(32)

Legend: *National institute of standard technology
†MSI: Metabolomics Standards Initiative
‡CAS Chemical abstract service
[&]NA: not applicable

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Table 4: Summary of the diagnostic accuracy of breath biomarker scores in identifying eosinophilic inflammation/eosinophilic cohorts in replication cohorts.

	Target patient profile	*Sensitivity*¥	*Specificity* ¥	*PPV* ¥	*NPV* ¥	*AUROC (95% CI)* ¥
EMBER: Blood eosinophilia	Acute asthma exacerbation	**0.31** (0.06-0.56)	**0.90** (0.82-0.98)	**0.44** (0.12-0.77)	**0.84** (0.74-0.94)	**0.59** (0.40-0.78)
EMBER: Sputum eosinophilia	Acute asthma exacerbation	**0.89** (0.68-1.00)	**0.80** (0.60-1.00)	**0.73** (0.46-0.99)	**0.92** (0.78-1.07)	**0.89** (0.76-1.00)
U-BIOPRED: Blood eosinophilia	Stable Severe Asthma	**0.75** (0.51-1.00)	**0.77** (0.62-0.92)	**0.56** (0.32-0.81)	**0.89** (0.76-1.01)	**0.79** (0.64-0.93)
U-BIOPRED: Sputum eosinophilia	Stable Severe Asthma	**0.93** (0.79-1.00)	**0.88** (0.65-1.00)	**0.93** (0.80-1.10)	**0.88** (0.65-1.10)	**0.90** (0.75-1.00)

Legend: SD = standard deviation, 95% CI = 95 % confidence interval. Positive predictive value (PPV), negative predictive value (NPV) and area under ROC curve (AUROC).

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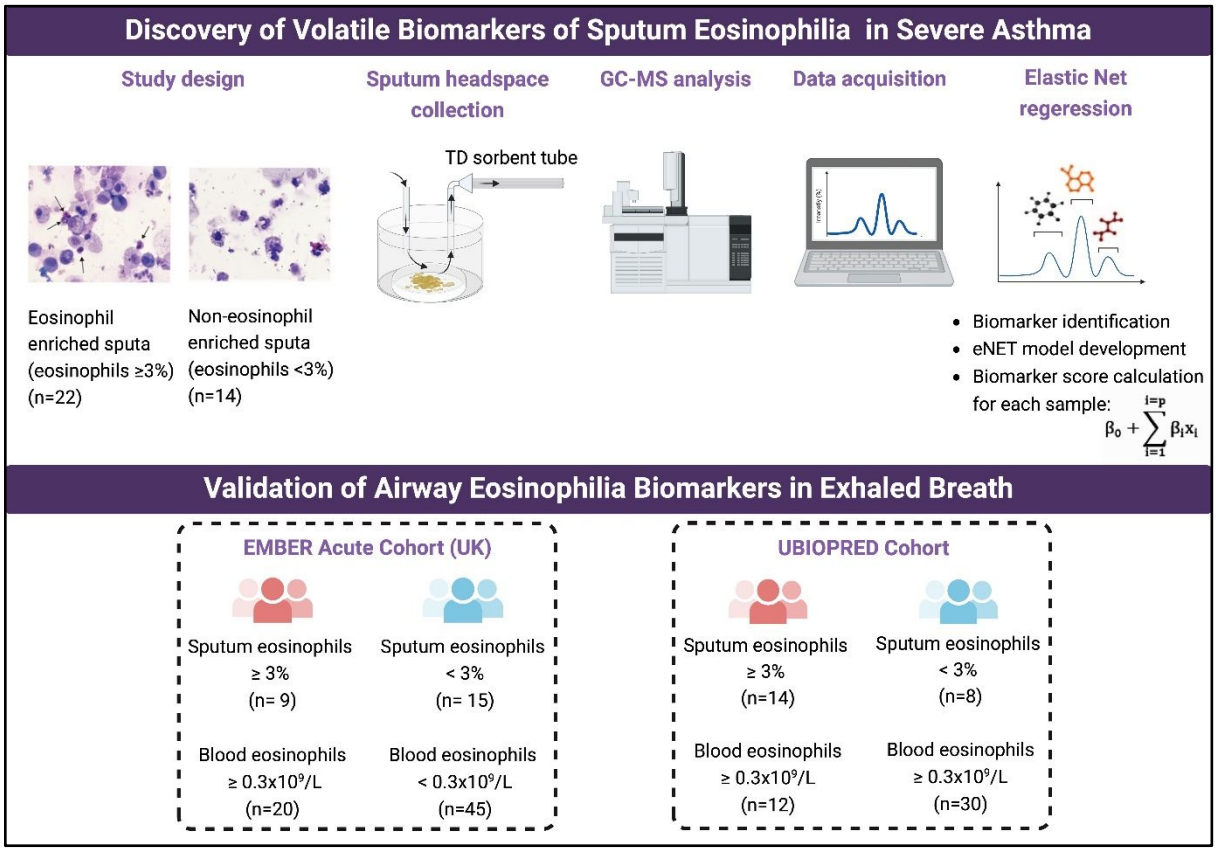
Figure 1



Legend: (A) Graphical summary of the discovery cohort and identification of canonical eosinophil associated VOCs using elastic net regression and generation of volatile biomarker score using the regression coefficient and VOC concentrations in tissue headspace. **(B)** Summary of EMBER and U-BIOPRED replication cohorts for exhaled breath validation of eosinophil associated VOCs identified in the discovery cohort.

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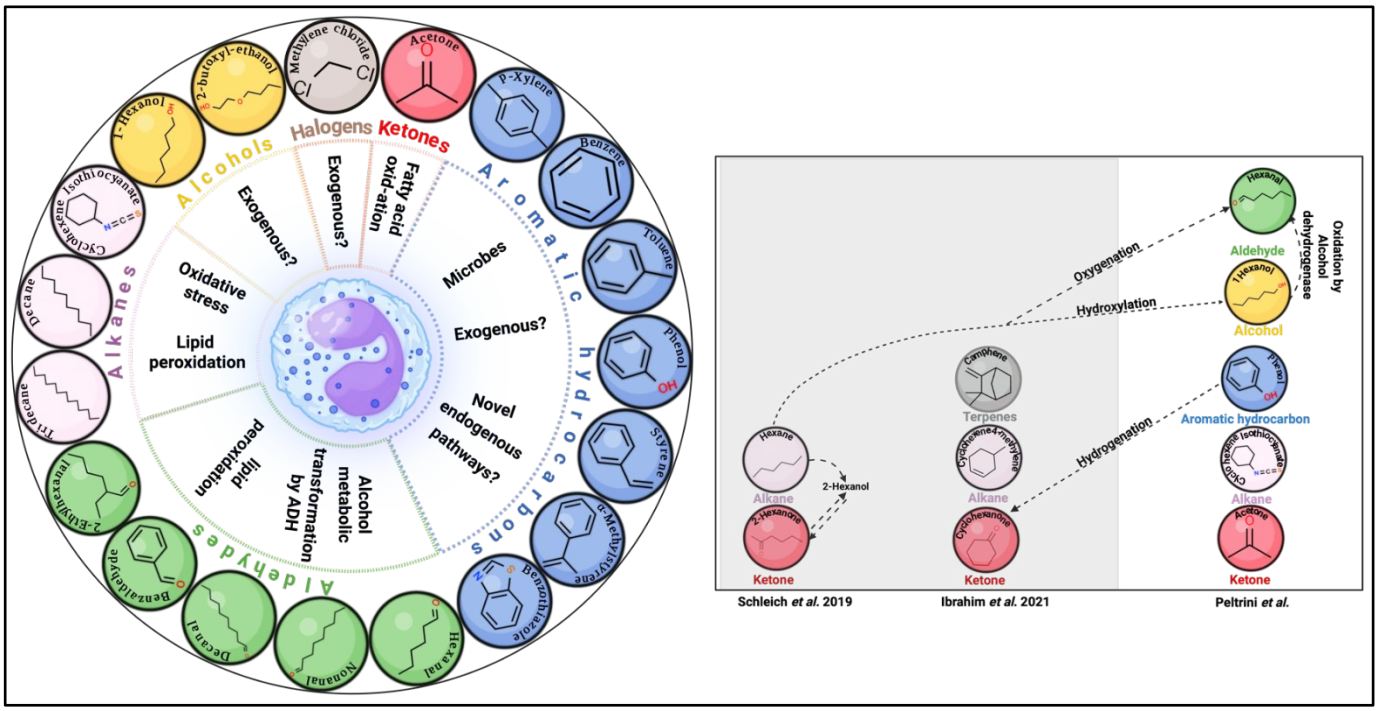
Figure 2



Legend: (A -left hand panel) An illustration of discovered eosinophilic breath volatile biomarker, their chemical structures and the metabolic pathways that could potentially be related to each chemical group. **(B-right hand panel)** previously identified breath biomarkers in severe asthma studies characterizing patients according to sputum eosinophilia and arranged to show the possible chemical relationship and similarities with the reported discovery biomarkers in this study.

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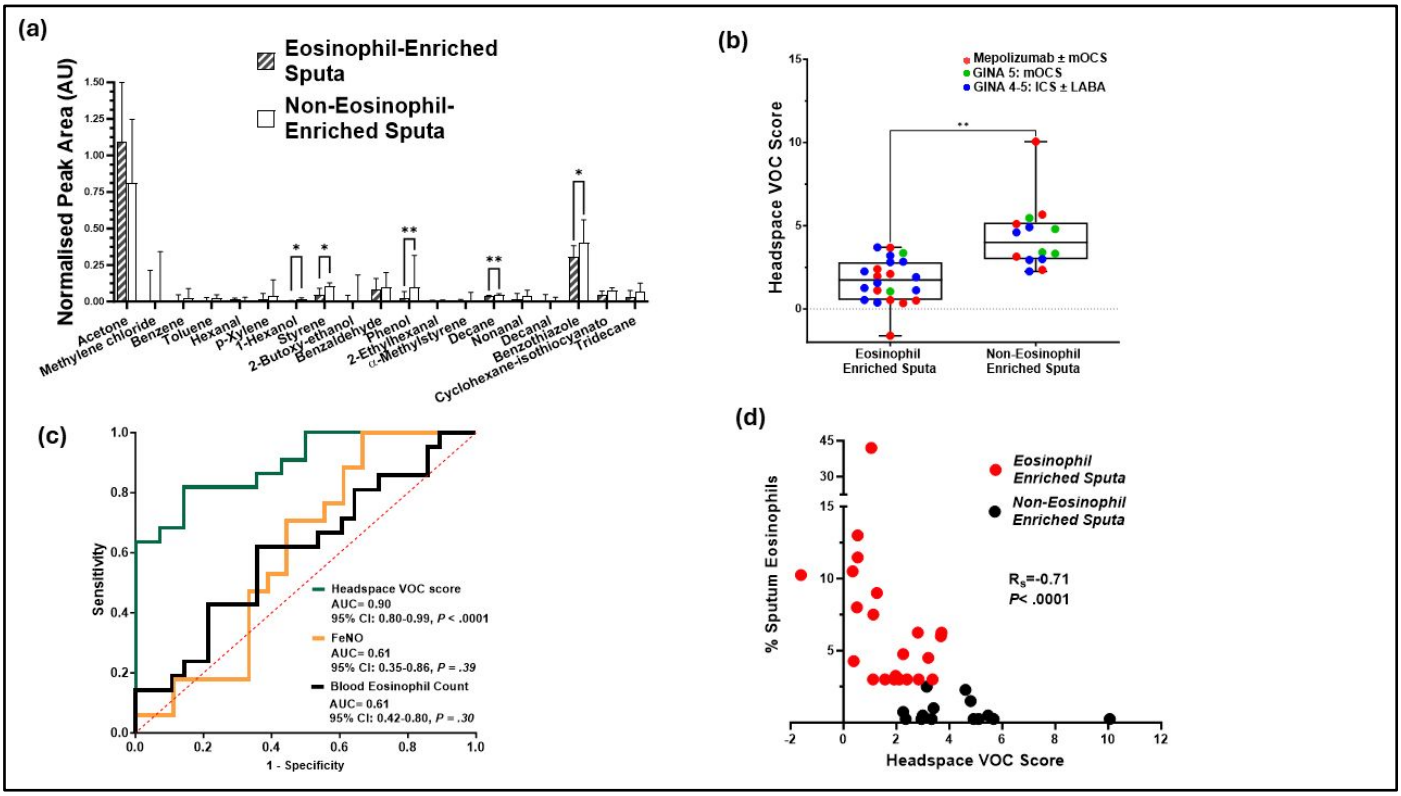
Figure 3



Legend: (A) Histogram of the normalised peak area values of the 19 discovered eosinophilic VOCs summarised as median and IQR and compared by Mann-Whitney test (**significance at p< 0.05). **(B)** Box plot of VOC biomarker score derived from elastic net regression in the eosinophil-enriched and non-eosinophil-enriched sputum samples (median, Q1, Q3, min and max) (Mann-Whitney test: **p<0.0001). **(C)** Receiver operating characteristic (ROC) curve to evaluate the discriminatory performance of VOC scores in differentiating between eosinophil-enriched and non-eosinophil enriched sputa. **(D & E)** Spearman's correlation coefficient between VOC biomarker scores and the percentage of sputum eosinophils ($\mathbf{r_s}$= -0.71; two-tailed t-test: p<0.0001).

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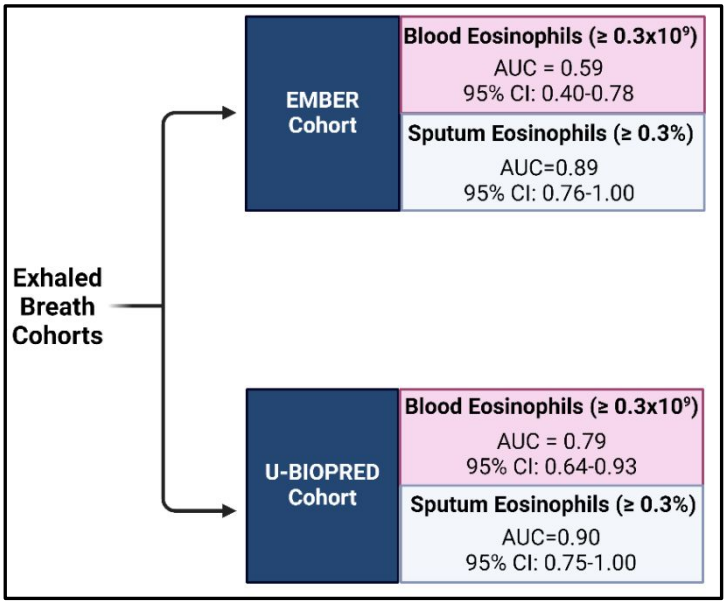
Figure 4



Legend: Visual summary of the diagnostic accuracy for both sputum and blood eosinophilia of eosinophil associated VOCs in exhaled breath in the EMBER and UBIOPRED cohorts.

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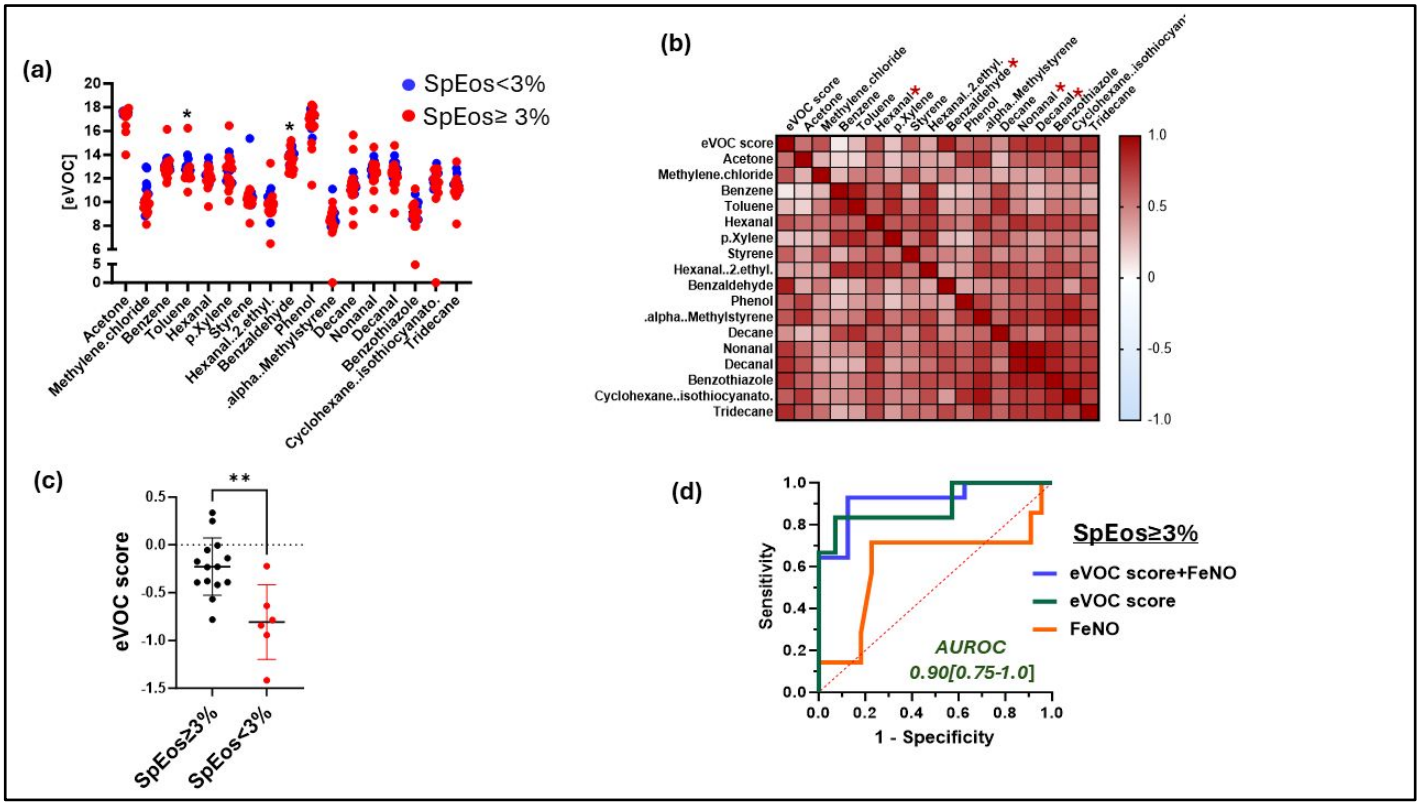
Figure 5



Legend: (a) Ln (x+1) transformed exhaled VOC biomarker concentrations (y-axis), eVOC (exhaled VOC), in eosinophilic (red) and non-eosinophilic (blue) U-BIOPRED patients characterized by sputum eosinophilia. *=p<0.05 (unadjusted) when comparing eosinophilic and non-eosinophilic participants. **(b)** correlation (Pearson r) heatmap for the exhaled VOCs that individually constitute the eVOC score. * (p<0.05) for the correlation with the eVOC biomarker score. **(c)** eNET biomarker score values for eosinophilic and non-eosinophilic patients according to sputum eosinophilia in U-BIOPRED. **(d)** ROC curves for classification of severe eosinophilic asthma, according to sputum eosinophilia with FeNO (orange), eVOC score (green) and combination of FeNO and eVOC score (blue). Area under ROC curve (AUROC) for eVOC score is presented in green.

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Discovery and Validation of a Volatile Signature of Eosinophilic Airway Inflammation in Asthma

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ONLINE DATA SUPPLEMENT

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1 Extended sampling methods

1.1 Sorbent tube preparation for VOC collection (Discovery and EMBER cohorts)

VOCs from both headspace and exhaled breath samples in the **Discovery and EMBER cohorts** - were concentrated onto stainless steel sorbent tubes, which were packed with a hydrophobic dual-bed sorbent mixture of Carbograph 1TD and Tenax® TA 60/40 (Markes International, Llantrisant), respectively a medium and a weak strength adsorbent material.

Prior to sample collection, sorbent tubes were weighed and conditioned. Uncapped tubes were weighed before each conditioning procedure, to check for possible sorbent mass degradation, and then were conditioned at 330℃ in a nitrogen flow at a pressure of 1.5 bar, for 150 minutes. The tube caps and their combined polytetrafluoroethylene (PTFE) ferrules were washed using an appropriate neutral detergent, rinsed in deionized water and dried in an oven at 200℃. Capped tubes were stored at room temperature and used for headspace collection within 15 days from their conditioning.

Prior to the TD-GC-MS analysis for the in vitro discovery study, sorbent tubes containing the VOCs collected from headspace samples were removed from the storage at 4°C and placed at room temperature to warm up. In order to ensure acceptable levels of inter-samples variability, an internal standard solution was loaded into the sorbent tube. This was performed using a calibration solution-loading rig (CSLR, Markes International Ltd, Llantrisant, UK). The sorbent tube was attached to an injector and 0.6µl of internal standard solution were introduced in a purified nitrogen flow (Zero grade, BOC) of 100 ml/min for 2 minutes to ensure an efficient transfer of the standard onto the adsorbent packing. The internal standard solution comprised a mixture of deuterated toluene (D8), octane (D18) and phenanthrene (D10) at a concentration of 10µg/ml.

Within 72 hours of breath collection, sorbent tubes were dry purged in a flow of nitrogen (CP grade with an in-line trap; BOC, Leicester, UK) at a rate of 50ml/min for 2 minutes, in the sampling direction. The dry purge was performed to reduce the amount of condensed water contained in them, and therefore to increase sample stability. GC-MS technologies are normally very sensitive to water, which can affect the integrity, and therefore reduce the working life, of some components such as the chromatographic column and the mass spectrometer detector. Furthermore, water can affect the analysis outcome quenching the detector response for those compounds that co-elute with it (1). The sorbents tubes had higher affinity for organic molecules (including highly polar compounds like light alcohols) than water, therefore during the dry purge step, water was selectively removed to vent without loss of the most volatile/polar compounds of interest. Dry purged tubes were placed at 4°C to be then analysed by GCxGC-FID/MS within 4 weeks. Before GCxGC/MS analysis, the tubes were loaded with 0.6 µL of the same internal standards solution used for headspace sample.

1.2 Sputum collection (Discovery cohort)

Spontaneously produced sputum samples were acquired at least six weeks post exacerbation from the severe asthmatic cohort employed in the *in vitro* study, and from cohorts 1 and 2, which were used for the biomarker validation studies. A spontaneous sputum production was preferred to sputum induction by inhalation of hypertonic saline solution in order to reduce possible contaminating sources, to standardize the sputum collection method, and to avoid patients' submission to this invasive procedure, encouraging therefore their participation.

Sputa were collected into small Petri dishes (Merck Chemicals Ltd., Nottingham, UK) in the clinical research facility testing room at the Glenfield hospital in Leicester.

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1.3 Sputum headspace VOC collection (Discovery cohort)

For the *in vitro* biomarker discovery study, VOCs were collected from the headspace of sputum samples, or just from control samples (blank). Collection was based on the generation of a dynamic headspace by flushing pure air (BTCA 178 air, BOC) above the uncovered Petri dish respectively in presence or absence of sputum. The air flow dragged the volatiles from the headspace onto a stainless-steel sorbent tube, where VOCs were concentrated to be subsequently analysed by TD-GC-MS. **Figure S1** summarises the collection method for headspace VOCs.
The system to collect the headspace from sputum and control (blank) samples consisted in a custom vessel (University of Leicester, UK) with a volume of 0.2 L, made of polyether ether ketone (PEEK), a semi-crystalline thermoplastic material with excellent mechanical and chemical resistance properties. The uncovered Petri dish that contained the sputum or a blank sample was placed into the vessel which was then sealed with a screw cap fitted with two ports; an inlet port, joined to a pure air cylinder (BTCA 1788 grade, BOC, UK), and an outlet port which was connected to the sorbent tube during the headspace collection (**Figure S1)**. The decision to flush the sample with pure air rather than nitrogen or other gasses originated from the intent to avoid possible modification of the sputum microbiome, preventing so potential chemical alterations in the volatilome composition.
In order to generate the dynamic headspace, the uncovered Petri dish in the vessel was flushed with pure filtered air at a flow rate of 200 ml min^{-1} for 5 minutes, sampling each time, 1 L of air.
Pure air was chosen in other to reduce possible contaminations that could originate from interaction of different gasses with biological sample and/or by the interaction of the carrier gas with the sorbet material into the sampling tube.
The airflow displaced the headspace above the Petri dish and concentrated VOCs from the headspace onto the sorbent tube, which were then capped and stored at 4°C for no longer than 15 days.
The flow rate of 200 mL min^{-1} was selected based on a previous optimisation of the sampling parameters for standardised the sampling procedure of volatile compounds. A flow rate of 200 mL min^{-1}, it was proved to enable optimum collection of VOCs whilst minimising sample collection time and the impact of environmental contamination. Furthermore, a speed of 200 ml min^{-1} was proved to reduce the risk of volatile back diffusion (when the speed is too low) or of volatiles lost (when the speed is too high and the compounds do not properly adsorb to the sorbent material) (2).
Headspace samples were collected also in absence of sputum samples with the aim to estimate the presence in the background signal of the selected volatile biomarkers.

1.4 Exhaled breath collection (EMBER cohort)

Detailed methods for sample collection and analysis in EMBER have previously been reported(3-5).
Exhaled breath samples for the volatile biomarker validation studies were collected using the ReCIVA sampler (Owlstone Medical, Cambridge, UK) shown in **Figure S2**, which collected exhaled breath samples from a silicon mask into thermal desorption stainless steel sorbent tubes for their subsequent analysis by GCxGC-FID/MS.
ReCIVA sampler allowed using up to four sorbent tubes, which were held within four ports located above two pumps and pressure sensors. When the patient started breathing into the mask, the pressure sensors were able to monitor patient's breath phases allowing the software to activate the pumps at the specific phase of breath cycle that was meant to collect. A method was set up to collect multiple alveolar breaths from severe asthmatics.

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The samples collection and analysis took place in a dedicated room in the NIHR Respiratory Biomedical Research Unit at the Glenfield hospital (Leicester). The patient was sat down, with the mask appropriately fitted to avoid leaks, and was asked to breath normally. During the sampling procedure, the patient breathed into the ReCIVA silicone mask. Sample collection lasted as long as it took to collect 1 L of exhaled breath at a pump flow rate of 200ml min^{-1} for a maximum of 15 minute. Patients' normal breathing, during the collection, was supported by a flow of 30 L/min of filtered purified air. The filters consisted in activated charcoal to adsorb environmental VOCs, and HEPA filters, which retained the particulate matter released from the charcoal. Only two sorbent tubes were used for each breath collection; they were placed in the two ReCIVA back ports, and two solid aluminium tubes were located in the two front ports. A sample of pure air was always collected before each patient's breath collection.

Silicon facemasks (Owlstone Medical Ltd) were conditioned at 180 °C overnight before being used in order to reduce the background level of siloxanes detected by TD-GCxGC-MS. The unused masks were reconditioned after two weeks.

1.5 Exhaled breath collection (U-BIOPRED cohort)

Exhaled breath was collected at eight participating sites using a previously described method (6, 7). In short, patients breathed for 5 mins at tidal volume through a two-way nonrebreathing valve and an organic compound filter (A2; North Safety, Middelburg, The Netherlands). Next, the subjects exhaled a single vital capacity volume into a 10 L Tedlar bag (SKC, Eighty-Four, PA, USA). The exhaled VOCs were then trapped on sorbent tubes containing Tenax (Tenax GR SS 6 mm×7 inch; Gerstel, Mülheim an der Ruhr, Germany) by pulling the air through the Tenax tube using a peristaltic pump at a flow rate of 250 $mL \cdot min^{-1}$.

2 Extended analytical methods

2.1 TD-GC-MS analysis (Discovery cohort)

The analytical method parameters which were used for headspace analysis, are summarised in **Table S1** and have previously been reported (8).

After being spiked with the internal standard solution, the sorbent tube containing VOCs from sputum/blank headspace sample was placed into the thermal desorption Unity-2 (Markes International, Cardiff, UK) where the volatiles were desorbed at 300°C for 5 minutes in a helium flow rate of 45 ml/min in splitless mode. The volatiles desorbed onto a 'hydrophobic, general' cold trap (matching the sorbent in the sample tube) which was held at -10 °C. The focussing trap was then heated at the maximum heating rate to 300 °C for 5 min with a desorption flow rate of 2 ml/min splitless and the compounds were introduced into the GC column.

The thermal desorption unit was linked to a gas chromatograph (Agilent, 7820A). Volatile compounds were separated on the fused silica capillary chromatographic column (Rxi-5 m s 60 m length x 0.25 mm ID x 0.25 μm film - (Restek, Bellefonte, PA, USA)) in a helium flow of 2 ml min-1, with an initial oven temperature of 40°C and a final oven temperature of 300°C held for 5 minutes (oven temperature ramp of 5°C min-1). The intensity of the signal generated from a component passing through the detector, was reported as function of the compound retention time (RT) generating a chromatogram. The gas chromatograph was coupled to a single quadrupole mass spectrometer (Agilent, 5977B). Mass spectrometer was fitted with an electron ionisation (EI) ion source in which a beam of electrons (at 70 eV) ionized the sample molecules at vacuum condition. Once ionized, the ions were

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repelled out from the ionization chamber and moved to a single quadrupole mass analyser, in which were separated based on their mass/charge ratio (m/z). The mass scanning was performed for a range of 40 – 450 m/z at a frequency of 3 Hz, and the separated ions entered an output detector that recorded and converted the electrical impulses into visual displays.

2.1.1 Chemometrics data processing and analysis (Discovery cohort)

Raw data generated from the TD-GC-MS analysis of headspace samples were analysed by AnalyzerPro (SpectralWorks, Runcorn, UK – version 5.7), a spectral pre-processing package for denoising, deconvolution, integration and alignment of the chromatographic peaks across the samples. AnalyzerPro is a vendor-independent software, which can perform the analysis of TD-GC-MS files using qualitative processing and proprietary algorithms to detect components.

Each day before the analysis of headspace samples a clean sorbent tube was spiked with 0.2 µl of a chemical standard mixture and analysed by TD-GC-MS. The standard solution (**Table S2**) comprised a range of n-alkanes (C8 to C20), alcohols and chlorinated compounds with known RIs, which were used to allocate the retention index (RI) for each chromatographic feature isolated from the sample.

The data analysis process always started with the analysis of all the TD-GC-MS raw data of the standard solutions. The software attributed a RT value to each compound with a known RI and spectra that was contained in the solution. This analysis allowed building a library of RIs, RTs and mass spectra (Table S2) that was subsequently used by the software to allocate RIs to the chromatographic features detected in the headspace samples.

The data analysis process continued with the simultaneous analysis of all the raw data generated from the TD-GC-MS analysis of the headspace samples. The software allowed to use the RI library previously generated to attribute a RI to each detected feature. AnalyzerPro detected all the different chromatographic features and aligned them across different samples; each feature was annotated with its RI, the first and second most abundant ion (m/z) and its RT, which together constituted a unique compound label. AnalyzerPro headspace data analysis generated a data matrix containing the chromatographic features (rows) identified across all the headspace samples (columns) and their chromatographic peak area values.

The data matrix was optimised through a refining process for the subsequent multivariate statistical analysis. The peak area value of each chromatographic feature was normalised by the internal standard toluene D8 – the most representative internal standard across all the samples, compared to the others spiked in sorbent tubes before thermal desorption.

Additionally, the data matrix was edited by removing the deuterated features deriving from the internal standard solution, the siloxanes, which are mostly volatiles that derived from the chromatographic column bleed, and the features present only in one headspace sample, in order to work on more stable and consistent compounds.

2.2 TD-GCxGC-FID-MS analysis (EMBER cohort)

Breath samples analysis was performed by a two-dimensional gas chromatograph (Agilent 7890 A) which was linked to a flow modulator (G3486 A CFT) and a three-way splitter plate coupled to a flame ionisation detector and to a single quadrupole mass spectrometer (Agilent HES 5977B) with electron ionisation ion source (Agilent Technologies Ltd, Stockport, UK)(4). The GCxGC-FID/MS analytical method and the used parameters are summarised in **Table S3**.

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For all the volatile biomarkers validation studies, chemical standards for the *in vitro* selected VOCs - which discriminated non-eosinophil-enriched sputa from eosinophil-enriched sputa -were analysed by TD-GCxGC/MS and the chromatographic data files were used for VOCs targeted analysis in breath samples.

Sampled tubes containing exhaled breath samples were capped with diffusion locking (DiffLok) caps (Markes International Ltd) after being spiked with the internal standard solution, and then placed on trays into a thermal desorption auto-sampler (Markes TD-100xr thermal desorption auto-sampler - Markes International Ltd, Llantrisant, UK). Diffusion locking caps prevented sample loss and contaminants entrance onto the tube. A tray comprised six samples, a reference mixture of n-alkanes and aromatics, and an analytical blank sample.

Reference solution containing saturated alkanes at a concentration of 10 µg/mL and aromatics calibration standards at a concentration of 20 µg/mL diluted in methanol was analysed by TD-GCxGC-FID/qMS in order to monitor the retention behaviour and to facilitate separation of different VOCs and their chemical identity assignment.

The auto-sampler loaded a tube which was purged with helium at a flow rate of 50 mL/min for 1 minute, and then desorbed at 300°C for 5 minutes into a general hydrophobic trap (Markes International Ltd, Llantrisant, UK) which was held at −10°C and that matched the tube sorbent material. The trap was purged for 2 minutes at 2 ml/min and then was heated for 5 minutes to reach 300°C with a split flow rate of 2 ml/min. An empty tube with no sorbent material was loaded by the auto-sampler between each breath sample. After the analysis of each tray batch, a trap blank was always analysed by purging the trap for 2 min at 20 ml/min and then desorbing it at 300 °C for 5 min with a split flow rate of 2 ml/min(4)

Two-dimensional gas chromatography allowed the separation of all the compounds according to their volatility and functional group along two GC columns containing different stationary phases. The primary column contained a non-polar stationary phase made of 5% phenyl 95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm Rxi-5Sil MS - (Restek Thames Ltd, Saunderton, UK)), and the second was a polar column whose configuration was a polyethylene glycol 4 m x 0.25 mm x 0.25 µm DB-WAX (Agilent Technologies Ltd, Stockport, UK).

2.2.1 Chemometrics data processing and analysis (EMBER cohort)

TD-GCxGC-MS data were acquired in MassHunter GC–MS Acquisition B.07.04.2260 (Agilent Technologies Ltd, Stockport, UK) and processed using GC Image™ v2.6 along with GC Project and Image Investigator (JSB Ltd, Horsham, UK). A reference mixture of *n*-alkane and aromatics was analysed at the beginning of each tray generating a chromatographic file, which was used to correct variation in the retention time position captured by the reference chromatograms in each sample batch (9). Reference mixtures analyses helped to separate different chemical classes and to perform VOCs class assignment, based on the analysis of reference compounds, their elution order and mass spectral library matching and interpretation. An example of a chromatogram (presented as a colour plot) generated from a breath sample analysis in shown in **Figure S3**. VOC chemical classes detected in breath samples by TD-GCxGC/MS had a typical distribution on the chromatograms. Branched and unsaturated alkenes and cycloalkanes are the most abundant non-polar compounds present in breath samples that were mostly separated on the first dimension. Polar compounds such as aromatic carbonyls, amines, alcohols and phthalates, were located at the top section of the chromatogram, which had a quite low peak intensity, as they eluted late in the second dimension. Saturated, unsaturated and cyclic carbonyls such as aldehydes ketones, furans, esters and sulphide occupied the middle region of the chromatogram, while siloxanes - which mostly originate from

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environmental air, facemask and chromatographic column bleeding - were located at the lower chromatographic region.

Chemical standards for the selected VOCs, which were identified in sputum headspace, were run on TD-GCxGC/MS and the chromatographic data files were used for VOCs targeted analysis in breath samples (in both the studies that involved exhaled breath analysis). Data matrix comprised the peak area values of the selected VOCs, which were log transformed and adjusted for batch effect dividing the product between VOC abundance and VOC abundance average by the VOC abundance standard deviation.

2.3 TD-GC-MS analysis (U-BIOPRED cohort)

At the Philips Research laboratory (Eindhoven, The Netherlands), Tenax tubes were thermally desorbed using helium as carrier gas. The sample was transmitted to a packed liner, heated to 300°C for 3 min and subsequently transferred to a Tenax TA cold trap (at −150°C), which was heated after 2 min to 280°C at 20°C·s^{-1} and splitless injected onto the chromatographic column. Compounds were separated using capillary GC with helium as a carrier gas at 1.2 mL·min^{-1} (7890 N GC; Agilent, Santa Clara, CA, USA) on a VF1-MS column (30 m×0.25 mm, film thickness 1 µm, 100% dimethylpolysiloxane; Varian Chrompack, Middelburg, The Netherlands). The temperature of the gas chromatograph was adjusted in three steps: 40°C for 5 min, increased until 300°C at 10°C·min^{-1} and finally held isothermally for 5 min. A time-of-flight mass spectrometer (Pegasus 4D; LECO, Sint Joseph, MI, USA), in electron ionisation mode at 70 eV, was used for the detection of product ions (ranging from 29 to 400 Da).

2.3.1 Chemometrics data processing and analysis (U-BIOPRED cohort)

For the purpose of this post hoc analysis in U-BIOPRED, raw mass spectral data was re analysed with a targeted analysis looking specifically at the 19 VOCs identified in the discovery cohort of this study.

Total ion chromatograms of the selected patients' samples from the U-BIOPRED dataset were subjected to peak detection using Masshunter-Unknowns Analysis software (Agilent Technologies) with an area cut-off of 1000, and deconvolution windows of 25, 50, 100 and 200. The resultant peaks were screened with a custom library of the 19 target discovery compounds. Where >5 samples had a library hit with a match factor of >85% compounds and retention times were added to a Masshunter Quantitative Analysis (Agilent Technologies) method, with the most abundant ion selected as the quantifier ion, and two further ions as qualifiers to confirm identity. The target analyte window was set to ±0.1 minutes. All samples were interrogated using this method and results were manually screened to ensure the correct peaks were selected. The integrated peak area of the quantifier ion channel was reported for each target compound and sample.

Target Compound	Quantifier Ion	Retention time (min)
Acetone	43	7.547
Methylene chloride	49	8.278
Benzene	78	10.965
Toluene	91	13.201
Hexanal	44	13.529
p-Xylene	91	15.296
Styrene	104	15.688
2-Ethylhexanal	57	16.651

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Benzaldehyde	77	16.834
Phenol	94	17.049
alpha. -Methylstyrene	117	17.371
Decane	43	17.632
Nonanal	41	19.180
Decanal	41	20.786
Benzothiazole	108	21.550
Cyclohexane-isothiocyanate-	55	21.558
Tridecane	57	22.282

N.B. 1-hexanol and 2-butoxyethanol were not found in any U-BIOPRED samples.

3 Extended statistical methods

3.1 Sample size calculation (discovery cohort)

Sample size calculations for sensitivity and specificity were performed using equations 6.6 and 6.7 of Tilaki (10), the equations were implemented in R version 4.2.1(11). The i*n vitro* diagnostic accuracy study was powered based upon a prevalence of sputum eosinophilia ≥ 3% in severe asthma of 40% (this was downsized from the estimates in Shaw *et al* (12), as the current population included patients on anti-IL-5 therapies), sensitivity and specificity of ≥90% with a maximal marginal error of ±15% at the 95% confidence interval, yielding a sample size of 38 participants.

3.2 Penalised regression methods: elastic net and biomarker sample score calculation (discover cohort)

3.2.1: Principal components analysis: Initially, an unsupervised multivariate technique, principal component analysis (PCA), was used in the background headspace study to achieve a simplified representation of the information in TD-GC-MS data. However, PCA was not the best approach for biomarker discovery in this pr project. Indeed, sputum headspace and breath samples analysed by TD-GC/MS and TD-GCxGC/MS data contained hundreds of chromatographic features, of which uninformative variables dominated. Too many redundant variables negatively affected the selection of projection directions, leading to overfitting and low prediction accuracy for clinically relevant groups (**Figure S4**). For that reason unsupervised data analysis was replaced by supervised analyses using elastic net regression that supported the screening of potential volatile biomarkers, and identification of reduced canonical set of VOCs for breath signature generation.

3.2.2: Penalised regression methods: elastic net and biomarker signature score calculation

A summary of the statistical approach for elastic net regression used is provided in **Figure S5**.

For the *in vitro* volatile biomarker discovery study a supervised multivariate analysis was performed using penalized regression techniques and providing class information (eosinophil-enriched and non-eosinophil-enriched sputa) for each observation. More specifically, an elastic net regression was fitted to a data set of 393 chromatographic features, which were detected in 36 sputum headspace samples, 22 classified as eosinophil-enriched and 14 as non-eosinophil-enriched sputa. An elastic net regression (eNET) was fitted to the sputum headspace data matrix in which the samples were classified according to the sputum eosinophil threshold of 3%. Elastic net has got the advantage of performing with variable multicollinearity as Ridge regression, and the ability of variable selection performed by Lasso, by combining L1 and L2 regularization penalties (13) and therefore it was the chosen method for this study.

Chromatographic feature matrices are likely to contain a number of highly correlated features. For data where there is high correlation between features the LASSO has the disadvantage that it will tend to randomly select one

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or a very small number from sets of such correlated features. The elastic net allows for grouped feature selection i.e. the selection of features that form natural groups will be preserved, the elastic net also performs variable selection (shrinkage to zero). We thus viewed the elastic net as a reasonable approach given the correlation structure within the chromatographic feature matrix. Alpha was set to 0.5 so that the penalization was midway between the two extremes of LASSO and Ridge. We selected alpha based on a simple practical compromise which is in accord with the approach taken by Tay, Narasimhan and Hastie(14) who prefer to treat alpha as a higher level hyperparameter depending on the type of prediction model. The optimal lambda was selected automatically by the cv.glmnet [*https://cran.r-project.org/web/packages/glmnet/index.html*] function based on 10 fold cross-validation.

A 10-fold cross validation (CV) was used to estimate how the model was expected to perform when it was used to make predictions on data that were not used during the model training. Ten represents the number of groups in which the data were split; therefore the set of observations was divided into 10 groups. Each group was taken as test data set once while the others were treated as training data set, then the model was fitted on the training set and assessed on the test data set.

In order to select those features with a non-zero regression coefficients in at least the 80 of 100 runs of a 10-fold cross validation. The estimated average value of the regression coefficient for each of the selected features (VOC biomarkers) and the intercept term were used in developing a score which was able to discriminate between eosinophil-enriched and non-eosinophil enriched sputum headspace samples. The biomarker score for each headspace sample was obtained by summing the regression intercept value and the sum of the products between the averaged regression coefficients of the selected features and their respective normalised peak area values where β_0 is the regression model intercept (constant), β_i is the average regression coefficient for the i^{th} feature obtained after running the regression model over 100 cross-validations, x_i is the peak area value for the i^{th} feature, and p is the number of features (VOCs). Biomarker signature generation is summarised in **Figure S6**, alongside the peak areas for each individual VOC comparing eosinophilia and non eosinophilic samples (**Figure S6**) and VOC concentrations relative to background (**Figure S6**). Correlations between individual discovery VOCS and sputum eosinophilia are summarised in **Table S7**. The eNET was fitted using the cv.glmnet function from the glmnet package in R.3.6.1, (R Core Team, https://www.R-project.org). This research used the SPECTRE High Performance Computing Facility at the University of Leicester.

Sensitivity analysis: In order to investigate the effect of the number of folds used in cross-validation for the elastic net regression models we fitted models using 5-fold cross-validation and leave-one-out cross-validation (LOOCV). The elastic net model for the discovery cohort as discussed in section 2.4 of the main manuscript used 10-fold cross-validation, we apply the same modelling procedure but use 5-fold cross-validation and LOOCV. We do not observe substantial differences in the performance metrics for the elastic net regression model between 5-fold cross-validation (AUROC 95% CI: 0.83[0.68-0.97]) and LOOCV(AUROC 95% CI: 0.82[0.67-0.96]).

The identities of the selected chromatographic features were compared to data acquired from TD-GC-MS analysis of the corresponding chemical standards (Sigma-Aldrich), assigning so to the features a level 1 of identification according to the Metabolomics Standard Initiative (MSI) guidelines (**Figure S9**, standard curves).

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3.3 Comparison of the selected volatile biomarker peak area values between sputum headspace and control headspace samples (discovery cohort)

Figure S7 reported the comparative analysis of the abundance values of the selected volatile biomarkers between sputum headspace and control headspace samples.

3.4 Evaluation of the model classification performance: ROC curve analysis: EMBER and U-BIOPRED exhaled breath cohorts.

ROC curve analysis was used for assessing the ability of the statistical model to discriminate samples between two classes (such as eosinophil-enriched and non-eosinophil-enriched sputa; exhaled breaths samples with blood eosinophils $\geq$ or $<$ $0.3x10^9$ cell/L, and exhaled breaths samples with sputum eosinophils $\geq$ 3% or $<$ 3%) – summarised in **Figure S5**

ROC curve was obtained by plotting on the y-axis the model sensitivity, and on the x-axis the model false positive rate (1-specificity), which were both measured across the range of all the possible score values.

Area under the ROC curve (AUC) represented the predictive accuracy of the model to distinguish between classes, and it was used to summarise the performance of each score (classifier) into a single measure.

According to Swets' classification to interpret the AUC values (15): a model with an AUC that ranges between 0.5 and 0.7 has a low discriminating accuracy, while an AUC oscillating between 0.7 and 0.9 indicates a model with a moderate discriminating accuracy, and an AUC comprised between 0.9 and 1.0 designates a model with a high discriminating accuracy. For the EMBER cohort biomarker concentrations, biomarker score values and ROC curves are summarised in **Figure S10**. The same modelling pipeline was deployed in U-BIOPRED, with results presented in the main manuscript.

Figure S11, reports the results of randomly assigning model labels using shuffling and compares the shuffled results to the true class labels. In both EMBER and U-BIOPRED exhaled air cohorts the diagnostic performance of the eNET model with shuffled labels was poor and in both cases the model the true class labels demonstrated a highly significant superior performance to the shuffled labels ($p<0.01$, permutation test).

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4 Extended clinical methods (Discovery cohort)

4.1 Clinical assessment, demographics and asthma diagnosis

A clinical assessment was performed to estimate the presence and recurrence of clinical symptoms and signs related to airway hyper-responsiveness and airway obstruction. The predictive value of a combination of different symptoms and signs was considered clinically relevant and more helpful in asthma diagnosis compared to the presence of single symptoms.

Typical clinical symptoms and signs considered for asthma diagnosis were recurrent wheezes, cough and/or breathlessness. Furthermore, the presence of triggers that made symptoms worse was estimated by skin prick test to aeroallergens, by increased serum IgE (total and specific) and of peripheral blood eosinophils. Skin prick test required the patients to stop any antihistaminic assumption 72 hours before the test. The test was performed using a panel of common allergens (such as grass pollen, tree, dog and cat fur, *Aspergillus fumigatus* and *Penicillum notatum* etc.) and saline solution and histamine as controls. After 15 minutes the formation of a wheal larger than 2mm compared to controls was considered a positive response. Additionally, several other pieces of information were recorded to characterise the asthmatic cohorts, such as baseline demographic information (age, gender, body mass index, smoking habits and exposure), disease history (age at onset, duration of asthma, exacerbation frequency and ITU admission), personal or family history of atopic disorders such as eczema and rhinitis that increase the probability of asthma, and current medications.

4.2 Spirometry and bronchodilator reversibility test

Obstructive spirometry was used to estimate the ration between FEV^1 and FVC, where FEV_1 represented the exhaled volume at the end of the first second of a forced expiration, and FVC the vital capacity from a maximally forced expiratory effort. Patients were required to stop their ICS 6/8 hours prior the test and not to smoke. Their nose was closed so that they were forced to breath into a mouthpiece linked to the spirometer. They were asked to perform a full inhalation followed by a forced exhalation. The procedure was repeated eight times with one minute pause between each repetition. The best values for FEV_1 and FVC were recorded and used to calculate the reference value and the lower limit of normality for each patient according to the global lung function equation (16). According to GINA guidelines, the FEV_1/FVC ratio cut-off of normality ranges between 0.75–0.80 in adults, and it usually decreases in asthmatics due to the airway obstruction.

The bronchodilator reversibility test was performed to measure, through spirometry, the improvement of the expiratory airflow 15 minutes after patient treatment with a bronchodilator (salbutamol). It was performed after the normal spirometry to assess patient expiratory airflow limitation and to perform a differential diagnosis of asthma and COPD, since flow-related bronchodilator reversibility is more strongly associated with asthma than COPD. A positive response to salbutamol was usually given by an increase of FEV_1 of 200ml.

4.3 Asthma inflammatory biomarkers

4.3.1 FeNO

Exhaled fractional exhaled nitric oxide (FeNO) was measured in exhaled breath samples from all the four independent cohorts of asthmatics in order to assess the airway eosinophilic inflammation although not always the amount of FeNO is strictly associated with airway eosinophilic inflammation. FeNO levels indeed can increase in case of allergic rhinitis, rhinovirus infection in healthy individuals, in men, tall people and in case of

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consumption of dietary nitrates, and can be lower in children, in cigarette smokers or following exposure to inhaled or oral corticosteroids.

FeNO levels were measured using an electrochemical analyser (Niox Vero, Aerocrine, Sweden), at a flow rate of 50ml/sec before taking any inhaled drug.

4.3.2 Total and differential cell count in sputum samples

Sputum samples were used not only to collect the headspace in the *in vitro* study, but also to estimate the sputum cellularity and to assess the granulocytic airway inflammatory phenotype.

After the immediate headspace collection for the in vitro study, and directly after sputum production for the validation studies, the sputa were processed to estimate the total and differential count of inflammatory cells.

Sputum plugs were selected, weighted and diluted in a homogenization solution made of 0.1% DTT (dithiothreitol), and after 15 minutes were diluted in cold PBS with Ca^{2+} and Mg^{2+} and centrifuged. The cells were stained with Trypan Blue and counted using a haemocytometer to record the cell viability (equation 1: L= live cells, D= dead cells), the squamous cells count (equation 2: Sq. = squamous cells), and to calculate the number of total non-squamous cells, which was expressed as millions per gram of selected sputum plugs (equation 3).

$$\textbf{Eq.1}: \text{Viability } (\%) = \left(\frac{\mathrm{L}}{\mathrm{L}+\mathrm{D}}\right)\mathrm{x}100$$

$$\textbf{Eq. 2}: \text{Squamous cells } (\%) = \left(\frac{\mathrm{Sq.}}{\mathrm{L}+\mathrm{D}+\mathrm{Sq.}}\right)\mathrm{x}100$$

$$\textbf{Eq.3}: \frac{\text{Total cells x } 10^6}{\text{g (selected sputum)}} = \frac{\text{Total cells x } 10^6\text{x}1000}{\text{sputumplugs weight (mg)}}$$

The sample was centrifuged, and the pellet was re-suspended in PBS and then placed into a cytospin, where it was centrifuged again at 459xg for six minutes in order to have the cells laid on a slide. The slide underwent to Rapidoff-2 Romanovsky staining to perform the differential cell count for eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells. Differential cell counts were reported as percentage of the total non-squamous cell count. Sputa with a percentage of non-squamous cells lower than 20 and a viability higher than 50% were classified as good samples.

In the *in vitro* biomarker discovery study, sputum differential cell count was used to classify patients' sputa in eosinophil-enriched and non-eosinophil-enriched sputa. The eosinophil and neutrophil upper limit values used for asthmatics were 3% and 65% respectively (17). Therefore, sputum samples with an eosinophil differential count $\geq$ 3% and a neutrophil count $\geq 65\%$ or $< 65\%$ were considered as eosinophil-enriched, while all the other samples that did not meet these criteria were classified as non-eosinophilic sputa. **Table S5**, summarises the sputum cell counts in the discovery cohort.

4.3.3 Peripheral blood eosinophils

Blood eosinophil count was performed for all the cohorts employed in the study. Patients with asthma and blood eosinophil count greater than $0.3x10^9$cells/L are usually subject to frequent exacerbations and poorer asthma control (18). A blood eosinophil threshold ($0.3x10^9$cells/L) was used to classify eosinophil patients in blood across the three cohorts.

4.4 Asthma severity-GINA guidelines

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All the patients were classified as severe asthmatics according to ATS/ERS criteria (19) and /or corresponding to GINA 2023 treatment steps.

4.5 Asthma control and quality of life questionnaires

Patient answered the Juniper asthma questionnaire (ACQ) and the Juniper asthma quality of life questionnaire (AQLQ) in order to assess asthma controls. ACQ included seven questions, five related to symptoms, one on the rescue treatment use and one on pre-bronchodilator % predicted FEV_1 finding. In this study a modified and validated version of ACQ, which was characterised by the absence of point seven, was used as the pre-bronchodilator test was assessed only during the first visit. Both the control of symptoms and the % predicted FEV_1 were described on a severity scale of 0-6. The overall score was given dividing by six the total score of symptoms and rescue treatment usage questions. The minimal clinically significant difference in the score value between visits was 0.5.

AQLQ assessed the asthma-specific health-related quality of life measures. It comprised 32 items that covered four domains: symptoms (11 items), activity limitation (12 items), emotional function (5 items), and environmental exposure (4 items). The overall score was given by the arithmetic average of the scores of each domain. 0.5 was the minimally relevant difference in score for overall quality of life and for each of the individual domains.

4.6 Clinical characteristics of breath analysis cohorts within EMBER and UBIOPRED.

Clinical characteristics of the EMBER and U-BIOPRED cohorts have previously been reported (3, 12) . Clinical characteristics of the breath analysis sub cohorts from EMBER and U-BIOPRED for the replication performed in this study are summarised in **Tables S6-S9**.

4.7 Rhinovirus 16 PCR in sputum: EMBER acute cohort

To assess the relations ship between exhaled VOCs and viral infection, qPCR for Rhinovirus 16 was performed in archival sputum plugs in the EMBER asthma acute cohort. RNA was extracted from sputum samples (QIAamp viral RNA mini kit; Qiagen) and reverse-transcribed (omniscript RT kit, Qiagen) with random hexamers. qPCR was performed on 1 µl of cDNA was performed on unprocessed plugs of induced sputum using Amplitaq Gold DNA polymerase (PE Biosystems ABI Prism 7700). A standard curve was produced by using serially diluted cloned product and results expressed as viral copies/ml. The sensitivity of this assay was 104 copies/ml.

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Figures

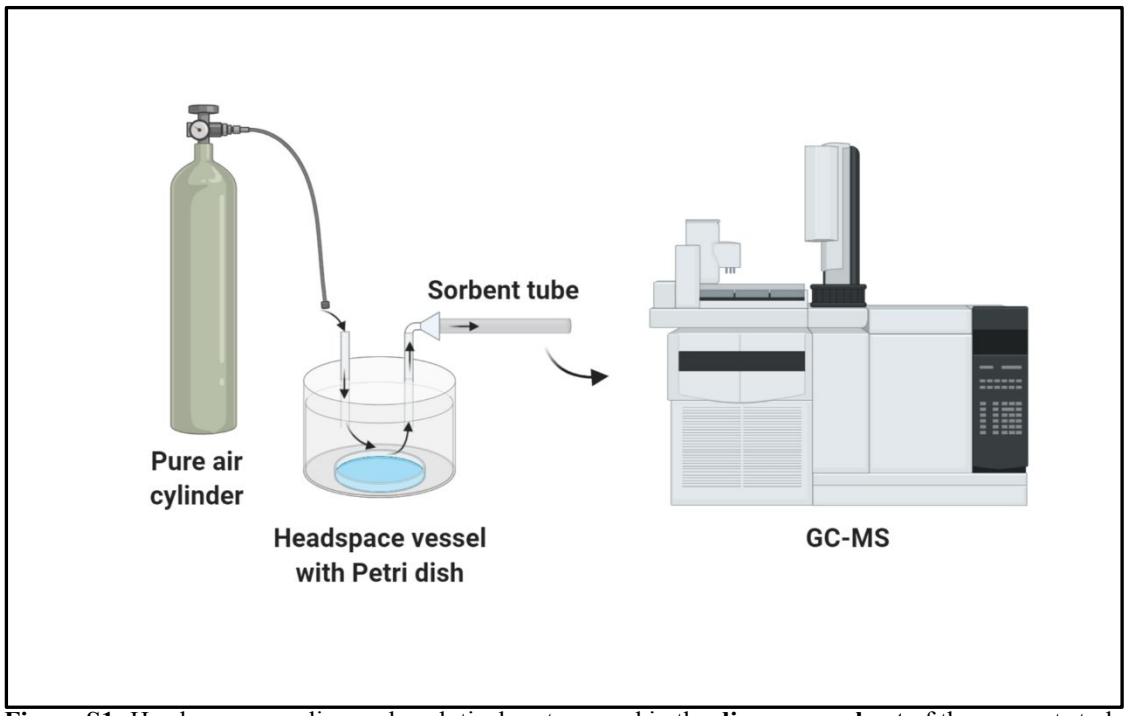

Figure S1: Headspace sampling and analytical system used in the **discovery cohort** of the current study(8).

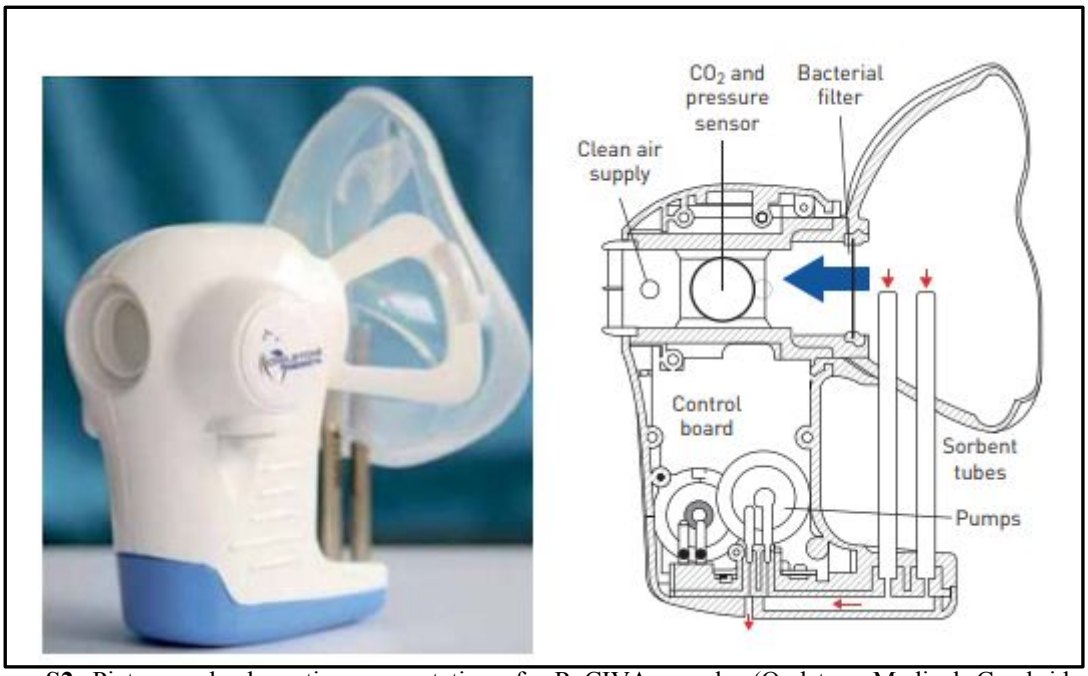

Figure S2: Picture and schematic representation of a ReCIVA sampler (Owlstone Medical, Cambridge, UK). (Adapted with permission from Owlstone Medical Ltd, Cambridge, UK).

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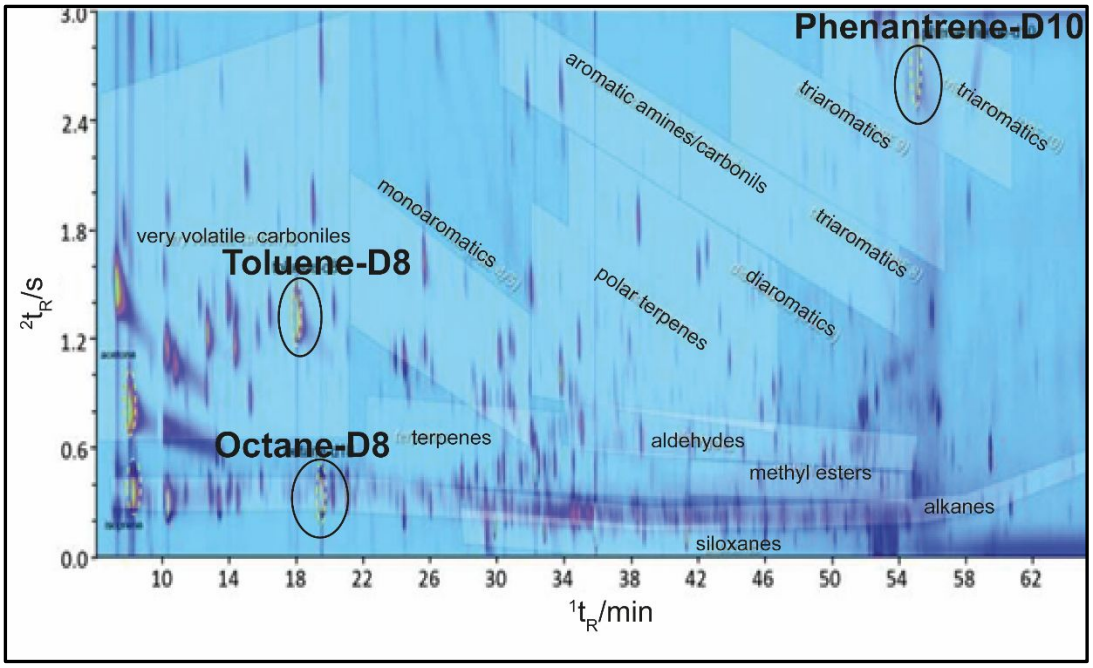

Figure S3: Example of GCxGC chromatogram for an exhaled breath sample in the **EMBER cohort**, showing volatile organic compounds separation, the deuterated internal standards and the chromatographic region occupied by different chemical classes. Adapted from(4).

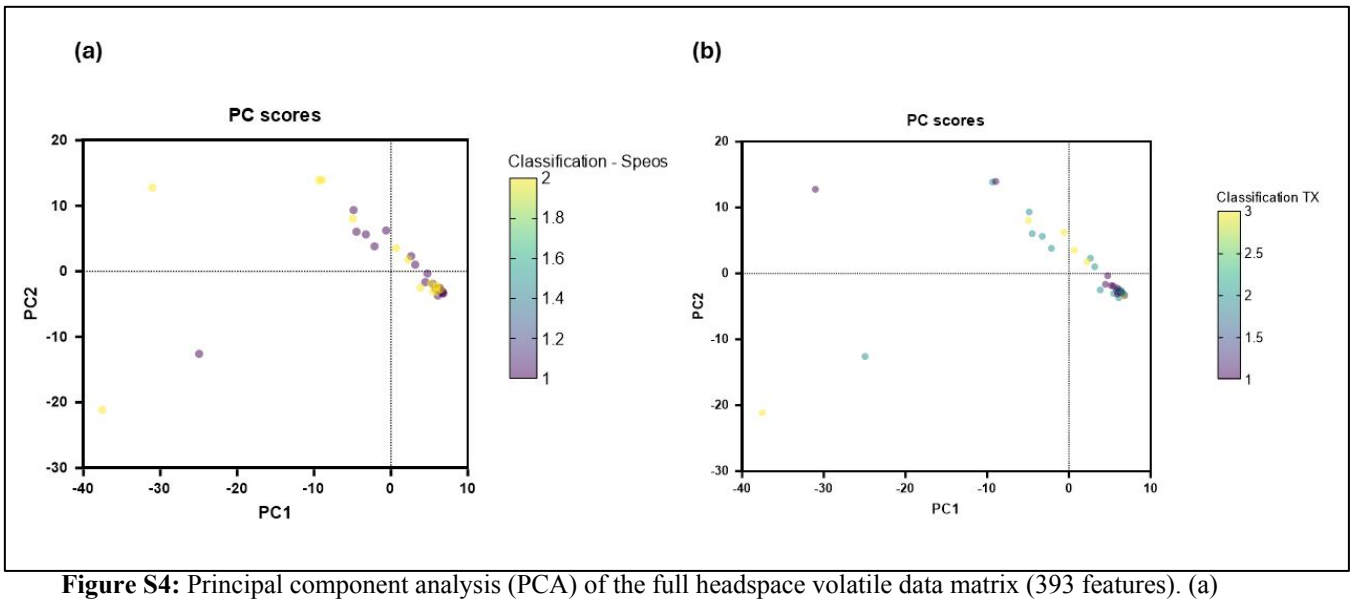

Figure S4: Principal component analysis (PCA) of the full headspace volatile data matrix (393 features). (a) PCA of headspace VOCs according to the presence (1, on colour bar), or absence (2) on colour bar. First two principal components account for 42% of the variance of the data. (b) PCA of headspace VOCs according to treatment classification (1= Mepolizumab ± mOCS, 2-ICS±LABA alone, 3=mOCS+ ICS±LABA).mOCS= maintenance oral steroids, ICS = inhaled corticosteroids, LABA = long acting β_2 agonist.

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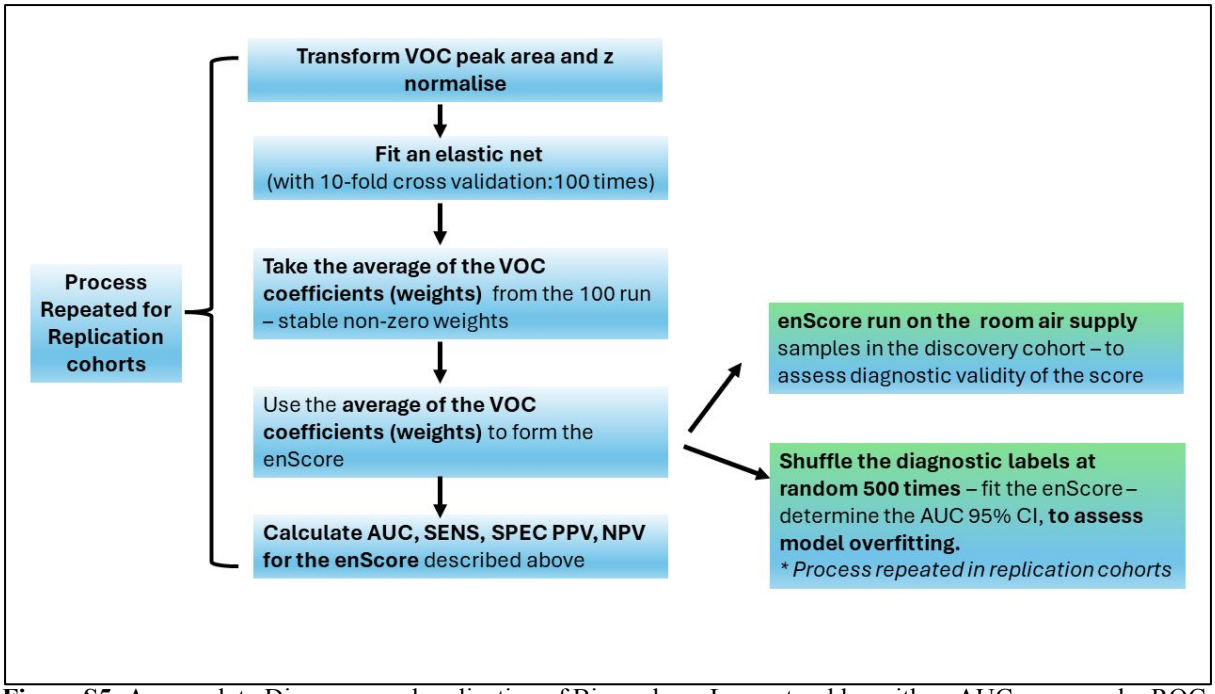

Figure S5: Approach to Discovery and replication of Biomarkers. Ln= natural logarithm, AUC= area under ROC curve, CI: confidence interval, enScore= elastic net regression-based VOC biomarker score. **NB:** in the headspace discovery cohort - peak areas were z-normalised without ln(x+1) transformation.

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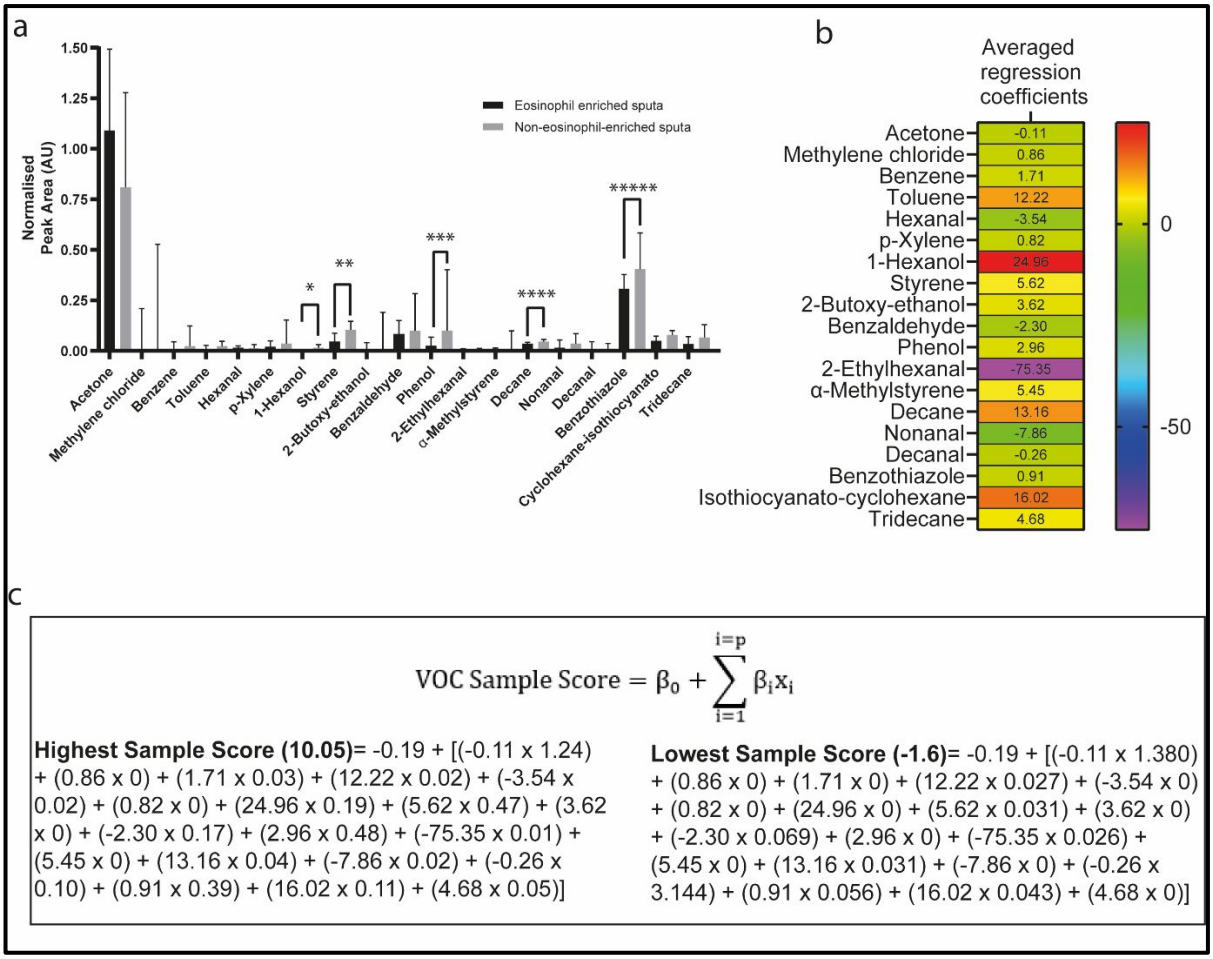

Figure S6: (a) Histogram of the normalised peak area values in the **discovery cohort** summarised as median and IQR and compared by Mann-Whitney test (significance at $p < 0.05$). **(b)** Heat map of the average regression coefficients for the 19 selected VOCs detected in sputum headspace of severe asthmatics as feature able to discriminate eosinophil-enriched from non-eosinophil-enriched sputa. **(c)** Equation used to calculate the sample scores (β_0 = intercept, β_i= average regression coefficient for the i^{th} VOC, x_i= normalised peak value for the i^{th} VOC, p = VOCs) and calculation of the highest (10.05) and lowest (-1.6) sample scores.

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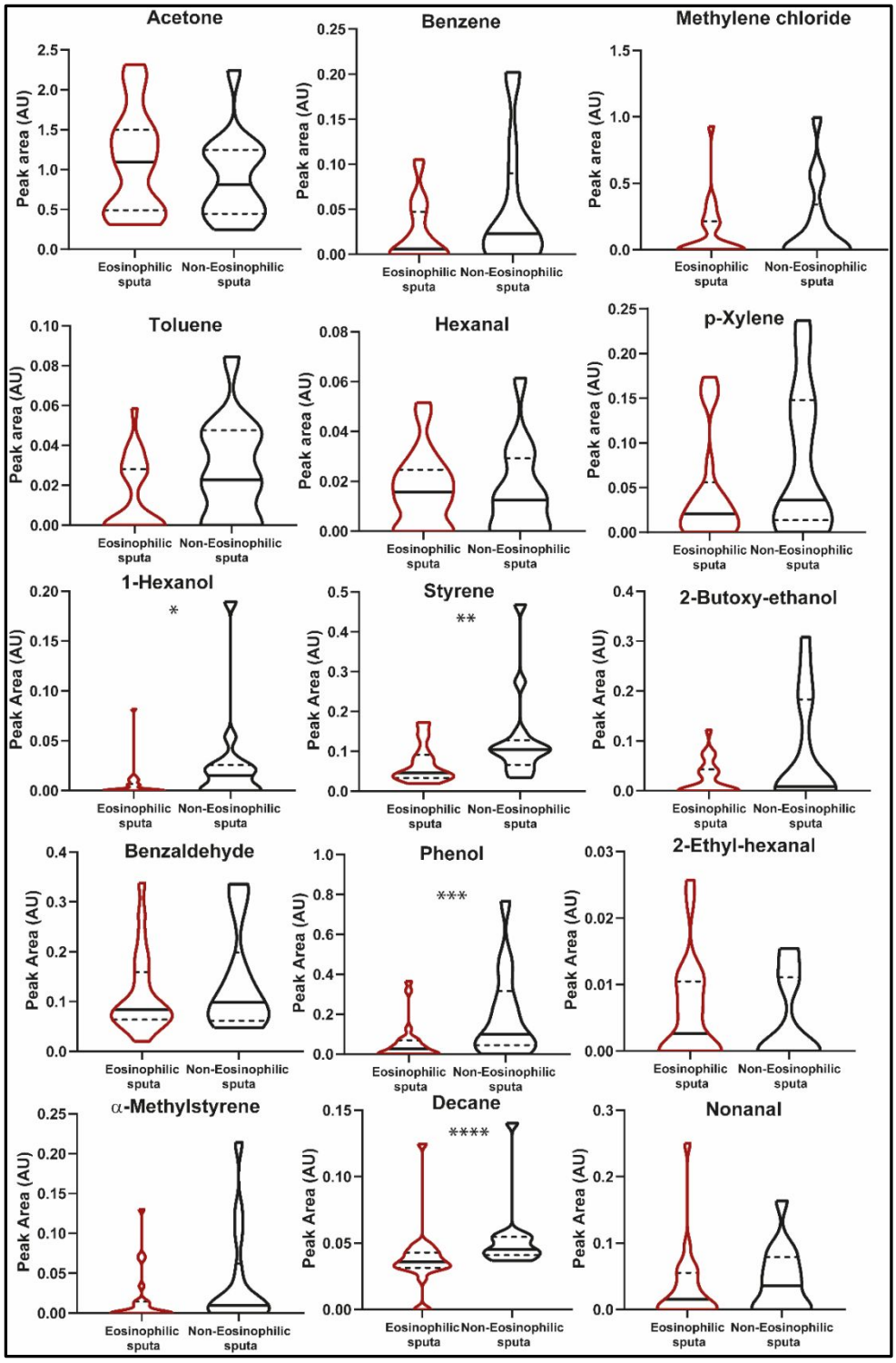

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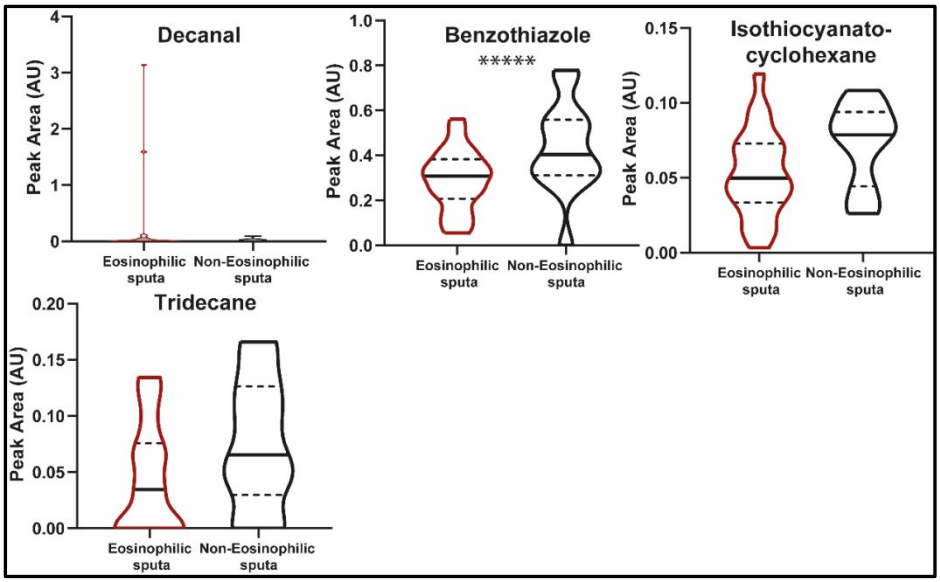

Figure S7: Violin plots of the normalised peak area values of the selected 19 x VOCs compared between eosinophil-enriched and non-eosinophil-enriched sputum headspace samples. Two-tailed Mann Whitney test 1-hexanol *p= 0.02, styrene **p= 0.017, ***phenol p= 0.005, ****decane p= 0.019, benzothiazole *****p= 0.03.

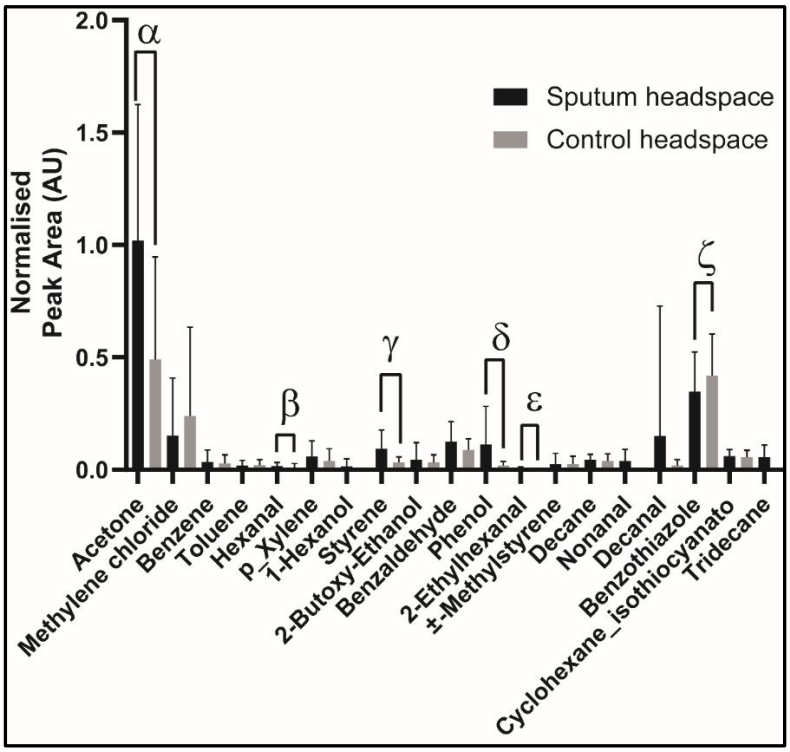

Figure S8: Bar chart of the abundance values (median; IQR) of the 19 selected biomarkers in sputum and control headspace samples in the **discovery cohort**. Two-tailed non-parametric test compared the peak area values of each compound between sputum and control headspace samples. The comparison revealed significant differences in peak area values for acetone ($p<0.0001$), hexanal ($p= 0.013$), styrene ($p<0.0001$), phenol ($p= 0.002$), benzothiazole ($p= 0.04$) and 2-ethylhexanal ($p= 0.006$); benzothiazole was the only compound to show a significant increase in control headspace samples compared to sputum headspace samples.

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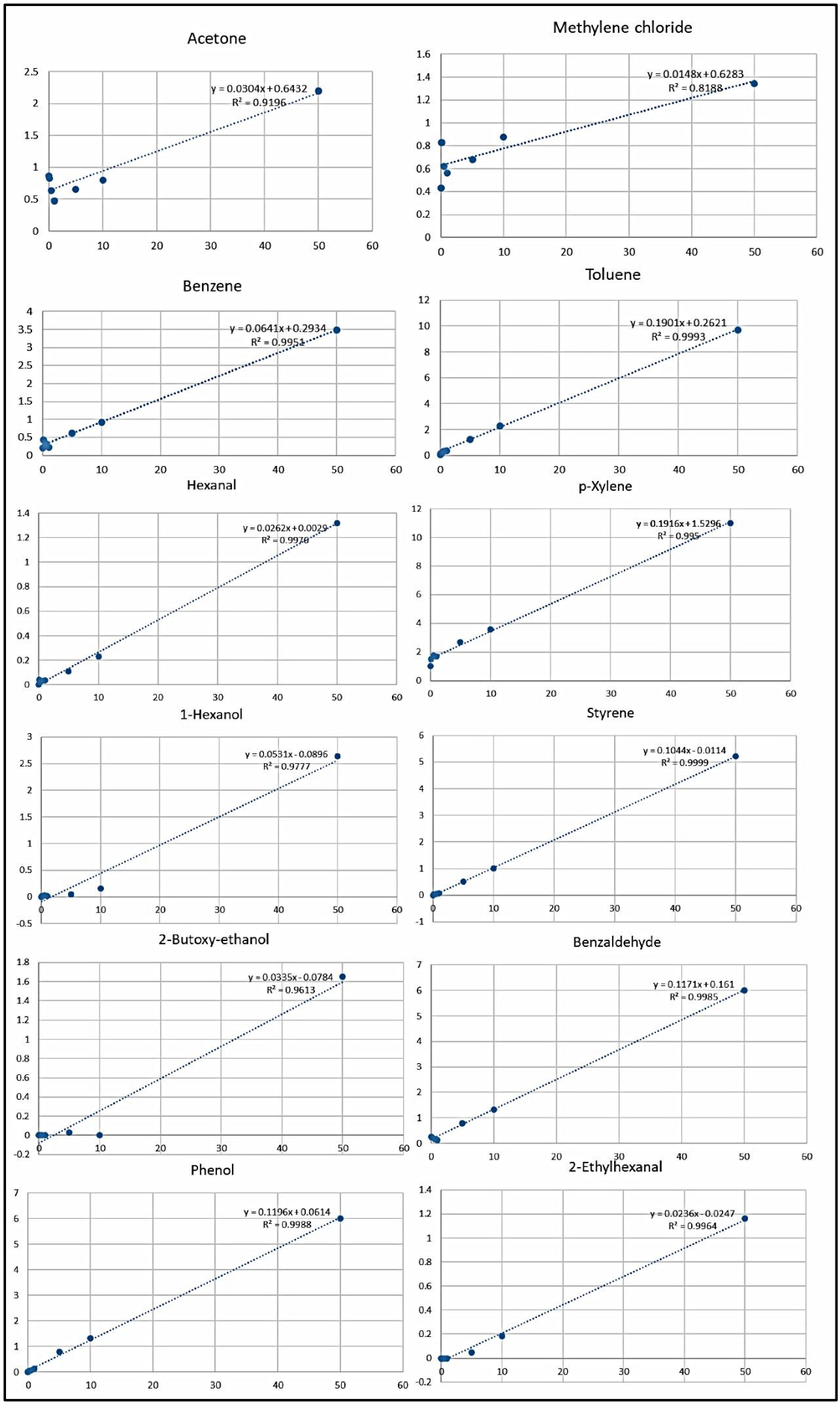

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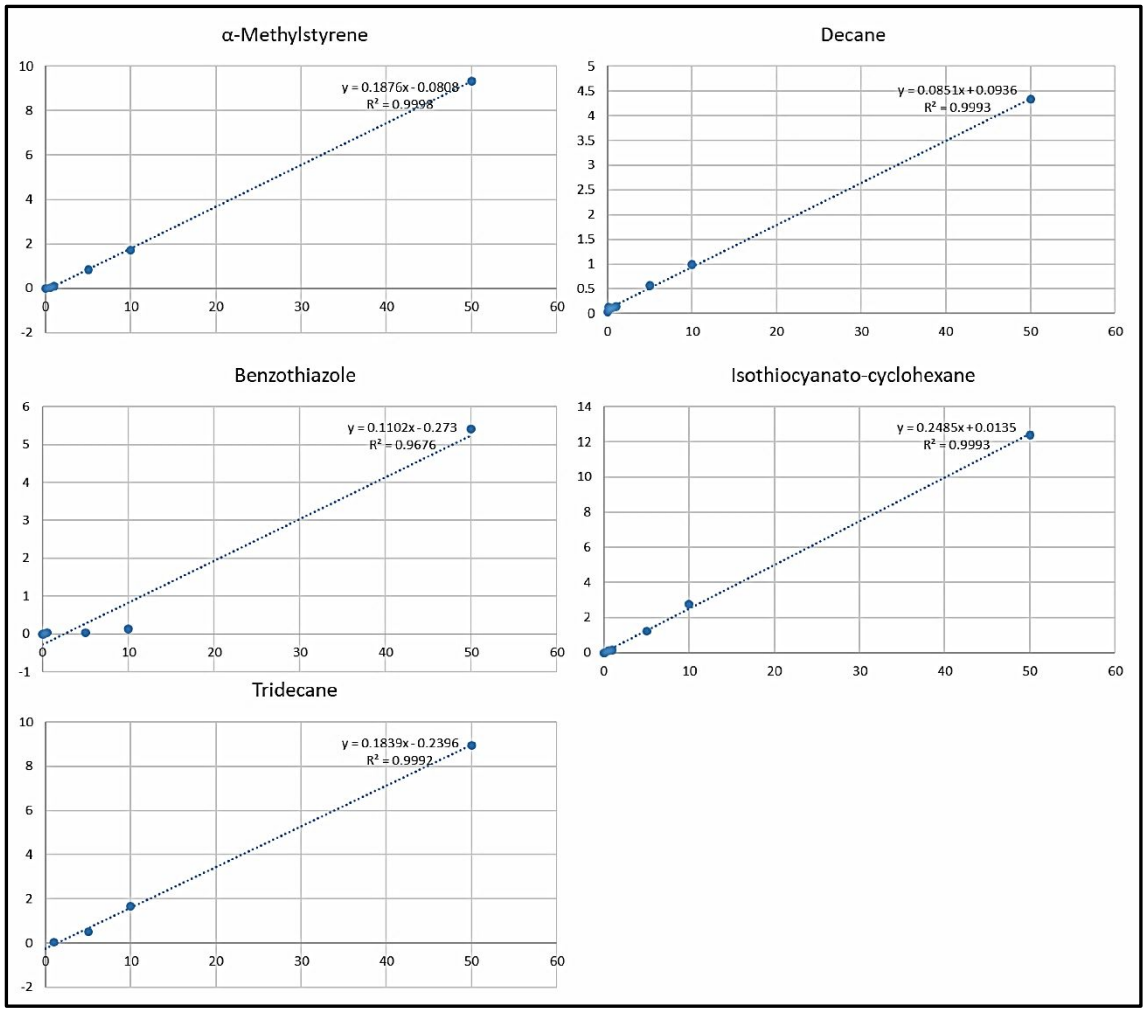

Figure S9: Calibration curves for each analytical standard analysed by GC-MS for the 19 VOCs identified in the discovery cohort. Units: **ng** on the x axis and **internal standard ratioed response** on the y axis.

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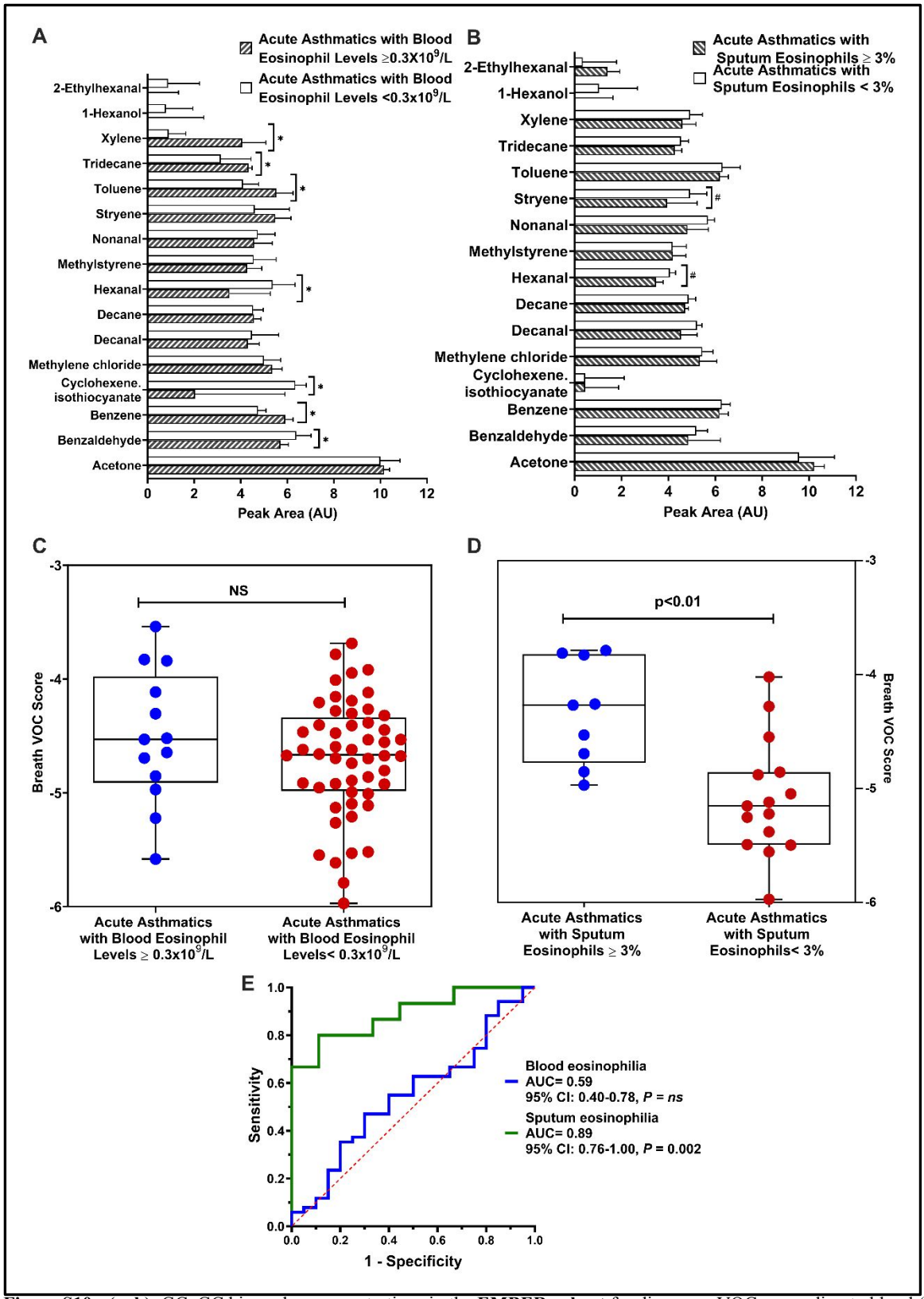

Figure S10: (a, b) GCxGC biomarker concentrations in the **EMBER cohort** for discovery VOCs according to blood (a) or sputum (b) eosinophilia. **(c, d)** breath biomarker signature values according to blood (a) or sputum (b) eosinophilia. (e) ROC analysis of biomarker score values and their ability to predict blood or sputum eosinophilia. * p<0.05 adjusted for multiple comparisons using Mann Whitney test and two-stage step-up (Benjamini, Krieger, and Yekutieli). #p <0.05 non-adjusted p-value

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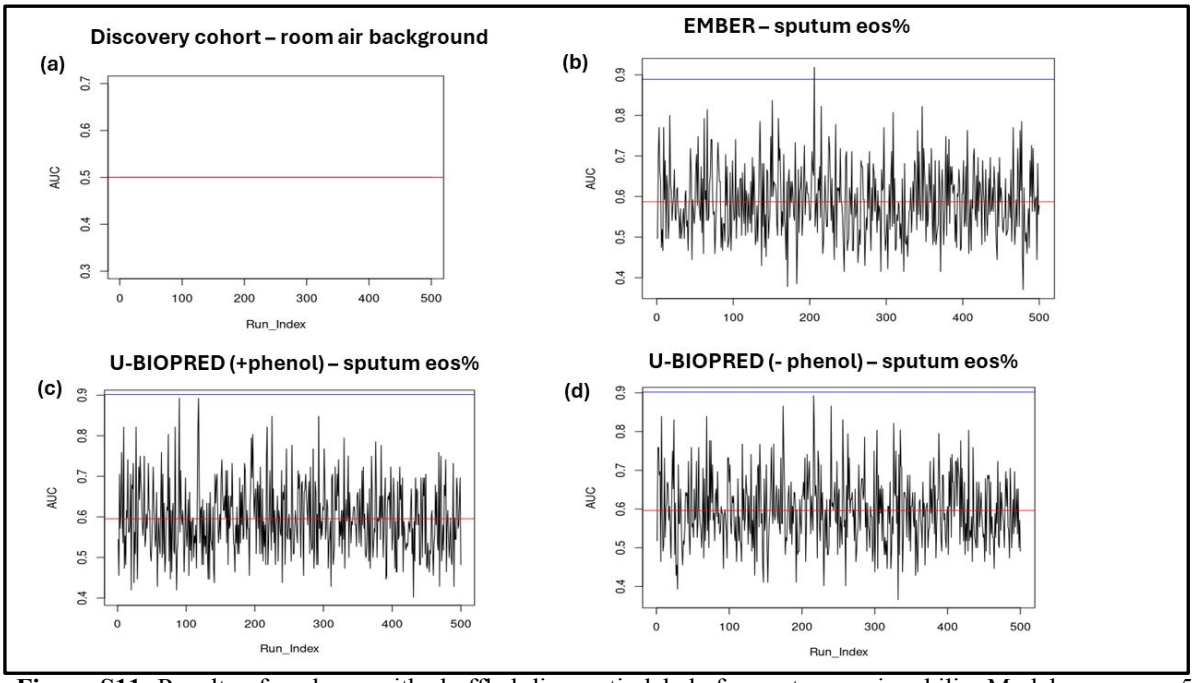

Figure S11: Results of analyses with shuffled diagnostic labels for sputum eosinophilia. Models were run 500 times (x-axis), with shuffled diagnostic labels, elastic net regression score based AUROC for each model run is summarised on the y-axis. **(a)** analysis for the room air in the discovery headspace cohort (note none of the VOCs had non zero coefficients on the e-NET, leaving the intercept term only, hence the AUC is constant at 0.50): **(b-d)** shuffled label analysis for **(b)** eosinophilic sputum in EMBER, **(c)** eosinophilic sputum in U-BIOPRED with phenol included in the marker panel and **(d)** eosinophilic sputum in U-BIOPRED with phenol excluded from the marker panel. Blue line is AUC for model with true labels, red line is the average AUC for model with randomly shuffled labels. The difference between the AUC for the model with true labels and AUC for model with randomly shuffled labels is statistically significant ,p-value perm test <0.01 for when comparing the model with true labels and shuffled labels in (**b,c & d**).

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Tables

Table S1: Analytical method parameters for TD-GC-MS headspace analysis in **discovery cohort**

Thermal desorption	
Tube desorption temperature	300°C
Tube desorption time	5min
Tube desorption flow	45 mL min^{-1}
Cold trap temperature	-10°C
Trap desorption temperature	300°C
Trap desorption time	5 min
Trap desorption flow	2 mL min^{-1}
Flow path temperature	200 °C
Mode	Splitless
Gas chromatography	
Column	Rxi-5ms 60mx0.25mm i.d.x0.25µm
Initial oven temperature	40°C
Carrier gas	Helium
Carrier gas flow	2 mL min^{-1}
Oven temperature ramp	5°C min^{-1}
Final oven temperature	300°C
Final temperature holding time	5 min
Mass spectrometry	
Scan type	Full scan (+ve)
Mass range	40 to 550 m/z
Ionisation type	EI
Scan frequency	3Hz
Transfer line temperature	300°C
Quadrupole temperature	150°C
Manifold temperature	230°C
Solvent delay time	5 min

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Table S2: Library of RTs, RIs, molecular weights, formula, CAS numbers and base peaks for the components of the RI standards solution – **discovery cohort**

Name	RT	RI	Formula	Molecular weight	CAS number	Base peak
Toluene D8	5·50	777·9	C7D8	100	2037-26-5	98
n-Octane	6·10	800·0	C8H18	114	111-65-9	43
1-Chlorohexene	7·33	856·3	C6H13Cl	120	544-10	91
1-Hexanol	7·62	867·4	C6H14O	102	111-27-3	56
1-Chloroheptane	10·03	957·0	C7H15Cl	134	619-06-1	91
n-Decane	11·18	1000·0	C10H22	142	124-18-5	57
1-Chloroctane	12·93	1056·4	C8H17Cl	148	111-85-3	91
5-Nonanol	13·74	1087·2	C9H20O	144	623-93-8	69
n-Undecane	14·04	1100·0	C11H24	156	1120-21-4	57
1-Chlorononane	15·91	1163·6	C9H19Cl	162	2473-01-0	91
n-Dodecane	16·95	1200·0	C12H26	170	112-40-3	57
1-Chloroundecane	18·79	1267·7	C10H21Cl	190	1002-69-3	91
n-Tetradecane	22·35	1400·0	C14H30	198	629-59-4	57
n-Heptadecane	29·.44	1700·.0	C17H36	240	629-50-5	57
n-Eicosane	35·52	2000·0	C20H42	282	17312-73-1	57
Dioctyl-phthalate	44·75	2435·1	C16H22O4	390	4376-20-9	149

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Table S3: TD-GCxGC-FID-qMS analytical method parameters – **EMBER cohort**

DRY PURGE ABD THRMAL DESORPTION	
Tube dry purge helium flow	50mL min^{-1}
Tube dry purge time	1 min
Tube desorption temperature	300°C
Tube desorption time	5min
Tube desorption flow	45 mL min^{-1}
Cold trap temperature	-10°C
Trap desorption temperature	300°C
Trap desorption time	2 min
Trap desorption flow	2 mL min^{-1}
Mode	Splitless
TWO-DIMENSIONAL GAS CHROMATOGRAPHY	
First chromatographic column	5% phenyl 95% dimethylpolysiloxane (30 m x 0.25 mm x 0.25 µm Rxi-5Sil MS)
Second chromatographic column	Polyethylene-glycol (4 m x 0.25 mm x 0.25 µm DB-WAX)
Carrier gas	Helium
First column-Carrier gas flow	0.6 mL min^{-1}
Modulation period	3 sec
Load time	2.799 sec
Injection time (to second column)	0.201 sec
Second column-Carrier gas flow	0.3 mL min^{-1}
Initial oven temperature	30°C – held for 5min
Oven temperature ramp	3°C min^{-1}
Intermediate oven temperature	80°C – held for 5min
Oven temperature ramp	5°C min^{-1}
Final oven temperature	250°C – held for 10min
RESTRICTORS	
Restrictor to FID	1.2 m x 0.25mm; fused silica
Carrier gas flow rate	23 $mLmin^{-1}$
Restrictor to single quadrupole mass spectrometer	0.76 m x 0.10 mm; fused silica
FID	
FID heater temperature	250°C
Make-up gas flow	Nitrogen; 25mL min^{-1}
Pure air flow	400 mL min^{-1}
Hydrogen flow	35 mL min^{-1}
Acquisition rate	100 Hz
SINGLE QUADRUPOLE MASS SPECTOMETER	
Mass scan range	40 to 300 m/z
Ionisation type	EI
Acquisition rate	21.5Hz
Transfer line temperature	300°C
Ion source temperature	250°C
Quadrupole temperature	150°C

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Table S4: Results of Spearman correlation between sputum eosinophil percentage and normalised peak area values of the 19-sputum headspace VOCs identified in the **discovery cohort**

Compounds	Spearman correlation coefficient	p-value
Acetone	0.1933	0.2659
Methylene chloride	-0.1719	0.3235
Benzene	-0.3372	**0.0476**
Toluene	-0.2009	0.2472
Hexanal	-0.05478	0.7546
p-Xylene	-0.1721	0.3227
1-Hexanol	-0.2647	0.1244
Styrene	-0.4083	**0.0149**
2-Butoxy-ethanol	-0.1817	0.2962
Benzaldehyde	-0.1717	0.3241
Phenol	-0.4603	**0.0054**
2-Ethylhexanal	0.163	0.3496
α-Methylstyrene	-0.2221	0.1998
Decane	-0.5751	**0.0003**
Decanal	-0.1397	0.4234
Nonanal	-0.01294	0.9412
Benzothiazole	-0.4522	**0.0064**
Isothiocyanato-cyclohexane	-0.3071	0.0728
Tridecane	-0.3084	0.0715

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Table S5: Severe asthmatics sputum samples in the discovery cohort according to inflammatory phenotype.

	Eosinophil-Enriched Sputa (n=22)		**Non-Eosinophil-Enriched Sputa (n=14)**	
	Eosinophilic sputa	**Mixed-granulocytic sputa**	**Neutrophilic sputa**	**Pauci-granulocytic Sputa**
Eosinophils ≥ 3% and Neutrophils < 65%	6	/	/	/
Eosinophils ≥ 3% and Neutrophils ≥ 65%	/	16	/	/
Eosinophils < 3% and Neutrophils ≥ 65%	/	/	10	/
Eosinophils < 3% and Neutrophils < 65%	/	/	/	4

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Table S6: Clinical characteristics and demographics of **EMBER acute cohort**, **grouped according to the blood eosinophil threshold of 0·3x10^9/L**.

	N	EMBER BEC ≥ 0.3 x10^9/L (n=20/65)	N	EMBER sputum BEC < 0.3 x10^9/L (n=40/65)	p-value
Age	20	39 (24.2-64.5)	45	43 (28.5-59)	ns
Sex (Number-male)	20	12/20	45	12/45	0.01
BMI (Kg/m^2)	20	26.1 (23.1-39)	45	32.3 (25.7-38.4)	0.02
Never-Ex-Current (%)	19	10/7/2	45	22/11/12	ns
Pre exacerbation background GINA 2023 treatment step	19	**GINA 1:** 3 **GINA 2-3**: 10 **GINA 4-5:** 6	43	**GINA 1:** 7 **GINA 2-3**: 7 **GINA 4-5:** 29	0.009
Blood Eosinophils (x10^9/L)	20	0·61(0·43-1.1)	45	0·09 (0·050-0·21)	<0·0001
CRP level (mg/L)	20	6.5(5.0-19.8)	43	11(5.0-23.0)	ns
Forced oscillation technique R_5-R_{19} (kPa.s. L^{-1})	17	1·18 (0.47-1.87)	43	1·18 (0·47-2·09)	ns
Visual analogue breathlessness score (100 mm scale)	18	77 (61-83)	42	78.0 (70-87.2)	ns
Visual analogue cough score (100 mm scale)	18	58.0(31.5-79.5)	42	73(53.5-86.8)	ns
Visual analogue wheeze (100 mm scale)	18	71.0(43-81.25)	42	75.0(52.5-87.25)	ns
Respiratory rate/min	20	20(18-20)	44	20(18-23)	
Spo2 at the time of breath sampling (%)	18	96.5(94-98)	41	96.0(95-98)	ns
Requiring supplemental oxygen during acute admission	19	5/19	43	2/43	0.01
Length of hospital stay (days)	17	2,0(1-3)	42	2.0 (1-3)	ns
Oral steroid or antibiotic exposure for exacerbation with 2 weeks of admission	13	3/13	44	23/44	ns

Legend: Date presented as median (Q1-Q3). Maan-Whitney test was performed for continuous variables. Chi squared test was performed for categorical variables. Statistically significant differences were reported for: (BEC) baseline blood. GINA Global Initiative for Asthma 2023 guideline. CRP = C-reactive protein. BMI = body mass index. Spo2= pulse oximetry oxygen saturations.

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Table S7: Clinical characteristics and demographics of **EMBER acute cohort**, **grouped according to the sputum eosinophil threshold of 3%.**

	N	EMBER sputum eosinophils ≥ 3% (n=9/65)	N	EMBER sputum eosinophils < 3% (n=15/65)	p-value
Age	9	50 (29-55)	15	43 (26-55)	ns
Sex (Number-male)	9	6/9	15	4/15	-
BMI (Kg/m²)	9	30 (24-39)	15	34 (26-44)	ns
Never-Ex-Current (%)	9	3/5/1	15	7/4/4	ns
Pre exacerbation background GINA 2023 treatment step	9	**GINA 1:** 1/9 **GINA 2-3**:5/9 **GINA 4-5:**3/9	15	**GINA1:** 1/15 **GINA 2-3:**4/15 **GINA 4-5:**10/15	ns
Blood Eosinophils (x10⁹/L)	8	0·59(0·22-1.0)	15	0·07 (0·030-0·20)	<0·002
Sputum eosinophils (%)	9	9·5 (5.4-35)	15	0·0 (0·0-0·50)	<0.0001
CRP level (mg/ml)	8	13.5(5.2-43.2)	14	7.0(5.0-17.5)	ns
RhV-16 PCR Log_{10} RV_{16} copy number in sputum	3/6	1.1 (0-3.0)	10/12	3.0 (2.1-4.9)	ns
Forced oscillation technique R_5-R_{19} (kPa.s. L^{-1})	8	1·94 (0.68-2·9)	14	1·20 (0·65-2·31)	ns
Visual analogue breathlessness score (100 mm scale)	7	70 (46-92)	14	76.0 (67-82)	ns
Visual analogue cough score (100 mm scale)	8	72.5(30.75-92)	14	77.0 (58-93)	ns
Visual analogue cough wheeze (100 mm scale)	8	84.0(52-91)	14	74.5 (71-84)	ns
Respiratory rate/min	9	18(18-20)	15	24(20-24)	0.002
Spo2 at the time of breath sampling (%)	9	95(94-98)	14	97(94-98)	ns
Requiring supplemental O_2 during acute admission	9	2/9	15	1/15	ns
Length of hospital stay (days)	8	2.5 (1.25-4.8)	15	3.0 (2-3)	ns
Oral steroid or antibiotic exposure for exacerbation with 2 weeks of admission	9	2/9	15	8/15	ns

Legend: Mann-Whitney test was performed for continuous variables. Chi-squared test was performed for categorical variables. Spo2= pulse oximetry oxygen saturations. GINA 2023 = Global initiative for Asthma guideline. BMI = body mass index. OCS= oral corticosteroids. PCR= polymerase chain reaction. RV_{16} = Rhinovirus 16.

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Table S8: Clinical characteristics and demographics of **U-BIOPRED severe asthma cohort**, **grouped according to the sputum eosinophil threshold of 3%.**

	U-BIOPRED Sputum eosinophils ≥ 3%	U-BIOPRED Sputum eosinophils <3%	p-value
n	14	8	
Age (years)	53.5(47.5-57.5,14)	54(44.25-59.5,8)	0.578
Age of onset (years)	38.5(13.5-48.5,14)	26.5(9.5-44,8)	0.785
Female	8(57%,14)	4(50%,8)	1
BMI (kg/m^2)	30.2(27.45-33.58,14)	32.58(26.33-35.04,8)	0.698
Smoker	5(36%,14)	6(75%,8)	0.191
Nasal polyps(yes)	8(57%,14)	2(25%,8)	0.199
Allergic rhinitis (yes)	2(14%,14)	2(29%,7)	0.585
Eczema (yes)	2(14%,14)	2(25%,8)	0.608
Oral corticosteroid use (yes)	11(79%,14)	3(38%,8)	0.086
Atopy (yes)	11(79%,14)	5(62%,8)	0.64
Exacerbations previous year	1(0-1.75,14)	1(0.75-1.25,8)	0.829
FEV_1 (L)	1.77(1.43-2.51,14)	2.63(1.64-3.26,8)	0.33
Total IgE (Ku/L)	215(60.2-782.75,14)	80.95(35.53-203.25,8)	0.297
Blood leukocytes (x 10^9/L)	9.29(6.1-11.13,14)	7.54(6.02-8.33,8)	0.228
Blood eosinophils (x 10^9/L)	0.31(0.19-0.44,14)	0.11(0.09-0.14,8)	0.004
Blood neutrophils (x 10^9/L)	6.06(3.47-7.83,14)	4.7(3.78-5.08,8)	0.187
Sputum eosinophils (%)	20.355(12.5-24.2,14)	0.58(0.135-1.905,8)	0.000151
Sputum neutrophils (%)	55.8(37.7-65.48,14)	80.8(52.69-85.96,8)	0.076
Sputum macrophages (%)	25.28(13-29.45,14)	15.96(11.76-46.17,8)	1
FeNO (ppb)	49.5(31.25-71.75,14)	20(17.12-25.25,8)	0.082
Serum periostin (ng/mL)	48.13(37.7-57.2,12)	51.28(42.74-54.7,7)	0.902
CRP (mg/L)	1.65(0.94-3,14)	4.15(2.88-5.38,8)	0.109
Combined.Atopy.Regional.Aeroallergens	1(7%,14)	2(25%,8)	0.513
History. Pneumonia (+)	0(0%,14)	1(12%,8)	0.349

Legend: Unpaired two-tailed t test was performed for continuous variables. Chi squared test was performed for categorical variables. Statistically significant difference was reported.

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Table S9: Clinical characteristics and demographics of **U-BIOPRED severe asthma cohort**, **grouped according to the blood eosinophil threshold of 0·3x10^9/L**.

	U-BIOPRED BEC ≥ 0·3x10^9/L	**U-BIOPRED BEC <0·3x10^9/L**	**p-value**
n	**12**	**30**	
Age (years)	57(51.25-62,12)	48(40.5-58.75,30)	0.02
Age of onset (years)	47(37.5-55.5,11)	14(4-32.5,27)	0.012
Female	7(58%,12)	16(53%,30)	1
BMI (Kg/m^2)	28.17(26.67-33.39,12)	32.33(25.46-35.66,30)	0.539
Smoker (yes)	3(25%,12)	23(77%,30)	0.003
Nasal polyp s(yes)	6(50%,12)	12(40%,30)	0.738
Allergic rhinitis (yes)	5(42%,12)	9(31%,29)	0.729
Eczema (yes)	2(17%,12)	8(27%,30)	0.69
Oral corticosteroid use (yes)	8(67%,12)	20(67%,30)	1
Atopy (yes)	10(83%,12)	19(63%,30)	0.279
Exacerbations previous year	1(1-2.25,12)	1(1-2,30)	0.522
FEV_1 (L)	1.71(1.42-2.17,12)	2.02(1.29-3.18,30)	0.791
Total IgE (Ku/L)	250.5(164.5-362,12)	104.5(45-356.2,30)	0.452
Blood leukocytes (x 10^9/L)	7.12(5.97-9.61,12)	9.08(7-10.68,30)	0.167
Blood eosinophils (x 10^9/L)	0.43(0.39-0.5,12)	0.11(0.07-0.16,30)	5.59E-07
Blood neutrophils (x 10^9/L)	4.42(3.28-5.2,12)	6.54(4.36-8.38,30)	0.033
Sputum eosinophils (%)	21.3(13.4-33.6075,8)	3.58(0.38-11.6,14)	0.013
Sputum neutrophils (%)	47.31(36.37-68.18,8)	62.16(47.8-78,14)	0.412
Sputum macrophages (%)	19.7(11.66-28.19,8)	21.4(13.3-47.1,14)	0.402
FeNO (ppb)	53(39-67,11)	21(13-42,29)	0.011
Serum periostin (ng/mL)	53.02(44.92-70.83,12)	47.1(39.6-55.4,26)	0.174
CRP (mg/L)	2.3(0.66-3.1,12)	3.25(1.12-4.72,30)	0.186
Combined.Atopy.Regional.Aeroallergens	2(17%,12)	13(45%,29)	0.145
History.Pneumonia (+)	0(0%,12)	2(7%,30)	0.582

Legend: Unpaired two-tailed t test was performed for continuous variables. Chi squared test was performed for categorical variables.

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