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# BMJ Open

## Assessment of Breath Volatile Organic Compounds in Acute Cardio-respiratory Breathlessness: A Prospective Real World Observational Study

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# 1 Assessment of Breath Volatile Organic Compounds in Acute Cardio- 2 respiratory Breathlessness: A Prospective Real World Observational Study

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## 69 **Abstract**

### 70 **Introduction**

71 Patients presenting with acute undifferentiated breathlessness are commonly encountered in  
72 admissions units across the United Kingdom. Existing blood biomarkers have clinical utility in  
73 distinguishing patients with single organ pathologies but have poor discriminatory power in multi  
74 factorial presentations. Evaluation of volatile organic compounds (VOC) in exhaled breath offers the  
75 potential to develop biomarkers of disease states that underpin acute cardio-respiratory breathlessness,  
76 owing to their proximity to the cardio-respiratory system. To date there has been no systematic  
77 evaluation of VOC in acute cardio-respiratory breathlessness. The proposed study will seek to use  
78 both offline and online VOC technologies to evaluate the predictive value of VOC in identifying  
79 common conditions that present with acute cardio-respiratory breathlessness.

### 80 **Methods and analysis**

81 A prospective real world observational study carried out across three acute admissions units within  
82 Leicestershire. Participants with self-reported acute breathlessness, with a confirmed primary  
83 diagnosis of either acute heart failure, community acquired pneumonia and acute exacerbation of  
84 asthma or COPD will be recruited within 24 hours of admission. Additionally, school age children  
85 admitted with severe asthma will be evaluated. All participants will undergo breath sampling on  
86 admission and upon recovery following discharge. A range of online technologies including: proton-  
87 transfer-reaction mass spectrometry (PTR-MS), gas chromatography ion mobility spectrometry (GC-  
88 IMS), atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) and offline  
89 technologies including gas chromatography mass spectroscopy (GC-MS) and comprehensive two-  
90 dimensional gas chromatography-mass spectrometry (GCxGC-MS) will be utilised for VOC  
91 discovery and replication. For offline technologies a standardised CE marked breath sampling device  
92 (ReCIVA<sup>®</sup>) will be used. All recruited participants will be characterised using existing blood  
93 biomarkers including C - reactive protein (CRP), brain derived natriuretic peptide (BNP), Troponin-I  
94 and blood eosinophil levels and further evaluated using a range of standardised questionnaires, lung  
95 function testing, sputum cell counts and other diagnostic tests pertinent to acute disease.

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3 96 **Ethics and dissemination**

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5 97 The National Research Ethics Service Committee East Midlands has approved the study protocol  
6  
7 98 (REC number: 16/LO/1747). IRAS 198921. Findings will be presented at academic conferences and  
8  
9 99 published in peer-reviewed scientific journals. Dissemination will be facilitated via a partnership with  
10  
11 100 the East Midlands Academic Health Sciences Network and via interaction with all UK funded  
12  
13 101 MRC/EPSRC molecular pathology nodes.  
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18 103 **Key words:** Breathlessness; Breath analysis; Volatile Organic Compound, Observational study  
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## 118 **Strengths and Limitations of this Study**

### 119 **Strengths**

- 120 • A pragmatic real world, prospective, observational study across three admission units that  
121 focuses on the systematic discovery and replication of VOC in acutely breathless patients  
122 using both online and offline technologies
- 123 • The study will evaluate populations that often present with diagnostic uncertainty  
124 including elderly multi-morbid patients and school age children
- 125 • The proposed study is the largest of its kind in acute disease to characterise VOC with a  
126 range of additional assessments that will build a comprehensive phenotype of acute  
127 cardio-respiratory exacerbations
- 128 • Benchmarking of candidate VOC to established blood based biomarkers utilised in  
129 clinical practice e.g. BNP, CRP will form an important method of validation against  
130 existing molecular pathology
- 131 • The proposed study will build an infrastructure for research and subsequent evaluation of  
132 VOC in interventional trials within acute cardio-respiratory exacerbations

### 133 **Limitations**

- 134 • Prior acute treatment exposure will need to be accounted for when evaluating potential  
135 discriminative biomarkers
- 136 • VOC technologies are not currently suited for deployment in patients that are of high clinical  
137 acuity e.g. severe respiratory failure and thus the study will self-select patients that can be  
138 safely sampled with online and offline methodologies
- 139 • Although the study will quantify and evaluate diagnostic uncertainty, the study does not  
140 enrich for these cases and the patient population will predominantly comprise of patients in  
141 whom a senior clinical decision maker could make the primary clinical diagnosis within 24  
142 hours of admission. As a consequence future studies will be required to evaluate the  
143 biomarkers developed in cardio-respiratory exacerbations where the diagnosis is unclear.



## 1. Introduction:

Breathlessness is a common symptom of cardio-respiratory illnesses that has a significant direct impact on patients' wellbeing as well as a substantial economic burden on healthcare systems [1]. Although its etiologies can be variable, exacerbations of common complex chronic cardio-respiratory conditions account for approximately 70% of acute presentations with breathlessness, namely exacerbations of asthma and COPD, acute heart failure and community acquired pneumonia [2]. Moreover, moderate and severe breathlessness is significantly associated with all-cause, cardiovascular and COPD mortality[3]. As a consequence symptomatic breathlessness warrants rapid evaluation and targeted diagnostics at presentation.

Diagnostic evaluation of acute breathlessness is heavily reliant on blood based biomarkers e.g. CRP, BNP, Troponin and on occasions blood eosinophil levels. These biomarkers have clinical utility primarily in patients with single pathologies, but have poor discriminatory power in patients with multifactorial presentations of acute breathlessness[4]. There is therefore an unmet need for the development of sensitive and specific biomarkers that differentiate acute breathlessness from its recovery and the common cardio-respiratory conditions that present with acute breathlessness.

CRP plays an important role in diagnosing breathlessness caused by an underlying bacterial pneumonia[5], as well as predicting mortality in patients with COPD [6]. BNP is routinely utilised in acute settings to support the diagnosis of acute heart failure [7].The European Society of Cardiology (ESC) recommends BNP threshold values of <100 pg/mL to rule out acute congestive cardiac failure and values > 500 pg/ml as diagnostic of acute exacerbations of heart failure [8].

The role of peripheral blood eosinophil count in airway inflammation was poorly understood up until the second half of the 19<sup>th</sup> century when Paul Ehrlich, a German physician and Nobel prize winner, introduced eosin in his technique for white cell differentiation in 1879[9] . Considerable advances in the field of airway inflammation and the role of eosinophils have taken place since [10-12]. More

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3 171 recently Bafadhel *et al* suggested that peripheral blood eosinophil count can be used to direct  
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5 172 corticosteroid therapy during COPD exacerbations in single centre study [13].  
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7 173 Currently, blood biomarkers together with clinical, physiological and imaging parameters are used in  
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9 174 diagnosing the cause of acute breathlessness. Blood biomarkers may be less specific as they originate  
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11 175 far from the target organs of interest (the heart and the lungs in cardio-respiratory disease). Sputum,  
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13 176 although potentially a more definitive lung specific matrix, is comparatively difficult to obtain  
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15 177 particularly in acutely unwell patients, limiting its use in acute disease and highlighting the need for  
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17 178 better biomarkers. Ideally these biomarkers would have the following characteristics, (i) they would  
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19 179 originate from the target organ of interest, (ii) they would significantly add value to conventional risk  
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21 180 scoring and diagnostic algorithms in acute breathlessness, (iii) they would be minimally invasive and  
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23 181 suitable for rapid point of care diagnosis in emergency rooms and acute admissions units (iv) they  
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25 182 would have diagnostic value in patients with multifactorial acute breathlessness.

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29 184 Exhaled breath contains thousands of volatile organic compounds (VOC) that reflect biological  
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31 185 processes occurring in the host both locally in the airways and systematically offering the potential to  
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33 186 develop more effective biomarkers in acutely breathless patients (**Figure 1**).

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37 188 The proposed program of research will use a combination of offline and online technologies to  
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39 189 identify and evaluate the diagnostic and prognostic value of VOC in patients with acute cardio-  
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41 190 respiratory related breathlessness (**Figure 2**).

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45 192 Exhaled breath analysis of volatile chemicals offers the potential to develop more effective biomarkers  
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47 193 in acutely breathless patients. The use of breath analysis for disease diagnostics dates back to ancient  
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49 194 Greeks where physicians used exhaled breath to diagnose different diseases. Breath odours allow  
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51 195 correct associations to certain diseases. For example, the sweet smell of diabetic ketoacidosis, the  
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53 196 fishy smell of breath associated to liver illness, the urine-like odour of kidney disease and the smell of  
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55 197 the breath of patients with lung abscesses, caused by the proliferation of anaerobic bacteria [14-17].

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3 198 More recently, exhaled breath analysis has demonstrated early proof of concept in the diagnosis of  
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5 199 acute heart failure, ventilator associated pneumonia[18] and stable state airways disease [15]. The  
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7 200 validity of breath analysis has also been demonstrated in breathless children[19]. This population is  
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9 201 likely to prefer breath-based tests, as these are minimally invasive. Importantly, a variety of point of  
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11 202 care sensors are now available to evaluate potential exhaled breath biomarkers in emergency care  
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13 203 settings.

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17 205 Despite the novelty of non-invasive sampling technology and the growing interest in exhaled breath  
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19 206 analysis there remains a disappointing level of comparability across studies due to the lack of  
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21 207 standardisation and appropriate data analysis methods.

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23 208 A recent systemic review by Anders Christiansen *et al* compared eleven publications reporting very  
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25 209 heterogeneous designs, methods, patient group sizes, data analytics and, consequently, quite varying  
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27 210 results [20].

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31 212 To our knowledge, no other large studies exploring the use of breath biomarkers in profiling acute  
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33 213 breathlessness have been completed. Few studies have explored the use of electronic nose in stable  
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35 214 disease with good discriminatory power in COPD [21], Pneumonia [22] and heart failure[23] with  
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37 215 relatively small sample size. The focus of the current research study will be to evaluate acutely  
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39 216 breathless cardio-respiratory patients using a combination of ‘discovery’ and near-patient care breath  
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41 217 sampling technologies.

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43 218 Medical Research Council (MRC) and Engineering and Physical Sciences Research Council (EPSRC)  
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45 219 have commissioned a series of molecular pathology nodes aimed at developing molecular signatures  
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47 220 relevant to disease diagnosis and progression. This was triggered by the clear need for alliance  
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49 221 between academic institutions, industry and NHS partners to enhance the benefits of stratified  
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51 222 medicine for patients[24, 25].

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53 223 University of Leicester and Loughborough University were awarded a joint molecular pathology node  
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55 224 East Midlands Breathomics Pathology Node (EMBER) which this study forms a key part of.

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## 226 **2. Methods and Analysis**

### 227 **2.1. Study design**

228 A prospective real world observational study across three acute admissions units within Leicestershire  
229 (two adult admissions units and one children's assessment unit). The acute units routinely assess and  
230 treat cardio-respiratory admissions due to breathlessness in adults and children.

231 Participants with self-reported acute breathlessness, either requiring admission or a change in baseline  
232 treatment, will be screened for the study. Informed consent will be obtained in all participants  
233 following a clinical review by a senior decision maker within 24 hours of acute admission (**Figure 3**).

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### 236 **2.2. Objectives**

#### 237 **2.2.1. Primary objective**

- 238
- 239 • To evaluate the sensitivity, specificity, positive and negative predictive value of exhaled  
240 breath VOC biomarkers to differentiate acute breathlessness in cardio-respiratory patients.

#### 241 **2.2.2. Secondary objectives:**

- 242
- 243 • To replicate selected breath VOC biomarkers identified in acute breathlessness.
  - 244 • To discover and replicate breath VOC biomarkers that differentiate the common cardio-  
245 respiratory conditions that cause acute breathlessness, specifically (i) acute heart failure, (ii)  
246 community acquired pneumonia, (iii) adult exacerbations of asthma and COPD and age-  
247 matched adults that do not have cardio-respiratory disease or breathlessness.
  - 248 • To quantify the level of clinical uncertainty in the primary diagnosis using a 100 mm visual  
249 analogue scale and independent clinical adjudication of case notes blinded to the following  
250 blood biomarkers (i) CRP (ii) BNP (iii) Troponin-I (iv) blood eosinophils, but not clinical  
251 history and acute presentation nor chest x-ray imaging. Potential discriminatory breath VOC  
252 biomarkers will be adjusted for clinical uncertainty in statistical models.
  - 253 • To identify and replicate exhaled breath VOC biomarkers in school age children treated in  
254 hospital for severe asthma attacks and compare these to age-matched healthy controls.

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3 255 **2.2.3. Exploratory end points (where applicable):**  
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- 5 257 • To evaluate the dynamic profile of selected breath VOC between the acute state and the  
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7 258 recovery state post exacerbation.  
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9 259 • To evaluate the relationship between exhaled VOC biomarkers and clinical outcomes  
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11 260 including (i) hospital readmission at 30 and 60 days post event (ii) all-cause mortality over a 2  
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13 261 year period post admission.  
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15 262 • To evaluate the relationship between breath VOC biomarkers and functional measures  
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17 263 e.g. physical performance and activity  
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19 264 • To explore potential breath VOC biomarkers of multifactorial acute breathlessness  
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21 265 • To evaluate the relationship between diet, lifestyle and environment upon breath VOC  
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23 266 biomarkers  
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27 268 **2.3. Sample size estimation**  
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31 270 Preliminary data was utilised to conduct sample size estimates from a cohort of acutely breathless  
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33 271 patients admitted to acute admissions units over a 6 month period (February 2017 to August 2017).  
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35 272 Hundred and twelve adult participants (asthma 46, community acquired pneumonia 26, COPD 22)  
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37 273 and eighteen healthy controls were utilised for the analysis.  
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39 274 A panel of ten pre-specified aldehydes, based on literature search [26-28], were extracted from breath  
40  
41 275 using gas chromatography-mass spectrometry (GC-MS). The aldehydes were normalised to a  
42  
43 276 common internal standard and were not background-subtracted.  
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45  
46 277 A closed formula from Hsieh *et al*[29], relating sample size to observable effect size, was used to  
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48 278 calculate sample size from logistic regression models of the ten aldehydes with acute breathlessness  
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50 279 as the outcome measure. The sample size estimates are also relevant to acute class comparisons vs.  
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52 280 the sum of other acute classes.  
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54 281 Based upon the sample size estimates we would have an 80% power, with a type 1 error rate of 5%, to  
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56 282 detect an odds ratio of association of 1.2 between two disease classes with 55 patients per class. Given  
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3 283 the fact that study seeks to discover and replicate breath VOC amongst five adult disease classes  
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5 284 (community acquired pneumonia, heart failure, COPD, asthma and healthy aged matched subjects) we  
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7 285 would require 110 adult patients per class – 550 patients across the program to achieve these aims.  
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10 286 The closed formulae by Tihaki *et al*,[30] were also utilised to understand the discriminatory power  
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12 287 that the samples sizes above would provide with respect to biomarker sensitivity and specificity; The  
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14 288 following assumptions were made:

- 15  
16 289 • That a sensitivity of 80 % with a precision of 5% would provide a useful biomarker capable  
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18 290 of ‘ruling out’ an acute class. The same target was applied to specificity.  
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20 291 • We assume a prevalence of acute breathlessness of 80% as the recruitment campaign uses  
21  
22 292 acute breathlessness as the initial stratification tool for recruitment and 1:5 patients recruited  
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24 293 will be non-breathless healthy controls  
25  
26 294 • We aim to balance group sizes across classes equally  
27

28 295 For a type 1 error rate of 0.05 and a 95% confidence interval

29  
30 296  $N_{\text{sensitivity}} = 307$

31  
32 297  $N_{\text{specificity}} = 1,230$

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34 298

35 299 For a type 1 error rate of 0.05 and a 90% confidence interval

36  
37 300  $N_{\text{sensitivity}} = 218$

38  
39 301  $N_{\text{specificity}} = 871$

40  
41 302

42 303 For a type 1 error rate of 0.05 and an 85% confidence interval

43  
44 304  $N_{\text{sensitivity}} = 166$

45  
46 305  $N_{\text{specificity}} = 664$

47  
48 306

49 307 For a type 1 error rate of 0.05 and an 80% confidence interval

50  
51 308  $N_{\text{sensitivity}} = 131$

52  
53 309  $N_{\text{specificity}} = 524$

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3 311 Therefore, we are powered to identify sensitive biomarkers ( $\geq 80\%$ ) of acute breathlessness with a  
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5 312 maximum marginal error in the estimate for sensitivity not exceeding 5% with 95% confidence.  
6  
7 313 Similarly, we are powered to identify specific biomarkers ( $\geq 80\%$ ) of acute breathlessness with a  
8  
9 314 maximum marginal error in the estimate for specificity not exceeding 5% with 80% confidence.  
10  
11 315 For the primary analysis the outcome will be treated as a nominal variable with levels (i) acute heart  
12  
13 316 failure (ii) community acquired pneumonia (iii) adult exacerbations of asthma and COPD and (iv)  
14  
15 317 acute exacerbations in school age children treated in hospital for severe asthma attacks.  
16  
17 318 The relationship between the primary outcome and the exhaled breath VOC biomarkers will be  
18  
19 319 modelled using multinomial logistic regression. In addition to metabolomics markers the following  
20  
21 320 independent variables will be included in the model: clinical uncertainty score on a 100 mm VAS  
22  
23 321 scale, age, and a validated co morbidity score (the Charlson comorbidity score)[31, 32].  
24  
25 322  
26  
27 323 Receiver operator analyses will be utilised to generate ROC curves for individual and multiple panels  
28  
29 324 VOC predictors in the primary analysis.  
30  
31 325  
32  
33  
34 326 To understand the dynamic profile of breath biomarkers during (i) the acute state and (ii) in the  
35  
36 327 chronic state up to 6 months post exacerbation, a repeated measures model with a random intercept  
37  
38 328 and random effect for time will be fitted, the random effects will be fitted for each patient. For the  
39  
40 329 repeated measures mixed model an unstructured covariance will be assumed. To evaluate the  
41  
42 330 relationship between breath biomarkers and hospital readmission at 30 and 60 days Cox proportional  
43  
44 331 hazards and frailty models will be utilised [33]. Analysis of Multivariate Survival Data, [CITE]  
45  
46 332 competing risk models and joint models will be fitted [34]. Relationship between death and breath  
47  
48 333 biomarkers will be evaluated using a logistic regression model. Changes in outcome measures will be  
49  
50 334 measured appropriately for each variable (e.g. paired t-test, Mann-Whitney, repeated measures  
51  
52 335 analysis). Tables of descriptive statistics will be compiled for all key variables  
53  
54  
55 336 All analysis will be performed using R 3.5.0 <https://www.r-project.org/>.  
56  
57 337

## 338 **2.4. Discovery and Replication studies**

339 Specific indicator conditions have been selected for targeted recruitment according to their high  
340 prevalence and unmet need, their high morbidity and mortality and the need to develop better  
341 diagnostic and prognostic algorithms in acute care pathways.

342 The indicator diagnoses of interest are (i) exacerbations of adult asthma and COPD, (ii) community  
343 acquired pneumonia (iii) acute heart Failure and (iv) exacerbation in school age children treated in  
344 hospital for severe asthma attacks.

345 Patient level clinico-pathological and outcome data (spanning the entire acute pathway) will be  
346 collected in parallel to breath sampling. In addition, breath samples will be acquired in the stable state  
347 post exacerbation (**Figure 3**).

348  
349 Age matched healthy volunteers will be recruited where possible at separate visits. For acute admission  
350 the study team will approach the spouse, parent or sibling of the index case and seek informed consent  
351 for study assessments. All healthy subjects will undergo two assessments separated by a duration of 8-  
352 16 weeks to match the acute and recovery time points elapsed in their index  
353 case/partner/spouse/sibling/child. Additional healthy volunteers will be identified from local  
354 recruitment databases and via advertising

### 356 **2.4.1. Discovery Phase (Project months 1-24):**

357 The aim of the discovery phase is to identify putative discriminatory breath VOC, using both offline  
358 and online technologies.

359 Pre-planned recruitment of acutely breathless patients will be enriched into the following disease  
360 strata following senior clinical decision maker assessment and within 24 hours of acute admission.

361 Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of  
362 asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=55).

363 Additional age matched healthy volunteers (n=55 adults and 50 children) will be identified as a non-  
364 disease reference group.



1  
2  
3 365 **2.4.2. Replication Phase (years 3-4)**

4  
5 366 The aim of the replication phase is to replicate putative discriminatory breath VOC /VOC signatures  
6  
7 367 identified in the discovery phase.

8  
9 368 The aim of the discovery phase is to identify putative discriminatory breath VOC, using both offline  
10  
11 369 and online technologies.

12  
13 370 Pre-planned recruitment of acutely breathless patients will be enriched into the following disease  
14  
15 371 strata following senior clinical decision maker assessment and within 24 hours of acute admission.

16  
17 372 Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of  
18  
19 373 asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=55).

20  
21 374 Additional age matched healthy volunteers (n=55 adults and 50 children) will be identified as a non-  
22  
23 375 disease reference group.

24  
25 376 **Total combined sample size of the discovery and replication phases = 650 participants**

26  
27 377

28 378 **2.5. Schedule of assessments**

29  
30  
31 379 A schedule of acute assessments is outlined below and aligns to the movement of acute patients  
32  
33 380 through the clinical care pathway and the overall aim of developing a complete phenotypic picture of  
34  
35 381 acutely breathless patients.

36  
37 382

38  
39 383 **2.5.1. Defining acute breathlessness**

40  
41 384 At presentation (within 24 hours of admission) to one of three acute admissions units potentially  
42  
43 385 eligible patients will be identified following confirmation of acute breathlessness, identified as (i)  
44  
45 386 patient defined acute breathlessness and/or (ii) 1 unit increase above patient reported baseline in the  
46  
47 387 extended medical research council (eMRC) dyspnoea score [35, 36] and at least one of the indicator  
48  
49 388 diagnoses identified as the primary clinical diagnosis by a senior clinical decision maker.

50  
51 389

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53 390

54  
55 391

### 392 **2.5.2 Informed consent**

393 Patients meeting the pre-specified definition of acute breathlessness will be approached for informed  
394 consent in to the breath VOC biomarker study. Only patients that are eligible to give full written  
395 informed consent will be recruited.

### 397 **2.5.3 Collection of blood based pathology markers**

398 Collection of the blood biomarkers CRP, BNP, Troponin-I and blood eosinophil count will be  
399 performed both acutely and following recovery, when not taken as part of clinical care pathway.  
400 These are currently used in profiling acutely breathless patients in clinical practice (**Table1**).

Test	ANALYSER/METHOD	LOWER LIMIT OF DETECTION	UPPER LIMIT OF DETECTION
C-Reactive protein (CRP)	Siemens Advia Chemistry XPT, PEG enhanced immunoturbidimetric. Siemens Advia 1800, PEG enhanced immunoturbidimetric	5 mg/L	Diluted to result
B-type natriuretic peptide (BNP)	Siemens Advia Centaur XPT, two-site sandwich immunoassay using direct chemiluminescent technology	2.0 pg/mL	1445 pg/mL
Troponin-I	Abbott Architect i2000SR, three-site sandwich immunoassay using direct chemiluminescent technology (CMIA).	5.0 ng/L	50,000 ng/L

401

402 **Table (1):** Type of analyser and methodology used for blood biomarker calculation. The table  
403 outlines analyser make, methodology, upper and lower limits of detection as per the University  
404 Hospitals of Leicester NHS Foundation trust laboratory guidelines.

405

### 406 **2.5.4 Breath VOC sampling**

407 Offline breath sampling using GC-MS coupled with a standardised and CE marked breath sampler-  
408 ReCIVA<sup>®</sup>[37] and comprehensive two-dimensional gas chromatography-mass spectrometry, coupled  
409 with a standardised and CE marked breath sampler (ReCIVA<sup>®</sup> GCxGC-MS) will be performed .  
410 Additionally the following online technologies, proton transfer mass spectrometry (PTR-MS), gas  
411 chromatography - ion mobility spectrometry (GC-IMS) and atmospheric pressure chemical ionisation-

412 mass spectrometry (APCI-MS) will be evaluated according to the sampling strategy outlined in

	COPD		Asthma		Pneumonia		Heart Failure		Healthy		Paediatrics	
Time point	1	2	1	2	1	2	1	2	1	2	1	2
Written informed consent	x		x		x		x		x		x	
<b>Volatile organic compound (VOC) sampling</b>												
ReCIVA- gas chromatography and mass spectrometry (GC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
ReCIVA- comprehensive two-dimensional gas chromatography (GCxGC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Proton transfer reaction mass spectrometry (PTRMS)		x		x		x		x	x	x	x	x
Gas chromatography - ion mobility spectrometry (GC-IMS)	x		x		x		x				x	x
<b>Pathology blood tests</b>												
Full blood count (including differential cell count)	x	x	x	x	x	x	x	x	x	x	x	x
Brain natriuretic peptide (BNP) [pg/mL]	x	x	x		x		x	x	x			
Troponin-I [ng/L]	x		x		x		x		x			
C-Reactive protein (CRP) [mg/L]	x	x	x	x	x	x	x	x	x	x	x	x
<b>Lung function tests</b>											x	x
Hand held forced oscillation technique (FOT)	x	x	x	x	x	x	x	x	x	x		
Fractional exhaled nitric oxide (FeNO) - Flow rate 50 [ml/s]			x	x							x	x
Spontaneous sputum sample	x	x	x	x	x	x	x	x	x	x		
Bio-banking (urine, serum, plasma, sputum supernatants & plugs)	x	x	x	x	x	x	x	x	x	x	x	x
Transthoracic echocardiography	x						x					

413 section 3, **Figure 3 and Table 2.**

414 **Table (2):** Summary of baseline and follow up assessments. The table summarises key assessments  
 415 carried out at different time points during the study. The participants may undertake any combination  
 416 of the investigations listed at any of these time points.

417

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8 421 **2.5.5. Collection of additional samples for future biomarker campaigns**9  
10 422 Collection of additional biomarkers for future biomarker discovery campaigns including (i) a urine11  
12 423 sample, (ii) blood samples, up to 85mls for DNA, RNA, plasma and serum and peripheral blood cell13  
14 424 flow cytometry in selected subjects and (iii) spontaneous or induced sputum samples (plugs and15  
16 425 supernatants) will be carried out (**Table 2**).17  
18 42619  
20 427 All samples will be collected at time point 1 and at 2 (**Figure 3**). The additional samples will be used21  
22 428 for future omics analyses, these may include detailed analysis of the metagenome in sputum and23  
24 429 proteomics applied to urine and serum samples.25  
26 43027  
28 431 **2.5.6. Physiological characterisation**29  
30  
31 432 Physiological measures of lung function will be performed in acutely ill participants and at32  
33 433 recovery including, (i) Hand held forced oscillation technique (FOT): an easily accessible34  
35 434 measure of lung function. Patients favour this to spirometry as it is effort independent, unlike36  
37 435 spirometry and requires less than a minute of quiet tidal breathing to obtain triplicate high38  
39 436 quality measurements [38], (ii) Fractional exhaled nitric oxide (FeNO): A measure of airway40  
41 437 inflammation in asthmatic patients[39, 40] (iii) Echocardiography: Two dimensional42  
43 438 transthoracic echocardiography was performed in heart failure and COPD patients using an44  
45 439 iE 33 system with S5-1 transducer (frequency transmitted 1.7 MHz, received 3.4 MHz;46  
47 440 Philips Medical Systems, Best, The Netherlands). Standard techniques as per American48  
49 441 Society of Echocardiography guidelines (ASE)[41] were used to acquire 2D, colour and50  
51 442 Doppler images in conventional parasternal long-axis, short-axis and apical 4-, 2- and 3-52  
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3 443 chamber views. Left ventricular ejection fraction (LVEF) was calculated using the biplane  
4  
5 444 method of discs formula (Simpson's rule) to derive left ventricular volume indices.  
6  
7

8 445

#### 10 446 **2.5.7 Recovery follow up**

12 447 The recovery from an acute exacerbation will be confirmed and identified as patient defined recovery,  
13  
14 448 at the recovery study visit (time point 2) up to six months post-acute event. The schedule of  
15  
16 449 assessments at the recovery visit is outlined (**Table 2**).  
17

18 450

#### 19 451 **2.6. Clinical Adjudication:**

20 452

21 453 In an effort to reduce data variability and minimise bias, an independent panel consisting of two senior  
22  
23 454 acute clinicians (SS & NG) will review all pertinent clinical and diagnostic source documentation,  
24  
25 455 whilst blinded to admission blood biomarkers and clinical diagnosis.  
26

27 456 All acutely breathless adult patients' notes will be sequentially divided for adjudication. The panel will  
28  
29 457 independently determine the primary diagnosis of highest probability from a list of the four potential  
30  
31 458 acute indicator diagnoses in section 2.4 and mark their level of clinical certainty on a 100 mm visual  
32  
33 459 analogue scale (VAS scale). The panel members will be able to review imaging, electrocardiograms  
34  
35 460 (ECGs), and other relevant information but not admission blood based pathology tests.  
36

37 461 In a subset of patients adjudication will be validated by separate panel member to ensure between  
38  
39 462 observer agreement using Bland-Altman analysis and inter-rater agreement of the primary diagnosis  
40  
41 463 using Kohen's kappa via repeated evaluation of a subset of cases (see statistical methods section 2.3).  
42

43 464

#### 45 465 **2.7. Clinical Informatics:**

46 466

47  
48 467 Clinical data collection will be undertaken using a securely-hosted bespoke database system (ADD)  
49  
50 468 developed within the NIHR Leicester Biomedical Research Centre – Respiratory (BRC). The system  
51  
52 469 links acute admission episodes to hospital pathology records; historical respiratory physiology tests;  
53  
54 470 and demographic information. The system provides functionality to validate data entry; manually  
55  
56 471 verify records and highlight incomplete records. A custom VOC 'module' has been created to  
57

1  
2  
3 472 support data collection within the study visits (1 and 2), and standardise diagnoses and medications  
4 473 through the use of clinical ontologies as well as linking hospital records/tests to patient visits.  
5  
6

7 474

8 475 Non-ADD based clinical data (e.g. hospital admissions, re-admissions and mortality) will be extracted  
9 476 from the hospital data warehouse using identifiable patient identifiers, and subsequently  
10 477 pseudonymised prior to integration.  
11  
12

13 478

14 479 An informatics pipeline will be created to facilitate the transfer of chemo-metric data from remote  
15 480 computers to the data repository. This will include tools to (i) enforce the correct labelling of data sets  
16 481 (e.g. study number, visit, type/source of sample) prior to automated validated transfer to the  
17 482 repository; (ii) record information about the sample process; (iii) search and extract data sets from the  
18 483 repository for subsequent analysis. Prior to analysis, clinical and chemo-metric data will be integrated  
19 484 using the study number and any potentially identifiable information will be removed.  
20  
21  
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29 485

### 30 486 **3. Breath profiling**

31 487

32 488 The technologies utilised in the VOC study during discovery and replication phases are:

33  
34  
35  
36 489 Offline technologies

- 37  
38 490 - ReCIVA+ GC-MS  
39 491 - ReCIVA + GC x GC-MS  
40 492 -

41  
42  
43 493 Online technologies

- 44  
45 494 - GC-IMS  
46 495 - PTR-MS  
47 496 - APCI-MS  
48  
49

50 497

51  
52 498 Offline technologies will underpin the discovery analyses owing to their ability to identify chemical  
53 499 identity and their recognition as the analytical gold standard in exhaled breath VOC analysis [42].  
54  
55  
56  
57  
58  
59

1  
2  
3 500 In contrast online technologies will be utilised for VOC biomarker replication and at the recovery  
4  
5 501 visits owing to their portability and potential for future point of care testing. **(Figure 4)**.

6  
7 502 A brief description of the core VOC platforms is provided below

8  
9 503 A CE marked breath sampling device (ReCIVA®) developed by Owlstone Medical, will be used to  
10  
11 504 sample breath onto adsorbent Tenax tubes. This effectively allows de-coupling of the breath sampling  
12  
13 505 from the breath sensor and analysis platforms in selected patients that are not able to mobilise to a real  
14  
15 506 time breath sampling device. The Owlstone ReCIVA sampler will be utilised in breath collection for  
16  
17 507 offline technologies namely GC-MS and GCxGC-MS. The ReCIVA sampler is capable of entraining  
18  
19 508 oxygen and is therefore suitable for patients with mild respiratory failure requiring low flow rates of  
20  
21 509 oxygen to maintain target oxygen saturations [37].  
22

23  
24 510 **3.1. Gas chromatography and mass spectroscopy (GC-MS):** is a commonly applied methodology  
25  
26 511 used to accurately measure trace gases in complex mixtures such as exhaled air [42]. Pre-  
27  
28 512 concentrating breath volatiles by various means and subsequent analysis constitute a reliable and  
29  
30 513 sensitive method for VOC analysis [43]. Despite its high sensitivity, it is however, a time consuming  
31  
32 514 technique and carries a risk of contamination at the pre-concentration step. It is also not suitable for  
33  
34 515 online and multiple measurements limiting its use as a point-of-care testing technology for VOC [44].  
35

36 516 **3.2. Comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS):** an  
37  
38 517 advanced analytical technique for the analysis of complex organic matrices; its main advantage is the  
39  
40 518 unparalleled separation power it affords over conventional one-dimensional chromatographic  
41  
42 519 techniques [45]. Previous research, albeit sparse, has demonstrated the potential of GCxGC-MS for  
43  
44 520 breath analysis with the number of VOC detected exceeding those detected by conventional GC-MS  
45  
46 521 [46, 47]. GCxGC-MS of breath metabolites has been used for the identification of biomarkers related  
47  
48 522 to glucose metabolism [48, 49], tuberculosis [50] and radiation response [51]. This has generated  
49  
50 523 interest within the breath research community, however, such studies were conducted on a small scale  
51  
52 524 (<50 patients) and involved the use of expensive detectors and modulators. Method development and  
53  
54 525 analysis of the data-rich GCxGC chromatograms, however, can be time-consuming and require  
55  
56 526 specialist knowledge.

1  
2  
3 527 **3.3. Proton-transfer-reaction mass spectrometry (PTRMS):** a real time technique, capable of  
4  
5 528 simultaneously measuring the evolution of multiple gas metabolites from a single breath. It has been  
6  
7 529 used for the identification of potential useful VOC biomarkers for diagnosis of a variety of diseases:  
8  
9 530 including various cancers[52-54], liver disease[55, 56] and respiratory disease[57]. It has several  
10  
11 531 advantages in clinical settings, such as the speed of sampling, the instant result achieved and the lack  
12  
13 532 of need for sample storage or shipping. However, owing to the lack of pre-concentration or  
14  
15 533 chromatographic separation, sensitivity and definitive compound identification can be somewhat  
16  
17 534 limited when compared to GC-MS.

18  
19 535 **3.4. Gas chromatography- ion mobility spectrometry (GC-IMS):** allows the detection of volatile  
20  
21 536 organic compounds down to ultra-trace level (ng/L - to pg/L - range). For years, IMS has been used to  
22  
23 537 discover potential discriminatory breath VOC in lung cancer [58, 59], COPD[60, 61] and asthma[61].  
24  
25 538 The technology's multiple advantages of ultra-sensitivity, portability, online sampling and short  
26  
27 539 analysis time (typical analysis time of 10 minutes) with real time detection, brings a promise to  
28  
29 540 provide immediate and potentially reliable results for point of care breath diagnostics. Another  
30  
31 541 concept with IMS devices is that once the required breath signatures have been discovered using GC-  
32  
33 542 MS, IMS offers the potential to be 'tuned' for selective detection of VOC.

34  
35  
36 543 **3.5. Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) semiportable**  
37  
38 544 **compact version:** is one of less sensitive but more affordable versions of mass spectrometers released  
39  
40 545 to the commercial market in recent years. The device uses APCI to produce ions. Although the most  
41  
42 546 common use of APCI-MS systems is the detection in liquid chromatography applications, the  
43  
44 547 technique has proven to be a valuable tool for direct measurement of VOC in air[62, 63] food[64, 65]  
45  
46 548 and breath[66, 67]. Recently, the technique has shown potential for online, real time profiling of  
47  
48 549 pseudo-metabolites in exhaled breath [68] with sensitivity comparable with other techniques. By  
49  
50 550 combining miniaturised MS technology with APCI techniques, adequate quality of on-site, real time  
51  
52 551 measurements with minimal or no sample preparation requirement can be provided. This is a desirable  
53  
54 552 outcome as it overcomes main limitation of using standard breath analysis method in clinical setting,  
55  
56  
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60



1  
2  
3 553 which is a need for breath sample collection followed by desorption and time-consuming laboratory  
4  
5 554 analysis.

6  
7 587 There remains an overall lack of standardisation and rigour across these technologies which hindered  
8  
9 588 previous advancements in breath discovery; something we intend to minimize.

#### 11 12 589 **4. Chemometric processing and data analysis:**

13  
14 590 GC-MS breath data will be aligned, deconvoluted and the features for each participant will be  
15  
16 591 extracted. The extracted features will be grouped and classified by retention index and mass spectrum.  
17  
18 592 The registered and aligned data will be linked to participant meta-data to generate a breath matrix.

19  
20  
21 593 The breath matrix is a  $n \times p$  matrix where  $n$  is the number of subjects and  $p$  is the number of VOC. The  
22  
23 594 breath matrix is high dimensional with  $p \gg n$  and many potentially correlated VOC. In view of this, we  
24  
25 595 will employ sparse partial least squares discriminant analysis (sPLS-DA)[69] to investigate which of  
26  
27 596 the VOC can identify breathlessness. We will also investigate which of the VOC can discriminate  
28  
29 597 between the different disease states including acute exacerbations of asthma and COPD and  
30  
31 598 Pneumonia. In addition to the supervised methods, unsupervised methods will be explored,  
32  
33 599 specifically sparse principle component analysis (sPCA)[70].

34  
35  
36 600 Extracted VOC will also be investigated. Relationships between VOC and patient reported acute  
37  
38 601 breathlessness will be analysed using logistic regression model. VOC associated with patient  
39  
40 602 associated acute breathlessness will be incorporated into multinomial logistic regression models in  
41  
42 603 conjunction with CRP, BNP, blood eosinophils and Troponin-I, pathology biomarkers currently in use  
43  
44 604 for diagnosing undifferentiated breathlessness. In addition to the conventional binary and multinomial  
45  
46 605 logistic regression models, regression models [71].

#### 47 48 49 606 **5. Ethics and dissemination:**

50  
51  
52 607 The study has obtained full ethical approval from the London South East Research ethics Committee,  
53  
54 608 REC reference 16/LO/1747. IRAS project ID 198921. Publications will be prepared according to the  
55  
56 609 MRC-EMBER consortium agreement and the University of Leicester publications policy. All

1  
2  
3 610 intended publications will be submitted to the EMBER executive board for review and comments  
4  
5 611 within 60 days of journal submission. Authorship will be according to contribution and internationally  
6  
7 612 recognised guidance on journal authorship.  
8  
9

10 613 **6. Study dates:** 01/2/2017 – 30/10/2020  
11

#### 12 614 **7. Authors' contributions:**

13  
14 615 WI and SS drafted the manuscript and all co-authors critically revised and contributed to the  
15  
16 616 manuscript. All co-authors contributed to the study design and development. SS is the Chief  
17  
18 617 investigator for the acute VOC study.  
19

20 618

21  
22 619 **Protocol version:** Version 4, 1<sup>st</sup> April 2018  
23  
24

25 620

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28  
29 622 This study has been funded by the Medical research Council (MRC) and Engineering and Physical  
30  
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32  
33 624 component of this study is also being supported by The Midlands Asthma and Allergy Research  
34  
35 625 Association (MAARA). Grant number [MR/N005880/1]  
36  
37

38 626 **Competing interests:** SS has performed advisory services for Owlstone Medical.  
39

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11 **Figure legends:**

12  
13 **Figure (1)** Relationship between lung proximity and degree of invasiveness of different lung  
14  
15 matrices. The figure plots the level of invasiveness of various lung matrices in relation to their  
16  
17 proximity to the lung. Given their pathological relevance, the degree of invasiveness of  
18  
19 bronchoalveolar lavage (BAL) and lung biopsy makes them less favourable in diagnosing respiratory  
20  
21 diseases.  
22

23  
24 **Figure (2)** Multi-instrument use in breath sampling. Figure illustrates the various combinations of  
25  
26 offline and online devices used in breath sampling and the relevant pros and cons. Offline and online  
27  
28 technologies are used for the discovery and validation phases of the study respectively.  
29

30  
31 **Figure (3)** Study flow chart. Figure outlines the patient journey from admission through to discharge  
32  
33 and follow up. Participants with self-reported acute breathlessness presenting to University Hospitals  
34  
35 of Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling  
36  
37 is carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge.  
38  
39 Patients are admitted through the standard operational emergency medical streaming and care  
40  
41 pathways at the University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission)  
42  
43 are measured at 30 and 60 days and late outcomes (mortality) including respiratory and all-cause  
44  
45 mortality are measured at 2 years. Assessments carried out at each time point are summarised in  
46

47 **Table 1.**

48  
49 **Figure (4)** Multi-instrument use in breath sampling. Operational space of the analytical technologies  
50  
51 used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including  
52  
53 proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-  
54  
55 mass spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas  
56  
57

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3 664 chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass  
4  
5 665 spectrometry (GCxGC). Comparing the typical molar mass range detectable; selectivity in detection  
6  
7 666 owing to the type of ionisation involved and the proton affinity of analytes; and the inclusion of a  
8  
9 667 chromatographic separation affecting total time of analysis. The online technologies involving  
10  
11 668 chemical ionisation (PTR-MS, CMS and GC-IMS) can be used in-clinic owing to short analysis  
12  
13 669 times, but only detect lower molar mass molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>.  
14  
15 670 Offline chromatographic techniques (GC-MS and GCxGC) detect a wider range of compounds  
16  
17 671 independent of proton affinity; however, the techniques have longer analysis times and involve  
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19 672 sample transportation and storage.

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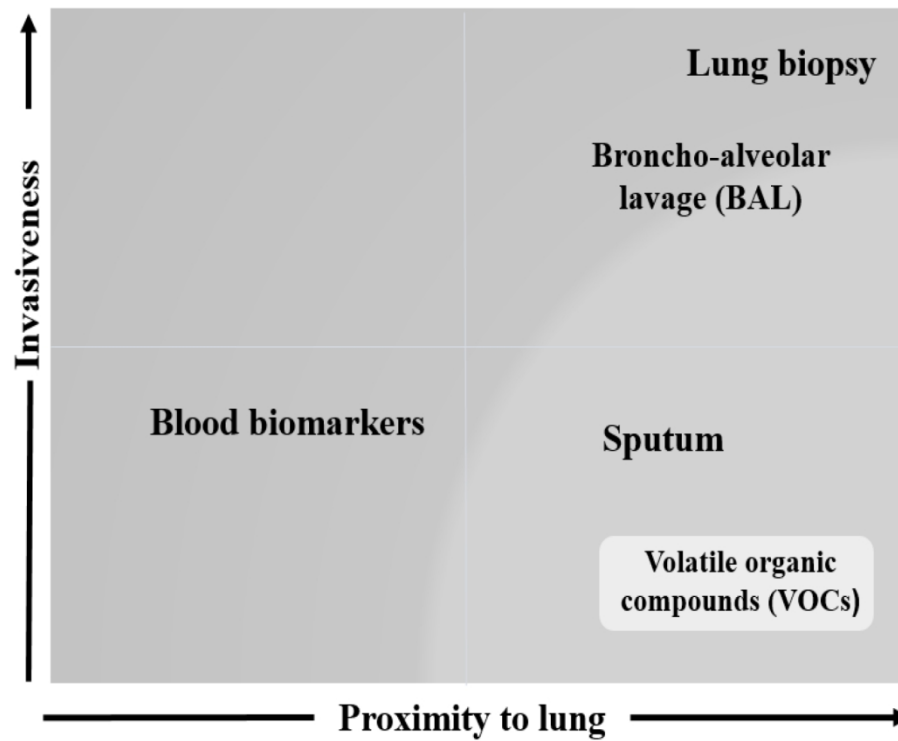


Figure (1): Relationship between lung proximity and degree of invasiveness of different lung matrices. The figure plots the level of invasiveness of various lung matrices in relation to their proximity to the lung. Given their pathological relevance, the degree of invasiveness of bronchoalveolar lavage (BAL) and lung biopsy makes them less favourable in diagnosing respiratory diseases.

144x115mm (300 x 300 DPI)

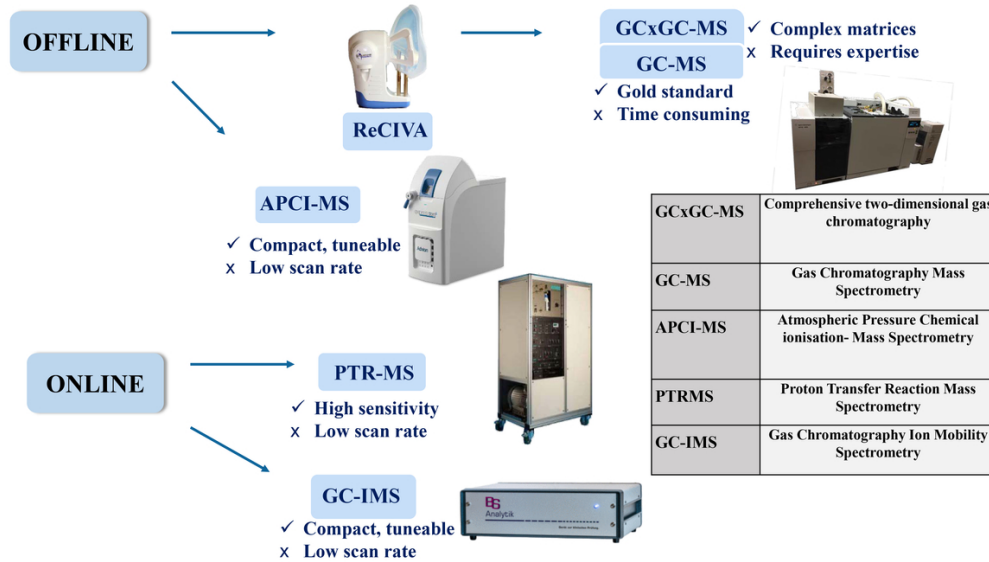
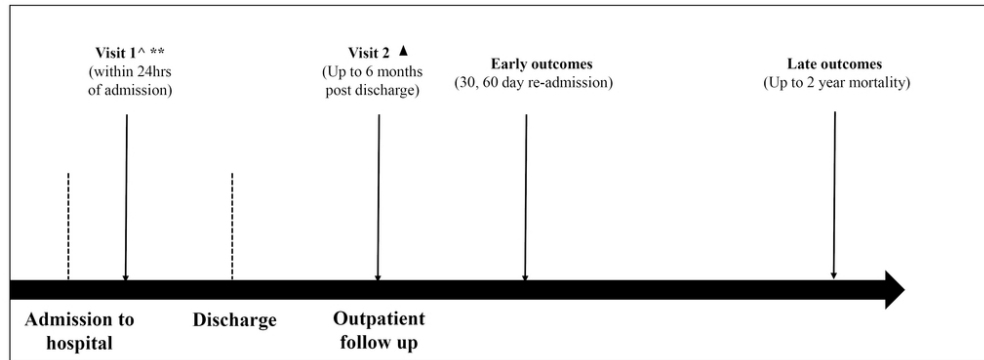


Figure (2): Multi-instrument use in breath sampling. Figure illustrates the various combinations of offline and online devices used in breath sampling and the relevant pros and cons. Offline and online technologies are used for the discovery and validation phases of the study respectively.

97x55mm (300 x 300 DPI)



- ^ Following senior decision maker review  
 \*\* Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, GC-IMS, APCI-MS)  
 ▲ Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, PTR-MS, APCI-MS)  
 ---- Following University Hospitals of Leicester streaming and clinical care pathways

Figure (3): Study flow chart. Figure outlines the patient journey from admission through to discharge and follow up. Participants with self-reported acute breathlessness presenting to University Hospitals of Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling is carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge. Patients are admitted through the standard operational emergency medical streaming and care pathways at the University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission) are measured at 30 and 60 days and late outcomes (mortality) including respiratory and all-cause mortality are measured at 2 years. Assessments carried out at each time point are summarised in Table 1.

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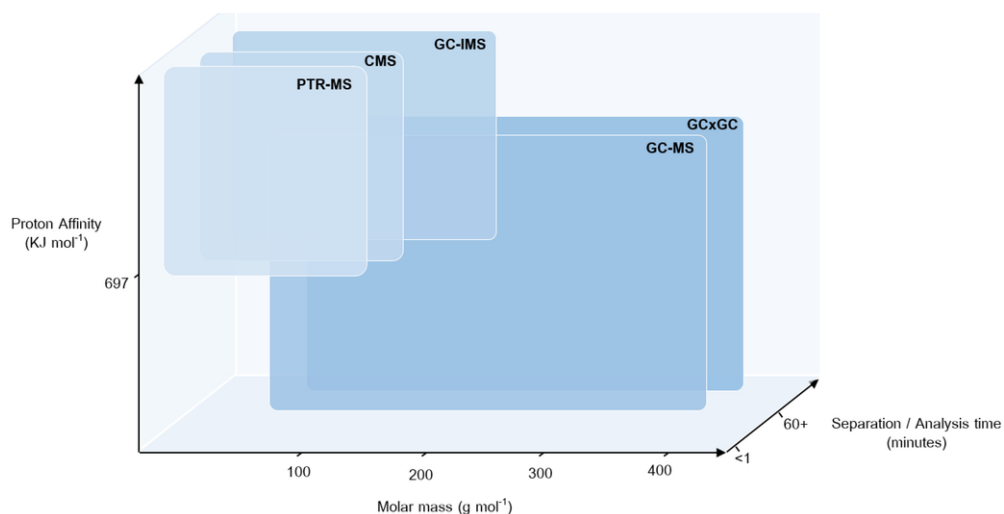


Figure (4): Multi-instrument use in breath sampling. Operational space of the analytical technologies used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-mass spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass spectrometry (GCxGC). Comparing the typical molar mass range detectable; selectivity in detection owing to the type of ionisation involved and the proton affinity of analytes; and the inclusion of a chromatographic separation affecting total time of analysis. The online technologies involving chemical ionisation (PTR-MS, CMS and GC-IMS) can be used in-clinic owing to short analysis times, but only detect lower molar mass molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>. Offline chromatographic techniques (GC-MS and GCxGC) detect a wider range of compounds independent of proton affinity; however, the techniques have longer analysis times and involve sample transportation and storage.

88x44mm (300 x 300 DPI)

# BMJ Open

## Assessment of Breath Volatile Organic Compounds in Acute Cardio-respiratory Breathlessness: A protocol describing a Prospective Real World Observational Study

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Manuscripts

# 1                   **Assessment of Breath Volatile Organic Compounds in Acute Cardio-** 2                   **respiratory Breathlessness: A protocol describing a Prospective Real World** 3                   **Observational Study**

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14 69 **Abstract**15  
16 70 **Introduction**

17 71 Patients presenting with acute undifferentiated breathlessness are commonly encountered in  
18 72 admissions units across the United Kingdom. Existing blood biomarkers have clinical utility in  
19 73 distinguishing patients with single organ pathologies but have poor discriminatory power in multi  
20 74 factorial presentations. Evaluation of volatile organic compounds (VOC) in exhaled breath offers the  
21 75 potential to develop biomarkers of disease states that underpin acute cardio-respiratory breathlessness,  
22 76 owing to their proximity to the cardio-respiratory system. To date there has been no systematic evaluation  
23 77 of VOC in acute cardio-respiratory breathlessness. The proposed study will seek to use both offline and  
24 78 online VOC technologies to evaluate the predictive value of VOC in identifying common conditions that  
25 79 present with acute cardio-respiratory breathlessness.

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37 80 **Methods and analysis**

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40 81 A prospective real world observational study carried out across three acute admissions units within  
41 82 Leicestershire. Participants with self-reported acute breathlessness, with a confirmed primary diagnosis of  
42 83 either acute heart failure, community acquired pneumonia and acute exacerbation of asthma or COPD  
43 84 will be recruited within 24 hours of admission. Additionally, school age children admitted with severe  
44 85 asthma will be evaluated. All participants will undergo breath sampling on admission and upon recovery  
45 86 following discharge. A range of online technologies including: proton-transfer-reaction mass  
46 87 spectrometry (PTR-MS), gas chromatography ion mobility spectrometry (GC-IMS), atmospheric pressure  
47 88 chemical ionisation- mass spectrometry (APCI-MS) and offline technologies including gas

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3 89 chromatography mass spectroscopy (GC-MS) and comprehensive two-dimensional gas chromatography-  
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5 90 mass spectrometry (GCxGC-MS) will be utilised for VOC discovery and replication. For offline  
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7 91 technologies a standardised CE marked breath sampling device (ReCIVA®) will be used. All recruited  
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9 92 participants will be characterised using existing blood biomarkers including C - reactive protein (CRP),  
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11 93 brain derived natriuretic peptide (BNP), Troponin-I and blood eosinophil levels and further evaluated  
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13 94 using a range of standardised questionnaires, lung function testing, sputum cell counts and other  
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15 95 diagnostic tests pertinent to acute disease.

### 18 96 **Ethics and dissemination**

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21 97 The National Research Ethics Service Committee East Midlands has approved the study protocol (REC  
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23 98 number: 16/LO/1747). IRAS 198921. Findings will be presented at academic conferences and published in  
24  
25 99 peer-reviewed scientific journals. Dissemination will be facilitated via a partnership with the East  
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27 100 Midlands Academic Health Sciences Network and via interaction with all UK funded MRC/EPSRC  
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29 101 molecular pathology nodes.

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35 103 **Key words:** Breathlessness; Breath analysis; Volatile Organic Compound, Observational study  
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18 **Strengths and Limitations of this Study**19  
20 116 • A pragmatic real world, prospective, observational study across three admission units that21  
22 117 focuses on the systematic discovery and replication of VOC in acutely breathless patients23  
24 118 using both online and offline technologies25  
26 119 • The proposed study is the largest of its kind in acute disease to characterise VOC with a27  
28 120 range of additional assessments that will build a comprehensive phenotype of acute cardio-29  
30 121 respiratory exacerbations31  
32 122 • The proposed study will build an infrastructure for research and subsequent evaluation of33  
34 123 VOC in interventional trials within acute cardio-respiratory exacerbations35  
36 124 • Prior acute treatment exposure will need to be accounted for when evaluating potential37  
38 125 discriminative biomarkers39  
40 126 • VOC technologies are not currently suited for deployment in patients that are of high clinical41  
42 127 acuity43  
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48 **1. Introduction:**49  
50 130 Breathlessness is a common symptom of cardio-respiratory illnesses that has a significant direct impact51  
52 131 on patients' wellbeing as well as a substantial economic burden on healthcare systems [1]. Although its53  
54 132 etiologies can be variable, exacerbations of common complex chronic cardio-respiratory conditions

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3 133 account for approximately 70% of acute presentations with breathlessness, namely exacerbations of  
4  
5 134 asthma and COPD, acute heart failure and community acquired pneumonia [2]. Moreover, moderate and  
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7 135 severe breathlessness is significantly associated with all-cause, cardiovascular and COPD mortality[3]. As  
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9 136 a consequence symptomatic breathlessness warrants rapid evaluation and targeted diagnostics at  
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11 137 presentation.  
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16 139 Diagnostic evaluation of acute breathlessness is heavily reliant on blood based biomarkers e.g. CRP,  
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18 140 BNP, Troponin and on occasions blood eosinophil levels. These biomarkers have clinical utility primarily  
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20 141 in patients with single pathologies, but have poor discriminatory power in patients with multifactorial  
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22 142 presentations of acute breathlessness[4]. There is therefore an unmet need for the development of  
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24 143 sensitive and specific biomarkers that differentiate acute breathlessness from its recovery and the  
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26 144 common cardio-respiratory conditions that present with acute breathlessness.  
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31 146 CRP plays an important role in diagnosing breathlessness caused by an underlying bacterial  
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33 147 pneumonia[5], as well as predicting mortality in patients with COPD [6]. BNP is routinely utilised in  
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35 148 acute settings to support the diagnosis of acute heart failure [7]. The European Society of Cardiology  
36  
37 149 (ESC) recommends BNP threshold values of <100 pg/mL to rule out acute congestive cardiac failure and  
38  
39 150 values > 500 pg/ml as diagnostic of acute exacerbations of heart failure [8].  
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41 151  
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43 152 The role of peripheral blood eosinophil count in airway inflammation was poorly understood up until the  
44  
45 153 second half of the 19<sup>th</sup> century when Paul Ehrlich, a German physician and Nobel prize winner,  
46  
47 154 introduced eosin in his technique for white cell differentiation in 1879[9]. Considerable advances in the  
48  
49 155 field of airway inflammation and the role of eosinophils have taken place since [10-12]. More recently  
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51 156 Bafadhel *et al* suggested that peripheral blood eosinophil count can be used to direct corticosteroid  
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53 157 therapy during COPD exacerbations in single centre study [13].  
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3 158 Currently, blood biomarkers together with clinical, physiological and imaging parameters are used in  
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5 159 diagnosing the cause of acute breathlessness. Blood biomarkers may be less specific as they originate far  
6  
7 160 from the target organs of interest (the heart and the lungs in cardio-respiratory disease). Sputum, although  
8  
9 161 potentially a more definitive lung specific matrix, is comparatively difficult to obtain particularly in  
10  
11 162 acutely unwell patients, limiting its use in acute disease and highlighting the need for better biomarkers.  
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13 163 Ideally these biomarkers would have the following characteristics, (i) they would originate from the target  
14  
15 164 organ of interest, (ii) they would significantly add value to conventional risk scoring and diagnostic  
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17 165 algorithms in acute breathlessness, (iii) they would be minimally invasive and suitable for rapid point of  
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19 166 care diagnosis in emergency rooms and acute admissions units (iv) they would have diagnostic value in  
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21 167 patients with multifactorial acute breathlessness.  
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26 169 Exhaled breath contains thousands of volatile organic compounds (VOC) that reflect biological processes  
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28 170 occurring in the host both locally in the airways and systematically offering the potential to develop more  
29  
30 171 effective biomarkers in acutely breathless patients (**Figure 1**).  
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35 173 The proposed program of research will use a combination of offline and online technologies to identify  
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37 174 and evaluate the diagnostic and prognostic value of VOC in patients with acute cardio-respiratory related  
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39 175 breathlessness (**Figure 2**).  
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43 177 Exhaled breath analysis of volatile chemicals offers the potential to develop more effective biomarkers in  
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45 178 acutely breathless patients. The use of breath analysis for disease diagnostics dates back to ancient Greeks  
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47 179 where physicians used exhaled breath to diagnose different diseases. Breath odours allow correct  
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49 180 associations to certain diseases. For example, the sweet smell of diabetic ketoacidosis, the fishy smell of  
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51 181 breath associated to liver illness, the urine-like odour of kidney disease and the smell of the breath of  
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53 182 patients with lung abscesses, caused by the proliferation of anaerobic bacteria [14-17].  
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3 183 More recently, exhaled breath analysis has demonstrated early proof of concept in the diagnosis of acute  
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5 184 heart failure, and ventilator associated pneumonia[18] . The validity of breath analysis has also been  
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7 185 demonstrated in breathless children[19]. This population is likely to prefer breath-based tests, as these are  
8  
9 186 minimally invasive. Importantly, a variety of point of care sensors are now available to evaluate potential  
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11 187 exhaled breath biomarkers in emergency care settings.  
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16 189 A study by Van Berkel et al demonstrated the ability to distinguish COPD subjects from controls solely  
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18 190 based on the presence of VOCs in breath, suggesting that analysis of VOC might be highly relevant for  
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20 191 diagnosis of COPD [20]. This established the basis of further studies of VOC in COPD [21-  
21  
22 192 25].recommending larger studies for validation.  
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24 193 Several other studies found that VOC profiling in diagnosing asthma is potentially feasible [26-32]. This  
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26 194 however has been done in relatively small numbers in stable disease.  
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28 195 Despite the novelty of non-invasive sampling technology and the growing interest in exhaled breath  
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30 196 analysis there remains a disappointing level of comparability across studies due to the lack of  
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32 197 standardisation and appropriate data analysis methods. A recent systemic review by Anders Christiansen  
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34 198 *et al* compared eleven publications reporting very heterogeneous designs, methods, patient group sizes,  
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36 199 data analytics and, consequently, quite varying results [33].  
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41 201 To our knowledge, no other large studies exploring the use of breath biomarkers in profiling acute  
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43 202 breathlessness have been completed. Several studies have explored the use of electronic nose (eNose) in  
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45 203 stable disease with good discriminatory power in COPD [34], Pneumonia [35] and heart failure[36] with  
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47 204 relatively small sample size. While eNose has now been widely used in detecting various VOC patterns,  
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49 205 GC-MS, a largely validated methodology, remains the gold standard technique for detecting VOCs in  
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51 206 exhaled breath. The focus of the current research study will be to evaluate acutely breathless cardio-  
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53 207 respiratory patients using a combination of ‘discovery’ and near-patient care breath sampling  
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55 208 technologies.  
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3 209 Medical Research Council (MRC) and Engineering and Physical Sciences Research Council (EPSRC)  
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5 210 have commissioned a series of molecular pathology nodes aimed at developing molecular signatures  
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7 211 relevant to disease diagnosis and progression. This was triggered by the clear need for alliance between  
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9 212 academic institutions, industry and NHS partners to enhance the benefits of stratified medicine for  
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11 213 patients[37, 38].

13 214 University of Leicester and Loughborough University were awarded a joint molecular pathology node  
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15 215 East Midlands Breathomics Pathology Node (EMBER) which this study forms a key part of.  
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## 20 217 **2. Methods and Analysis**

### 22 218 **2.1. Study design**

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25 219 A prospective real world observational study across three acute admissions units within Leicestershire  
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27 220 (two adult admissions units and one children's assessment unit). The acute units routinely assess and treat  
28  
29 221 cardio-respiratory admissions due to breathlessness in adults and children.

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31 222 Participants with self-reported acute breathlessness, either requiring admission or a change in baseline  
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33 223 treatment, will be screened for the study. Informed consent will be obtained in all participants following a  
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35 224 clinical review by a senior decision maker within 24 hours of acute admission (**Figure 3**).  
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### 41 227 **2.2. Objectives**

#### 42 228 **2.2.1. Primary objective**

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  - To evaluate the sensitivity, specificity, positive and negative predictive value of exhaled breath

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48 231 VOC biomarkers to differentiate acute breathlessness in cardio-respiratory patients.

#### 49 50 232 **2.2.2. Secondary objectives:**

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  - To replicate selected breath VOC biomarkers identified in acute breathlessness.

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3 235 • To discover and replicate breath VOC biomarkers that differentiate the common cardio- respiratory  
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5 236 conditions that cause acute breathlessness, specifically (i) acute heart failure, (ii) community  
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7 237 acquired pneumonia, (iii) adult exacerbations of asthma and COPD and age-matched adults that do  
8  
9 238 not have cardio-respiratory disease or breathlessness.  
10  
11 239 • To quantify the level of clinical uncertainty in the primary diagnosis using a 100 mm visual  
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13 240 analogue scale and independent clinical adjudication of case notes blinded to the following blood  
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15 241 biomarkers (i) CRP (ii) BNP (iii) Troponin-I (iv) blood eosinophils, but not clinical history and  
16  
17 242 acute presentation nor chest x-ray imaging. Potential discriminatory breath VOC biomarkers will  
18  
19 243 be adjusted for clinical uncertainty in statistical models.  
20  
21 244 • To identify and replicate exhaled breath VOC biomarkers in school age children treated in hospital  
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23 245 for severe asthma attacks and compare these to age-matched healthy controls.  
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26 246 **2.2.3. Exploratory end points (where applicable):**  
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29 248 • To evaluate the dynamic profile of selected breath VOC between the acute state and the recovery  
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31 249 state post exacerbation.  
32  
33 250 • To evaluate the relationship between exhaled VOC biomarkers and clinical outcomes including  
34  
35 251 (i) hospital readmission at 30 and 60 days post event (ii) all-cause mortality over a 2 year period  
36  
37 252 post admission.  
38  
39 253 • To evaluate the relationship between breath VOC biomarkers and functional measures  
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41 254 e.g. physical performance and activity  
42  
43 255 • To explore potential breath VOC biomarkers of multifactorial acute breathlessness  
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45 256 • To evaluate the relationship between diet, lifestyle and environment upon breath VOC  
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47 257 biomarkers  
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53 259 **2.3. Sample size estimation**  
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3 261 Preliminary data was utilised to conduct sample size estimates from a cohort of acutely breathless patients  
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5 262 admitted to acute admissions units over a 6 month period (February 2017 to August 2017). Hundred and  
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7 263 twelve adult participants (asthma 46, community acquired pneumonia 26, COPD 22) and eighteen healthy  
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9 264 controls were utilised for the analysis.

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12 265 A panel of ten pre-specified aldehydes, based on literature search [31, 39, 40], were extracted from breath  
13  
14 266 using gas chromatography-mass spectrometry (GC-MS). The aldehydes were normalised to a common  
15  
16 267 internal standard and were not background-subtracted.

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19 268 A closed formula from Hsieh *et al*[41], relating sample size to observable effect size, was used to  
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21 269 calculate sample size from logistic regression models of the ten aldehydes with acute breathlessness as the  
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23 270 outcome measure. The sample size estimates are also relevant to acute class comparisons vs. the sum of  
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25 271 other acute classes.

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28 272 Based upon the sample size estimates we would have an 80% power, with a type 1 error rate of 5%, to  
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30 273 detect an odds ratio of association of 1.2 between two disease classes with 55 patients per class. Given the  
31  
32 274 fact that study seeks to discover and replicate breath VOC amongst five adult disease classes (community  
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34 275 acquired pneumonia, heart failure, COPD, asthma and healthy aged matched subjects) we would require  
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36 276 110 adult patients per class – 550 patients across the program to achieve these aims.

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39 277 The closed formulae by Tihaki *et al*,[42] were also utilised to understand the discriminatory power that  
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41 278 the samples sizes above would provide with respect to biomarker sensitivity and specificity; The  
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43 279 following assumptions were made:

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45  
46 280 • That a sensitivity of 80 % with a precision of 5% would provide a useful biomarker capable of  
47  
48 281 ‘ruling out’ an acute class. The same target was applied to specificity.  
49  
50  
51 282 • We assume a prevalence of acute breathlessness of 80% as the recruitment campaign uses acute  
52  
53 283 breathlessness as the initial stratification tool for recruitment and 1:5 patients recruited will be  
54  
55 284 non-breathless healthy controls

285 • We aim to balance group sizes across classes equally

286 For a type 1 error rate of 0.05 and a 95% confidence interval

287  $N_{\text{sensitivity}} = 307$

288  $N_{\text{specificity}} = 1,230$

289

290 For a type 1 error rate of 0.05 and a 90% confidence interval

291  $N_{\text{sensitivity}} = 218$

292  $N_{\text{specificity}} = 871$

293

294 For a type 1 error rate of 0.05 and an 85% confidence interval

295  $N_{\text{sensitivity}} = 166$

296  $N_{\text{specificity}} = 664$

297

298 For a type 1 error rate of 0.05 and an 80% confidence interval

299  $N_{\text{sensitivity}} = 131$

300  $N_{\text{specificity}} = 524$

301

302 Therefore, we are powered to identify sensitive biomarkers ( $\geq 80\%$ ) of acute breathlessness with a  
303 maximum marginal error in the estimate for sensitivity not exceeding 5% with 95% confidence. Similarly,  
304 we are powered to identify specific biomarkers ( $\geq 80\%$ ) of acute breathlessness with a maximum  
305 marginal error in the estimate for specificity not exceeding 5% with 80% confidence.

306 For the primary analysis the outcome will be treated as a nominal variable with levels (i) acute heart failure  
307 (ii) community acquired pneumonia (iii) adult exacerbations of asthma and COPD and (iv) acute  
308 exacerbations in school age children treated in hospital for severe asthma attacks.

309 The relationship between the primary outcome and the exhaled breath VOC biomarkers will be modelled  
310 using multinomial logistic regression. In addition to metabolomics markers the following independent  
311 variables will be included in the model: clinical uncertainty score on a 100 mm VAS scale, age, and a

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3 312 validated co morbidity score (the Charlson comorbidity score)[43, 44].  
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6  
7 314 Receiver operator analyses will be utilised to generate ROC curves for individual and multiple panels VOC  
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9 315 predictors in the primary analysis.  
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14 317 To understand the dynamic profile of breath biomarkers during (i) the acute state and (ii) in the chronic  
15  
16 318 state up to 6 months post exacerbation, a repeated measures model with a random intercept and random  
17  
18 319 effect for time will be fitted, the random effects will be fitted for each patient. For the repeated measures  
19  
20 320 mixed model an unstructured covariance will be assumed. To evaluate the relationship between breath  
21  
22 321 biomarkers and hospital readmission at 30 and 60 days Cox proportional hazards and frailty models will  
23  
24 322 be utilised [45]. Analysis of Multivariate Survival Data, [CITE] competing risk models and joint models  
25  
26 323 will be fitted [46]. Relationship between death and breath biomarkers will be evaluated using a logistic  
27  
28 324 regression model. Changes in outcome measures will be measured appropriately for each variable (e.g.  
29  
30 325 paired t-test, Mann-Whitney, repeated measures analysis). Tables of descriptive statistics will be compiled  
31  
32 326 for all key variables  
33  
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36 327 All analysis will be performed using R 3.5.0 <https://www.r-project.org/>.  
37

38 328

#### 39 329 **2.4. Discovery and Replication studies**

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42 330 Specific indicator conditions have been selected for targeted recruitment according to their high  
43  
44 331 prevalence and unmet need, their high morbidity and mortality and the need to develop better diagnostic  
45  
46 332 and prognostic algorithms in acute care pathways.  
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48  
49 333 The indicator diagnoses of interest are (i) exacerbations of adult asthma and COPD, (ii) community  
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51 334 acquired pneumonia (iii) acute heart Failure and (iv) exacerbation in school age children treated in hospital  
52  
53 335 for severe asthma attacks.  
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3 336 Patient level clinico-pathological and outcome data (spanning the entire acute pathway) will be collected  
4  
5 337 in parallel to breath sampling. In addition, breath samples will be acquired in the stable state post  
6  
7 338 exacerbation (**Figure 3**).  
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9 339  
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11 340 Age matched healthy volunteers will be recruited where possible at separate visits. For the purposes of this  
12  
13 341 study, healthy volunteers will be defined as participants who have no prior history of asthma, COPD, heart  
14  
15 342 failure and have not been admitted to hospital with community acquired pneumonia within 6 weeks of the  
16  
17 343 baseline study visit. For acute admission the study team will approach the spouse, parent or sibling of the  
18  
19 344 index case and seek informed consent for study assessments. All healthy subjects will undergo two  
20  
21 345 assessments separated by a duration of 8-16 weeks to match the acute and recovery time points elapsed in  
22  
23 346 their index case/partner/spouse/sibling/child. Additional healthy volunteers will be identified from local  
24  
25 347 recruitment databases and via advertising  
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#### 30 349 **2.4.1. Discovery Phase (Project months 1-24):**

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33 350 The aim of the discovery phase is to discover putative discriminatory breath VOC, using both offline and  
34  
35 351 online technologies.

36  
37 352 Pre-planned recruitment of acutely breathless patients will be enriched into the following disease strata  
38  
39 353 following senior clinical decision maker assessment and within 24 hours of acute admission.

40  
41 354 Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of  
42  
43 355 asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=50).

44  
45 356 Additional age matched healthy volunteers (n=55 adults and 50 children) will be identified as a non-  
46  
47 357 disease reference group (**Table 1**).  
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#### 51 360 **2.4.2. Replication Phase (years 3-4)**

361 The aim of the replication phase is to replicate putative discriminatory breath VOC /VOC signatures  
 362 identified in the discovery phase.

363 Similar to the discovery phase, recruitment of acutely breathless patients will be enriched into the  
 364 following disease strata following senior clinical decision maker assessment and within 24 hours of acute  
 365 admission.

366 Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of  
 367 asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=25) (**Table 1**).

368 Additional age matched healthy volunteers (n=55 adults and 25 children) will be identified as a non-  
 369 disease reference group.

370 **Total combined sample size of the discovery and replication phases = 700 participants**

<b>Disease Category</b>	<b>Discovery</b>	<b>Replication</b>
Acute Adult Asthma	<b>55</b>	<b>55</b>
Acute COPD	<b>55</b>	<b>55</b>
Acute Heart Failure	<b>55</b>	<b>55</b>
Community Acquired Pneumonia	<b>55</b>	<b>55</b>
Adult healthy volunteers	<b>55</b>	<b>55</b>
Acute paediatrics Asthma	<b>50</b>	<b>25</b>
Paediatrics healthy volunteers	<b>50</b>	<b>25</b>
<b>Total sample</b>	<b>375</b>	<b>325</b>

371 **Table (1): Table summarising recruitment targets for both adult and paediatric groups.**

## 372 **2.5. Schedule of assessments**

373 A schedule of acute assessments is outlined below and aligns to the movement of acute patients through  
 374 the clinical care pathway and the overall aim of developing a complete phenotypic picture of acutely  
 375 breathless patients.

### 377 **2.5.1. Defining acute breathlessness**

378 At presentation (within 24 hours of admission) to one of three acute admissions units potentially eligible  
 379 patients will be identified following confirmation of acute breathlessness, identified as (i) patient defined  
 380 acute breathlessness and/or (ii) 1 unit increase above patient reported baseline in the extended medical

381 research council (eMRC) dyspnoea score [47, 48] and at least one of the indicator diagnoses identified as  
 382 the primary clinical diagnosis by a senior clinical decision maker.

### 383 **2.5.2 Informed consent**

384 Patients meeting the pre-specified definition of acute breathlessness will be approached for informed  
 385 consent in to the breath VOC biomarker study. Only patients that are eligible to give full written informed  
 386 consent will be recruited.

### 387 **2.5.3 Collection of blood based pathology markers**

388 Collection of the blood biomarkers CRP, BNP, Troponin-I and blood eosinophil count will be performed  
 389 both acutely and following recovery, when not taken as part of clinical care pathway. These are currently  
 390 used in profiling acutely breathless patients in clinical practice (**Table2**).

391

Test	ANALYSER/METHOD	LOWER LIMIT OF DETECTION	UPPER LIMIT OF DETECTION
C-Reactive protein (CRP)	Siemens Advia Chemistry XPT, PEG enhanced immunoturbidimetric. Siemens Advia 1800, PEG enhanced immunoturbidimetric	5 mg/L	Diluted to result
B-type natriuretic peptide (BNP)	Siemens Advia Centaur XPT, two-site sandwich immunoassay using direct chemiluminescent technology	2.0 pg/mL	1445 pg/mL
Troponin-I	Abbott Architect i2000SR, three-site sandwich immunoassay using direct chemiluminescent technology (CMIA).	5.0 ng/L	50,000 ng/L

392 **Table (2):** Type of analyser and methodology used for blood biomarker calculation. The table outlines  
 393 analyser make, methodology, upper and lower limits of detection as per the University Hospitals of  
 394 Leicester NHS Foundation trust laboratory guidelines.

395

### 396 **2.5.4 Breath VOC sampling**

397 Offline breath sampling using GC-MS coupled with a standardised and CE marked breath sampler-  
 398 ReCIVA<sup>®</sup>[49] and comprehensive two-dimensional gas chromatography-mass spectrometry, coupled  
 399 with a standardised and CE marked breath sampler (ReCIVA<sup>®</sup> GCxGC-MS) will be performed. Gas  
 400 chromatography is considered a gold standard technique in detecting volatile organic compounds and as

401 such its sampling will be prioritised. Additionally the following online technologies, proton transfer  
 402 mass spectroscopy (PTR-MS), gas chromatography - ion mobility spectroscopy (GC-IMS) and  
 403 atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) will be evaluated according to  
 404 the sampling strategy outlined in section 3, **Figure 3 and Table 3.**

	COPD		Asthma		Pneumonia		Heart Failure		Healthy		Paediatrics	
Time point	1	2	1	2	1	2	1	2	1	2	1	2
Written informed consent	x		x		x		x		x		x	
<b>Volatile organic compound (VOC) sampling</b>												
ReCIVA- gas chromatography and mass spectrometry (GC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
ReCIVA- comprehensive two-dimensional gas chromatography (GCxGC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Proton transfer reaction mass spectrometry (PTRMS)		x		x		x		x	x	x	x	x
Gas chromatography - ion mobility spectrometry (GC-IMS)	x		x		x		x				x	x
<b>Pathology blood tests</b>												
Full blood count (including differential cell count)	x	x	x	x	x	x	x	x	x	x	x	x
Brain natriuretic peptide (BNP) [pg/mL]	x	x	x		x		x	x	x			
Troponin-I [ng/L]	x		x		x		x		x			
C-Reactive protein (CRP) [mg/L]	x	x	x	x	x	x	x	x	x	x	x	x
<b>Lung function tests</b>											x	x
Hand held forced oscillation technique (FOT)	x	x	x	x	x	x	x	x	x	x		
Fractional exhaled nitric oxide (FeNO) - Flow rate 50 [ml/s]			x	x							x	x
Spontaneous sputum sample	x	x	x	x	x	x	x	x	x	x		
Bio-banking (urine, serum, plasma. sputum supernatants & plugs)	x	x	x	x	x	x	x	x	x	x	x	x

Transthoracic echocardiography	x						x					
--------------------------------	---	--	--	--	--	--	---	--	--	--	--	--

405 **Table (3):** Summary of baseline and follow up assessments. The table summarises key assessments  
 406 carried out at different time points during the study. The participants may undertake any combination of  
 407 the investigations listed at any of these time points.

408

409

### 410 **2.5.5. Collection of additional samples for future biomarker campaigns**

411 Collection of additional biomarkers for future biomarker discovery campaigns including (i) a urine  
 412 sample, (ii) blood samples, up to 85mls for DNA, RNA, plasma and serum and peripheral blood cell flow  
 413 cytometry in selected subjects and (iii) spontaneous or induced sputum samples (plugs and supernatants)  
 414 will be carried out (**Table 3**).

415

416 All samples will be collected at time point 1 and at 2 (**Figure 3**). The additional samples will be used for  
 417 future omics analyses, these may include detailed analysis of the metagenome in sputum and proteomics  
 418 applied to urine and serum samples.

419

### 420 **2.5.6. Physiological characterisation**

421 Physiological measures of lung function will be performed in acutely ill participants and at  
 422 recovery including, (i) Hand held forced oscillation technique (FOT): an easily accessible  
 423 measure of lung function. Patients favour this to spirometry as it is effort independent, unlike  
 424 spirometry and requires less than a minute of quiet tidal breathing to obtain triplicate high quality  
 425 measurements [50], (ii) Fractional exhaled nitric oxide (FeNO): A measure of airway  
 426 inflammation in asthmatic patients[51, 52] (iii) Echocardiography: Two dimensional  
 427 transthoracic echocardiography will be performed in heart failure and COPD patients using an iE  
 428 33 system with S5-1 transducer (frequency transmitted 1.7 MHz, received 3.4 MHz; Philips  
 429 Medical Systems, Best, The Netherlands). Standard techniques as per American Society of



1  
2  
3 430 Echocardiography guidelines (ASE)[53] were used to acquire 2D, colour and Doppler images in  
4  
5 431 conventional parasternal long-axis, short-axis and apical 4-, 2- and 3-chamber views. Left  
6  
7 432 ventricular ejection fraction (LVEF) was calculated using the biplane method of discs formula  
8  
9 433 (Simpson's rule) to derive left ventricular volume indices.  
10  
11  
12

13 434 All participants are encouraged to report any testing related discomfort or concerns to the research team to  
14  
15 435 terminate the sampling process.  
16

### 17 436 **2.5.7 Recovery follow up**

- 18 437 • Patient recovery will be defined as:  
19 438 (i) Patient reported recovery from the acute exacerbation spell and back to their  
20 439 baseline extended MRC score or clinician defined recovery from the acute  
21 440 exacerbation spell  
22 441 and  
23 442 (ii) At least 6 weeks post exacerbation event (up to 6 months).  
24 443

25 444 Patients that re admit to hospital between visits 1 and 2, can have additional visit 1 assessments. Visit 2  
26 445 will be taken as recovery following the subsequent admission. If a patient is admitted to hospital after visit  
27 446 2 then they will be eligible to be recruited as a new study participant.  
28 447

29 448 The schedule of assessments at the recovery visit is outlined (**Table 3**).  
30  
31  
32

### 33 449 **2.6. Clinical Adjudication:**

34 450  
35 451 In an effort to reduce data variability and minimise bias, an independent panel consisting of two senior  
36 452 acute clinicians (SS & NG) will review all pertinent clinical and diagnostic source documentation, whilst  
37 453 blinded to admission blood biomarkers and clinical diagnosis.  
38 454

39 455 All acutely breathless adult patients' notes will be sequentially divided for adjudication. The panel will  
40 456 independently determine the primary diagnosis of highest probability from a list of the four potential acute  
41 457 indicator diagnoses in section 2.4 and mark their level of clinical certainty on a 100 mm visual analogue  
42 458 scale (VAS scale). The panel members will be able to review imaging, electrocardiograms (ECGs), and  
43 459 other relevant information but not admission blood based pathology tests.  
44

45 460 In a subset of patients adjudication will be validated by separate panel member to ensure between observer  
46 461 agreement using Bland-Altman analysis and inter-rater agreement of the primary diagnosis using Kohen's  
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1  
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3 462 kappa via repeated evaluation of a subset of cases (see statistical methods section 2.3).  
4  
5 463

## 6 7 464 **2.7. Clinical Informatics:** 8 9 465

10 466 Clinical data collection will be undertaken using a securely-hosted bespoke database system (ADD)  
11  
12 467 developed within the NIHR Leicester Biomedical Research Centre – Respiratory (BRC). The system links  
13  
14 468 acute admission episodes to hospital pathology records; historical respiratory physiology tests; and  
15  
16 469 demographic information. The system provides functionality to validate data entry; manually verify  
17  
18 470 records and highlight incomplete records. A custom VOC ‘module’ has been created to support data  
19  
20 471 collection within the study visits (1 and 2), and standardise diagnoses and medications through the use of  
21  
22 472 clinical ontologies as well as linking hospital records/tests to patient visits.  
23  
24 473

25  
26 474 Non-ADD based clinical data (e.g. hospital admissions, re-admissions and mortality) will be extracted  
27  
28 475 from the hospital data warehouse using identifiable patient identifiers, and subsequently pseudonymised  
29  
30 476 prior to integration.  
31  
32 477

33  
34 478 An informatics pipeline will be created to facilitate the transfer of chemo-metric data from remote  
35  
36 479 computers to the data repository. This will include tools to (i) enforce the correct labelling of data sets  
37  
38 480 (e.g. study number, visit, type/source of sample) prior to automated validated transfer to the repository;  
39  
40 481 (ii) record information about the sample process; (iii) search and extract data sets from the repository for  
41  
42 482 subsequent analysis. Prior to analysis, clinical and chemo-metric data will be integrated using the study  
43  
44 483 number and any potentially identifiable information will be removed.  
45  
46 484

## 47 48 49 50 51 485 **3. Breath profiling** 52 486

53  
54 487 The technologies utilised in the VOC study during discovery and replication phases are:  
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1  
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3 488 Offline technologies

4  
5 489 - ReCIVA+ GC-MS

6  
7 490 - ReCIVA + GC x GC-MS

8  
9 491 -

10  
11 492 Online technologies

12  
13 493 - GC-IMS

14  
15 494 - PTR-MS

16  
17 495 - APCI-MS

18  
19 496

20 497 Offline technologies will underpin the discovery analyses owing to their ability to identify chemical  
21  
22 498 identity and their recognition as the analytical gold standard in exhaled breath VOC analysis [54].

23  
24 499 In contrast online technologies will be utilised for VOC biomarker replication and at the recovery visits  
25  
26 500 owing to their portability and potential for future point of care testing. **(Figure 4)**.

27  
28 501 A brief description of the core VOC platforms is provided below

29  
30  
31 502 A CE marked breath sampling device (ReCIVA®) developed by Owlstone Medical, will be used to  
32  
33 503 sample breath onto two adsorbent Tenax tubes. Participants will be asked to breathe through the ReCIVA  
34  
35 504 face mask for a maximum of 300 seconds, aiming for collection of  $\geq 80\%$  of the target sample volume of  
36  
37 505 1 litre, after which the Tenax tubes will be transferred to the laboratory for analysis. This effectively  
38  
39 506 allows de-coupling of the breath sampling from the breath sensor and analysis platforms in selected  
40  
41 507 patients that are not able to mobilise to a real time breath sampling device. The Owlstone ReCIVA  
42  
43 508 sampler will be utilised in breath collection for offline technologies namely GC-MS and GCxGC-MS.  
44  
45 509 The ReCIVA sampler is capable of entraining oxygen and is therefore suitable for patients with mild  
46  
47 510 respiratory failure requiring low flow rates of oxygen to maintain target oxygen saturations [49].  
48  
49  
50

51 511 **3.1. Gas chromatography and mass spectroscopy (GC-MS):** is a commonly applied methodology used to  
52  
53 512 accurately measure trace gases in complex mixtures such as exhaled air [54]. Pre-concentrating breath  
54  
55 513 volatiles by various means and subsequent analysis constitute a reliable and sensitive method for VOC

1  
2  
3 514 analysis [55]. Despite its high sensitivity, it is however, a time consuming technique and carries a risk of  
4  
5 515 contamination at the pre-concentration step. It is also not suitable for online and multiple measurements  
6  
7 516 limiting its use as a point-of-care testing technology for VOC [56].  
8  
9

10 517 **3.2. Comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS):** an  
11  
12 518 advanced analytical technique for the analysis of complex organic matrices; its main advantage is the  
13  
14 519 unparalleled separation power it affords over conventional one-dimensional chromatographic techniques  
15  
16 520 [57]. Previous research, albeit sparse, has demonstrated the potential of GCxGC-MS for breath analysis  
17  
18 521 with the number of VOC detected exceeding those detected by conventional GC-MS [58, 59]. GCxGC-  
19  
20 522 MS of breath metabolites has been used for the identification of biomarkers related to glucose metabolism  
21  
22 523 [60, 61], tuberculosis [62] and radiation response [63]. This has generated interest within the breath  
23  
24 524 research community, however, such studies were conducted on a small scale (<50 patients) and involved  
25  
26 525 the use of expensive detectors and modulators. Method development and analysis of the data-rich GCxGC  
27  
28 526 chromatograms, however, can be time-consuming and require specialist knowledge.  
29  
30

31  
32 527 **3.3. Proton-transfer-reaction mass spectrometry (PTRMS):** a real time technique, capable of  
33  
34 528 simultaneously measuring the evolution of multiple gas metabolites from a single breath. It has been used  
35  
36 529 for the identification of potential useful VOC biomarkers for diagnosis of a variety of diseases: including  
37  
38 530 various cancers[64-66], liver disease[67, 68] and respiratory disease[69]. It has several advantages in  
39  
40 531 clinical settings, such as the speed of sampling, the instant result achieved and the lack of need for sample  
41  
42 532 storage or shipping. However, owing to the lack of pre-concentration or chromatographic separation,  
43  
44 533 sensitivity and definitive compound identification can be somewhat limited when compared to GC-MS.  
45  
46  
47 534 Two breath sampling devices will be used. The first device is a Loccioni SOFIA GSI-S; the subject is  
48  
49 535 required to exhale a single breath, five times (three if providing five samples proves too difficult) into a  
50  
51 536 sterile mouthpiece connected to an electrostatic bacterial/viral filter whilst wearing a nose clip (all CE  
52  
53 537 marked). Flow from the mouthpiece passes into a gas sampling interface capnograph (Loccioni GSI-S –  
54  
55 538 CE marked) and real-time user feedback of flow is provided on screen, allowing the regulation of the  
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3 539 breath sampling rate. The gas sampling interface acts to simultaneously trigger the acquisition of the  
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5 540 PTR-ToF-MS data and the exhaled breath travels through the capnograph down a heated sample line into  
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7 541 the ion source of the PTR-ToF-MS  
8  
9

10 542 The second breath sampling device is a ReCIVA breath sampler (Owlstone) with one of the adsorbent  
11  
12 543 Tenax tubes replaced with an outlet tube adapted for online sampling. The exhaled breath is transferred to  
13  
14 544 the PTR-ToF-MS via a heated transfer line connected to the outlet tube, continuously drawn at a constant  
15  
16 545 flow rate by the PTR-ToF-MS. The online adaptation of the consumable adsorbent tube does not affect  
17  
18 546 the CE mark of the ReCIVA sampling device.  
19  
20

21 547 Once the breath sample reaches the PTR-ToF-MS, via either breath sampler, the breath mixes with  
22  
23 548 protonated water ( $H_3O^+$ ) inducing proton transfer to the target volatile organic compounds (VOCs)  
24  
25 549 present, resulting in their ionisation. Sample ions are then guided into the time of flight mass  
26  
27 550 spectrometer and mass spectra, showing the abundance and mass of the VOCs present, are collected  
28  
29 551 throughout the exhalation. Following sampling, mouthpieces, filters and nose clips are disposed of and all  
30  
31 552 patient contacted surfaces wiped down with antiseptic cleaning wipes in preparation for the next patient.  
32  
33

34  
35 553  
36  
37 554 **3.4. Gas chromatography- ion mobility spectrometry (GC-IMS) (B&S Analytik):** Allows the detection  
38  
39 555 of volatile organic compounds down to ultra-trace level (ng/L - to pg/L - range). For years, IMS has been  
40  
41 556 used to discover potential discriminatory breath VOC in lung cancer [70, 71], COPD[72, 73] and  
42  
43 557 asthma[73]. Sampling takes place using a Spiroscout spirometer. The patients exhale through a disposable  
44  
45 558 mouth piece connected to a Teflon tube. A piezoelectric pressure sensor is used to monitor the breathing  
46  
47 559 profile, this opens the sampling valve at the appropriate point in the breath profile to collect end-tidal  
48  
49 560 breath in a sample loop of 10 mL volume. After filling this loop, the collected sample air is then  
50  
51 561 transferred to a multicapillary column for a chromatographic separation, which is achieved in 12 min. The  
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3 562 separated molecules are then transferred into the IMS, ionised and then separated according to their  
4  
5 563 mobility in a weak electric field.  
6  
7 564 The technology's multiple advantages of ultra-sensitivity, portability, online sampling and short analysis  
8  
9 565 time (typical analysis time of 10 minutes) with real time detection, brings a promise to provide immediate  
10  
11 566 and potentially reliable results for point of care breath diagnostics. Another concept with IMS devices is  
12  
13 567 that once the required breath signatures have been discovered using GC-MS, IMS offers the potential to  
14  
15 568 be 'tuned' for selective detection of VOC.

16  
17  
18 569 **3.5. Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) semiportable compact**  
19  
20 **version (Advion):** is one of less sensitive but more affordable versions of mass spectrometers released to  
21  
22 570 the commercial market in recent years. The device uses APCI to produce ions. Although the most  
23  
24 571 common use of APCI-MS systems is the detection in liquid chromatography applications, the technique  
25  
26 572 has proven to be a valuable tool for direct measurement of VOC in air[74, 75] food[76, 77] and breath[78,  
27  
28 573 79]. Recently, the technique has shown potential for online, real time profiling of pseudo-metabolites in  
29  
30 574 exhaled breath [80] with sensitivity comparable with other techniques. By combining miniaturised MS  
31  
32 575 technology with APCI techniques, adequate quality of on-site, real time measurements with minimal or  
33  
34 576 no sample preparation requirement can be provided. This is a desirable outcome as it overcomes main  
35  
36 577 limitation of using standard breath analysis method in clinical setting, which is a need for breath sample  
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38 578

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40 579  
41  
42 580  
43 608 There remains an overall lack of standardisation and rigour across these technologies which hindered  
44  
45 609 previous advancements in breath discovery; something we intend to minimize.

#### 47 610 **4. Chemometric processing and data analysis:**

48  
49  
50 611 GC-MS breath data will be aligned, deconvoluted and the features for each participant will be extracted.  
51  
52 612 The extracted features will be grouped and classified by retention index and mass spectrum. The  
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3 613 registered and aligned data will be linked to participant meta-data to generate a breath matrix. Data  
4  
5 614 handling and analysis will be performed by a senior statistician.  
6  
7

8 615 The breath matrix is a  $n \times p$  matrix where  $n$  is the number of subjects and  $p$  is the number of VOC. The  
9  
10 616 breath matrix is high dimensional with  $p \gg n$  and many potentially correlated VOC. In view of this, we  
11  
12 617 will employ sparse partial least squares discriminant analysis (sPLS-DA)[81] to investigate which of the  
13  
14 618 VOC can identify breathlessness. We will also investigate which of the VOC can discriminate between  
15  
16 619 the different disease states including acute exacerbations of asthma and COPD and Pneumonia. In  
17  
18 620 addition to the supervised methods, unsupervised methods will be explored, specifically sparse principle  
19  
20 621 component analysis (sPCA)[82].  
21  
22  
23

24 622 Extracted VOC will also be investigated. Relationships between VOC and patient reported acute  
25  
26 623 breathlessness will be analysed using logistic regression model. VOC associated with patient associated  
27  
28 624 acute breathlessness will be incorporated into multinomial logistic regression models in conjunction with  
29  
30 625 CRP, BNP, blood eosinophils and Troponin-I, pathology biomarkers currently in use for diagnosing  
31  
32 626 undifferentiated breathlessness. In addition to the conventional binary and multinomial logistic regression  
33  
34 627 models, regression models [83].  
35  
36  
37

## 38 628 **5. Ethics and dissemination:**

39  
40  
41 629 The study has obtained full ethical approval from the London South East Research ethics Committee, REC  
42  
43 630 reference 16/LO/1747. IRAS project ID 198921. Publications will be prepared according to the MRC-  
44  
45 631 EMBER consortium agreement and the University of Leicester publications policy. All intended  
46  
47 632 publications will be submitted to the EMBER executive board for review and comments within 60 days of  
48  
49 633 journal submission. Authorship will be according to contribution and internationally recognised guidance  
50  
51 634 on journal authorship.  
52  
53

## 54 55 635 **6. Study dates:** 01/2/2017 – 30/10/2020



1  
2  
3 **636 7. Authors' contributions:**  
4

5 637  
6  
7

8 638 S.S, C.E.B, N.Gr, P.Th and P.Mo conceived the study, obtained funding, wrote the study protocol,  
9  
10 639 obtained ethical and MHRA approvals for the study and coordinated the deployment of analytical testing  
11  
12 640 methods for breath analysis. W.I took the lead in writing the manuscript with support from S.S. Planning  
13  
14 641 and recruitment of adult participants was carried out by W.I, S.Jo, B.Pa, A.Aw, R.Ph, G.Fo, A.Yo, R. J. R  
15  
16 642 and C.Wh. Paediatrics study design was conceived by E.Ga and C.Be and participants recruited by T. Mc  
17  
18 643 and C. Fo. Analytical chemistry team formed of M.Wi, R.Co, D.Sa, D.Ru and L.Br expertly handled all  
19  
20 644 the breath samples and planned an analysis structure. M.Ri, a senior statistician, constructed a statistics  
21  
22 645 and data analysis plan in conjunction with SS. Bioinformatics pipeline and electronic CRFs developed by  
23  
24 646 R.Fr and B.Zh. All authors, including R.Pe, H. Bh, B.Ha, A. Si, K. Ry, H. Pa, T. Su, L. L Ng, contributed  
25  
26 647 to the study design and study protocol.  
27  
28  
29

30  
31 **648 8. Protocol version:** Version 4, 1<sup>st</sup> April 2018  
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34 649  
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36 **650 9. Public and patient involvement:**  
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38 651 A series of consultations have taken place with our patient involvement team within the NIHR Biomedical  
39  
40 652 Research Centre (Respiratory Theme) and across the wider BRC PPI group. Representations from the  
41  
42 653 paediatrics team were also present. This group was sent copies of the participant documentation for review and  
43  
44 654 discussion. Various revisions have been made following on from these discussions.  
45  
46  
47

48 **655 10. Funding statement:**  
49

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51  
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53  
54 658 Health Research (NIHR) Leicester Biomedical Research Centre and NIHR, Leicester Clinical Research  
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2  
3 659 Facility and the Midlands Asthma and Allergy Research Association (MAARA) to whom we are  
4  
5 660 extremely grateful. The authors would like to acknowledge the invaluable efforts of the research nurses  
6  
7 661 responsible for the in-clinic sample collection as well as the input from the wider EMBER consortium  
8  
9 662 (Members list can be found at: <https://ember.le.ac.uk/web>). The views expressed are those of the  
10  
11 663 author(s) and not necessarily those of the NHS and NIHR or the Department of Health  
12  
13  
14  
15 664

16  
17 665 **11. Competing interests:** SS has performed advisory services for Owlstone Medical.  
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#### 41 678 **Figure legends:**

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43 679 **Figure (1)** Relationship between lung proximity and degree of invasiveness of different lung matrices.  
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45 680 The figure plots the level of invasiveness of various lung matrices in relation to their proximity to the  
46  
47 681 lung. Given their pathological relevance, the degree of invasiveness of bronchoalveolar lavage (BAL) and  
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49 682 lung biopsy makes them less favourable in diagnosing respiratory diseases.  
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3 683 **Figure (2)** Multi-instrument use in breath sampling. Figure illustrates the various combinations of offline  
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5 684 and online devices used in breath sampling and the relevant pros and cons. Offline and online  
6  
7 685 technologies are used for the discovery and validation phases of the study respectively.  
8  
9

10 686 **Figure (3)** Study flow chart. Figure outlines the patient journey from admission through to discharge and  
11  
12 687 follow up. Participants with self-reported acute breathlessness presenting to University Hospitals of  
13  
14 688 Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling is  
15  
16 689 carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge. Patients  
17  
18 690 are admitted through the standard operational emergency medical streaming and care pathways at the  
19  
20 691 University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission) are measured at 30  
21  
22 692 and 60 days and late outcomes (mortality) including respiratory and all-cause mortality are measured at 2  
23  
24 693 years. Assessments carried out at each time point are summarised in **Table 1**.  
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26  
27

28 694 **Figure (4)** Multi-instrument use in breath sampling. Operational space of the analytical technologies  
29  
30 695 used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including  
31  
32 696 proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-mass  
33  
34 697 spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas chromatography-  
35  
36 698 mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass spectrometry (GCxGC).  
37  
38 699 Comparing the typical molar mass range detectable; selectivity in detection owing to the type of  
39  
40 700 ionisation involved and the proton affinity of analytes; and the inclusion of a chromatographic separation  
41  
42 701 affecting total time of analysis. The online technologies involving chemical ionisation (PTR-MS, CMS  
43  
44 702 and GC-IMS) can be used in-clinic owing to short analysis times, but only detect lower molar mass  
45  
46 703 molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>. Offline chromatographic techniques (GC-MS  
47  
48 704 and GCxGC) detect a wider range of compounds independent of proton affinity; however, the techniques  
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50 705 have longer analysis times and involve sample transportation and storage.  
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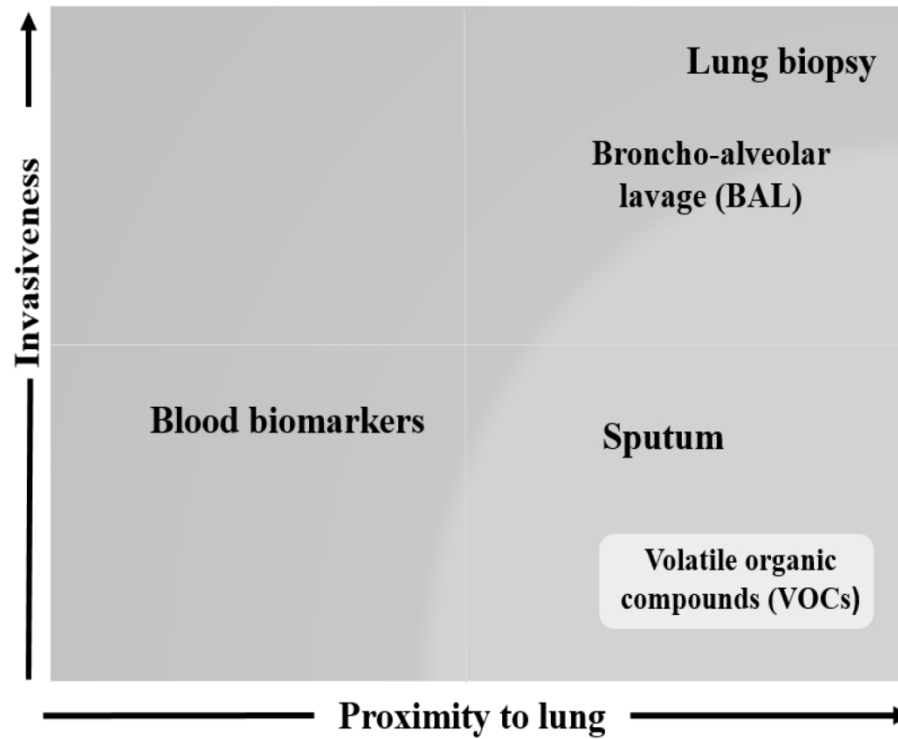


Figure (1): Relationship between lung proximity and degree of invasiveness of different lung matrices. The figure plots the level of invasiveness of various lung matrices in relation to their proximity to the lung. Given their pathological relevance, the degree of invasiveness of bronchoalveolar lavage (BAL) and lung biopsy makes them less favourable in diagnosing respiratory diseases.

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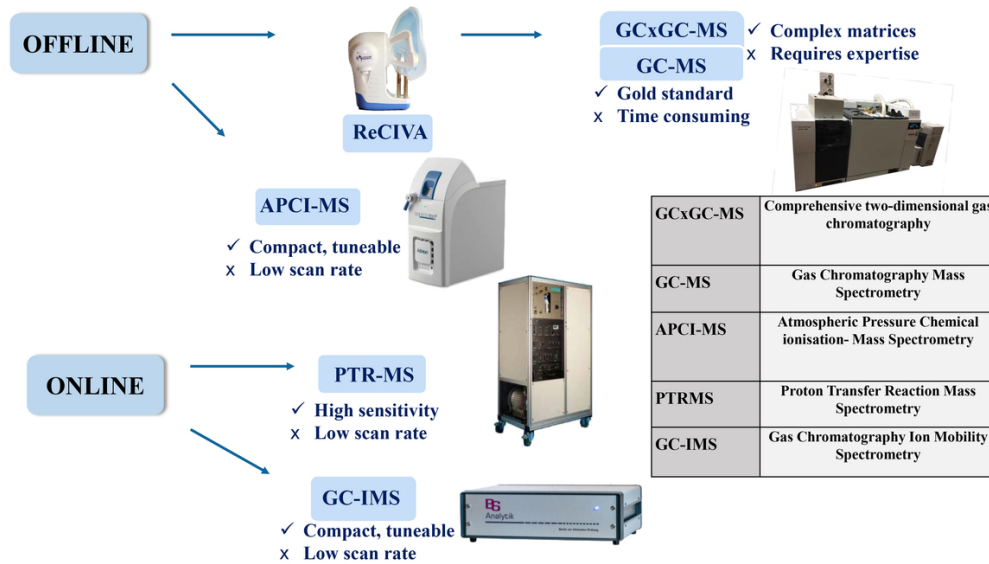
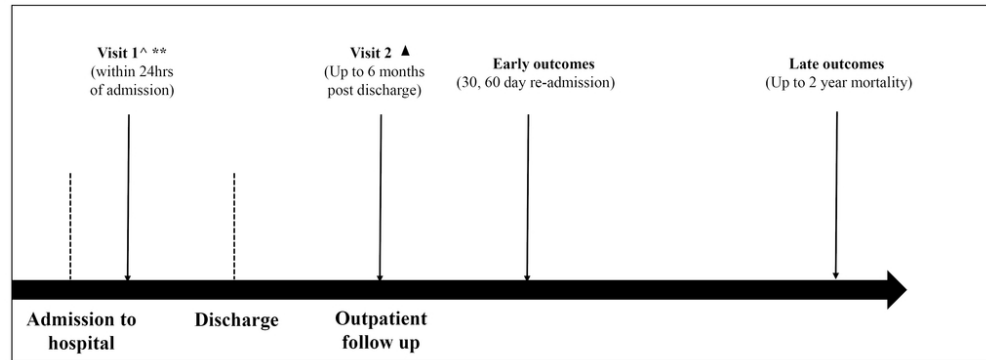


Figure (2): Multi-instrument use in breath sampling. Figure illustrates the various combinations of offline and online devices used in breath sampling and the relevant pros and cons. Offline and online technologies are used for the discovery and validation phases of the study respectively.

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- ^ Following senior decision maker review  
 \*\* Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, GC-IMS, APCI-MS)  
 ▲ Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, PTR-MS, APCI-MS)  
 ---- Following University Hospitals of Leicester streaming and clinical care pathways

Figure (3): Study flow chart. Figure outlines the patient journey from admission through to discharge and follow up. Participants with self-reported acute breathlessness presenting to University Hospitals of Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling is carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge. Patients are admitted through the standard operational emergency medical streaming and care pathways at the University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission) are measured at 30 and 60 days and late outcomes (mortality) including respiratory and all-cause mortality are measured at 2 years. Assessments carried out at each time point are summarised in Table 1.

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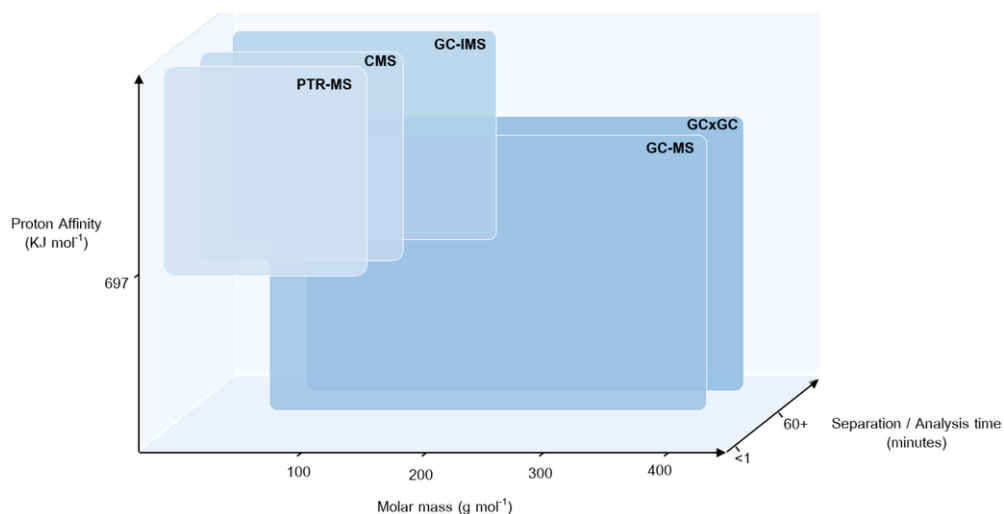


Figure (4): Multi-instrument use in breath sampling. Operational space of the analytical technologies used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-mass spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass spectrometry (GCxGC). Comparing the typical molar mass range detectable; selectivity in detection owing to the type of ionisation involved and the proton affinity of analytes; and the inclusion of a chromatographic separation affecting total time of analysis. The online technologies involving chemical ionisation (PTR-MS, CMS and GC-IMS) can be used in-clinic owing to short analysis times, but only detect lower molar mass molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>. Offline chromatographic techniques (GC-MS and GCxGC) detect a wider range of compounds independent of proton affinity; however, the techniques have longer analysis times and involve sample transportation and storage.

88x44mm (300 x 300 DPI)

# BMJ Open

## Assessment of Breath Volatile Organic Compounds in Acute Cardio-respiratory Breathlessness: A protocol describing a Prospective Real World Observational Study

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<b>Primary Subject Heading</b>:	Respiratory medicine
Secondary Subject Heading:	Research methods, Respiratory medicine
Keywords:	Breathlessness, Observational study, Volatile Organic Compound, Breath analysis

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# 1           **Assessment of Breath Volatile Organic Compounds in Acute Cardio-** 2           **respiratory Breathlessness: A protocol describing a Prospective Real** 3           **World Observational Study**

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review only

69

**Abstract****Introduction**

Patients presenting with acute undifferentiated breathlessness are commonly encountered in admissions units across the United Kingdom. Existing blood biomarkers have clinical utility in distinguishing patients with single organ pathologies but have poor discriminatory power in multi factorial presentations. Evaluation of volatile organic compounds (VOC) in exhaled breath offers the potential to develop biomarkers of disease states that underpin acute cardio-respiratory breathlessness, owing to their proximity to the cardio-respiratory system. To date there has been no systematic evaluation of VOC in acute cardio-respiratory breathlessness. The proposed study will seek to use both offline and online VOC technologies to evaluate the predictive value of VOC in identifying common conditions that present with acute cardio-respiratory breathlessness.

**Methods and analysis**

A prospective real world observational study carried out across three acute admissions units within Leicestershire. Participants with self-reported acute breathlessness, with a confirmed primary diagnosis of either acute heart failure, community acquired pneumonia and acute exacerbation of asthma or COPD will be recruited within 24 hours of admission. Additionally, school age children admitted with severe asthma will be evaluated. All participants will undergo breath sampling on admission and upon recovery following discharge. A range of online technologies including: proton-transfer-reaction mass spectrometry (PTR-MS), gas chromatography ion mobility spectrometry (GC-IMS), atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) and offline technologies including gas chromatography mass spectroscopy (GC-MS) and comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS) will be utilised for VOC discovery and replication. For offline technologies a standardised CE marked breath sampling device (ReCIVA<sup>®</sup>) will be used. All recruited participants will be characterised using existing blood biomarkers including C - reactive protein (CRP), brain derived natriuretic peptide (BNP), Troponin-I



1  
2  
3 95 and blood eosinophil levels and further evaluated using a range of standardised questionnaires, lung  
4  
5 96 function testing, sputum cell counts and other diagnostic tests pertinent to acute disease.  
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7

### 8 97 **Ethics and dissemination**

9  
10 98 The National Research Ethics Service Committee East Midlands has approved the study protocol  
11  
12 99 (REC number: 16/LO/1747). IRAS 198921. Findings will be presented at academic conferences and  
13  
14 100 published in peer-reviewed scientific journals. Dissemination will be facilitated via a partnership with  
15  
16 101 the East Midlands Academic Health Sciences Network and via interaction with all UK funded  
17  
18 102 MRC/EPSRC molecular pathology nodes.  
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25 104 **Key words:** Breathlessness; Breath analysis; Volatile Organic Compound, Observational study  
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## 116 **Strengths and Limitations of this Study**

- 117 • A pragmatic real world, prospective, observational study across three admission units that  
118 focuses on the systematic discovery and replication of VOC in acutely breathless patients  
119 using both online and offline technologies
- 120 • The proposed study is the largest of its kind in acute disease to characterise VOC with a  
121 range of additional assessments that will build a comprehensive phenotype of acute  
122 cardio-respiratory exacerbations
- 123 • The proposed study will build an infrastructure for research and subsequent evaluation of  
124 VOC in interventional trials within acute cardio-respiratory exacerbations
- 125 • Prior acute treatment exposure will need to be accounted for when evaluating potential  
126 discriminative biomarkers
- 127 • VOC technologies are not currently suited for deployment in patients that are of high  
128 clinical acuity

## 130 **1. Introduction:**

131 Breathlessness is a common symptom of cardio-respiratory illnesses that has a significant direct  
132 impact on patients' wellbeing as well as a substantial economic burden on healthcare systems [1].  
133 Although its etiologies can be variable, exacerbations of common complex chronic cardio-respiratory  
134 conditions account for approximately 70% of acute presentations with breathlessness, namely  
135 exacerbations of asthma and COPD, acute heart failure and community acquired pneumonia [2].  
136 Moreover, moderate and severe breathlessness is significantly associated with all-cause,  
137 cardiovascular and COPD mortality[3]. As a consequence symptomatic breathlessness warrants rapid  
138 evaluation and targeted diagnostics at presentation.

139

140 Diagnostic evaluation of acute breathlessness is heavily reliant on blood based biomarkers e.g. CRP,  
141 BNP, Troponin and on occasions blood eosinophil levels. These biomarkers have clinical utility  
142 primarily in patients with single pathologies, but have poor discriminatory power in patients with

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2  
3 143 multifactorial presentations of acute breathlessness[4]. There is therefore an unmet need for the  
4  
5 144 development of sensitive and specific biomarkers that differentiate acute breathlessness from its  
6  
7 145 recovery and the common cardio-respiratory conditions that present with acute breathlessness.  
8  
9 146  
10  
11 147 CRP plays an important role in diagnosing breathlessness caused by an underlying bacterial  
12  
13 148 pneumonia[5], as well as predicting mortality in patients with COPD [6]. BNP is routinely utilised in  
14  
15 149 acute settings to support the diagnosis of acute heart failure [7]. The European Society of Cardiology  
16  
17 150 (ESC) recommends BNP threshold values of <100 pg/mL to rule out acute congestive cardiac failure  
18  
19 151 and values > 500 pg/ml as diagnostic of acute exacerbations of heart failure [8].  
20  
21  
22 152

23  
24 153 The role of peripheral blood eosinophil count in airway inflammation was poorly understood up until  
25  
26 154 the second half of the 19<sup>th</sup> century when Paul Ehrlich, a German physician and Nobel prize winner,  
27  
28 155 introduced eosin in his technique for white cell differentiation in 1879[9]. Considerable advances in  
29  
30 156 the field of airway inflammation and the role of eosinophils have taken place since [10-12]. More  
31  
32 157 recently Bafadhel *et al* suggested that peripheral blood eosinophil count can be used to direct  
33  
34 158 corticosteroid therapy during COPD exacerbations in single centre study [13].

35  
36  
37 159 Currently, blood biomarkers together with clinical, physiological and imaging parameters are used in  
38  
39 160 diagnosing the cause of acute breathlessness. Blood biomarkers may be less specific as they originate  
40  
41 161 far from the target organs of interest (the heart and the lungs in cardio-respiratory disease). Sputum,  
42  
43 162 although potentially a more definitive lung specific matrix, is comparatively difficult to obtain  
44  
45 163 particularly in acutely unwell patients, limiting its use in acute disease and highlighting the need for  
46  
47 164 better biomarkers. Ideally these biomarkers would have the following characteristics, (i) they would  
48  
49 165 originate from the target organ of interest, (ii) they would significantly add value to conventional risk  
50  
51 166 scoring and diagnostic algorithms in acute breathlessness, (iii) they would be minimally invasive and  
52  
53 167 suitable for rapid point of care diagnosis in emergency rooms and acute admissions units (iv) they  
54  
55 168 would have diagnostic value in patients with multifactorial acute breathlessness.  
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3 170 Exhaled breath contains thousands of volatile organic compounds (VOC) that reflect biological  
4  
5 171 processes occurring in the host both locally in the airways and systematically offering the potential to  
6  
7 172 develop more effective biomarkers in acutely breathless patients (**Figure 1**).

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9 173  
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11 174 The proposed program of research will use a combination of offline and online technologies to  
12  
13 175 identify and evaluate the diagnostic and prognostic value of VOC in patients with acute cardio-  
14  
15 176 respiratory related breathlessness (**Figure 2**).

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19  
20 178 Exhaled breath analysis of volatile chemicals offers the potential to develop more effective biomarkers  
21  
22 179 in acutely breathless patients. The use of breath analysis for disease diagnostics dates back to ancient  
23  
24 180 Greeks where physicians used exhaled breath to diagnose different diseases. Breath odours allow  
25  
26 181 correct associations to certain diseases. For example, the sweet smell of diabetic ketoacidosis, the  
27  
28 182 fishy smell of breath associated to liver illness, the urine-like odour of kidney disease and the smell of  
29  
30 183 the breath of patients with lung abscesses, caused by the proliferation of anaerobic bacteria [14-17].  
31  
32 184 More recently, exhaled breath analysis has demonstrated early proof of concept in the diagnosis of  
33  
34 185 acute heart failure, and ventilator associated pneumonia[18] . The validity of breath analysis has also  
35  
36 186 been demonstrated in breathless children[19]. This population is likely to prefer breath-based tests, as  
37  
38 187 these are minimally invasive. Importantly, a variety of point of care sensors are now available to  
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40 188 evaluate potential exhaled breath biomarkers in emergency care settings.

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45 190 A study by Van Berkel et al demonstrated the ability to distinguish COPD subjects from controls  
46  
47 191 solely based on the presence of VOCs in breath, suggesting that analysis of VOC might be highly  
48  
49 192 relevant for diagnosis of COPD [20]. This established the basis of further studies of VOC in COPD  
50  
51 193 [21-25].recommending larger studies for validation.

52  
53 194 Several other studies found that VOC profiling in diagnosing asthma is potentially feasible [26-32].

54  
55 195 This however has been done in relatively small numbers in stable disease.

56  
57 196 Despite the novelty of non-invasive sampling technology and the growing interest in exhaled breath  
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59 197 analysis there remains a disappointing level of comparability across studies due to the lack of

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3 198 standardisation and appropriate data analysis methods. A recent systemic review by Anders  
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5 199 Christiansen *et al* compared eleven publications reporting very heterogeneous designs, methods,  
6  
7 200 patient group sizes, data analytics and, consequently, quite varying results [33].  
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9 201  
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11 202 To our knowledge, no other large studies exploring the use of breath biomarkers in profiling acute  
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13 203 breathlessness have been completed. Several studies have explored the use of electronic nose (eNose)  
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15 204 in stable disease with good discriminatory power in COPD [34], Pneumonia [35] and heart failure[36]  
16  
17 205 with relatively small sample size. While eNose has now been widely used in detecting various VOC  
18  
19 206 patterns, GC-MS, a largely validated methodology, remains the gold standard technique for detecting  
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21 207 VOCs in exhaled breath. The focus of the current research study will be to evaluate acutely breathless  
22  
23 208 cardio-respiratory patients using a combination of ‘discovery’ and near-patient care breath sampling  
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25 209 technologies.

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27  
28 210 Medical Research Council (MRC) and Engineering and Physical Sciences Research Council (EPSRC)  
29  
30 211 have commissioned a series of molecular pathology nodes aimed at developing molecular signatures  
31  
32 212 relevant to disease diagnosis and progression. This was triggered by the clear need for alliance  
33  
34 213 between academic institutions, industry and NHS partners to enhance the benefits of stratified  
35  
36 214 medicine for patients[37, 38].

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38  
39 215 University of Leicester and Loughborough University were awarded a joint molecular pathology node  
40  
41 216 East Midlands Breathomics Pathology Node (EMBER) which this study forms a key part of.  
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## 44 45 218 **2. Methods and Analysis**

### 46 47 219 **2.1. Study design**

48  
49 220 A prospective real world observational study across three acute admissions units within Leicestershire  
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51 221 (two adult admissions units and one children’s assessment unit). The acute units routinely assess and  
52  
53 222 treat cardio-respiratory admissions due to breathlessness in adults and children.  
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3 223 Participants with self-reported acute breathlessness, either requiring admission or a change in baseline  
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5 224 treatment, will be screened for the study. Informed consent will be obtained in all participants  
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7 225 following a clinical review by a senior decision maker within 24 hours of acute admission (**Figure 3**).  
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## 13 228 **2.2. Objectives**

### 14 229 **2.2.1. Primary objective**

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16 230  
17 231
- 18 231 • To evaluate the sensitivity, specificity, positive and negative predictive value of exhaled  
19  
20 232 breath VOC biomarkers to differentiate acute breathlessness in cardio-respiratory patients.

### 21 233 **2.2.2. Secondary objectives:**

- 22 234  
23 235
- 24 235 • To replicate selected breath VOC biomarkers identified in acute breathlessness.
  - 25 236 • To discover and replicate breath VOC biomarkers that differentiate the common cardio-  
26  
27 237 respiratory conditions that cause acute breathlessness, specifically (i) acute heart failure, (ii)  
28  
29 238 community acquired pneumonia, (iii) adult exacerbations of asthma and COPD and age-  
30  
31 239 matched adults that do not have cardio-respiratory disease or breathlessness.
  - 32 240 • To quantify the level of clinical uncertainty in the primary diagnosis using a 100 mm visual  
33  
34 241 analogue scale and independent clinical adjudication of case notes blinded to the following  
35  
36 242 blood biomarkers (i) CRP (ii) BNP (iii) Troponin-I (iv) blood eosinophils, but not clinical  
37  
38 243 history and acute presentation nor chest x-ray imaging. Potential discriminatory breath VOC  
39  
40 244 biomarkers will be adjusted for clinical uncertainty in statistical models.
  - 41 245 • To identify and replicate exhaled breath VOC biomarkers in school age children treated in  
42  
43 246 hospital for severe asthma attacks and compare these to age-matched healthy controls.

### 44 247 **2.2.3. Exploratory end points (where applicable):**

- 45 248  
46 249
- 47 249 • To evaluate the dynamic profile of selected breath VOC between the acute state and the  
48  
49 250 recovery state post exacerbation.
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3 251 • To evaluate the relationship between exhaled VOC biomarkers and clinical outcomes  
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5 252 including (i) hospital readmission at 30 and 60 days post event (ii) all-cause mortality over a 2  
6  
7 253 year period post admission.  
8  
9 254 • To evaluate the relationship between breath VOC biomarkers and functional measures  
10  
11 255 e.g. physical performance and activity  
12  
13 256 • To explore potential breath VOC biomarkers of multifactorial acute breathlessness  
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15  
16 257 • To evaluate the relationship between diet, lifestyle and environment upon breath VOC  
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18 258 biomarkers  
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### 22 260 **2.3. Sample size estimation**

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24 261  
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26 262 Preliminary data was utilised to conduct sample size estimates from a cohort of acutely breathless  
27  
28 263 patients admitted to acute admissions units over a 6 month period (February 2017 to August 2017).  
29  
30 264 Hundred and twelve adult participants (asthma 46, community acquired pneumonia 26, COPD 22)  
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32 265 and eighteen healthy controls were utilised for the analysis.  
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34

35 266 A panel of ten pre-specified aldehydes, based on literature search [31, 39, 40], were extracted from  
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37 267 breath using gas chromatography-mass spectrometry (GC-MS). The aldehydes were normalised to a  
38  
39 268 common internal standard and were not background-subtracted.  
40  
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42 269 A closed formula from Hsieh *et al*[41], relating sample size to observable effect size, was used to  
43  
44 270 calculate sample size from logistic regression models of the ten aldehydes with acute breathlessness  
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46 271 as the outcome measure. The sample size estimates are also relevant to acute class comparisons vs.  
47  
48 272 the sum of other acute classes.  
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50

51 273 Based upon the sample size estimates we would have an 80% power, with a type 1 error rate of 5%, to  
52  
53 274 detect an odds ratio of association of 1.2 between two disease classes with 55 patients per class. Given  
54  
55 275 the fact that study seeks to discover and replicate breath VOC amongst five adult disease classes  
56  
57 276 (community acquired pneumonia, heart failure, COPD, asthma and healthy aged matched subjects) we  
58  
59 277 would require 110 adult patients per class – 550 patients across the program to achieve these aims.  
60

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3 278 The closed formulae by Tihaki *et al*,<sup>[42]</sup> were also utilised to understand the discriminatory power  
4  
5 279 that the samples sizes above would provide with respect to biomarker sensitivity and specificity; The  
6  
7 280 following assumptions were made:

- 10 281 • That a sensitivity of 80 % with a precision of 5% would provide a useful biomarker capable  
11  
12 282 of ‘ruling out’ an acute class. The same target was applied to specificity.
- 14 283 • We assume a prevalence of acute breathlessness of 80% as the recruitment campaign uses  
16 284 acute breathlessness as the initial stratification tool for recruitment and 1:5 patients recruited  
18 285 will be non-breathless healthy controls
- 21 286 • We aim to balance group sizes across classes equally

23 287 For a type 1 error rate of 0.05 and a 95% confidence interval

24  
25 288  $N_{\text{sensitivity}} = 307$

26  
27 289  $N_{\text{specificity}} = 1,230$

28  
29 290

30 291 For a type 1 error rate of 0.05 and a 90% confidence interval

31  
32 292  $N_{\text{sensitivity}} = 218$

33  
34 293  $N_{\text{specificity}} = 871$

35  
36 294

37 295 For a type 1 error rate of 0.05 and an 85% confidence interval

38  
39 296  $N_{\text{sensitivity}} = 166$

40  
41 297  $N_{\text{specificity}} = 664$

42  
43 298

44 299 For a type 1 error rate of 0.05 and an 80% confidence interval

45  
46 300  $N_{\text{sensitivity}} = 131$

47  
48 301  $N_{\text{specificity}} = 524$

49  
50 302

51 303 Therefore, we are powered to identify sensitive biomarkers ( $\geq 80\%$ ) of acute breathlessness with a  
52  
53 304 maximum marginal error in the estimate for sensitivity not exceeding 5% with 95% confidence.

54  
55 305 Similarly, we are powered to identify specific biomarkers ( $\geq 80\%$ ) of acute breathlessness with a  
56  
57 306 maximum marginal error in the estimate for specificity not exceeding 5% with 80% confidence.



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3 307 For the primary analysis the outcome will be treated as a nominal variable with levels (i) acute heart  
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5 308 failure (ii) community acquired pneumonia (iii) adult exacerbations of asthma and COPD and (iv) acute  
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7 309 exacerbations in school age children treated in hospital for severe asthma attacks.  
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9

10 310 The relationship between the primary outcome and the exhaled breath VOC biomarkers will be  
11  
12 311 modelled using multinomial logistic regression. In addition to metabolomics markers the following  
13  
14 312 independent variables will be included in the model: clinical uncertainty score on a 100 mm VAS  
15  
16 313 scale, age, and a validated co morbidity score (the Charlson comorbidity score)[43, 44].  
17  
18 314

19  
20 315 Receiver operator analyses will be utilised to generate ROC curves for individual and multiple panels  
21  
22 316 VOC predictors in the primary analysis.  
23  
24 317

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26  
27 318 To understand the dynamic profile of breath biomarkers during (i) the acute state and (ii) in the  
28  
29 319 chronic state up to 6 months post exacerbation, a repeated measures model with a random intercept  
30  
31 320 and random effect for time will be fitted, the random effects will be fitted for each patient. For the  
32  
33 321 repeated measures mixed model an unstructured covariance will be assumed. To evaluate the  
34  
35 322 relationship between breath biomarkers and hospital readmission at 30 and 60 days Cox proportional  
36  
37 323 hazards and frailty models will be utilised [45]. Analysis of Multivariate Survival Data, [CITE]  
38  
39 324 competing risk models and joint models will be fitted [46]. Relationship between death and breath  
40  
41 325 biomarkers will be evaluated using a logistic regression model. Changes in outcome measures will be  
42  
43 326 measured appropriately for each variable (e.g. paired t-test, Mann-Whitney, repeated measures  
44  
45 327 analysis). Tables of descriptive statistics will be compiled for all key variables  
46  
47  
48

49 328 All analysis will be performed using R 3.5.0 <https://www.r-project.org/>.

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51 329

## 52 330 **2.4. Discovery and Replication studies**

53  
54  
55 331 Specific indicator conditions have been selected for targeted recruitment according to their high  
56  
57 332 prevalence and unmet need, their high morbidity and mortality and the need to develop better  
58  
59 333 diagnostic and prognostic algorithms in acute care pathways.  
60

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3 334 The indicator diagnoses of interest are (i) exacerbations of adult asthma and COPD, (ii) community  
4  
5 335 acquired pneumonia (iii) acute heart Failure and (iv) exacerbation in school age children treated in  
6  
7 336 hospital for severe asthma attacks.  
8  
9

10 337 Patient level clinico-pathological and outcome data (spanning the entire acute pathway) will be  
11  
12 338 collected in parallel to breath sampling. In addition, breath samples will be acquired in the stable state  
13  
14 339 post exacerbation (**Figure 3**).  
15

16 340

17  
18 341 Age matched healthy volunteers will be recruited where possible at separate visits. For the purposes of  
19  
20 342 this study, healthy volunteers will be defined as participants who have no prior history of asthma,  
21  
22 343 COPD, heart failure and have not been admitted to hospital with community acquired pneumonia  
23  
24 344 within 6 weeks of the baseline study visit. For acute admission the study team will approach the  
25  
26 345 spouse, parent or sibling of the index case and seek informed consent for study assessments. All  
27  
28 346 healthy subjects will undergo two assessments separated by a duration of 8-16 weeks to match the  
29  
30 347 acute and recovery time points elapsed in their index case/partner/spouse/sibling/child. Additional  
31  
32 348 healthy volunteers will be identified from local recruitment databases and via advertising  
33  
34

35 349

#### 37 350 ***2.4.1. Discovery Phase (Project months 1-24):***

39 351 The aim of the discovery phase is to discover putative discriminatory breath VOC, using both offline  
40  
41 352 and online technologies.  
42

43  
44 353 Pre-planned recruitment of acutely breathless patients will be enriched into the following disease  
45  
46 354 strata following senior clinical decision maker assessment and within 24 hours of acute admission.  
47

48 355 Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of  
49  
50 356 asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=50).  
51

52 357 Additional age matched healthy volunteers (n=55 adults and 50 children) will be identified as a non-  
53  
54 358 disease reference group (**Table 1**).  
55

56 359

58 360  
59  
60

## 2.4.2. Replication Phase (years 3-4)

The aim of the replication phase is to replicate putative discriminatory breath VOC /VOC signatures identified in the discovery phase.

Similar to the discovery phase, recruitment of acutely breathless patients will be enriched into the following disease strata following senior clinical decision maker assessment and within 24 hours of acute admission.

Acute adult heart failure (n=55), adult community acquired pneumonia (n=55), adult exacerbations of asthma (n=55) and COPD (n=55), acute severe asthma attacks in school age children (n=25) (Table 1).

Additional age matched healthy volunteers (n=55 adults and 25 children) will be identified as a non-disease reference group.

**Total combined sample size of the discovery and replication phases = 700 participants**

Disease Category	Discovery	Replication
Acute Adult Asthma	55	55
Acute COPD	55	55
Acute Heart Failure	55	55
Community Acquired Pneumonia	55	55
Adult healthy volunteers	55	55
Acute paediatrics Asthma	50	25
Paediatrics healthy volunteers	50	25
<b>Total sample</b>	<b>375</b>	<b>325</b>

**Table (1): Table summarising recruitment targets for both adult and paediatric groups.**

## 2.5. Schedule of assessments

A schedule of acute assessments is outlined below and aligns to the movement of acute patients through the clinical care pathway and the overall aim of developing a complete phenotypic picture of acutely breathless patients.

### 2.5.1. Defining acute breathlessness

At presentation (within 24 hours of admission) to one of three acute admissions units potentially eligible patients will be identified following confirmation of acute breathlessness, identified as (i) patient defined acute breathlessness and/or (ii) 1 unit increase above patient reported baseline in the extended medical research council (eMRC) dyspnoea score [47, 48] and at least one of the indicator

384 diagnoses identified as the primary clinical diagnosis by a senior clinical decision maker. eMRC will  
385 be completed by all patients and healthy volunteers at each research visit.

### 386 **2.5.2 Informed consent**

387 Patients meeting the pre-specified definition of acute breathlessness will be approached for informed  
388 consent in to the breath VOC biomarker study. Only patients that are eligible to give full written  
389 informed consent will be recruited.

### 390 **2.5.3 Collection of blood based pathology markers**

391 Collection of the blood biomarkers CRP, BNP, Troponin-I and blood eosinophil count will be  
392 performed both acutely and following recovery, when not taken as part of clinical care pathway.

393 These are currently used in profiling acutely breathless patients in clinical practice (**Table2**).

394

Test	ANALYSER/METHOD	LOWER LIMIT OF DETECTION	UPPER LIMIT OF DETECTION
C-Reactive protein (CRP)	Siemens Advia Chemistry XPT, PEG enhanced immunoturbidimetric. Siemens Advia 1800, PEG enhanced immunoturbidimetric	5 mg/L	Diluted to result
B-type natriuretic peptide (BNP)	Siemens Advia Centaur XPT, two-site sandwich immunoassay using direct chemiluminescent technology	2.0 pg/mL	1445 pg/mL
Troponin-I	Abbott Architect i2000SR, three-site sandwich immunoassay using direct chemiluminescent technology (CMIA).	5.0 ng/L	50,000 ng/L

395 **Table (2):** Type of analyser and methodology used for blood biomarker calculation. The table  
396 outlines analyser make, methodology, upper and lower limits of detection as per the University  
397 Hospitals of Leicester NHS Foundation trust laboratory guidelines.

398

### 399 **2.5.4 Breath VOC sampling**

400 Offline breath sampling using GC-MS and comprehensive two-dimensional gas chromatography-  
401 mass spectrometry coupled with a standardised and CE marked breath sampler-ReCIVA<sup>®</sup>[49], will be  
402 performed. Gas chromatography is considered a gold standard technique in detecting volatile organic  
403 compounds and as such its sampling will be prioritised. Additionally the following online  
404 technologies, proton transfer mass spectroscopy (PTR-MS), gas chromatography - ion mobility

405 spectroscopy (GC-IMS) and atmospheric pressure chemical ionisation- mass spectrometry (APCI-  
 406 MS) will be evaluated according to the sampling strategy outlined in section 3, **Figure 3 and Table 3.**

	COPD		Asthma		Pneumonia		Heart Failure		Healthy		Paediatrics	
	1	2	1	2	1	2	1	2	1	2	1	2
Time point												
Written informed consent	x		x		x		x		x		x	
<b>Volatile organic compound (VOC) sampling</b>												
ReCIVA- gas chromatography and mass spectrometry (GC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
ReCIVA- comprehensive two-dimensional gas chromatography (GCxGC-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS)	x	x	x	x	x	x	x	x	x	x	x	x
Proton transfer reaction mass spectrometry (PTRMS)		x		x		x		x	x	x	x	x
Gas chromatography - ion mobility spectrometry (GC-IMS)	x		x		x		x				x	x
<b>Pathology blood tests</b>												
Full blood count (including differential cell count)	x	x	x	x	x	x	x	x	x	x	x	x
Brain natriuretic peptide (BNP) [pg/mL]	x	x	x		x		x	x	x			
Troponin-I [ng/L]	x		x		x		x		x			
C-Reactive protein (CRP) [mg/L]	x	x	x	x	x	x	x	x	x	x	x	x
<b>Lung function tests</b>												
Hand held forced oscillation technique (FOT)	x	x	x	x	x	x	x	x	x	x		
Fractional exhaled nitric oxide (FeNO) - Flow rate 50 [ml/s]			x	x							x	x
Spontaneous sputum sample	x	x	x	x	x	x	x	x	x	x		
Bio-banking (urine, serum, plasma. sputum supernatants & plugs)	x	x	x	x	x	x	x	x	x	x	x	x
Transthoracic echocardiography	x						x					

407 **Table (3):** Summary of baseline and follow up assessments. The table summarises key assessments  
 408 carried out at different time points during the study. The participants may undertake any combination  
 409 of the investigations listed at any of these time points.

410

411

### 2.5.5. Collection of additional samples for future biomarker campaigns

Collection of additional biomarkers for future biomarker discovery campaigns including (i) a urine sample, (ii) blood samples, up to 85mls for DNA, RNA, plasma and serum and peripheral blood cell flow cytometry in selected subjects and (iii) spontaneous or induced sputum samples (plugs and supernatants) will be carried out (**Table 3**).

All samples will be collected at time point 1 and at 2 (**Figure 3**). The additional samples will be used for future omics analyses, these may include detailed analysis of the metagenome in sputum and proteomics applied to urine and serum samples.

### 2.5.6. Physiological characterisation

Physiological measures of lung function will be performed in acutely ill participants and at recovery including, (i) Hand held forced oscillation technique (FOT): an easily accessible measure of lung function. Patients favour this to spirometry as it is effort independent, unlike spirometry and requires less than a minute of quiet tidal breathing to obtain triplicate high quality measurements [50], This will be completed using Tremoflo®, Thorasys Thoracic Medical Systems Inc. (ii) Fractional exhaled nitric oxide (FeNO): A measure of airway inflammation in asthmatic patients[51, 52]. This instrument used for this will be NIOX VERO®, registered trademark of Circassia AB (PP-VERO-UK-0022-v1.0) (iii) Echocardiography: Two dimensional transthoracic echocardiography will be performed in heart failure and COPD patients using an iE 33 system with S5-1 transducer (frequency transmitted 1.7 MHz, received 3.4 MHz; Philips Medical Systems, Best, The Netherlands). Standard techniques as per American Society of Echocardiography guidelines (ASE)[53] will be used to acquire 2D, colour and Doppler images in conventional parasternal long-axis, short-axis and apical 4-, 2- and 3-chamber views. Left ventricular ejection fraction (LVEF)

1  
2  
3 437 will be calculated using the biplane method of discs formula (Simpson's rule) to derive left  
4  
5 438 ventricular volume indices.  
6  
7  
8

9 439 All participants are encouraged to report any testing related discomfort or concerns to the research  
10  
11 440 team to terminate the sampling process.  
12

### 13 441 **2.5.7 Recovery follow up**

14  
15 442 • Patient recovery will be defined as:

16  
17 443 (i) Patient reported recovery from the acute exacerbation spell and back to their  
18  
19 444 baseline extended MRC score or clinician defined recovery from the acute  
20  
21 445 exacerbation spell  
22

23  
24 446 and

25  
26 447 (ii) At least 6 weeks post exacerbation event (up to 6 months).  
27

28 448

29  
30 449 Patients that re admit to hospital between visits 1 and 2, can have additional visit 1 assessments. Visit  
31  
32 450 2 will be taken as recovery following the subsequent admission. If a patient is admitted to hospital  
33  
34 451 after visit 2 then they will be eligible to be recruited as a new study participant.  
35

36 452

37  
38 453 The schedule of assessments at the recovery visit is outlined (**Table 3**).  
39

40 454

### 41 42 455 **2.6. Clinical Adjudication:**

43  
44 456

45 457 In an effort to reduce data variability and minimise bias, an independent panel consisting of two senior  
46  
47 458 acute clinicians (SS & NG) will review all pertinent clinical and diagnostic source documentation,  
48  
49 459 whilst blinded to admission blood biomarkers and clinical diagnosis.  
50

51  
52 460 All acutely breathless adult patients' notes will be sequentially divided for adjudication. The panel will  
53  
54 461 independently determine the primary diagnosis of highest probability from a list of the four potential  
55  
56 462 acute indicator diagnoses in section 2.4 and mark their level of clinical certainty on a 100 mm visual  
57  
58 463 analogue scale (VAS scale). The panel members will be able to review imaging, electrocardiograms  
59  
60

1  
2  
3 464 (ECGs), and other relevant information but not admission blood based pathology tests.  
4

5 465 In a subset of patients adjudication will be validated by separate panel member to ensure between  
6  
7 466 observer agreement using Bland-Altman analysis and inter-rater agreement of the primary diagnosis  
8  
9 467 using Kohen's kappa via repeated evaluation of a subset of cases (see statistical methods section 2.3).  
10

11 468

## 14 469 **2.7. Clinical Informatics:**

15 470

17 471 Clinical data collection will be undertaken using a securely-hosted bespoke database system (ADD)  
18  
19 472 developed within the NIHR Leicester Biomedical Research Centre – Respiratory (BRC). The system  
20  
21 473 links acute admission episodes to hospital pathology records; historical respiratory physiology tests;  
22  
23 474 and demographic information. The system provides functionality to validate data entry; manually  
24  
25 475 verify records and highlight incomplete records. A custom VOC 'module' has been created to  
26  
27 476 support data collection within the study visits (1 and 2), and standardise diagnoses and medications  
28  
29 477 through the use of clinical ontologies as well as linking hospital records/tests to patient visits.  
30

31 478

33 479 Non-ADD based clinical data (e.g. hospital admissions, re-admissions and mortality) will be extracted  
34  
35 480 from the hospital data warehouse using identifiable patient identifiers, and subsequently  
36  
37 481 pseudonymised prior to integration.  
38

39 482

42 483 An informatics pipeline will be created to facilitate the transfer of chemo-metric data from remote  
43  
44 484 computers to the data repository. This will include tools to (i) enforce the correct labelling of data sets  
45  
46 485 (e.g. study number, visit, type/source of sample) prior to automated validated transfer to the  
47  
48 486 repository; (ii) record information about the sample process; (iii) search and extract data sets from the  
49  
50 487 repository for subsequent analysis. Prior to analysis, clinical and chemo-metric data will be integrated  
51  
52 488 using the study number and any potentially identifiable information will be removed.  
53

54 489

56 490

57 491

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60



### 3. Breath profiling

The technologies utilised in the VOC study during discovery and replication phases are:

#### Offline technologies

- ReCIVA+ GC-MS
- ReCIVA + GC x GC-MS
- 

#### Online technologies

- GC-IMS
- PTR-MS
- APCI-MS

Offline technologies will underpin the discovery analyses owing to their ability to identify chemical identity and their recognition as the analytical gold standard in exhaled breath VOC analysis [54].

In contrast online technologies will be utilised for VOC biomarker replication and at the recovery visits owing to their portability and potential for future point of care testing. **(Figure 4)**.

A brief description of the core VOC platforms is provided below

A CE marked breath sampling device (ReCIVA®) developed by Owlstone Medical, will be used to sample breath onto two adsorbent Tenax tubes. Participants will be asked to breathe through the ReCIVA face mask for a maximum of 300 seconds, aiming for collection of  $\geq 80\%$  of the target sample volume of 1 litre, after which the Tenax tubes will be transferred to the laboratory for analysis. This effectively allows de-coupling of the breath sampling from the breath sensor and analysis platforms in selected patients that are not able to mobilise to a real time breath sampling device. The Owlstone ReCIVA sampler will be utilised in breath collection for offline technologies namely GC-MS and GCxGC-MS. The ReCIVA sampler is capable of entraining oxygen and is therefore suitable for patients with mild respiratory failure requiring low flow rates of oxygen to maintain target oxygen saturations [49].

1  
2  
3 519 **3.1. Gas chromatography and mass spectroscopy (GC-MS):** is a commonly applied methodology  
4  
5 520 used to accurately measure trace gases in complex mixtures such as exhaled air [54]. Pre-  
6  
7 521 concentrating breath volatiles by various means and subsequent analysis constitute a reliable and  
8  
9 522 sensitive method for VOC analysis [55]. Despite its high sensitivity, it is however, a time consuming  
10  
11 523 technique and carries a risk of contamination at the pre-concentration step. It is also not suitable for  
12  
13 524 online and multiple measurements limiting its use as a point-of-care testing technology for VOC [56].  
14  
15  
16 525 The instrument used will be an Agilent 7890A gas chromatogram with a 5977a quadrupole mass  
17  
18 526 spectrometer (Agilent Technologies Ltd, Stockport, UK), interfaced with a Markes Unity 2 thermal  
19  
20 527 desorptionunit (Markes International Ltd, Llantrisant, UK).

21  
22  
23 528 **3.2. Comprehensive two-dimensional gas chromatography-mass spectrometry (GCxGC-MS):** an  
24  
25 529 advanced analytical technique for the analysis of complex organic matrices; its main advantage is the  
26  
27 530 unparalleled separation power it affords over conventional one-dimensional chromatographic  
28  
29 531 techniques [57]. Previous research, albeit sparse, has demonstrated the potential of GCxGC-MS for  
30  
31 532 breath analysis with the number of VOC detected exceeding those detected by conventional GC-MS  
32  
33 533 [58, 59]. GCxGC-MS of breath metabolites has been used for the identification of biomarkers related  
34  
35 534 to glucose metabolism [60, 61], tuberculosis [62] and radiation response [63]. This has generated  
36  
37 535 interest within the breath research community, however, such studies were conducted on a small scale  
38  
39 536 (<50 patients) and involved the use of expensive detectors and modulators. Method development and  
40  
41 537 analysis of the data-rich GCxGC chromatograms, however, can be time-consuming and require  
42  
43 538 specialist knowledge.

44  
45  
46  
47 539 The instrument used will be an Agilent 7890A gas chromatogram, fitted with a G3486A CFT flow  
48  
49 540 modulator and a three-way splitter plate coupled to a flame ionisation detector and a HES 5977B  
50  
51 541 quadrupole mass spectrometer (Agilent Technologies Ltd, Stockport, UK), interfaced with a Markes  
52  
53 542 TD-100xr thermal desorption autosampler (Markes International Ltd, Llantrisant, UK).

54  
55  
56 543 **3.3. Proton-transfer-reaction mass spectrometry (PTRMS):** a real time technique, capable of  
57  
58 544 simultaneously measuring the evolution of multiple gas metabolites from a single breath. It has been  
59  
60

1  
2  
3 545 used for the identification of potential useful VOC biomarkers for diagnosis of a variety of diseases:  
4  
5 546 including various cancers[64-66], liver disease[67, 68] and respiratory disease[69]. It has several  
6  
7 547 advantages in clinical settings, such as the speed of sampling, the instant result achieved and the lack  
8  
9 548 of need for sample storage or shipping. However, owing to the lack of pre-concentration or  
10  
11 549 chromatographic separation, sensitivity and definitive compound identification can be somewhat  
12  
13  
14 550 limited when compared to GC-MS.

15  
16 551 Two breath sampling devices will be used. The first device is a Loccioni SOFIA GSI-S; the subject is  
17  
18 552 required to exhale a single breath, five times (three if providing five samples proves too difficult) into  
19  
20 553 a sterile mouthpiece connected to an electrostatic bacterial/viral filter whilst wearing a nose clip (all  
21  
22 554 CE marked). Flow from the mouthpiece passes into a gas sampling interface capnograph (Loccioni  
23  
24 555 GSI-S – CE marked) and real-time user feedback of flow is provided on screen, allowing the  
25  
26 556 regulation of the breath sampling rate. The gas sampling interface acts to simultaneously trigger the  
27  
28 557 acquisition of the PTR-ToF-MS data and the exhaled breath travels through the capnograph down a  
29  
30 558 heated sample line into the ion source of the PTR-ToF-MS

31  
32  
33  
34 559 The second breath sampling device is a ReCIVA breath sampler (Owlstone) with one of the adsorbent  
35  
36 560 Tenax tubes replaced with an outlet tube adapted for online sampling. The exhaled breath is  
37  
38 561 transferred to the PTR-ToF-MS via a heated transfer line connected to the outlet tube, continuously  
39  
40 562 drawn at a constant flow rate by the PTR-ToF-MS. The online adaptation of the consumable  
41  
42 563 adsorbent tube does not affect the CE mark of the ReCIVA sampling device.

43  
44  
45 564 Once the breath sample reaches the PTR-ToF-MS, via either breath sampler, the breath mixes with  
46  
47 565 protonated water ( $H_3O^+$ ) inducing proton transfer to the target volatile organic compounds (VOCs)  
48  
49 566 present, resulting in their ionisation. Sample ions are then guided into the time of flight mass  
50  
51 567 spectrometer and mass spectra, showing the abundance and mass of the VOCs present, are collected  
52  
53 568 throughout the exhalation. Following sampling, mouthpieces, filters and nose clips are disposed of  
54  
55 569 and all patient contacted surfaces wiped down with antiseptic cleaning wipes in preparation for the  
56  
57  
58 570 next patient.

1  
2  
3 571 The instrument used will be a Kore Series II high performance proton transfer reaction-time of flight-  
4  
5 572 mass spectrometer (Kore Technology Ltd, Cambridge, UK).  
6  
7

8 573  
9

10 574 **3.4. Gas chromatography- ion mobility spectrometry (GC-IMS) (B&S Analytik):** Allows the  
11  
12 575 detection of volatile organic compounds down to ultra-trace level (ng/L - to pg/L - range). For years,  
13  
14 576 IMS has been used to discover potential discriminatory breath VOC in lung cancer [70, 71],  
15  
16 577 COPD[72, 73] and asthma[73]. Sampling takes place using a SpiroScout spirometer. The patients  
17  
18 578 exhale through a disposable mouth piece connected to a Teflon tube. A piezoelectric pressure sensor  
19  
20 579 is used to monitor the breathing profile, this opens the sampling valve at the appropriate point in the  
21  
22 580 breath profile to collect end-tidal breath in a sample loop of 10 mL volume. After filling this loop, the  
23  
24 581 collected sample air is then transferred to a multicapillary column for a chromatographic separation,  
25  
26 582 which is achieved in 12 min. The separated molecules are then transferred into the IMS, ionised and  
27  
28 583 then separated according to their mobility in a weak electric field.  
29  
30 584 The technology's multiple advantages of ultra-sensitivity, portability, online sampling and short  
31  
32 585 analysis time (typical analysis time of 10 minutes) with real time detection, brings a promise to  
33  
34 586 provide immediate and potentially reliable results for point of care breath diagnostics. Another  
35  
36 587 concept with IMS devices is that once the required breath signatures have been discovered using GC-  
37  
38 588 MS, IMS offers the potential to be 'tuned' for selective detection of VOC.  
39  
40  
41  
42

43 589 The instrument used will be a BioScout a multi-capillary column gas chromatogram-ion mobility  
44  
45 590 spectrometer, with a <sup>63</sup>Ni ion source, interfaced with a SpiroScout breath sampler (BS Analytik,  
46  
47 591 Dortmund, Germany).  
48  
49

50 592  
51  
52

53 593 **3.5. Atmospheric pressure chemical ionisation- mass spectrometry (APCI-MS) semiportable**  
54  
55 594 **compact version (Advion):** is one of less sensitive but more affordable versions of mass spectrometers  
56  
57 595 released to the commercial market in recent years. The device uses APCI to produce ions. Although  
58  
59 596 the most common use of APCI-MS systems is the detection in liquid chromatography applications,  
60

1  
2  
3 597 the technique has proven to be a valuable tool for direct measurement of VOC in air[74, 75] food[76,  
4  
5 598 77] and breath[78, 79]. Recently, the technique has shown potential for online, real time profiling of  
6  
7 599 pseudo-metabolites in exhaled breath [80] with sensitivity comparable with other techniques. By  
8  
9 600 combining miniaturised MS technology with APCI techniques, adequate quality of on-site, real time  
10  
11 601 measurements with minimal or no sample preparation requirement can be provided. This is a desirable  
12  
13 602 outcome as it overcomes main limitation of using standard breath analysis method in clinical setting,  
14  
15 603 which is a need for breath sample collection followed by desorption and time-consuming laboratory  
16  
17  
18 604  
19  
20 605  
21 637 There remains an overall lack of standardisation and rigour across these technologies which hindered  
22  
23 638 previous advancements in breath discovery; something we intend to minimize.

24  
25 639 The instrument used will be an Advion Compact Mass Spectrometer Express, with atmospheric  
26  
27 640 pressure chemical ionisation, interfaced with a heated breath sampling line (Advion, New York,  
28  
29 641 USA).

30  
31  
32 642

#### 33 34 35 643 **4. Chemometric processing and data analysis:**

36  
37 644 GC-MS breath data will be aligned, deconvoluted and the features for each participant will be  
38  
39 645 extracted. The extracted features will be grouped and classified by retention index and mass spectrum.  
40  
41 646 The registered and aligned data will be linked to participant meta-data to generate a breath matrix.  
42  
43 647 Data handling and analysis will be performed by a senior statistician.

44  
45  
46 648 The breath matrix is a  $n \times p$  matrix where  $n$  is the number of subjects and  $p$  is the number of VOC. The  
47  
48 649 breath matrix is high dimensional with  $p \gg n$  and many potentially correlated VOC. In view of this, we  
49  
50 650 will employ sparse partial least squares discriminant analysis (sPLS-DA)[81] to investigate which of  
51  
52 651 the VOC can identify breathlessness. We will also investigate which of the VOC can discriminate  
53  
54 652 between the different disease states including acute exacerbations of asthma and COPD and  
55  
56  
57  
58  
59  
60

1  
2  
3 653 Pneumonia. In addition to the supervised methods, unsupervised methods will be explored,  
4  
5 654 specifically sparse principle component analysis (sPCA)[82].  
6  
7  
8 655 Extracted VOC will also be investigated. Relationships between VOC and patient reported acute  
9  
10 656 breathlessness will be analysed using logistic regression model. VOC associated with patient  
11  
12 657 associated acute breathlessness will be incorporated into multinomial logistic regression models in  
13  
14 658 conjunction with CRP, BNP, blood eosinophils and Troponin-I, pathology biomarkers currently in use  
15  
16 659 for diagnosing undifferentiated breathlessness. In addition to the conventional binary and multinomial  
17  
18 660 logistic regression models, regression models [83].  
19  
20  
21

## 22 661 **5. Ethics and dissemination:**

23  
24 662 The study has obtained full ethical approval from the London South East Research ethics Committee,  
25  
26 663 REC reference 16/LO/1747. IRAS project ID 198921. Publications will be prepared according to the  
27  
28 664 MRC-EMBER consortium agreement and the University of Leicester publications policy. All intended  
29  
30 665 publications will be submitted to the EMBER executive board for review and comments within 60 days  
31  
32 666 of journal submission. Authorship will be according to contribution and internationally recognised  
33  
34 667 guidance on journal authorship.  
35  
36  
37

38  
39 668 **6. Study dates:** 01/2/2017 – 30/10/2020

## 40 669 **7. Authors' contributions:**

41  
42  
43 670  
44  
45  
46 671 S.S, C.E.B, N.Gr, P.Th and P.Mo conceived the study, obtained funding, wrote the study protocol,  
47  
48 672 obtained ethical and MHRA approvals for the study and coordinated the deployment of analytical  
49  
50 673 testing methods for breath analysis. W.I took the lead in writing the manuscript with support from  
51  
52 674 S.S. Planning and recruitment of adult participants was carried out by W.I, S.Jo, B.Pa, A.Aw, R.Ph,  
53  
54 675 G.Fo, A.Yo, R. J. R and C.Wh. Paediatrics study design was conceived by E.Ga and C.Be and  
55  
56 676 participants recruited by T. Mc and C. Fo. Analytical chemistry team formed of M.Wi, R.Co, D.Sa,  
57  
58 677 D.Ru and L.Br expertly handled all the breath samples and planned an analysis structure. M.Ri, a  
59  
60

1  
2  
3 678 senior statistician, constructed a statistics and data analysis plan in conjunction with SS.  
4  
5 679 Bioinformatics pipeline and electronic CRFs developed by R.Fr and B.Zh. All authors, including  
6  
7 680 R.Pe, H. Bh, B.Ha, A. Si, K. Ry, H. Pa, T. Su, L. L Ng, T. Co contributed to the study design and  
8  
9 681 study protocol.  
10  
11  
12

13 682 **8. Protocol version:** Version 4, 1<sup>st</sup> April 2018  
14  
15  
16 683

17  
18 684 **9. Public and patient involvement:**  
19

20 685 A series of consultations have taken place with our patient involvement team within the NIHR Biomedical  
21  
22 686 Research Centre (Respiratory Theme) and across the wider BRC PPI group. Representations from the  
23  
24 687 paediatrics team were also present. This group was sent copies of the participant documentation for review  
25  
26 688 and discussion. Various revisions have been made following on from these discussions.  
27  
28  
29

30 689 **10. Funding statement:**  
31

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33  
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35  
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37  
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39  
40 694 we are extremely grateful. The authors would like to acknowledge the invaluable efforts of the  
41  
42 695 research nurses responsible for the in-clinic sample collection as well as the input from the wider  
43  
44 696 EMBER consortium (Members list can be found at: <https://ember.le.ac.uk/web>). The views expressed  
45  
46 697 are those of the author(s) and not necessarily those of the NHS and NIHR or the Department of Health  
47  
48  
49

50 698

51  
52 699 **11. Competing interests:** SS has performed advisory services for Owlstone Medical.  
53  
54  
55 700

56 701

57 702

58  
59  
60



1  
2  
3 **703 Figure legends:**  
4

5 **704 Figure (1)** Relationship between lung proximity and degree of invasiveness of different lung  
6  
7 **705** matrices. The figure plots the level of invasiveness of various lung matrices in relation to their  
8  
9 **706** proximity to the lung. Given their pathological relevance, the degree of invasiveness of  
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11 **707** bronchoalveolar lavage (BAL) and lung biopsy makes them less favourable in diagnosing respiratory  
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13 **708** diseases.  
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16 **709 Figure (2)** Multi-instrument use in breath sampling. Figure illustrates the various combinations of  
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18 offline and online devices used in breath sampling and the relevant pros and cons. Offline and online  
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20 technologies are used for the discovery and validation phases of the study respectively.  
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24 **712 Figure (3)** Study flow chart. Figure outlines the patient journey from admission through to discharge  
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26 and follow up. Participants with self-reported acute breathlessness presenting to University Hospitals  
27  
28 of Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling  
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30 is carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge.  
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32 **716** Patients are admitted through the standard operational emergency medical streaming and care  
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34 pathways at the University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission)  
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36 are measured at 30 and 60 days and late outcomes (mortality) including respiratory and all-cause  
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38 mortality are measured at 2 years. Assessments carried out at each time point are summarised in  
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41 **720 Table 1.**  
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44 **721 Figure (4)** Multi-instrument use in breath sampling. Operational space of the analytical technologies  
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46 used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including  
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48 proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-  
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50 mass spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas  
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52 chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass  
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54 spectrometry (GCxGC). Comparing the typical molar mass range detectable; selectivity in detection  
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56 owing to the type of ionisation involved and the proton affinity of analytes; and the inclusion of a  
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58 chromatographic separation affecting total time of analysis. The online technologies involving  
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3 729 chemical ionisation (PTR-MS, CMS and GC-IMS) can be used in-clinic owing to short analysis  
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5 730 times, but only detect lower molar mass molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>.  
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7 731 Offline chromatographic techniques (GC-MS and GCxGC) detect a wider range of compounds  
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9 732 independent of proton affinity; however, the techniques have longer analysis times and involve  
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11 733 sample transportation and storage.  
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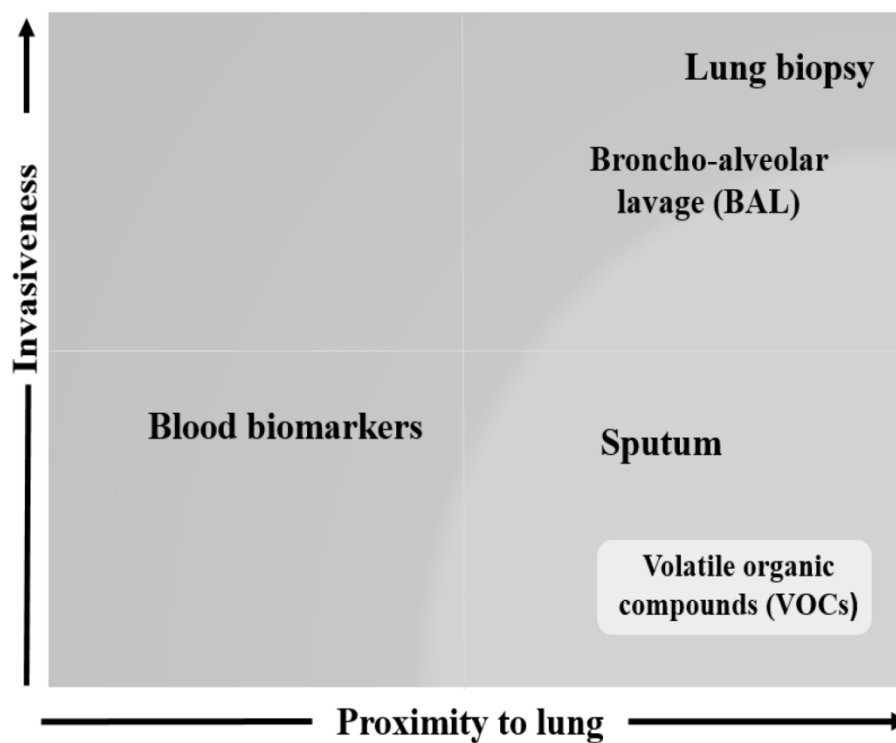


Figure (1): Relationship between lung proximity and degree of invasiveness of different lung matrices. The figure plots the level of invasiveness of various lung matrices in relation to their proximity to the lung. Given their pathological relevance, the degree of invasiveness of bronchoalveolar lavage (BAL) and lung biopsy makes them less favourable in diagnosing respiratory diseases.

144x115mm (300 x 300 DPI)

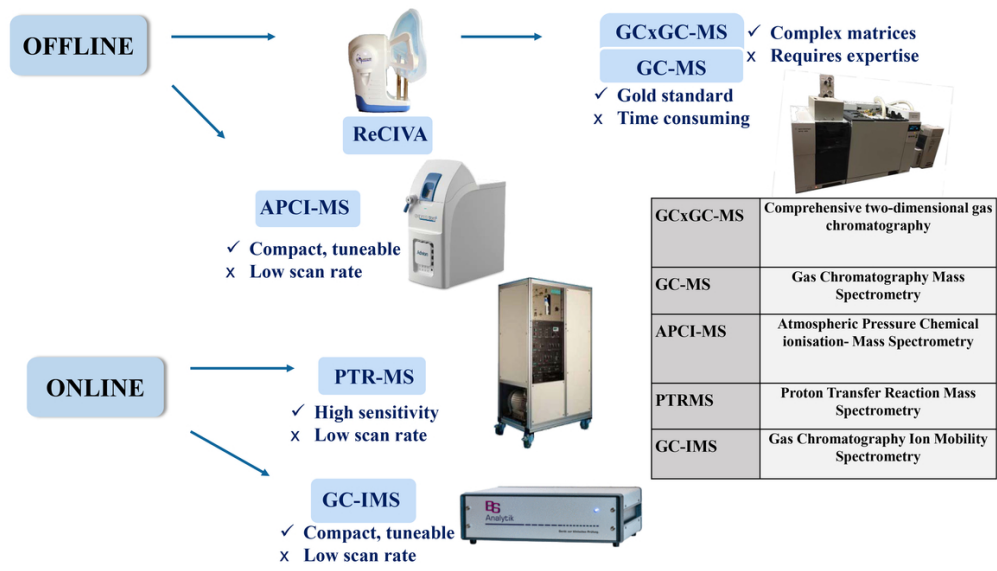
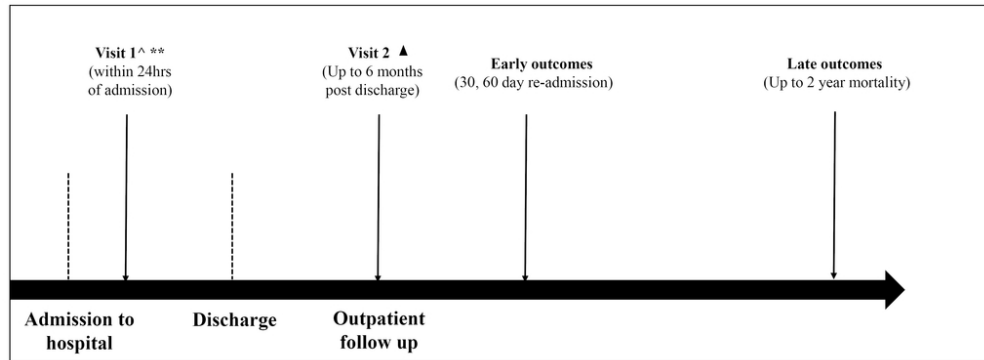


Figure (2): Multi-instrument use in breath sampling. Figure illustrates the various combinations of offline and online devices used in breath sampling and the relevant pros and cons. Offline and online technologies are used for the discovery and validation phases of the study respectively.

97x55mm (300 x 300 DPI)



^ Following senior decision maker review  
 \*\* Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, GC-IMS, APCI-MS)  
 ▲ Breath sampling (ReCIVA-GC-MS, ReCIVA- GCxGC-MS, PTR-MS, APCI-MS)  
 ---- Following University Hospitals of Leicester streaming and clinical care pathways

Figure (3): Study flow chart. Figure outlines the patient journey from admission through to discharge and follow up. Participants with self-reported acute breathlessness presenting to University Hospitals of Leicester are recruited within 24 hours following a senior decision maker review. Breath sampling is carried out on the first visit, at recruitment, and the second visit, up to 6 months after discharge. Patients are admitted through the standard operational emergency medical streaming and care pathways at the University Hospitals of Leicester NHS Trust. Early outcomes (hospital readmission) are measured at 30 and 60 days and late outcomes (mortality) including respiratory and all-cause mortality are measured at 2 years. Assessments carried out at each time point are summarised in Table 1.

86x43mm (300 x 300 DPI)



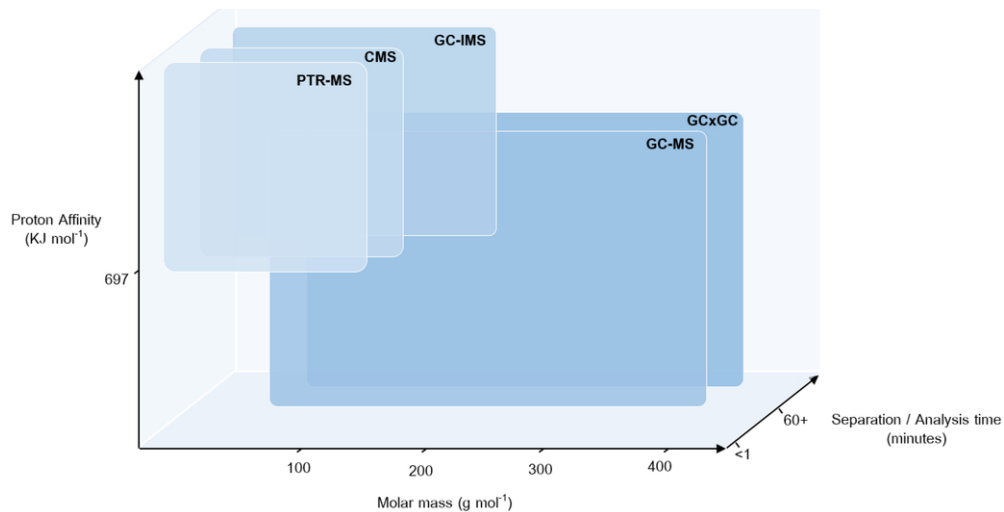


Figure (4): Multi-instrument use in breath sampling. Operational space of the analytical technologies used in EMBER for the analysis of volatile organic compounds (VOC) in exhaled breath, including proton transfer reaction-mass spectrometry (PTR-MS), atmospheric pressure chemical ionisation-mass spectrometry (CMS), gas chromatography-ion mobility spectrometry (GC-IMS), gas chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography-mass spectrometry (GCxGC). Comparing the typical molar mass range detectable; selectivity in detection owing to the type of ionisation involved and the proton affinity of analytes; and the inclusion of a chromatographic separation affecting total time of analysis. The online technologies involving chemical ionisation (PTR-MS, CMS and GC-IMS) can be used in-clinic owing to short analysis times, but only detect lower molar mass molecules with a proton affinity higher than 697 KJ mol<sup>-1</sup>. Offline chromatographic techniques (GC-MS and GCxGC) detect a wider range of compounds independent of proton affinity; however, the techniques have longer analysis times and involve sample transportation and storage.

88x44mm (300 x 300 DPI)