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(51) INT CL:
G01N 33/497 (2006.01)

(56) Documents Cited:
ERJ Open Research, Vol. 8 (2), 2021, Ibrahim W. et al., "A systematic review of the diagnostic accuracy of volatile organic compounds in airways diseases and their relation to markers of type-2 inflammation".
Molecules, Vol. 26 (6), 2021, Monedeiro F. et al., "Needle Trap Device-GC-MS for Characterization of Lung Diseases Based on Breath VOC Profiles".
Paediatric Asthma, Vol. 42, 2013, Robroeks C.M. et al., "Exhaled volatile organic compounds predicts exacerbations of childhood asthma in a 1-year prospective study", pp. 98-106.

(58) Field of Search:
INT CL **G01N**
Other: **WPI, EPODOC, MEDLINE, BIOSIS, Patent Fulltext, CAS ONLINE**

(54) Title of the Invention: **Biomarker**
Abstract Title: **A method of diagnosing a cardiorespiratory disease using exhaled breath biomarkers**

(57) A method of diagnosing a cardiorespiratory disease in a subject comprises detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled breath from the subject, where if one or more of the VOC biomarkers is present in the sample, the subject may have a cardiorespiratory disease. The cardiorespiratory disease may be one ore more diseases selected from asthma, COPD, heart failure and pneumonia. The one or more asthma-VOC biomarkers may be selected from one or more of: 3-methylpentane, 2-methylnonane, decane, 1-nonene, methyldecanal isomer, undecanal, 3-methylbenzaldehyde, 2-ethylhexanol, 1,4-dioxane, beta-bisabolene, and N,N-dimethyl-1-dodecanamine. The one or more COPD-VOC biomarkers may be selected from one or more of: 4-methylundecane, 1-decanol, menthol, camphene, galaxolide, 3-methyl thiophene, and N,N-dimethyl-1-dodecanamine. The one or more heart-failure-VOC biomarkers may be selected from one or more of: undecane, cyclohexene, butanal, 2-methyl-2-propenal, tridecanal, 1,3-dioxolane, beta myrcene, ethylbenzene, and decyl isobutyl ether. The one or more pneumonia-VOC biomarkers may be selected from one or more of: 2,6-dimethyloctane, dimethylundecane, dimethylundecane isomer, 1-decene, 3-buten-2-one, 1-(methylthio)-1-propene, 1-methylthio-propane, and dodecylacrylate.

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At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

Figure 1

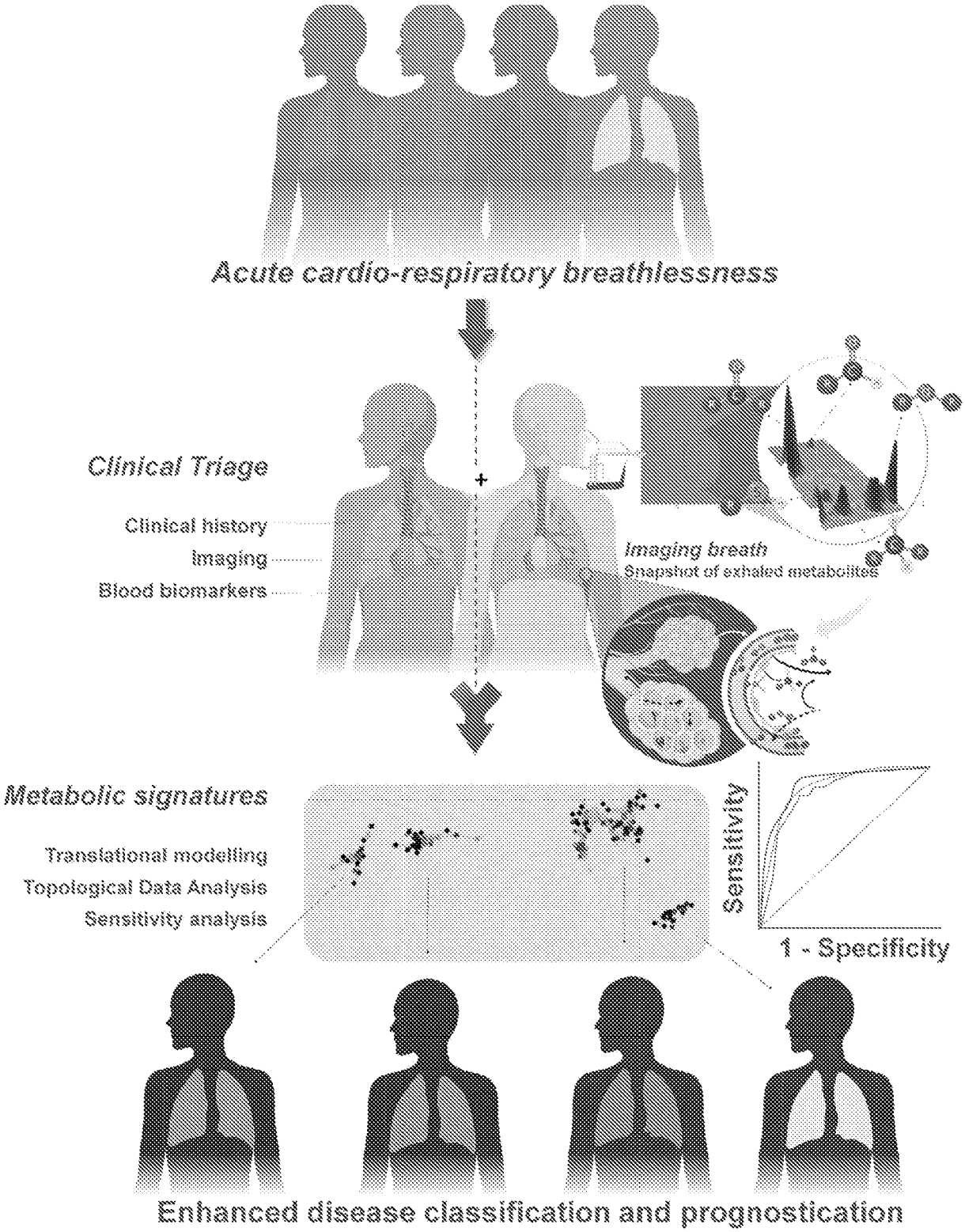

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Figure 2

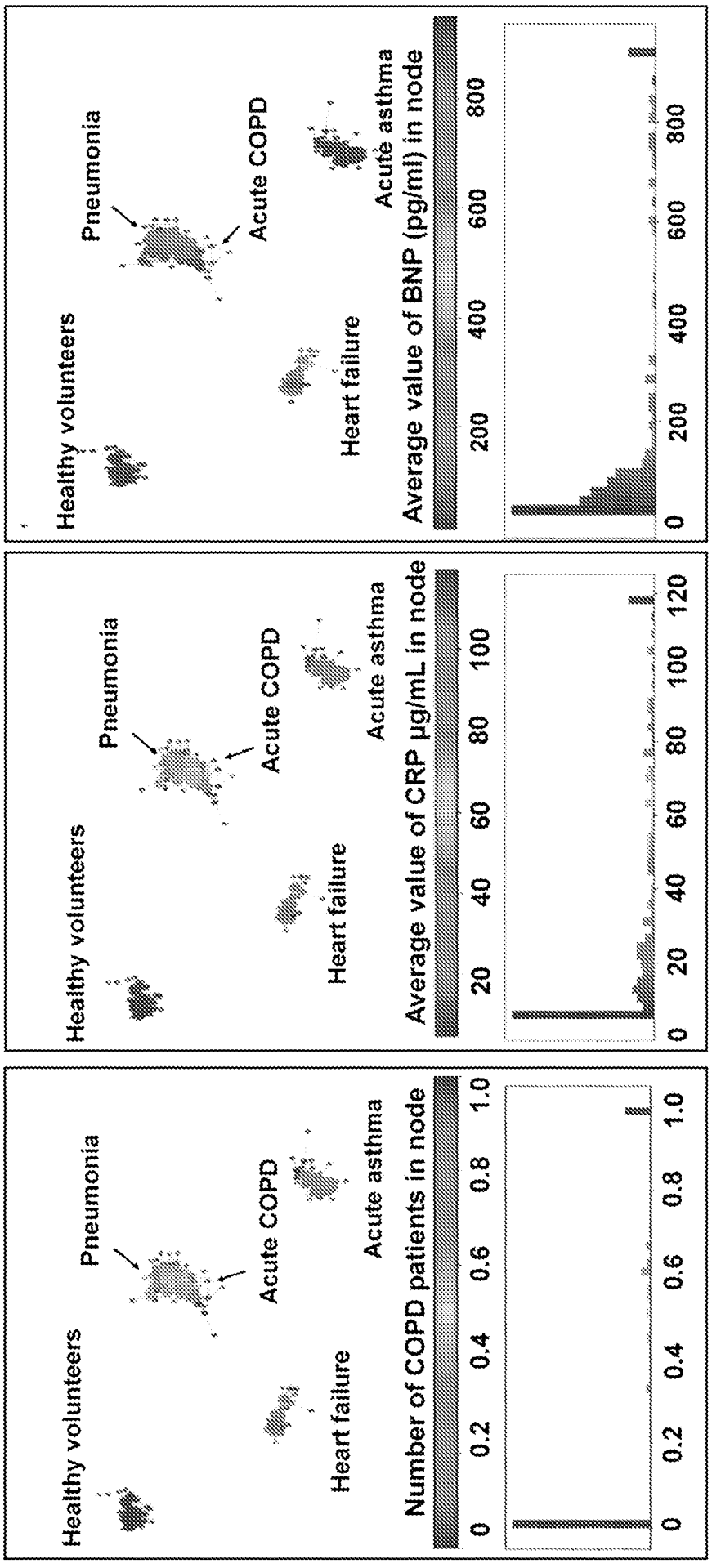

Figure 2 (continued)

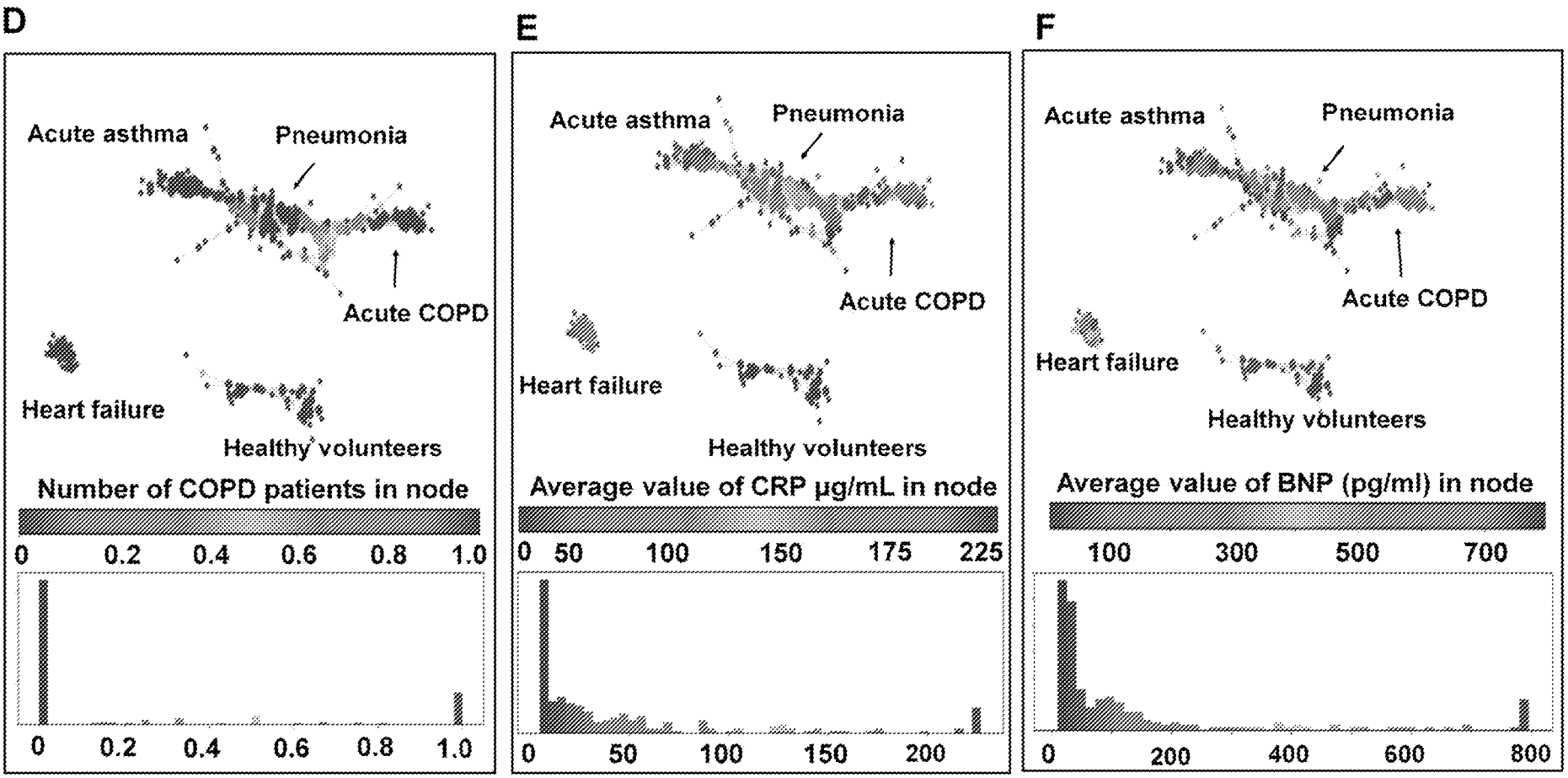

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Figure 3

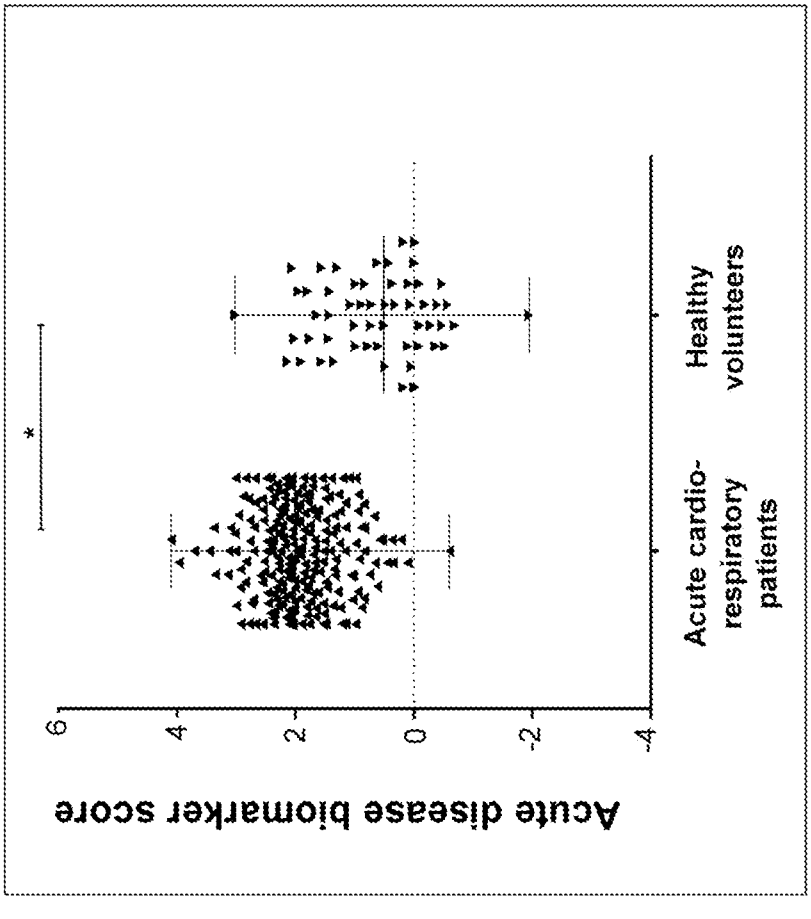

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Figure 3 (continued)

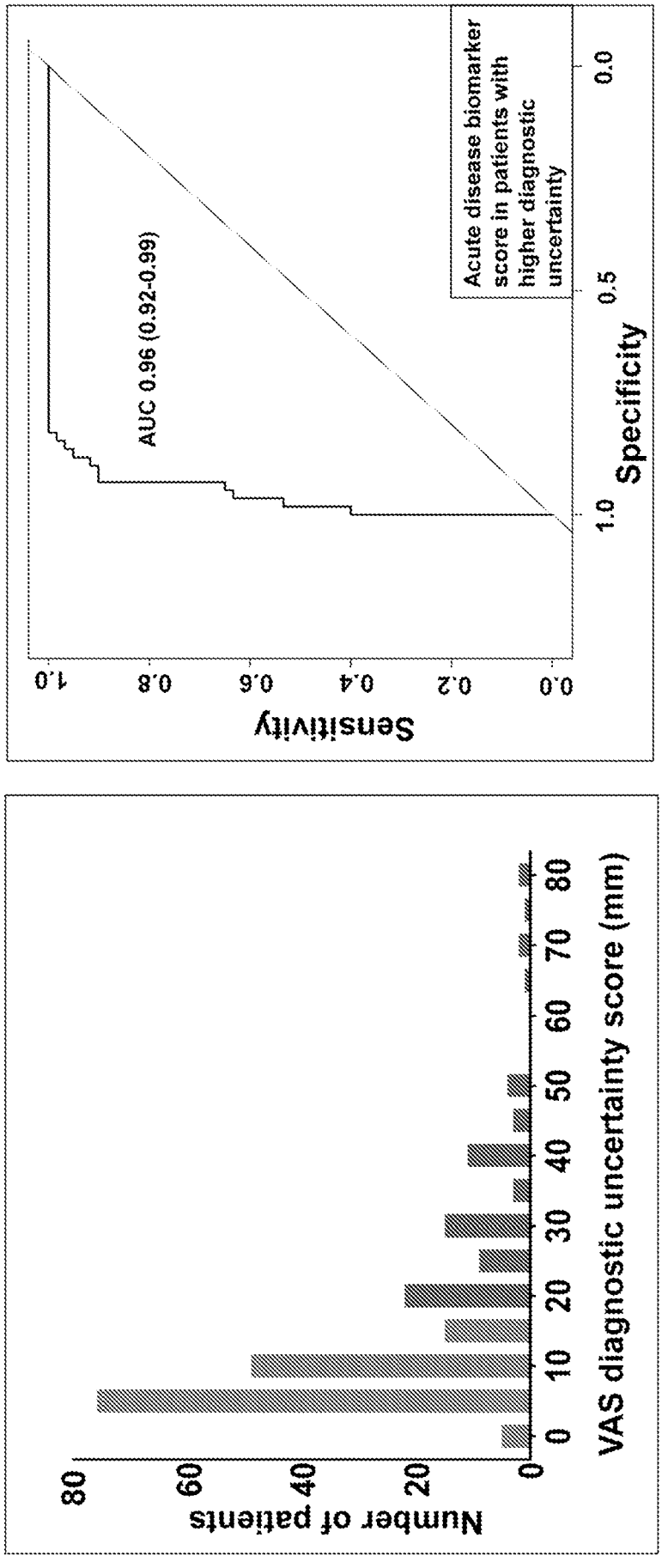

Figure 4

(A)

	VOC biomarker score					
	Acute disease	Healthy controls	Acute asthma	Acute COPD	Pneum-onia	Heart failure
CRP (mg/L)	0.131*	-0.159*	0.021	-0.031	0.338*	-0.144*
BNP (pg/L)	0.216*	-0.218*	-0.115	0.072	-0.053	0.339*
Troponin I (ng/L)	0.090	-0.082	-0.084	-0.030	-0.002	0.151*
Blood eosinophil count (x10^9/L)	-0.006	0.027	0.139*	-0.056	-0.084	0.008

Pearson correlation: 0.2, 0, - 0.2

(B)

	VOC biomarker score					
	Acute disease	Healthy controls	Acute asthma	Acute COPD	Pneum-onia	Heart failure
Breathlessness VAS score (mm)	0.485*	-0.495*	0.113	0.305*	0.182*	0.010
Body temperature (°C)	0.290*	-0.291*	-0.005	0.178*	0.288*	-0.090
Oxygen saturation SpO2 (%)	-0.253*	0.263*	-0.023	-0.215*	-0.240*	0.022
mNEWS-2 score	0.268*	-0.252*	0.018	0.105	0.098	0.096

Pearson correlation: 0.3, 0, - 0.3

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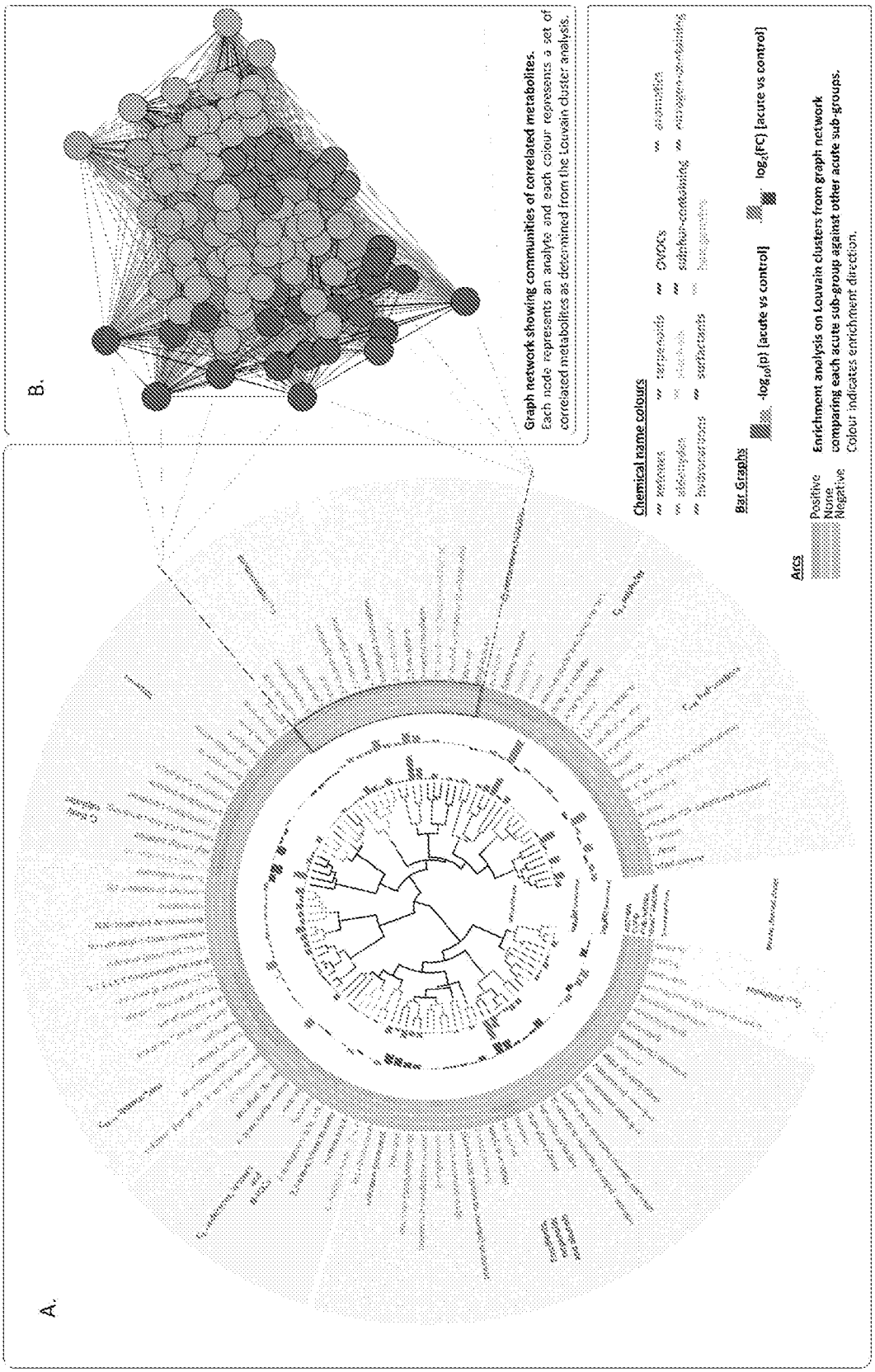

Figure 5

Figure 5 (continued)

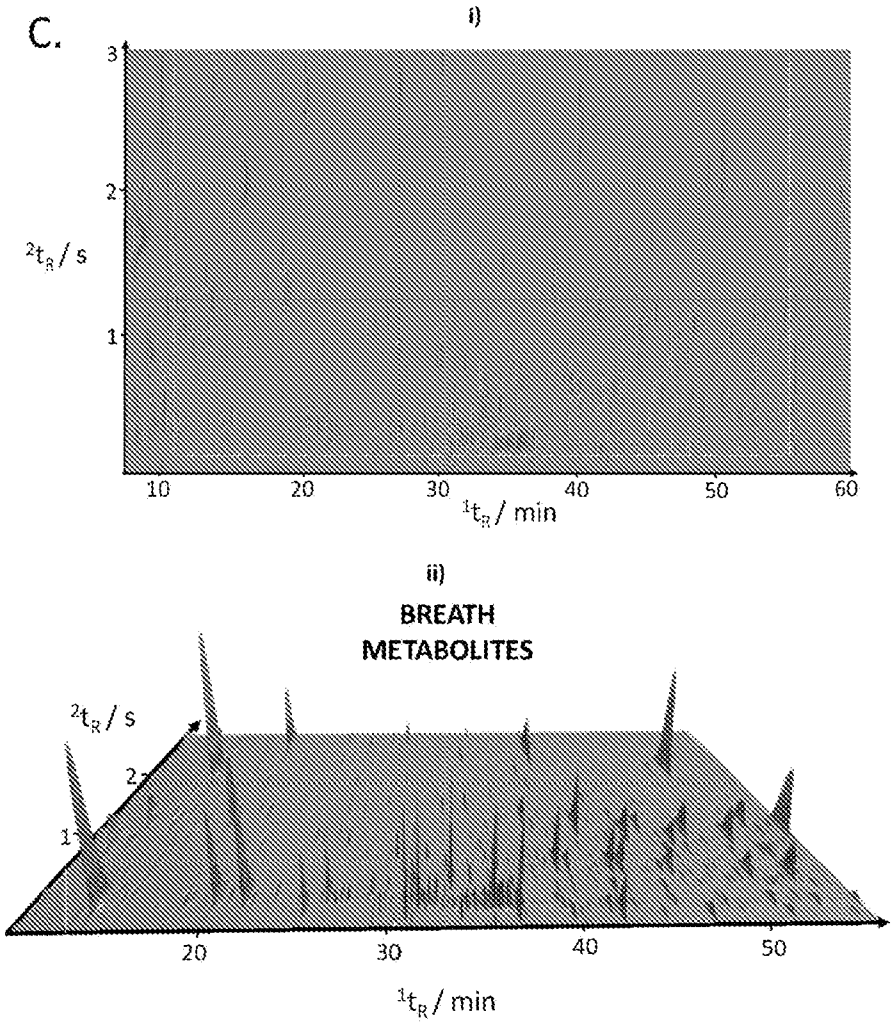

Figure 6

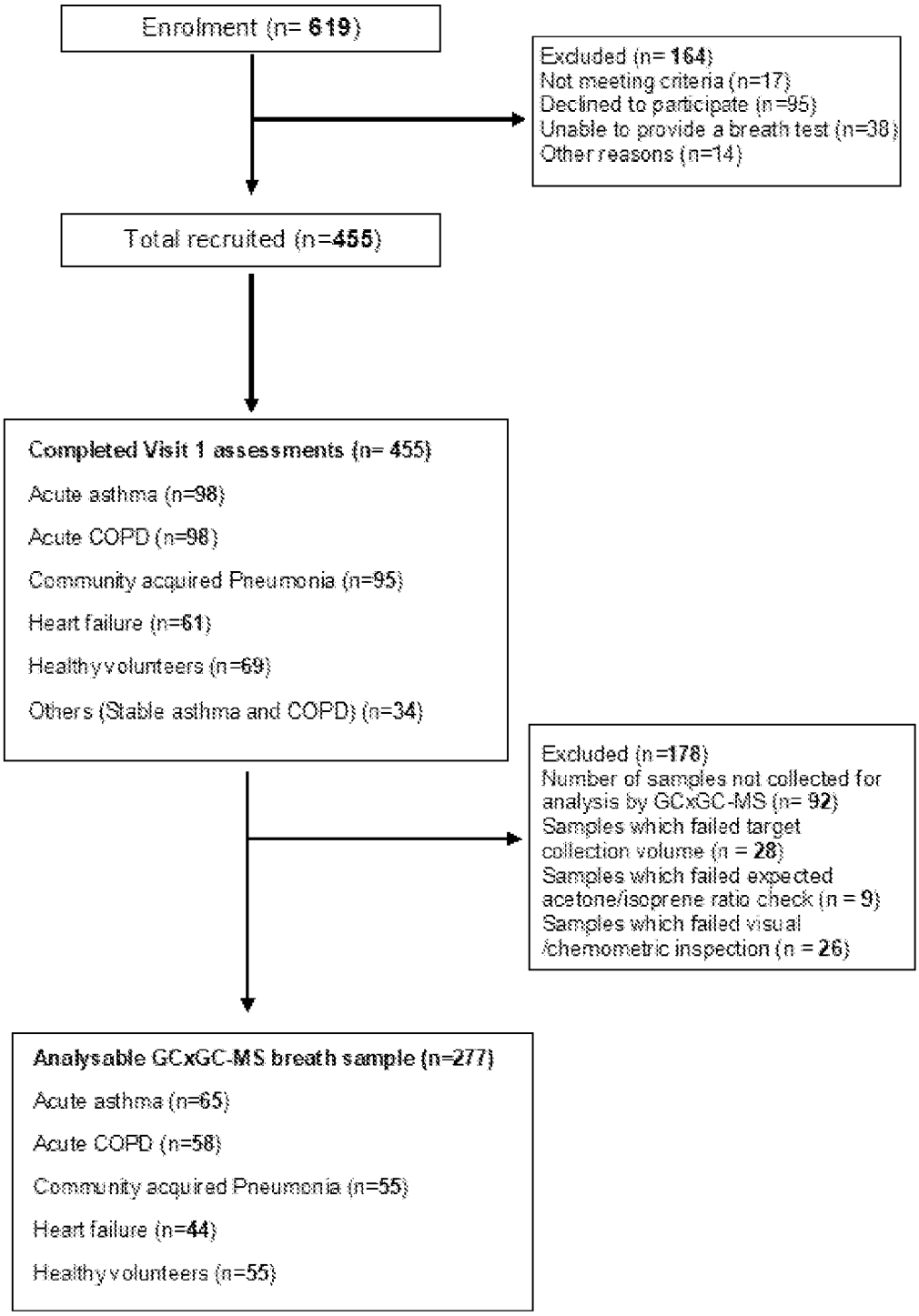

Figure 7

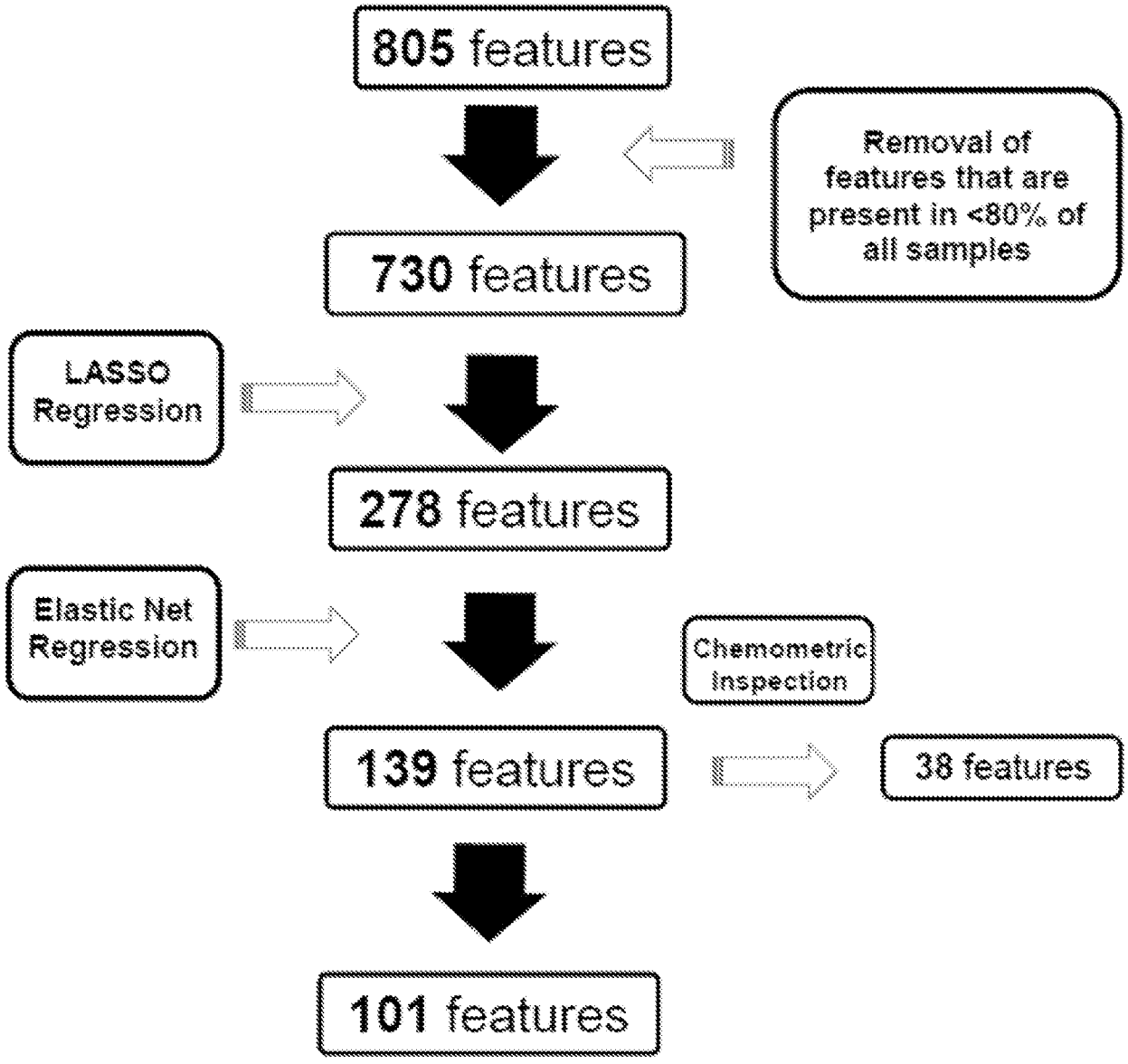

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Figure 8

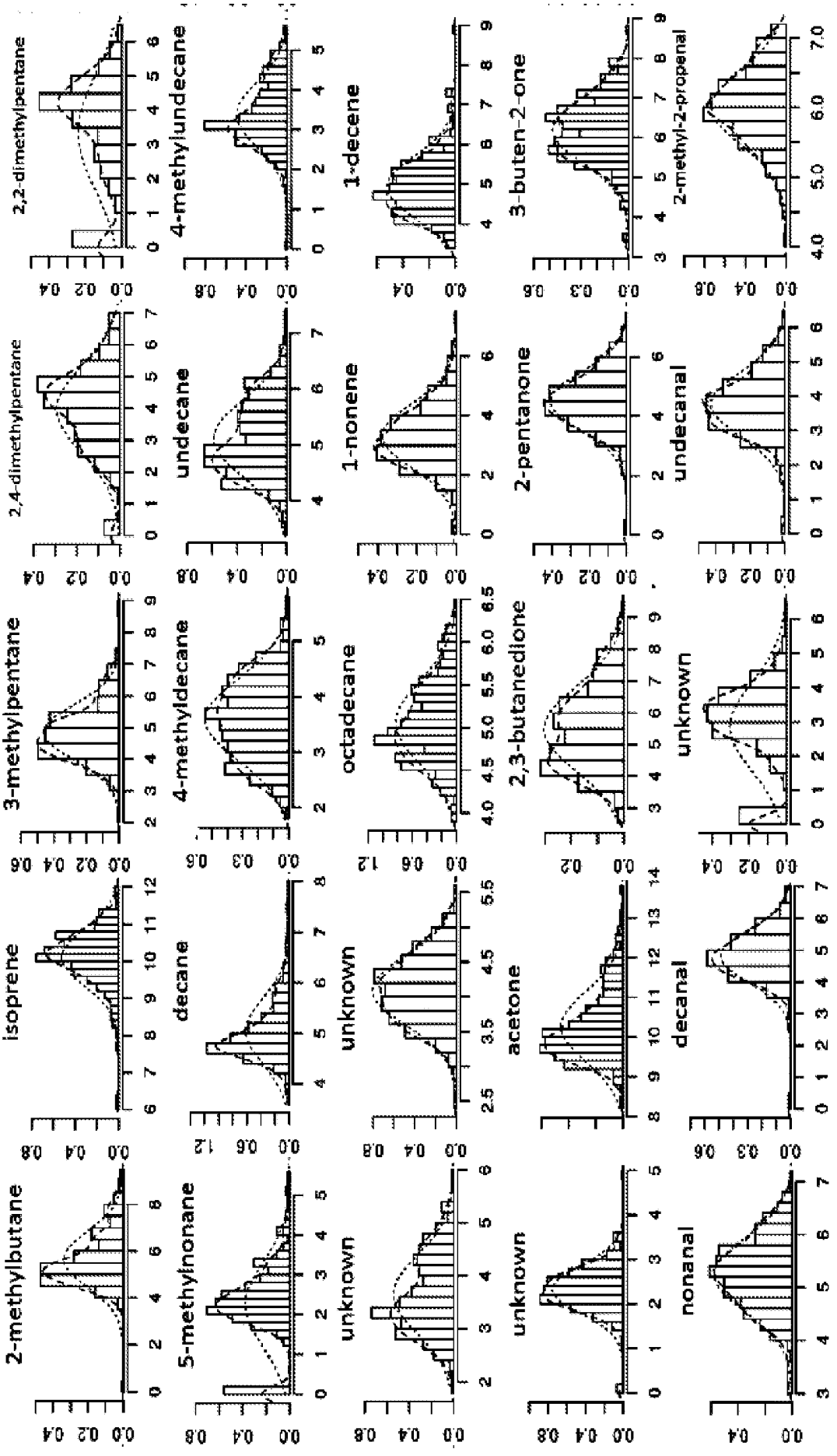

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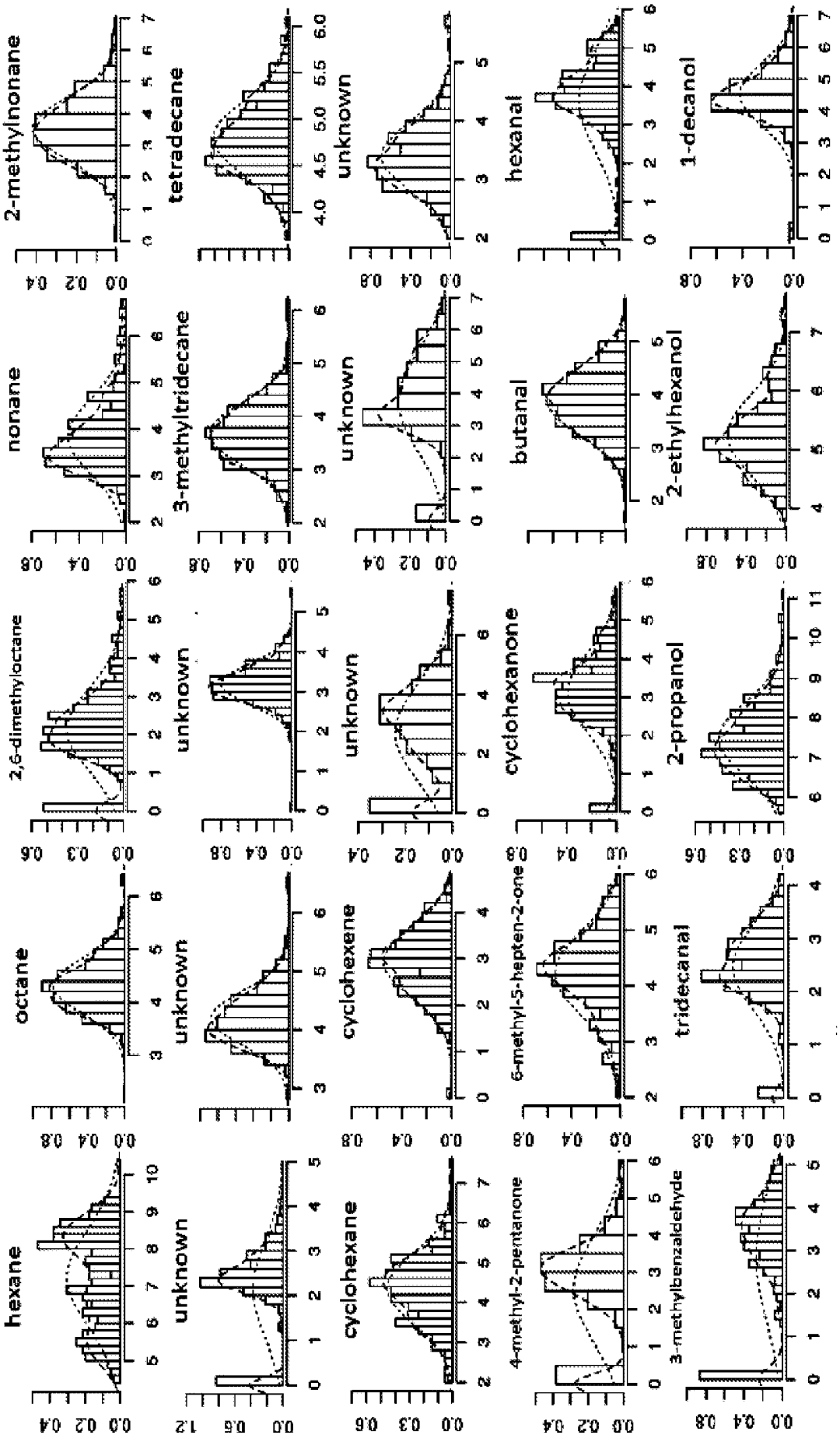
Figure 8 (continued)



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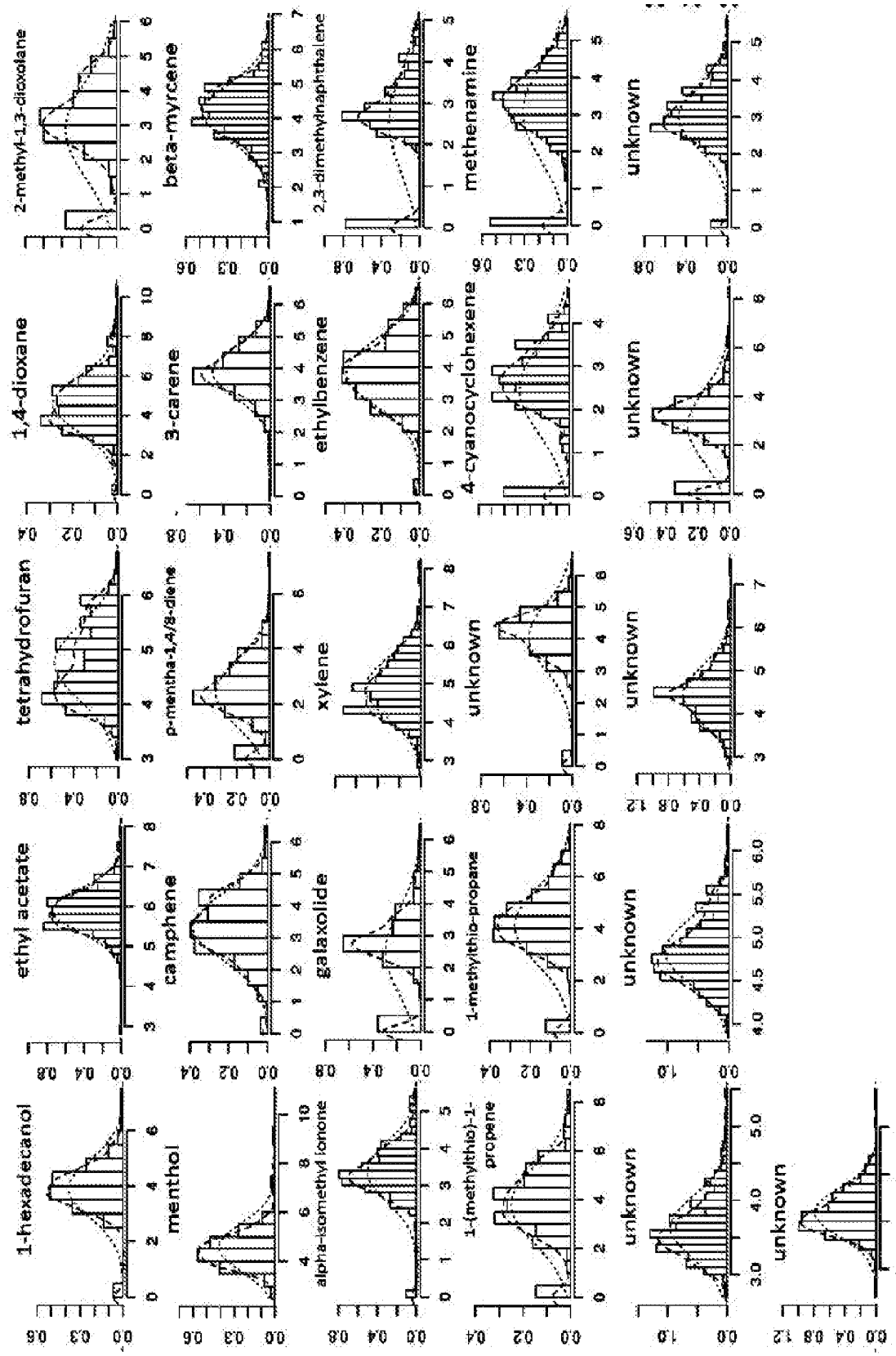

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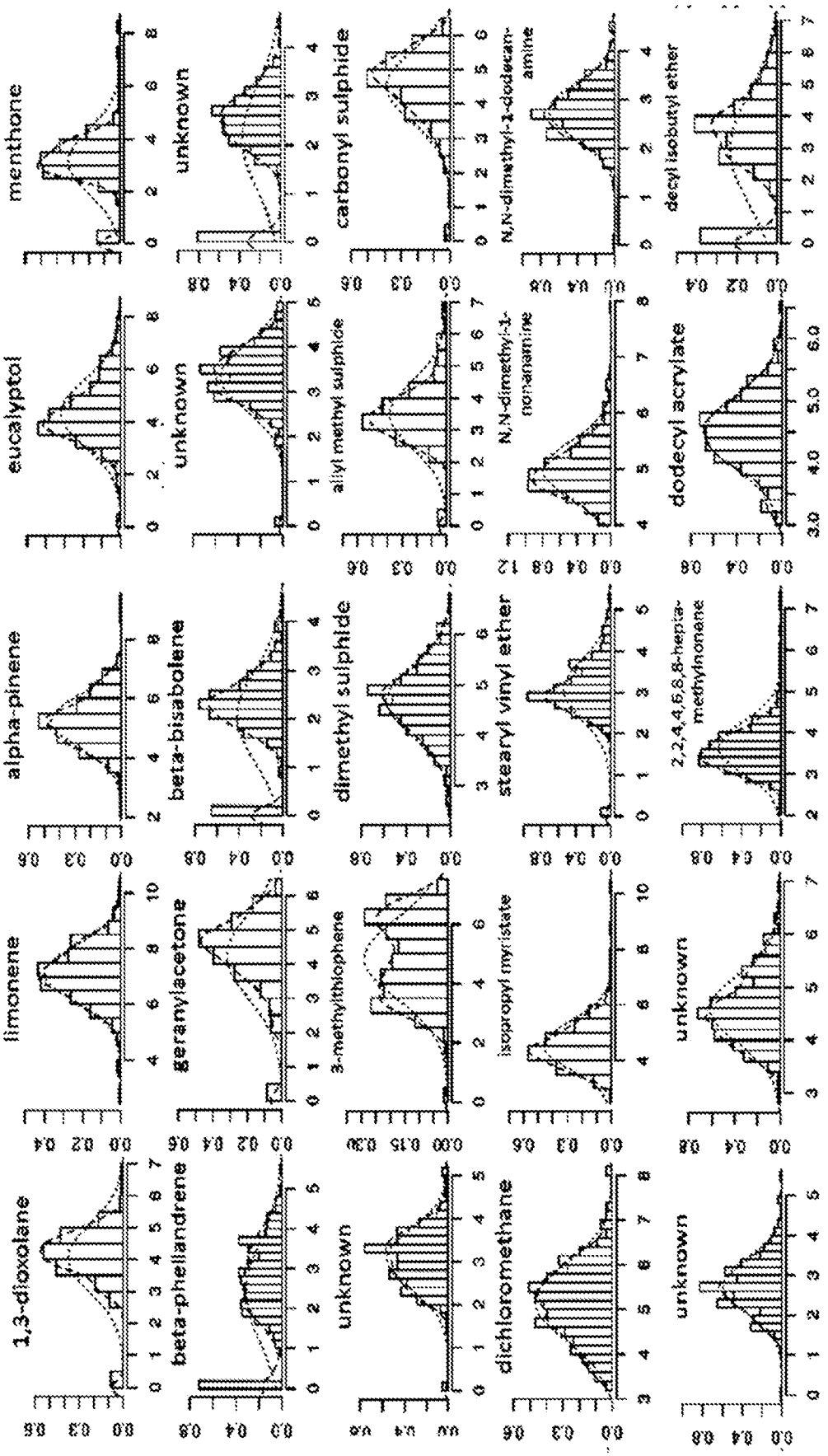

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Figure 9

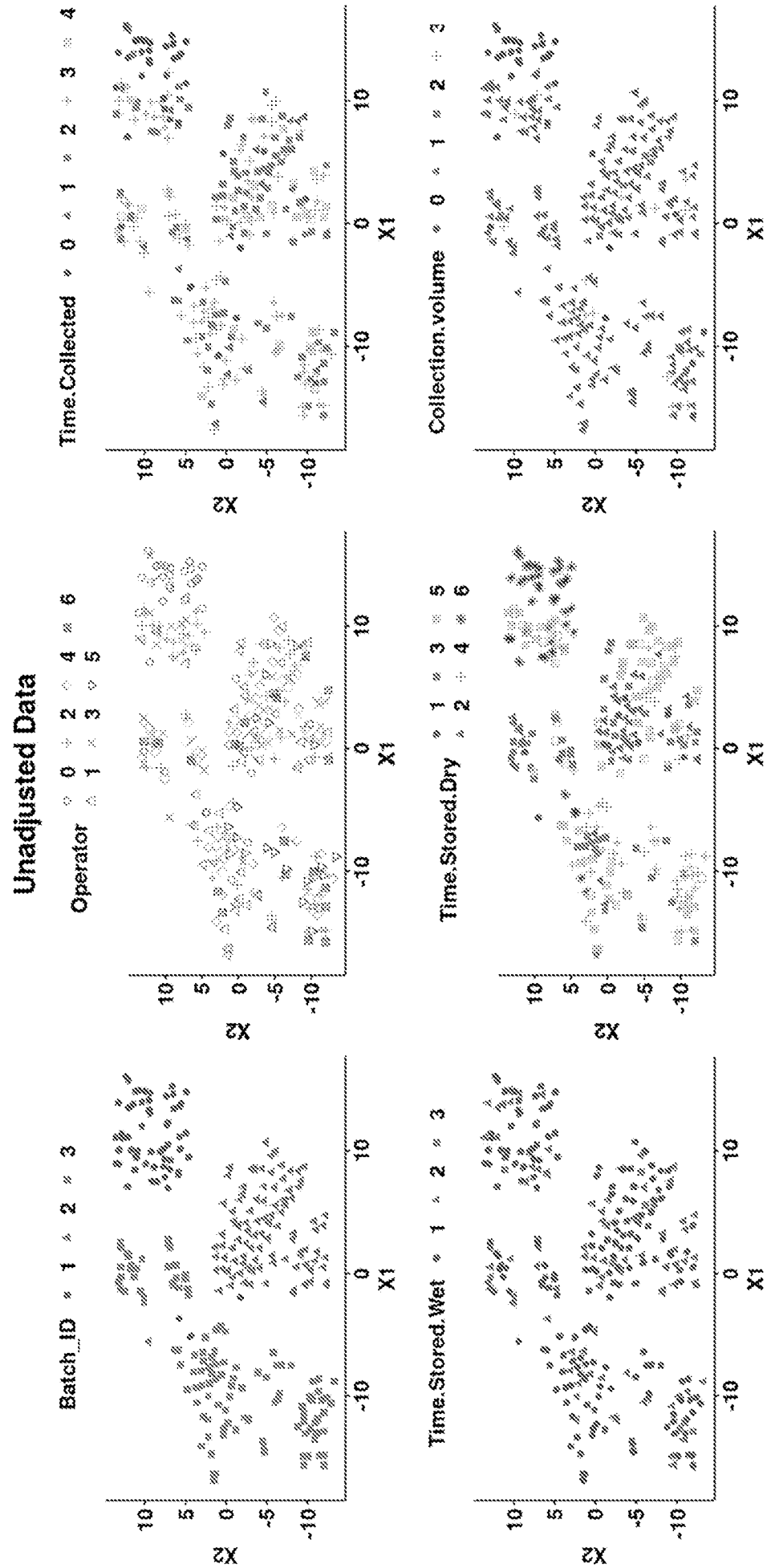


Figure 10

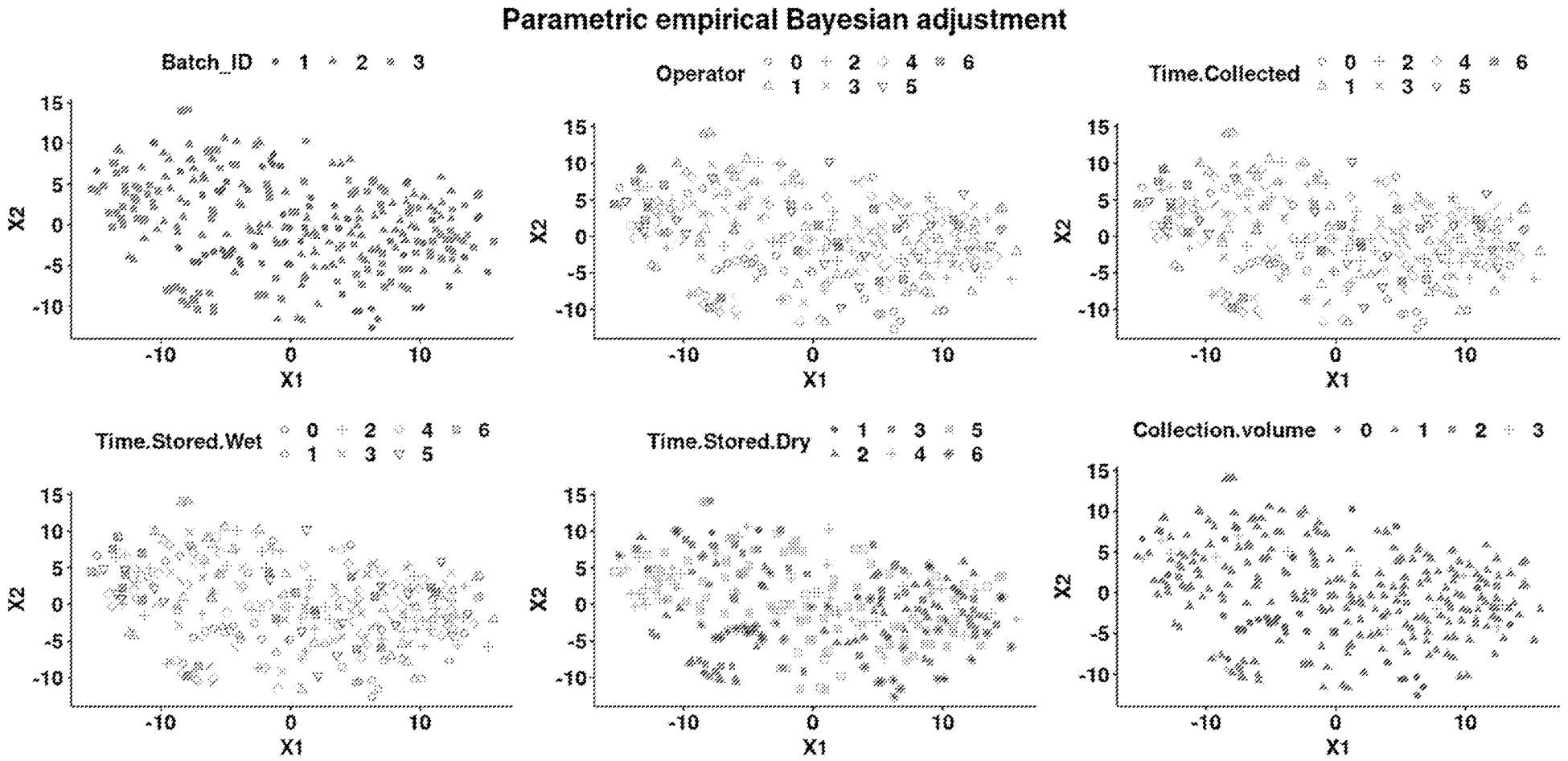

Parametric empirical Bayesian adjustment
Batch_ID 1 2 3
Operator 0 1 2 3 4 5 6
Time.Collected 0 1 2 3 4 5 6
Time.Stored.Wet 0 1 2 3 4 5 6
Time.Stored.Dry 1 2 3 4 5 6
Collection.volume 0 1 2 3
X1
X2

Figure 11

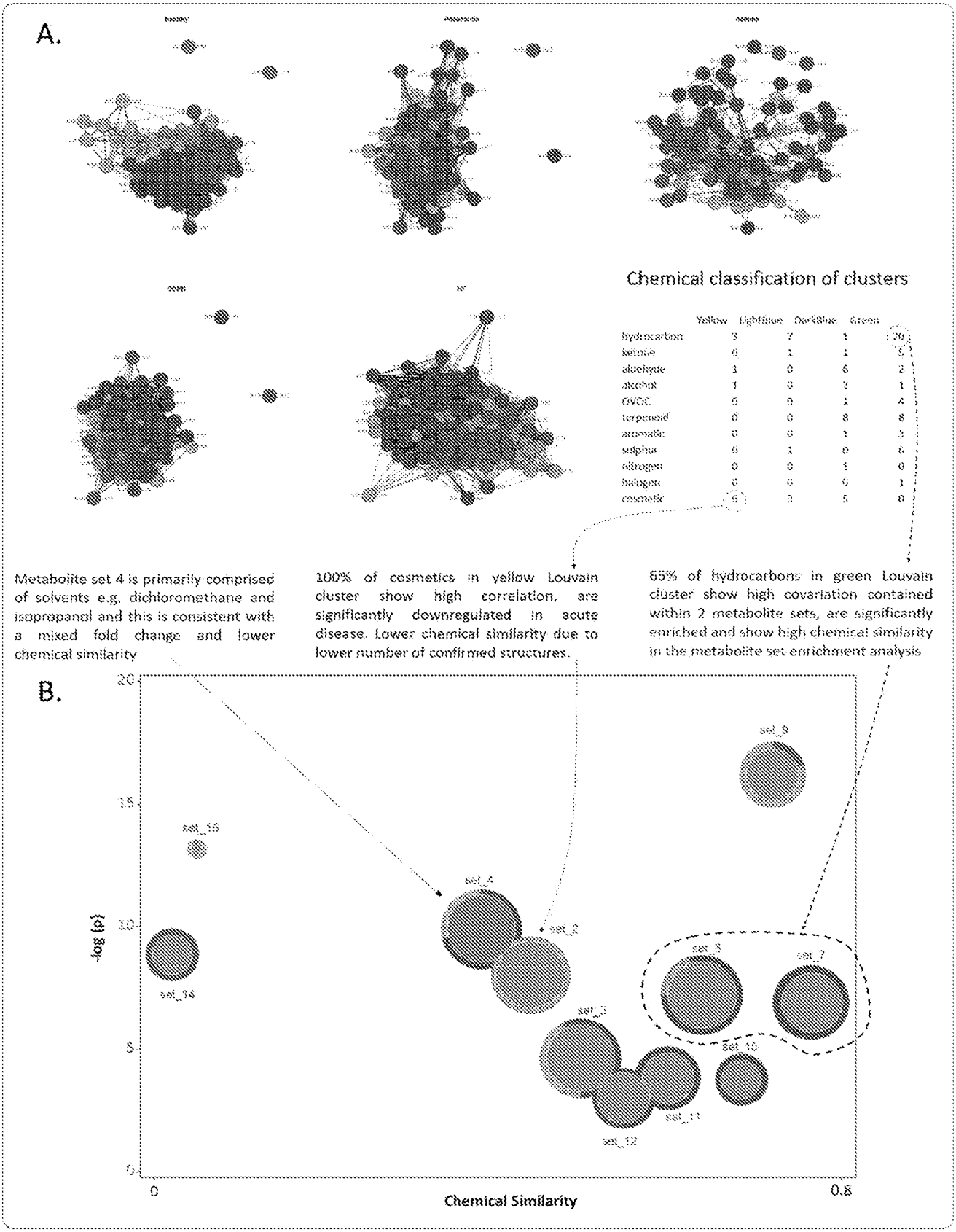
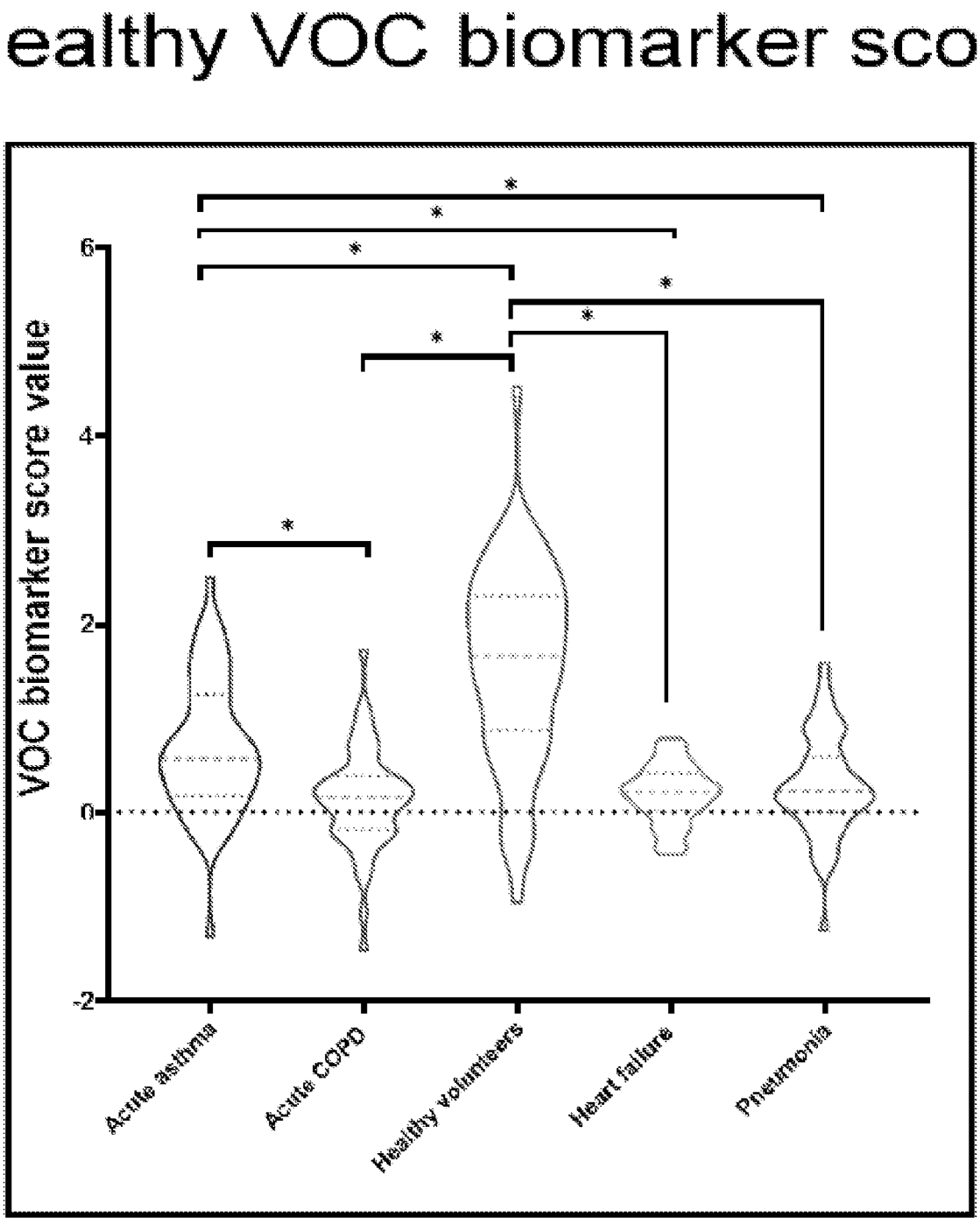
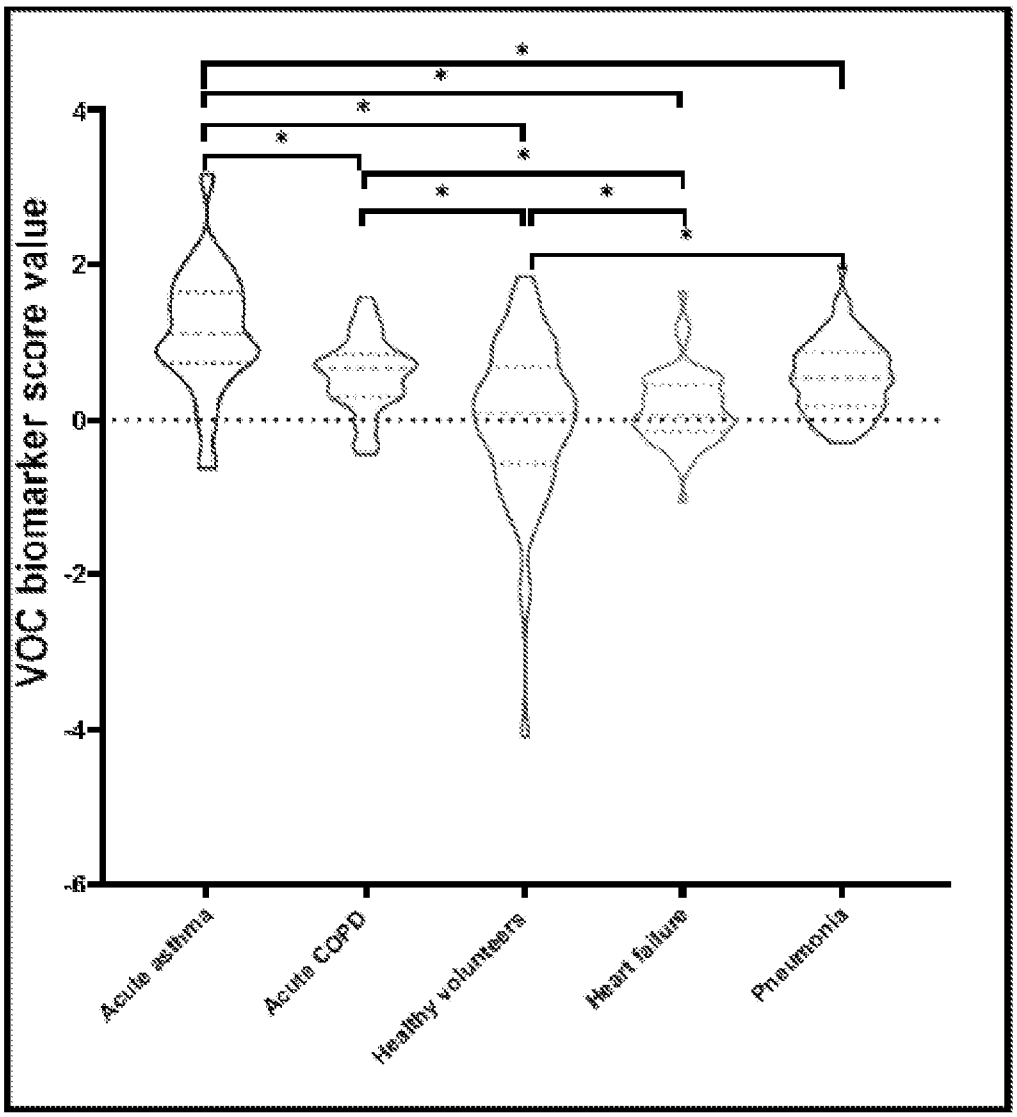


Figure 12



Asthma VOC biomarker score



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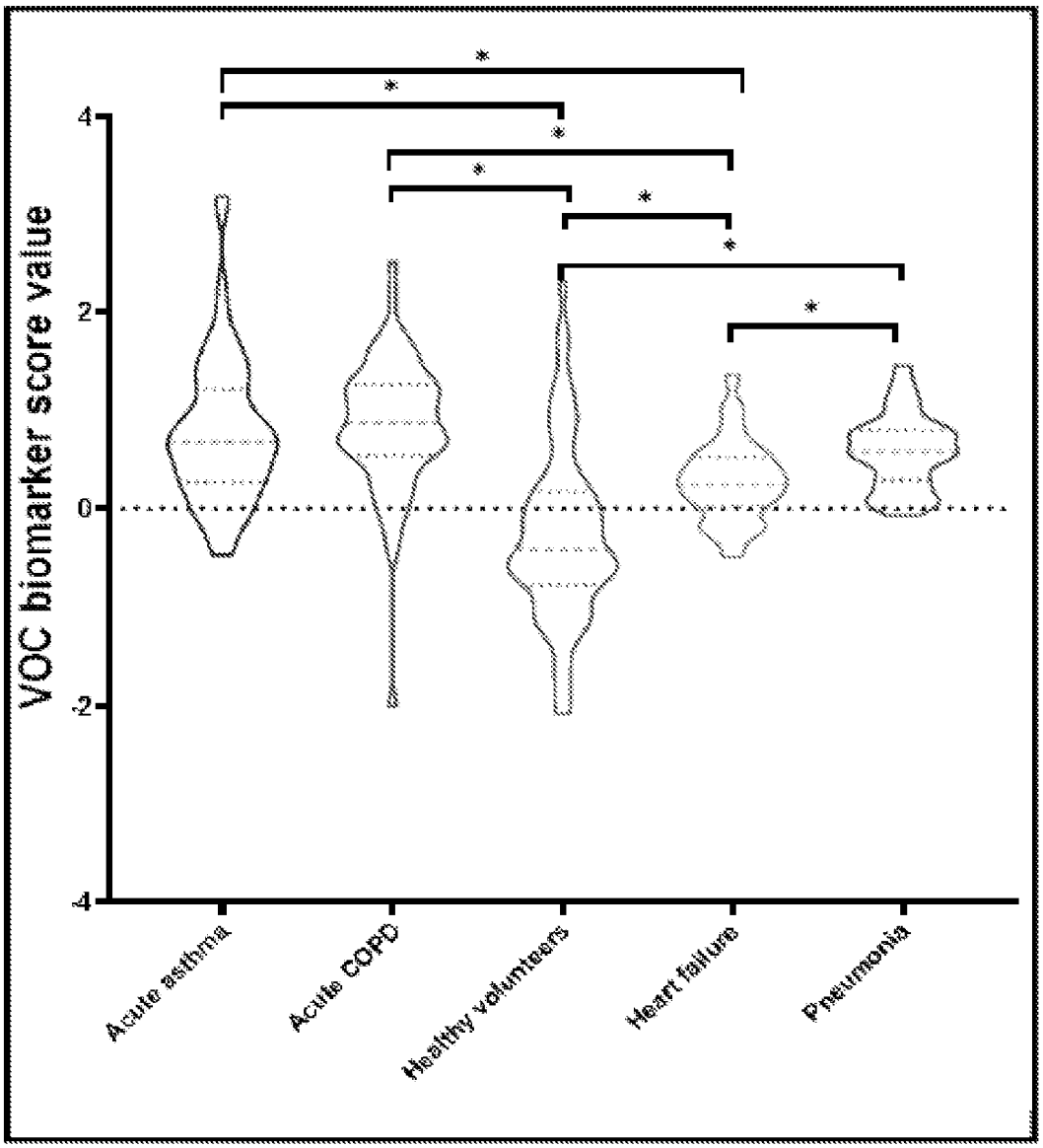
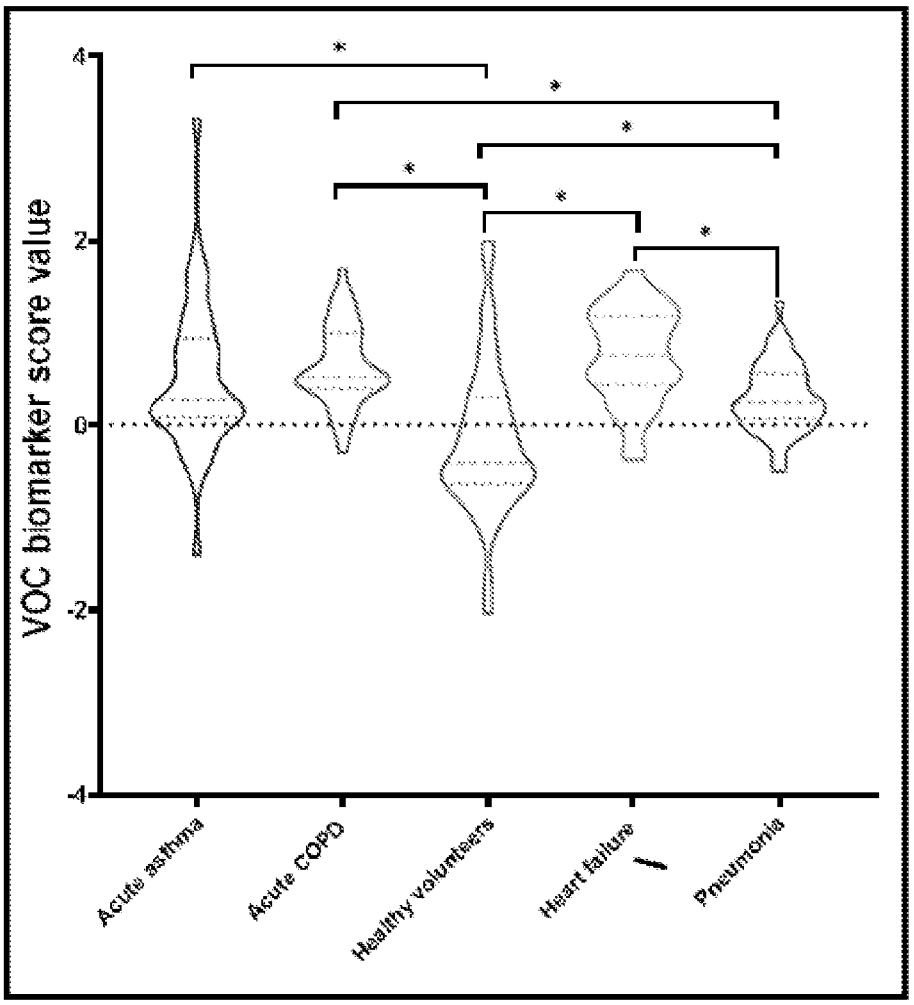
COPD VOC biomarker score



Heart failure VOC biomarker score



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Pneumonia VOC biomarker score

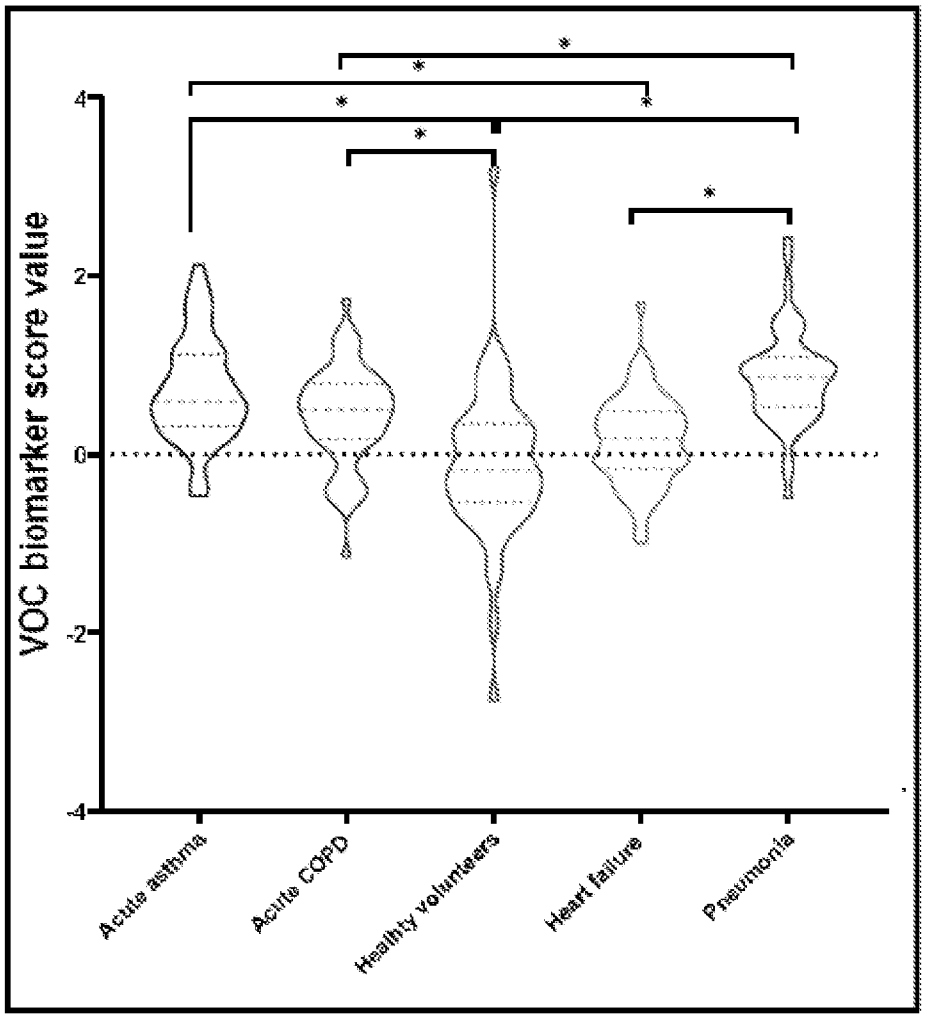

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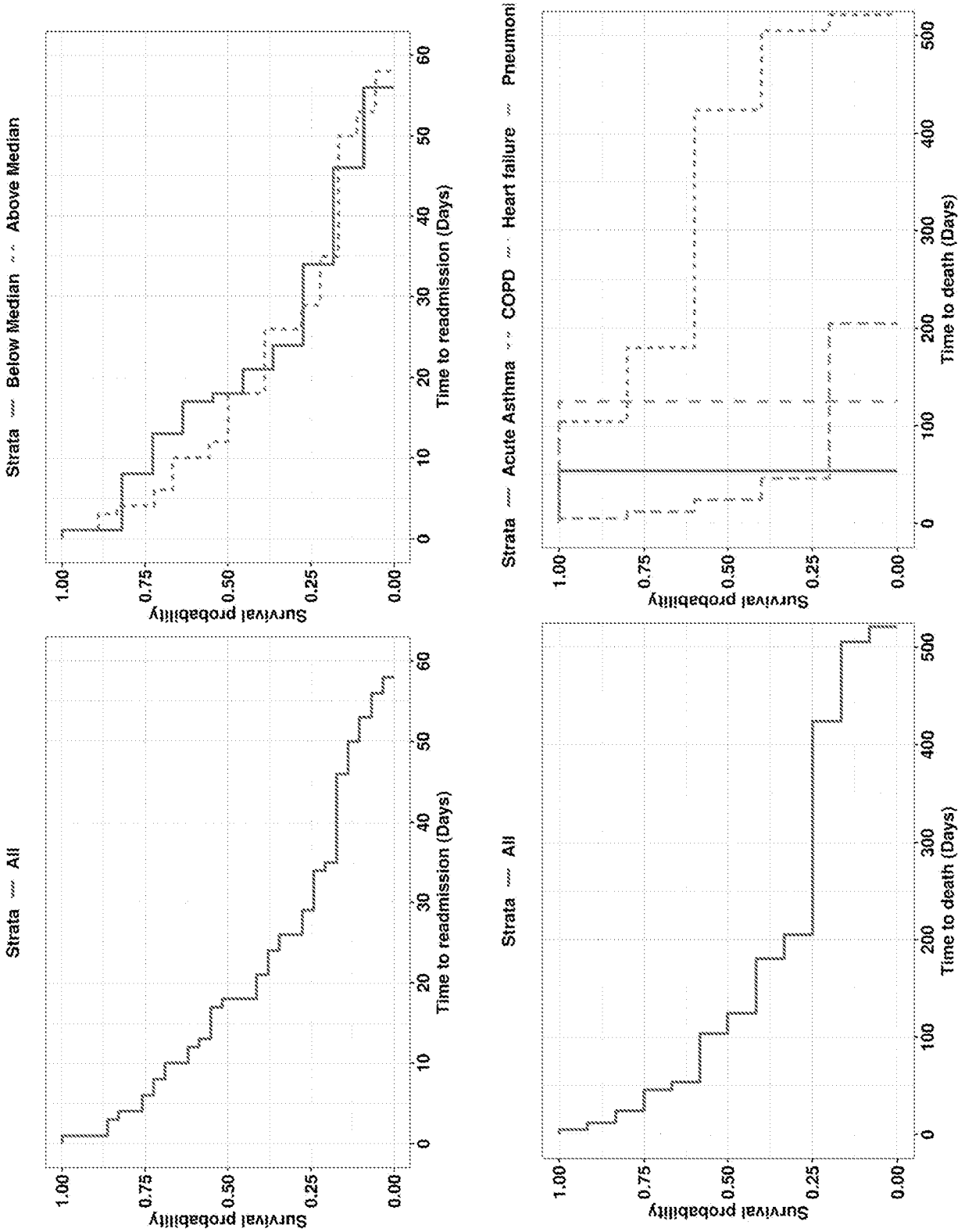
Figure 13



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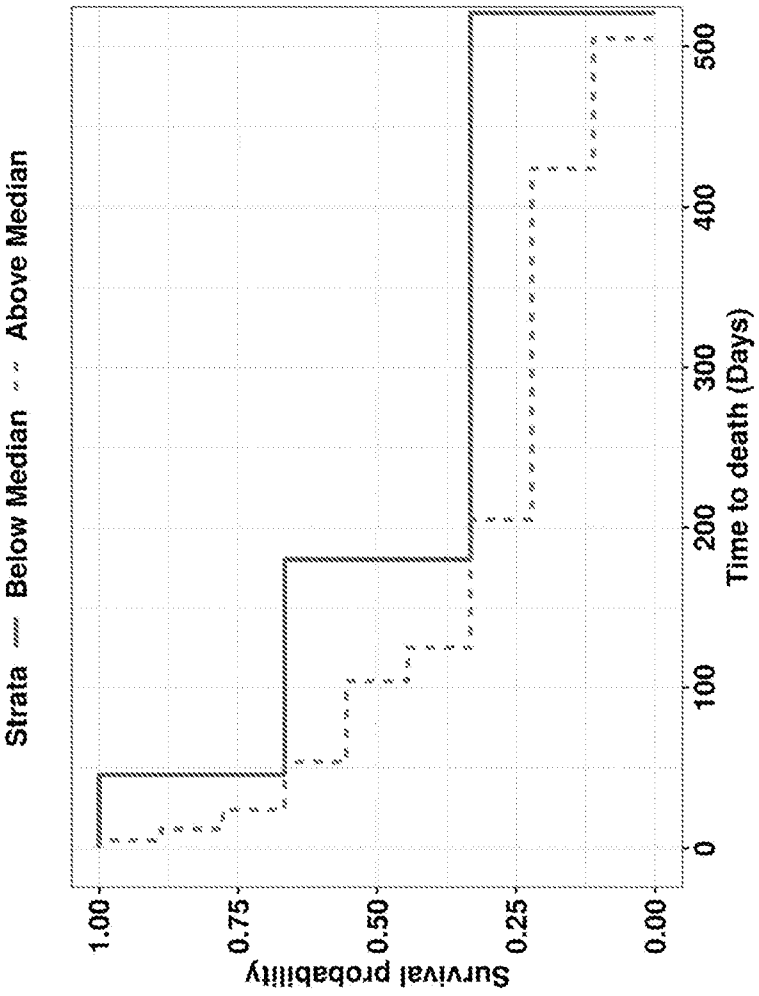
Strata ---- Below Median - - Above Median
Survival probability
1.00
0.75
0.50
0.25
0.00
0
100
200
300
400
500
Time to death (Days)

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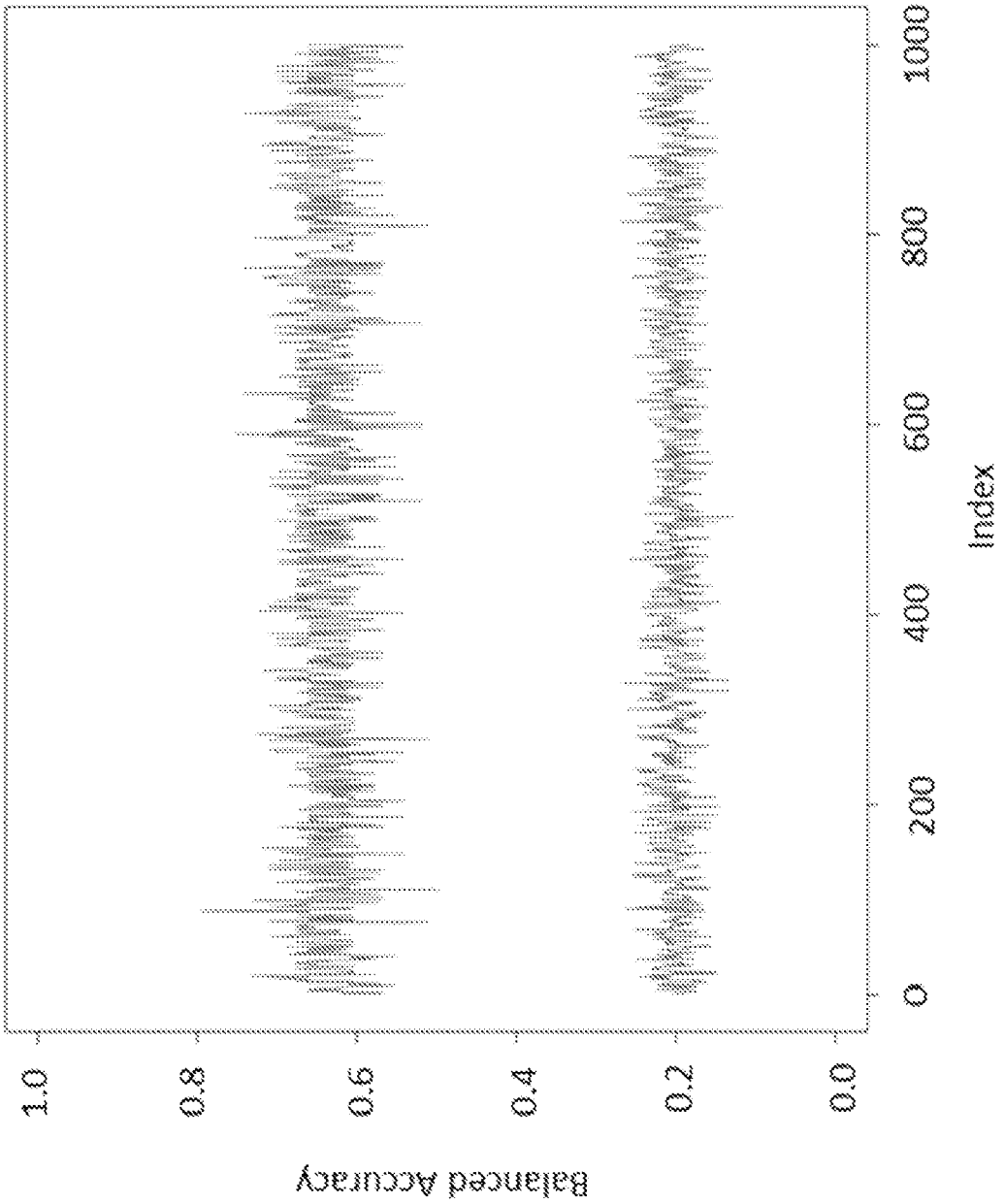

Figure 14

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Figure 15

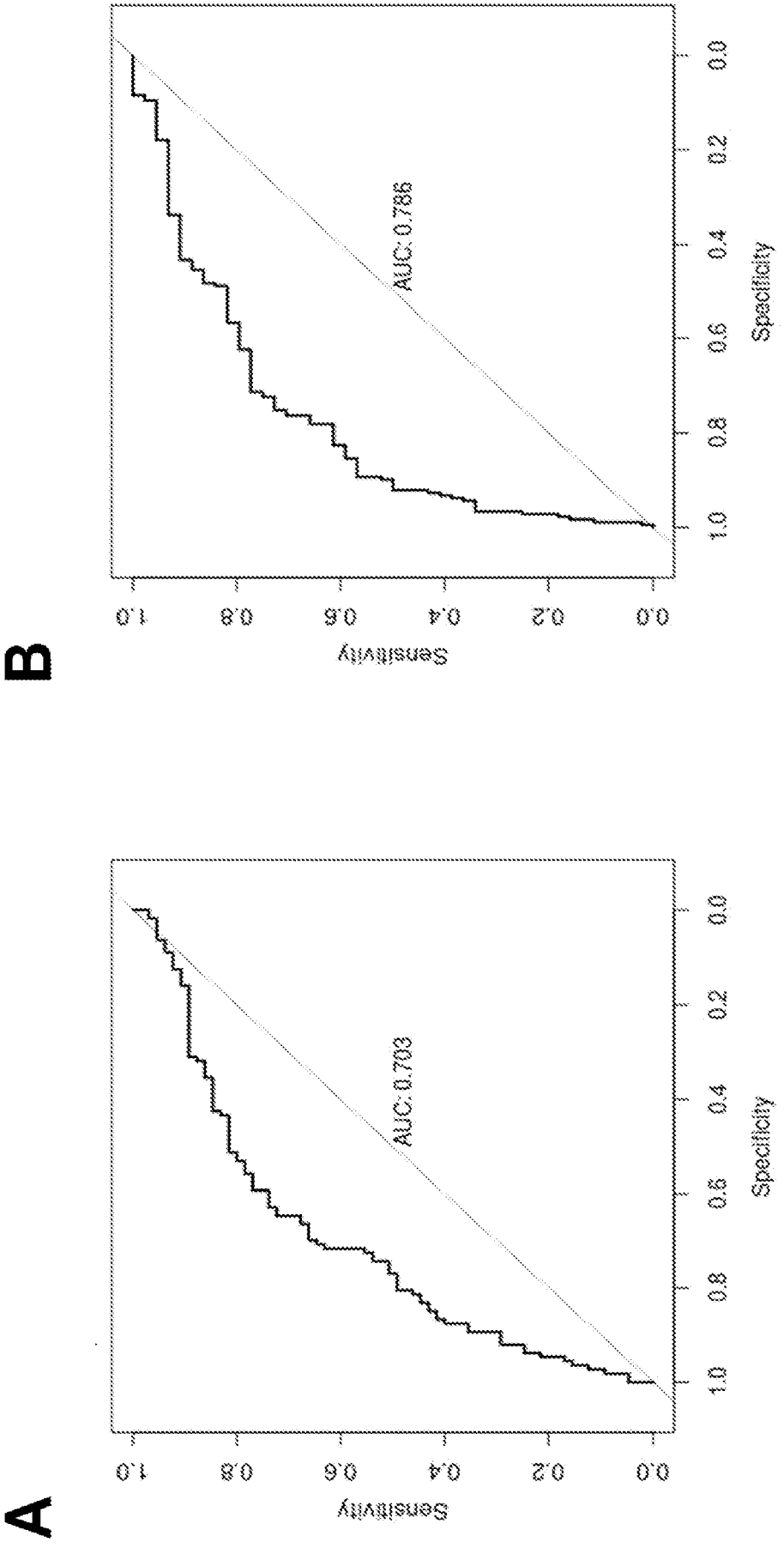


Figure 16

Chemical Name	CAS-RN	KEGG, C- / HMBD, H-	ChEBI	MSI Level	conc. µg/m^3	Log$_2$(FC)	Acute risk score classification
Hydrocarbons							
2-methylbutane	78-78-4	-	30362	1	0-186	-0.032	pneumonia
isoprene	78-79-5	C16521	35194	1	7-1,494	0.015	heart failure
3-methylpentane	96-14-0	HMDB0061885	88373	1	-	0.056	asthma
2,4-dimethylpentane	108-08-7	-	-	1	0-15	0.091	pneumonia
2,2-dimethylpentane	590-35-2	-	-	2	-	0.284†	pneumonia
hexane	110-54-3	C11271, HMDB0029600	29021	1	0-781	0.083†	asthma, pneumonia, heart failure
octane	111-65-9	C01387, HMDB0001485	17590	1	0-2	-0.030	pneumonia, COPD
2,6-dimethyloctane	2051-30-1	-	-	1	0-1	0.056	pneumonia
nonane	111-84-2	C02445, HMDB0029595	32892	1	0-3	-0.062	COPD
2-methylnonane	871-83-0	-	-	1	0-3	0.042	asthma
5-methylnonane	15869-85-9	-	-	2	-	0.102	heart failure
decane	124-18-5	-	41808	1	0-8	0.017	asthma
4-methyldecane	2847-72-5	HMDB0037268	88816	1	0-1	0.049	heart failure
undecane	1120-21-4	HMDB0031445	46342	1	0-4	0.036	heart failure
4-methylundecane	2980-69-0	-	-	2	-	0.045	COPD

unknown (branched C12)	-	-	-	3	-	0.277†	COPD
unknown (branched C12)	-	-	-	3	-	0.049†	heart failure, control
unknown (dimethylundecane isomer)	-	-	-	3	-	0.005	pneumonia
3-methyltridecane	6418-41-3	-	-	2	-	0.102†	control
tetradecane	629-59-4	HMDB0059907	41253	1	0-1	0.048†	asthma, pneumonia
unknown (branched C14)	-	-	-	3	-	0.103†	pneumonia, control
unknown (branched C14)	-	-	-	3	-	0.042	pneumonia
unknown (branched C15)	-	-	-	3	-	0.046†	heart failure
octadecane	593-45-3	HMDB0033721	32926	1	-	-0.061†	control
1-nonene	124-11-8	C08452, HMDB0031270	77443	1	0-4	-0.050	asthma
1-decene	872-05-9	-	87315	1	0-29	0.029	pneumonia
cyclohexane	110-82-7	C11249, HMDB0029597	29005	1	1-9	0.132†	COPD, control
cyclohexene	110-83-8	-	36404	1	0-1	0.096	heart failure
unknown (cyclohexadiene isomer)	-	-	-	3	-	0.639†	control
unknown (methylcyclopentadiene)	-	-	-	3	-	0.416†	COPD, control
unknown (hexadecene isomer)	-	-	-	3	-	0.003	control
unknown	-	-	-	3	-	0.010	pneumonia, COPD
Ketones							
acetone	67-64-1	C00207, HMDB0001659	15347	1	38-10,077	0.062†	heart failure, control
2,3-butanedione	431-03-8	C00741, HMDB0003407	16583	1	0-113	0.289†	asthma, COPD

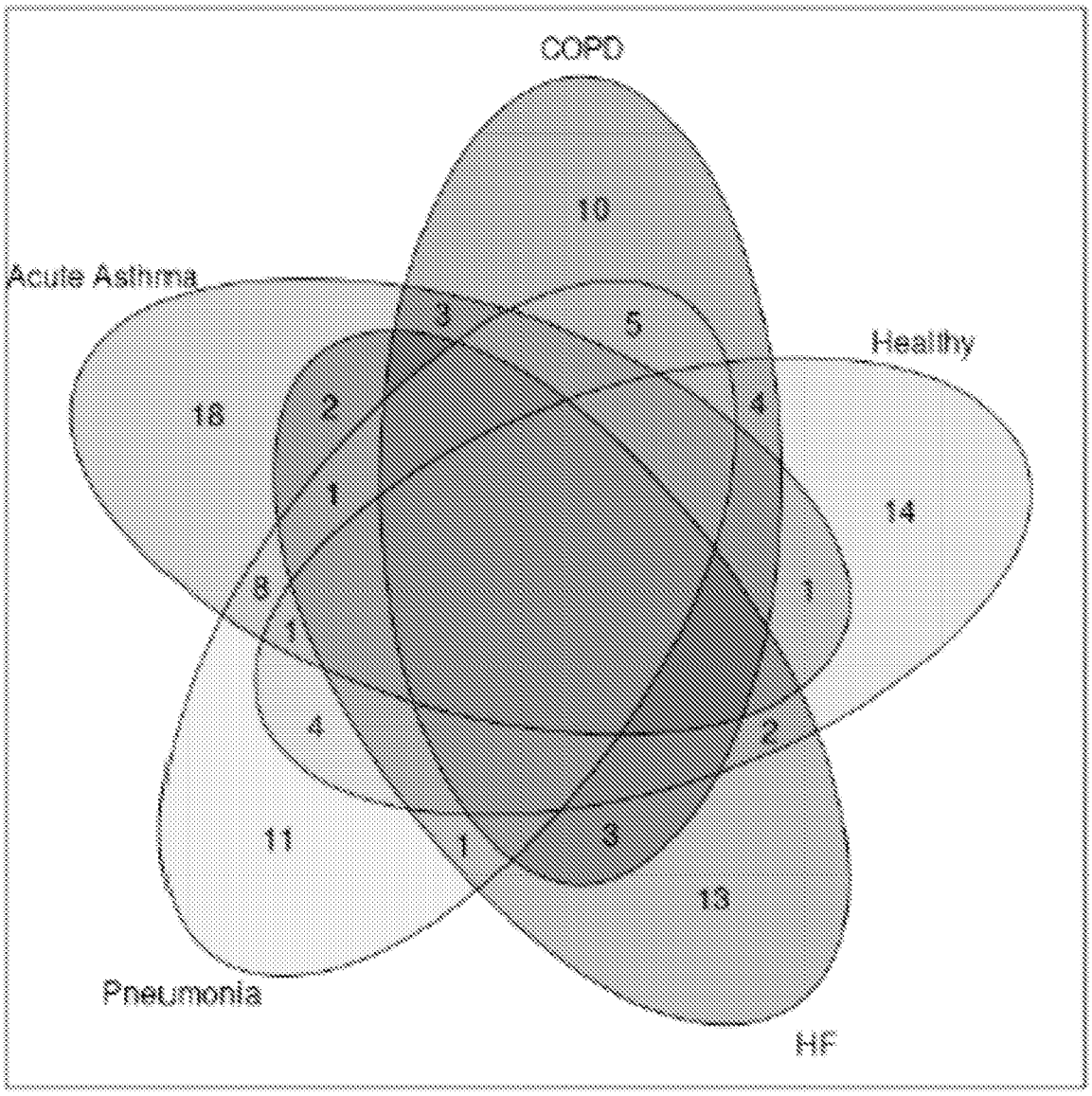
2-pentanone	107-87-9	C01949, HMDB0034235	16472	1	0-6	0.106†	asthma
3-buten-2-one (methyl vinyl ketone)	78-94-4	C20701, HMDB0061873	48058	1	0-52	0.078†	pneumonia
4-methyl-2-pentanone	108-10-1	C19263, HMDB0002939	142806	1	0-3	-0.126	control
6-methyl-5-hepten-2-one	110-93-0	C07287, HMDB0035915	16310	1	0-1	0.115†	COPD, control
cyclohexanone	108-94-1	C00414, HMDB0003315	17854	1	0-2	0.263†	pneumonia, control
Aldehydes							
butanal	123-72-8	C01412, HMDB0003543	15743	1	-	-0.007	heart failure
hexanal	66-25-1	C02373, HMDB0005994	121338	1	0-1	0.002	asthma, pneumonia
nonanal	124-19-6	HMDB0059835	84268	1	0-7	0.004	asthma
decanal	112-31-2	C12307, HMDB0011623	31457	1	0-5	-0.031	asthma
unknown (methyldecanal isomer)	-	-	-	3	-	0.004	asthma
undecanal	112-44-7	HMDB0030941	46202	1	0-4	-0.127†	asthma
2-methyl-2-propenal (methacrolein)	78-85-3	HMDB0061874	88384	1	0-2	0.016	pneumonia, heart failure
3-methylbenzaldehyde	620-23-5	C07209, HMDB0029637	28476	1	-	0.107	asthma
tridecanal	10486-19-8	HMDB0030928	89816	2	-	-0.108†	heart failure
Alcohols							
2-propanol	67-63-0	C01845, HMDB0000863	17824	1	2-719	-0.041†	pneumonia, control
2-ethylhexanol	104-76-7	C02498, HMDB0031231	16011	1	0-3	-0.014	asthma
1-decanol	112-30-1	C01633, HMDB0011624	28903	1	0-5	-0.013	COPD
1-hexadecanol	36653-82-4	C00823, HMDB0003424	16125	1	0-14	-0.046	asthma, pneumonia

Other oxygen-containing VOCs (OVOCs)							
ethyl acetate	141-78-6	C00849, HMDB0031217	27750	1	1-40	0.022	heart failure
tetrahydrofuran	109-99-9	HMDB0000246	26911	1	0-5	-0.017	asthma
1,4-dioxane	123-91-1	C14440	47032	1	0-276	0.105	asthma
2-methyl-1,3-dioxolane	497-26-7	-	-	2	-	0.080	asthma, COPD
1,3-dioxolane	646-06-0	-	87597	2	-	0.199†	heart failure
Terpenes / Terpenoids							
limonene	5989-27-5	C06099, HMDB0003375	15382	1	0-75	0.062†	COPD, heart failure
alpha-pinene	7785-26-4	C06308, HMDB0035658	28660	1	0-53	-0.066	pneumonia, control
eucalyptol	470-82-6	C09844, HMDB0004472	27961	1	0-16	0.008	asthma, COPD
menthone	14073-97-3	C00843, HMDB0035162	15410	1	0-5	-0.097	pneumonia, COPD
menthol	2216-51-5	C00400, HMDB0003352	15409	1	0-160	0.023	COPD
camphene	79-92-5	C06076, HMDB0059839	3830	1	0-2	-0.056	COPD
p-mentha-1,4/8-diene	99-85-4	C09900, HMDB0038150	10577	2	-	-0.057	asthma, pneumonia
3-carene	13466-78-9	C11382, HMDB0035619	35661	1	0-2	-0.032	asthma, heart failure
beta myrcene	123-35-3	C06074, HMDB0038169	17221	1	0-2	-0.002	heart failure
beta-phellandrene	555-10-2	C19818, HMDB0036081	48741	2	-	0.008	asthma, pneumonia
geranylacetone	3796-70-1	C13297, HMDB0031846	67206	1	1-6	0.177†	control
beta-bisabolene	495-61-4	C16775, HMDB0035992	49249	2	-	-0.198	asthma
unknown (sesquiterpenoid)	-	-	-	3	-	-0.082	asthma, pneumonia

unknown	-	-	-	3	-	0.231	COPD
alpha isomethyl ionone	-	-	-	2	-	-0.030	control
galaxolide	1222-05-5	-	83784	2	-	-0.113	COPD
Aromatics							
xylene	106-42-3	C06756, HMDB0059924	27417	1	0-5	0.12†	asthma, pneumonia, control
ethylbenzene	100-41-4	C07111, HMDB0059905	16101	1	0-1	0.15†	heart failure
2,3-dimethylnaphthalene	581-40-8	-	48615	1	-	-0.05	COPD, heart failure
unknown (C9, substituted benzene)	-	-	-	3	-	0.235†	control
Sulphur-containing VOCs							
3-methyl thiophene	616-44-4	HMDB0033119	89007	1	0-7	0.040	COPD
dimethyl sulphide	75-18-3	C00580, HMDB0002303	17437	1	0-16	-0.044	control
allyl methyl sulphide	10152-76-8	HMDB0031653	89856	1	0-4	-0.230†	COPD, control
carbonyl sulphide	463-58-1	C07331	16573	2	-	0.122†	pneumonia, COPD
1-(methylthio)-1-propene	10152-77-9	HMDB0059843	89721	1	0-1,126	-0.186	pneumonia
1-methylthio-propane	3877-15-4	HMDB0061871	88383	2	-	-0.044	pneumonia
unknown (C4 thio-containing)	-	-	-	3	-	-0.007	asthma
Nitrogen-containing VOCs							
4-cyanocyclohexene	100-45-8	-	-	1	-	-0.039	asthma, pneumonia
methenamine	100-97-0	D00393,HMDB0029598	6824	2	-	0.014	asthma, pneumonia
Halogenates							

dichloromethane	75-09-2	C02271, HMDB0031548	15767	1	0-199	-0.007	pneumonia, COPD
Surfactants and emollients							
isopropyl myristate	110-27-0	D02296,HMDB0040392	90027	1	0-76	-0.189†	control
stearyl vinyl ether	930-02-9	-	-	2	-	-0.235†	asthma, control
N,N-dimethyl-1-nonanamine	17373-27-2	-	-	2	-	0.055†	asthma, heart failure
N,N-dimethyl-1-dodecanamine	112-18-5	-	-	2	-	0.056	COPD
unknown (alkenyl hexanoic acid ester)	-	-	-	3	-	-0.061	control
unknown (alkenyl hexanoic acid ester)	-	-	-	3	-	-0.042	control
unknown (alkenyl hexanoic acid ester)	-	-	-	3	-	-0.038	COPD, heart failure
unknown (surfactant)	-	-	-	3	-	0.134	asthma
unknown (emoillent)	-	-	-	3	-	-0.162	asthma
unknown (eicosanol)	-	-	-	3	-	-0.165†	control
unknown (emoillent)	-	-	-	3	-	-0.013	asthma
2,2,4,4,6,8,8-heptamethylnonane	4390-04-9	-	131383	2	-	0.045†	control
dodecyl acrylate	2156-97-0	-	-	2	-	-0.021	pneumonia
decyl isobutyl ether	-	-	-	2	-	0.002	heart failure

Figure 17



BIOMARKER

FIELD OF THE INVENTION

The invention relates to a method of diagnosing and a method of treating a cardiorespiratory disease in a subject experiencing breathlessness.

BACKGROUND

Breathlessness due to cardio-respiratory diseases accounts for more than 1 in 8 of all emergency admissions to hospital. Despite the same presenting symptom, the aetiology of acute breathlessness is highly varied, with diverse disease trajectories and treatment options. Diagnostic evaluation of acute breathlessness is heavily reliant on investigations such as blood-based biomarkers (e.g. C-reactive protein (CRP), B-type natriuretic peptide (NT-pro BNP)) and radiological procedures. These biomarkers have clinical utility primarily in patients with single pathologies, but have poor discriminatory power in patients with multifactorial presentations of acute breathlessness and are particularly challenging to interpret in the context of pre-admission treatment exposure (e.g. antibiotics for pneumonia and admission CRP values). Additionally, delays in blood sample processing at the point of triage can result in inappropriate treatment decisions and consequently harmful effects to patients. To address these issues, there have been considerable advancements in the field of metabolomics, underpinned by analytical technologies, which permit comprehensive identification and quantification of metabolite profiles in biological systems from samples acquired at the point of clinical care.

Nevertheless, there is a need for biomarkers that can be used to diagnose and distinguish between cardiorespiratory conditions that present with breathlessness as a symptom.

STATEMENTS OF INVENTION

According to a first aspect of the invention, there is provided a method of diagnosing a cardiorespiratory disease in a subject, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled breath from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have a cardiorespiratory disease.

In one embodiment, there is provided a method of diagnosing asthma in a subject, the method comprising:

detecting the presence of one or more asthma-VOC biomarkers in a sample of exhaled air from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have asthma.

In one embodiment, there is provided a method of diagnosing COPD in a subject, the method comprising:

detecting the presence of one or more COPD-VOC biomarkers in a sample of exhaled air from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have COPD.

In one embodiment, there is provided a method of diagnosing pneumonia in a subject, the method comprising:

detecting the presence of one or more pneumonia-VOC biomarkers in a sample of exhaled air from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have pneumonia.

In one embodiment, there is provided a method of diagnosing heart failure in a subject, the method comprising:

detecting the presence of one or more heart failure-VOC biomarkers in a sample of exhaled air from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have heart failure.

According to a second aspect, there is provided a method of treating a cardiorespiratory disease in a subject, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled air from the subject,

wherein the presence of one or more of the VOC biomarkers in the sample suggests the subject has a cardiorespiratory disease, and

administering a therapeutic agent to the subject, in order to treat the cardiorespiratory disease.

According to a third aspect, there is provided a method of treating a cardiorespiratory disease in a subject, the method comprising:

administering a therapeutic agent to the subject, who has been diagnosed with a cardiorespiratory disease using the method according to the invention.

According to fourth aspect, there is provided a method of selecting a subject for treatment with a therapeutic agent or composition for a cardiorespiratory disease, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled air from the subject,

wherein the presence of one or more of the VOC biomarkers in the sample suggests the subject has a cardiorespiratory disease, and

selecting the subject for treatment with a therapeutic agent or composition for the cardiorespiratory disease.

According to another aspect, there is provided a method of selecting a subject for treatment with a therapeutic agent or composition for a cardiorespiratory disease, the method comprising:

selecting a subject, who has been diagnosed with a cardiorespiratory disease using the method according to the invention, for treatment with a therapeutic agent or composition for a cardiorespiratory disease.

The invention provides a more patient-compliant method of diagnosing and treating a cardiorespiratory disorder. The invention enables a subject to be diagnosed without the use of invasive procedures, such as taking blood, or radiological processes. The method may not be performed on the subject.

Two important features of any biomarker that are used for diagnostic purposes are sensitivity and specificity. The higher the degree of sensitivity, the lower the probability of generating a false negative. The higher the degree of specificity, the lower the probability of generating a false positive. The biomarkers disclosed herein can surprisingly exhibit up to 79% sensitivity and 85% specificity (with an AUC of

0.89) when distinguishing between individuals with a cardiorespiratory disease and healthy individuals (controls).

The values for differentiating each acute cardiorespiratory disease group from the other acute cardiorespiratory disease groups (i.e. not against healthy patients) are as follows:

- Asthma - sensitivity 0.75 (0.63, 0.85), specificity 0.90 (0.85, 0.94);
- COPD - sensitivity 0.66 (0.52, 0.78), specificity 0.89 (0.85, 0.93);
- Heart failure - sensitivity 0.64 (0.48, 0.78), specificity 0.96 (0.92, 0.98); and
- Pneumonia - sensitivity 0.65 (0.51, 0.78), specificity 0.93 (0.89, 0.96).

The invention thus enables a clinician to make a more informed decision about the diagnosis and treatment of a subject experiencing breathlessness and suffering from a cardiorespiratory disorder.

According to a fifth aspect, there is provided a method of determining if a therapeutic agent or composition is effectively treating a cardiorespiratory disease in a subject, the method comprising:

determining the concentration of one or more cardiorespiratory disease-VOC biomarkers in a test sample that has been exhaled by the subject, and

comparing the concentration of the at least one or more VOCs in the test sample with the concentration in a reference sample,

wherein if the concentration of the one or more VOC biomarkers in the test sample is lower compared to the concentration in a reference sample, it is indicative that the therapeutic agent or composition is effectively treating the cardiorespiratory disease in the subject.

It will be appreciated that the concentration of a VOC biomarker in a test sample positively correlates with the magnitude/severity of the cardiorespiratory disease. Thus, for example, a reduction in concentration of a VOC biomarker in the test sample compared to the concentration in a reference sample may be indicative of a reduction in the magnitude/severity of the cardiorespiratory disease. Similarly, an increase in concentration of a VOC biomarker in the test sample compared to the concentration in a reference sample may be indicative of an increase in the magnitude/severity of the cardiorespiratory disease.

The concentration of the VOC biomarker in the test sample may be lower by (or reduced by at) least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% compared to the concentration in the reference sample.

The reference sample may have been taken from the same subject or a different subject. Preferably, the reference sample is a sample that has been taken from the same subject but at an earlier time point than the test sample. Preferably the earlier sample indicates that the subject has a cardiorespiratory disease.

Preferably the subject referred to herein is experiencing breathlessness. Preferably, a two-dimensional gas chromatography coupled with mass spectrometry is used to detect the presence of one or more VOC biomarkers in the sample.

A cardiorespiratory disease may be a disease or disorder of the cardiovascular system and/or a disease of the respiratory system. Examples of cardiorespiratory diseases include asthma, COPD, heart failure and a respiratory infection (e.g. pneumonia), bronchitis, emphysema, congestive heart failure, hypertension, angina, peripheral vascular disease and myocardial infarction. Preferably, the term *"cardiorespiratory disease"* refers to one or more diseases selected from the group comprising: asthma, COPD, heart failure and pneumonia.

A VOC (volatile organic compound) may be referred to as an organic compound that has a boiling point between about 50°C and about 250°C at a standard atmospheric pressure of 101.3 kPa.

A cardiorespiratory disease-VOC biomarker may be one or more selected from the group comprising: hydrocarbons, ketones, aldehydes, alcohols, oxygen-containing VOCs, terpenoids, aromatics, sulphur-containing VOCs, nitrogen-containing VOCs, a halogenate (e.g. dichloromethane) and surfactants and emollients.

It will be appreciated that the step of detecting the presence of one or more cardiorespiratory disease-VOC biomarkers may comprise using the method according to the invention. It will also be appreciated that the detection of one more

cardiorespiratory disease-VOC biomarkers in a sample is indicative that the subject (from which the sample has been taken) has a cardiovascular disease.

The hydrocarbon VOC may be one or more selected from the group comprising: 2-methylbutane; isoprene; 3-methylpentane; 2,4-dimethylpentane; 2,2-dimethylpentane; hexane; octane; 2,6-dimethyloctane; nonane; 2-methylnonane; 5-methylnonane; decane; 4-methyldecane; undecane; 4-methylundecane; dimethylundecane isomer; 3-methyltridecane; tetradecane; octadecane; 1-nonene; 1-decene; cyclohexane; a cyclohexadiene isomer; methylcyclopentadiene; and a hexadecene isomer. The hydrocarbon may be one or more selected from Figure 16. Preferably the hydrocarbon VOC may be one or more selected from the group comprising ; hexane; octane; 2,6-dimethyloctane; nonane; 2-methylnonane; decane; undecane; 4-methylundecane; dimethylundecane isomer; 3-methyltridecane; tetradecane; octadecane; 1-nonene; 1-decene; cyclohexane; a cyclohexadiene isomer; methylcyclopentadiene; and a hexadecene isomer. The hydrocarbon may be one or more selected from Figure 16.

The ketone VOC may be one or more selected from the group comprising: acetone; 2,3-butanedione; 2-pentanone; 3-buten-2-one (methyl vinyl ketone); 4-methyl-2-pentanone; 6-methyl-5-hepten-2-one; and cyclohexanone. The ketone may be one or more selected from Figure 16. Preferably the ketone VOC may be one or more selected from the group comprising 3-buten-2-one (methyl vinyl ketone); 4-methyl-2-pentanone; and 6-methyl-5-hepten-2-one.

The aldehyde VOC may be one or more selected from the group comprising: butanal; hexanal; nonanal; decanal; methyldecanal isomer; undecanal; 2-methyl-2-propenal (methacrolein); 3-methylbenzaldehyde; and tridecanal. The aldehyde may be one or more selected from Figure 16. Preferably the aldehyde VOC is one or more selected from the group comprising butanal; methyldecanal isomer; undecanal; 2-methyl-2-propenal (methacrolein); 3-methylbenzaldehyde; and tridecanal.

The alcohol VOC may be one or more selected from the group comprising 2-propanol; 2-ethylhexanol; 1-decanol; and 1-hexadecanol. The alcohol may be one or more selected from Figure 16.

The oxygen-containing VOCs may be one or more selected from the group comprising: ethyl acetate; tetrahydrofuran; 1,4-dioxane; 2-methyl-1,3-dioxolane; and 1,3-dioxolane. The oxygen-containing VOC may be one or more selected from Figure 16.

The terpenoid VOC may be one or more selected from the group comprising: limonene; alpha-pinene; eucalyptol; menthone; menthol; camphene; p-mentha-1,4/8-diene; 3-carene; beta myrcene; beta-phellandrene; geranylacetone; beta-bisabolene; alpha isomethyl ionone; and galaxolide. The terpenoids may be one or more selected from Figure 16.

The aromatic VOC may be one or more selected from the group comprising: xylene; ethylbenzene; 2,3-dimethylnaphthalene; and a substituted benzene. The aromatic may be one or more selected from Figure 16.

The sulphur-containing VOC may be one or more selected from the group comprising 3-methyl thiophene; dimethyl sulphide; allyl methyl sulphide; carbonyl sulphide; 1-(methylthio)-1-propene; and 1-methylthio-propane. The sulphur-containing VOC may be one or more selected from Figure 16. Preferably the sulphur-containing VOC may be one or more selected from the group comprising dimethyl sulphide; 1-(methylthio)-1-propene; and 1-methylthio-propane.

The nitrogen-containing VOC may be one or more selected from the group comprising: 4-cyanocyclohexene; and methenamine. The nitrogen-containing VOC may be one or more selected from Figure 16.

The surfactant and emollient VOC may be one or more selected from the group comprising: isopropyl myristate; stearyl vinyl ether; N,N-dimethyl-1-nonanamine; N,N-dimethyl-1-dodecanamine; an alkenyl hexanoic acid ester; 2,2,4,4,6,8,8-heptamethylnonane; dodecyl acrylate; and decyl isobutyl ether. The surfactant and emollient may be one or more selected from Figure 16.

A cardiorespiratory disease-VOC biomarker may be any combination of the VOC biomarkers disclosed in Figure 16. Thus, a cardiorespiratory disease-VOC biomarker may be one or more selected from Figure 16. The VOC may be an isomer of a VOC

disclosed in Figure 16. Thus, a cardiorespiratory disease-VOC biomarker may be one or more VOC biomarkers selected from Figure 16, or an isomer thereof. An isomer may be a structural isomer, a diastereomer (e.g. cis-trans isomer or a rotamer) or an enatiomer.

In one embodiment, a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose a cardiorespiratory disease in a subject: hexane; octane; tetradecane,2,3-butanedione; hexanal; 2-methyl-2-propenal; 1-hexadecanol; 2-methyl-1,3-dioxolane; limonene; eucalyptol; menthone; p-mentha-1,4/8-diene; 3-carene; beta phellandrene; sesquiterpenoid; xylene; 2,3-dimethylnapthalene; carbonyl sulphide; 4-cyanocylohexene; methenamine; dichloromethane; N,N-dimethyl-1-nonanamine; and a alkenyl hexanoic acid ester.

In another embodiment, a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose asthma in a subject: 3-methylpentane; hexane; 2-methylnonane; decane; tetradecane; 1-nonene; 2,3-butanedione; 2-pentanone; hexanal; nonanal; decanal; methyldecanal isomer; undecanal; 3-methylbenzaldehyde; 2-ethylhexanol; 1-hexadecanol; tetrahydrofuran; 1,4-dioxane; 2-methyl-1,3-dioxolane; eucalyptol; p-mentha-1,4/8-diene; 3-carene; beta-phellandrene; beta-bisabolene; sesquiterpenoid; xylene; 4-cyanocyclohexene; methenamine; stearyl vinyl ether; N,N-dimethyl-1-nonanamine; and N,N-dimethyl-1-dodecanamine.

A selection of one or more (e.g. all) of the following VOC biomarkers may be used to diagnose asthma in a subject: 3-methylpentane; hexane; 2-methylnonane; decane; tetradecane; 1-nonene; 2,3-butanedione; methyldecanal isomer; undecanal; 3-methylbenzaldehyde; 2-ethylhexanol; 1-hexadecanol; tetrahydrofuran; 1,4-dioxane; 2-methyl-1,3-dioxolane; eucalyptol; p-mentha-1,4/8-diene; 3-carene; beta-phellandrene; beta-bisabolene; sesquiterpenoid; xylene; 4-cyanocyclohexene; methenamine; stearyl vinyl ether; N,N-dimethyl-1-nonanamine; and N,N-dimethyl-1-dodecanamine.

Preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose asthma in a subject: 3-methylpentane; 2-methylnonane; decane; 1-nonene; 2-pentanone; nonanal; decanal; methyldecanal isomer; undecanal; 3-methylbenzaldehyde; 2-ethylhexanol; tetrahydrofuran; 1,4-dioxane; beta-bisabolene; and N,N-dimethyl-1-dodecanamine.

Most preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose asthma in a subject: 3-methylpentane; 2-methylnonane; decane; 1-nonene; methyldecanal isomer; undecanal; 3-methylbenzaldehyde; 2-ethylhexanol; tetrahydrofuran; 1,4-dioxane; beta-bisabolene; and N,N-dimethyl-1-dodecanamine.

In another embodiment, a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose COPD in a subject: octane; nonane; 4-methylundecane; cyclohexane; methylcyclopentadiene; 2,3-butanedione; 6-methyl-5-hepten-2-one; 1-decanol; eucalyptol; 2-methyl-1,3-dioxolane; limonene; menthol; camphene; menthone; galaxolide; 2,3-dimethylnapthalene; carbonyl sulphide; 3-methyl thiophene; alkenyl hexanoic acid ester; allyl methyl sulphide; dichloromethane; and N,N-dimethyl-1-dodecanamine.

A selection of one or more (e.g. all) of the following VOC biomarkers may be used to diagnose COPD in a subject: octane; nonane; 4-methylundecane; cyclohexane; methylcyclopentadiene; 6-methyl-5-hepten-2-one; 1-decanol; eucalyptol; 2-methyl-1,3-dioxolane; limonene; menthol; camphene; menthone; galaxolide; 2,3-dimethylnapthalene; 3-methyl thiophene; alkenyl hexanoic acid ester; dichloromethane; and N,N-dimethyl-1-dodecanamine.

Preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose COPD in a subject: nonane; 4-methylundecane; 1-decanol; menthol; camphene; galaxolide; 3-methyl thiophene; and N,N-dimethyl-1-dodecanamine.

In another embodiment, a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose heart failure in a subject: isoprene; hexane; 5-methylnonane; 4-methyldecane; undecane; cyclohexene; acetone; butanal; 2-methyl-2-propenal; tridecanal; ethyl acetate; 1,3-dioxolane; limonene; 3-carene; beta myrcene; ethylbenzene; 2,3-dimethylnapthalene; N,N-dimethyl-1-nonanamine; 2-methyl-2-propenal (methacrolein); alkenyl hexanoic acid ester; and decyl isobutyl ether.

A selection of one or more (e.g. all) of the following VOC biomarkers may be used to diagnose heart failure in a subject: hexane; undecane; cyclohexene; acetone; butanal; 2-methyl-2-propenal; tridecanal; ethyl acetate; 1,3-dioxolane; limonene; 3-carene;

beta myrcene; ethylbenzene; 2,3-dimethylnapthalene; N,N-dimethyl-1-nonanamine; 2-methyl-2-propenal (methacrolein); alkenyl hexanoic acid ester; and decyl isobutyl ether.

Preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose heart failure in a subject: isoprene; 5-methylnonane; 4-methyldecane; undecane; cyclohexene; butanal; 2-methyl-2-propenal; tridecanal; ethyl acetate; 1,3-dioxolane; beta myrcene; ethylbenzene; and decyl isobutyl ether.

Most preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose heart failure in a subject: undecane; cyclohexene; butanal; 2-methyl-2-propenal; tridecanal; ethyl acetate; 1,3-dioxolane; beta myrcene; ethylbenzene; and decyl isobutyl ether.

In another embodiment, a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose pneumonia in a subject: 2-methylbutane; 2,4-dimethylpentane; 2,2-dimethylpentane; hexane; octane; 2,6-dimethyloctane; diemthylundecane isomer; tetradecane; p-mentha-1,4/8-diene;1-decene; 3-buten-2-one (methyl vinyl ketone); cyclohexanone; hexanal; 2-methyl-2-propenal; 2-propanol; 1-hexadecanol; alpha-pinene; menthone; beta-phellandrene; sesquiterpenoid; xylene; carbonyl sulphide; 1-(methylthio)-1-propene; 2-methyl-2-propenal (methacrolein); 1-methylthio-propane; 4-cyanocyclohexene; methenamine; dichloromethane; and dodecylacryalte.

A selection of one or more (e.g. all) of the following VOC biomarkers may be used to diagnose pneumonia in a subject: hexane; octane; 2,6-dimethyloctane; diemthylundecane isomer; tetradecane; p-mentha-1,4/8-diene;1-decene; 3-buten-2-one (methyl vinyl ketone); hexanal; 2-methyl-2-propenal; 2-propanol; 1-hexadecanol; alpha-pinene; menthone; beta-phellandrene; sesquiterpenoid; xylene; carbonyl sulphide; 1-(methylthio)-1-propene; 2-methyl-2-propenal (methacrolein); 1-methylthio-propane; 4-cyanocyclohexene; methenamine; dichloromethane; and dodecylacryalte.

Preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose pneumonia in a subject: 2-methylbutane; 2,4-dimethylpentane; 2,2-dimethylpentane; 2,6-dimethyloctane; diemthylundecane isomer; 1-decene; 3-buten-

2-one (methyl vinyl ketone); 1-(methylthio)-1-propene; 1-methylthio-propane; and dodecylacryalte.

Most preferably a selection of one or more (e.g. all) of the following VOC biomarkers is used to diagnose pneumonia in a subject: 2,6-dimethyloctane; diemthylundecane isomer; 1-decene; 3-buten-2-one (methyl vinyl ketone); 1-(methylthio)-1-propene; 1-methylthio-propane; and dodecylacryalte.

One or more of the cardiorespiratory disease-VOC biomarkers disclosed herein (e.g. asthma-VOC biomarkers, COPD-VOC biomarkers, pneumonia-VOC biomarkers, or heart failure-VOC biomarkers) may be a selection of one or more of the biomarkers disclosed above for diagnosing a cardiorespiratory disease in a subject.

Preferably, the one or more (e.g. all) of the following VOC biomarkers is used to diagnose a cardiorespiratory disease in a subject experiencing breathlessness.

Detection of a single VOC biomarker may be used to diagnose a cardiorespiratory disease in a subject. However, it will be appreciated that the more VOC biomarkers that are used in the invention, the more reliably a cardiorespiratory disease can be diagnosed in subject. In other word, the more VOC biomarkers that are used in the invention, the higher the sensitivity and the higher the specificity of the invention. Thus, the invention may comprise detecting two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, 10 or more, 20 or more, 25 or more, 30 or more, 35 or more, 40 or more, 45 or more, 50 or more, 55 or more, 60 or more, 65 or more, 70 or more, 75 or more, 80 or more, 85 or more, 90 or more, 95 or more, or all of the VOC biomarkers disclosed herein (e.g. the biomarkers disclosed in Figure 16, or the biomarkers specific for each cardiorespiratory disease disclosed herein). Preferably, the invention comprises determining the presence of five or more, or 10 or more VOC biomarkers.

Detecting the presence of a biomarker may comprise detecting the presence, absence, or the level of the biomarkers. Detecting the presence of a biomarker may comprise the detecting of a level of the biomarker. Detecting the presence or level of a biomarker may comprise determining the concentration of the biomarker(s) in the sample.

It will be appreciated that the absence or presence and/or concentration of a VOC may be detected or determined using any suitable method/technique/technology known in the art, such as two-dimensional gas chromatography coupled with mass spectrometry (GCxGC-MS), gas chromatograph – ion mobility spectrometry (GC – IMS) technology, Gas Chromatograph (GC), Gas Chromatograph – Mass Spectrometry (GCMS), Mass Spectrometry (MS), Ion Mobility Spectrometry (IMS), Differential Mobility Spectrometry (DMS), light absorption Spectrometry, Field Asymmetric Ion Mobility Spectrometry (FAIMS), Electronic Nose, Selective-Ion Flow Tube Mass Spectrometry (SIFT-MS), Protein-transfer-reaction-MS, Optical absorbance/Non-dispersive Infra-red and gas sensors (individual or in an array). Preferably, detecting the absence, presence and/or concentration of a VOC in a sample of exhaled breath from a subject comprises two-dimensional gas chromatography coupled with mass spectrometry (GCxGC-MS). Using GCxGC-MS to detect the absence or presence and/or concentration of a VOC provides unparalleled separation of VOC biomarkers with definitive identification of VOC biomarkers.

It will be appreciated that the sample may be analysed immediately after being taken from the subject (i.e. it may be a fresh sample). The sample may be placed in a sealed container, such as a universal or a bijoux. The sample may be stored. Preferably, the sample is stored in a sealed/sealable container, such as a tube, universal or a bijoux. Preferably the container comprises/contains a sorbent material. Thus, the container may be a sealable container (e.g. tube) comprising/containing sorbent material. The sample may be stored for up to 48 hours. The sample may be stored at a temperature between about 2°C and about 8°C, or a temperature between about 3°C and about 6°C. Preferably the sample is stored at a temperature of about 4°C. Thus, the sample may be stored at a temperature between about 2°C and about 8°C, a temperature between about 2°C and about 5°C, a temperature between about 3°C and about 6°C, or at a temperature of about 4°C, for about 48 hours.

The sample may be dry purged in to reduce the water content of the sample to below 2 mg per tube. Dry purging may be performed by purging the sample with nitrogen gas. Preferably, the dry purging (e.g. dry purging using nitrogen gas) is performed within 48 hours of the sample being collected from the subject.

"selecting the subject for treatment" may refer to recording the name and/or an identifier of the subject so that a third party is aware that the subject must be treated with a therapeutic agent or composition for a cardiorespiratory disease.

The term *"recording"* can refer to fixing or storing in writing (e.g. typed) or digitally (e.g. as a video or voice recording, or on a computer).

The subject may be a person suspected of having a cardiorespiratory disease (e.g. asthma, COPD, heart failure and/or pneumonia). Preferably the subject is experiencing breathlessness. The term *"breathlessness"*, which is also known as dyspnoea, refers to difficulty breathing. This may be in the form of fast shallow breaths, noisy breathing, wheezing, or using your shoulders and/or muscles of your upper chest to help you breathe.

The *'subject'* may be a vertebrate, mammal or domestic mammal. Hence, the method according to the invention may be used to diagnose or treat any animal, for example, pigs, cats, dogs, horses, sheep or cows. Preferably, the subject is a human.

Some or all of the steps of the method of the invention may be carried out *in vitro*, *ex vivo* or *in vivo*.

The method according to the invention may comprise providing a sample obtained from a subject. Thus, the term *'sample of exhaled air/breath'* refers to gas and/or liquid exhaled by a subject, preferably gas and/or liquid (condensate) exhaled from the lungs of the subject. The sample is exhaled from the nose and/or mouth of the subject. Preferably, the sample is an exhaled gaseous sample. Thus, the method of the invention may not be performed on the subject. The amount of the sample may be an amount that provides sufficient biomarker to be measured, for example the sample may be of 500 mL to 1L.

The term *'treating'* can refer to preventing, eradicating or reducing the severity of a cardiorespiratory disease. Thus, the therapeutic agent or composition referred to herein may be any agent that prevents, eradicates or reduce the severity of asthma, COPD, heart failure or pneumonia.

The term *"comprising"* may refer to *"consisting of"* or *"consisting essentially of"*.

All of the embodiments and features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects or embodiments in any combination, unless stated otherwise with reference to a specific combinations, for example, combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show embodiments of the invention may be put into effect, reference will now be made, by way of example, to the accompanying drawings, in which:-

Figure 1 is a visual abstract representing the proposed breath testing and diagnostic pipeline. Acutely breathless patients with cardio-respiratory disease exacerbations are currently triaged on admission by means of clinical assessment, digital pathology, and blood biomarkers. Lower airway derived breath volatile organic compound biomarkers, visualised using state of the art GCxGC mass spectrometry, undergo a process of chemometric and translational modelling coupled. The resultant breath metabolic signatures, provide accurate disease classification in acute cardiorespiratory patients, with co-location of specific VOC profiles and VOC classes with individual exacerbation subgroups.

Figure 2 is a topological Data Analysis (TDA) representing the various acute disease groups annotated by blood biomarkers. Each circle or 'node' in the TDA graph represents a subject or group of subjects. Similar subjects are grouped together in the same node and the relative similarity of the subjects is represented by the proximity of the nodes and the size of each node is determined by the number of subjects within it. **(A)** Visual mapping of the acute disease groups in the discovery cohort (n=139), based on the discriminatory 805 features and coloured by proportion of acute COPD exacerbations in each node. **(B)** The network is colour coded by the average values of CRP in each node in the discovery cohort (n=139). Higher CRP values corresponded topologically with the COPD and pneumonia patients. **(C)** The network is colour coded by the average values of BNP in each node in the discovery cohort (n=139). Higher BNP values corresponded topologically with the heart failure patients. **(D)** The

network is coloured by proportion of acute COPD exacerbations in each node in the replication cohort (n=138). In replication cohort, Pneumonia and COPD exacerbation subjects occupied polar ends of the same TDA network. **(E)** The networks are coloured by the average values of CRP in each node. High CRP values corresponded topologically with the pneumonia subjects. **(F)** The networks are coloured by the average values of BNP in each node. High BNP values corresponded topologically with the heart failure subjects.

Figure 3 is: **(A)** scatter plot demonstrating significant difference between breath VOC biomarker score values in acute cardio-respiratory patients compared to healthy volunteers. The black horizontal line within the scatter plot represents the median value of the biomarker score. Mann Whitney test p-value <0.0001. **(B)** Receiver operating characteristic (ROC) curve of participants in the discovery (black line) - AUC 1.00 (1.00-1.00), and replication cohorts (blue line) - AUC 0.89 (0.82-0.95) p<0.0001. **(C)** Histogram showing the number of patients with higher diagnostic uncertainty (blue bars with values > upper quartile value of 20mm). (D) ROC curve assessing the discriminatory power of exhaled breath VOCs in participants with higher diagnostic uncertainty. AUC 0.96 (0.92-0.99) p<0.0001.

Figure 4 is: (A) a Pearson's correlation of disease-specific VOC scores and blood-based biomarkers. Pearson correlation demonstrating the positive and negative correlations between breath VOC scores and blood-based biomarkers. * Significant correlations, p-value <0.05; and (B) a Pearson's correlation of disease-specific VOC scores and admission observations. Pearson correlation between the VOC biomarker score and admission vital signs. VAS: Visual Analogue Scale (100mm), participants were asked to rate their breathlessness on a 100mm VAS on admission.

Figure 5 is: **(A)** a circular correlation tree generated based on metabolite set enrichment and chemical similarity analysis on of 101 breath volatiles associated with acute breathlessness. Branches depict metabolite sets derived using the ChemRICH **(Methods)** bar graphs portray $-\log_{10}(p)$ and $\log_2$(fold change) values of 101 features extracted using LASSO regression Figure 16 in acute breathlessness compared with control group. The arcs represent the Louvain clusters, derived from the correlation graph (green for upregulated, red for not significant, blue for downregulated according to K-S test result). Chemical names are coloured based on their chemical classification

and coloured regions used to summarise broader chemical groups; and **(B)** a correlation graph showing metabolite communities identified using Louvain clustering, with the identity and location of the cluster significantly enriched in heart failure, projected onto the circular dendrogram. **(C)** i) Example GCxGC chromatogram showing complex profile of breath metabolites, ii) 3D render of chromatogram showing visualisation of breath markers and iii) phenotypic differences based on features included in the risk scores Figure 16 (yellow, asthma; red, pneumonia; magenta, COPD; cyan, heart failure).

Figure 6 is a consort diagram outlining the acute study recruitment and number of analysable GCxGC-MS breath samples.

Figure 7 is a flow chart demonstrating the removal of exhaled breath features from 805 to 101. Least Absolute Shrinkage and Selection Operator (LASSO) and Elastic Net regularized regression models were adopted as the feature selection methods of choice owing to the high variables to subject ratio and the potential correlations among the candidate features

Figure 8 is a graphical probability distribution of the final 101 exhaled breath features in the GCxGC-MS peak table. The features largely follow a similar distribution. Some features contained a mixture of zero and non-zero values, which have arisen owing to the measurement being below the instrument's lower limit of detection. Constant features (all zero values) were removed prior to fitting the main model.

Figure 9 is a 2-dimensional visualization of the high dimensional peak table before adjustment for batch effects. Clustering by date of collection 'Batch_ID' in the first panel can be clearly seen, compared to other variables (operators, time of collection, time of wet and dry storage, and collection volume) where no batch effects are apparent.

Figure 10 is a 2-dimensional visualization of the high dimensional peak table after adjustment for date of collection 'Batch_ID'. Clustering is no longer visible following parametric empirical Bayesian adjustment.

Figure 11 is **A)** Correlation graphs showing how the breath metabolites (panel of 101) are correlated within each of the casual subgroups, coloured based on Louvain clusters to highlight differences across the networks. Visual differences highlighted include the green Louvain cluster, being highly compact in the control group and dispersed in the acute groups. **B)** Output of the ChemRICH analysis, showing metabolite sets (circles) significantly enriched during acute breathlessness (size indicative of fold change; red = upregulated; blue = downregulated). The upregulated metabolite sets with high chemical similarity (based on Tanimoto coefficient) consisted predominantly of acyclic and branched hydrocarbons, belonging to the green Louvain cluster (indicated by outer ring colour). The quantitative output of the ChemRICH analysis complements the visual differences in the graph networks

Figure 12 shows violin plots demonstrating significant differences between VOC biomarker scores values across the different disease sub-groups. * Kruskal-Wallis test comparing non-parametric data. * Significant *p value* <0.0001.

Figure 13 shows a Kaplan-Meier survival analysis. **(A)** Total of 29 patients were readmitted within 60 days of hospital discharge. **(B)** Total number of patients readmitted classified by their acute disease VOC score median value, showing no significant difference in the readmission rate based on the underlying VOC score p value of 0.77 *(log – rank test for equality f survivor function)*. **(C)** Total of (n= 12) deaths in the 2 years follow-up period **(D)** Kaplan-Meier survival analysis for all-cause 2 year mortality, classified by disease groups. **(E)** Kaplan-Meier survival analysis for all-cause 2 year mortality, classified by acute disease VOC score median value.

There was no significant difference between groups, p value of 0.07 *(log – rank test for equality f survivor function)*

Figure 14 is a graph that demonstrates the overall classification accuracy using all 5 biomarker scores.

Figure 15 is (A) a comparative ROC analysis demonstrating the diagnostic value of asthma VOC score against the predominantly infection-driven acute disease groups (pneumonia and COPD) in the pooled (discovery and replication) cohorts. **(B)**

Comparative ROC analysis demonstrating the diagnostic value of heart failure VOC score against other acute disease subgroups (asthma, COPD and pneumonia) in the pooled cohorts.

Figure 16 shows the chemical assignment of selected predictive markers from the regression model detailing chemical name, CAS registry number, KEGG, Human Metabolome Database and ChEBI identifiers and MSI-compliant metabolite identification level, concentration range and fold change (expressed as log_2) between acute and control groups, and compound contribution towards disease-specific biomarker risk scores (†adjusted p-value <0.05).

Figure 17 is a Venn diagram demonstrating the distribution of the final panel of 101 exhaled 362 breath biomarkers across the different disease groups.

EXAMPLES

Disclosed herein is a real-world, prospective study of acutely unwell hospitalised patients presenting with breathlessness due to severe exacerbations of cardio-respiratory aetiology (asthma, chronic obstructive pulmonary disease (COPD), heart failure or pneumonia) and healthy controls. It has now been demonstrated that breath biomarkers can reliably and repeatedly identify acute cardio-respiratory breathlessness; including in the presence of diagnostic uncertainty.

Methods

Trial design, participants and ethical approval

The clinical study was a prospective, real-world, observational study, carried out in a tertiary cardio-respiratory centre in Leicester, United Kingdom. Participants were recruited all year-round from May 2017 through to December 2018.

Patients with self-reported acute breathlessness, requiring admission and/or a change in baseline treatment, presenting within University Hospitals of Leicester (UHL) were approached for study participation. Following triage and senior clinical assessment, if a primary clinical diagnosis of (i) acute decompensation of heart failure, (ii) exacerbation of asthma/COPD, or (iii) adult community acquired pneumonia was suspected by the triage nurse/attending clinician at triage, members of the research team would evaluate patients against predefined eligibility criteria for study

participation. Informed consent was obtained in all participants within 24 hours of hospital admission. Age and/or home environment matched healthy volunteers were recruited. Where environment-matched controls were unsuitable, healthy volunteers were recruited from local recruitment databases and via advertising. Further details of healthy volunteers' comorbidities and medication use are outlined in **Table 1**.

The trial was conducted in accordance with the ethics and principles of the deceleration of Helsinki and Good Clinical Practice Guidelines. All patients provided written consent. The National Research Ethics Service Committee East Midlands has approved the study protocol (REC number: 16/LO/1747). Integrated Research Approval System (IRAS) 198921.

Table 1: *demonstrates comorbidities and medications used by study participants, classified by disease and health. Values expressed as N (%). Table includes comorbidities occurring in >5% of participants and medications used by >5% of participants.*

Comorbidities	**Healthy controls N (%)**	**Acute disease group N (%)**
Anxiety/Depression	5 (9)	26 (11.7)
Diabetes Mellitus	4 (7.2)	45 (20)
Essential hypertension	16 (29)	58 (26)
Ischaemic heart disease	4 (7.2)	13 (5.8)
Arthritis	5 (9)	12 (5.4)
Thyroid disorder	3 (5)	15 (6.7)
COPD	0(0)	58(26)
Asthma	0(0)	65(29)
Heart failure	0(0)	44(19.8)
Medications		
Inhaled therapies		

Salbutamol	1 (1.8)	153 (68)
ICS/LABA	0 (0)	53 (23)
Lipid lowering agents		
Atrovastatin	9 (16)	57 (25.6)
Simvastatin	7 (12)	23 (10)
GORD medications		
Lansoprazole	9 (16)	49 (22)
Blood pressure lowering agents		
Amlodipine	5 (9)	25 (11.2)
Lisinopril	4 (7)	11 (5)
Ramipril	8 (14)	37 (16.6)
Antidepressants		
Citalopram	3 (5)	13 (6)
Sertraline	3 (5)	15 (6.7)
Thyroid medications		
Levothyroxine	5 (9)	15 (6.7)
Cardiac medications		
Aspirin	5 (9)	36 (16.2)
Analgesics		
Paracetamol	6 (10)	103 (46)

Recruitment started in February 2017 and following analytical method development and optimisation of a robust sample pathway for achieving continual deployment, collection and analysis of sorbent tubes to-and-from clinic, the analysis of samples by GCxGC-MS was set up and brought online later that year (August 2017). The denominator for the entire study was 455 participants and for the GCxGC-MS study presented here is 363 participants, with a 76% GCxGC-MS completion rate (Figure 6).

A detailed Survival analysis of study participants is demonstrated in **Figure 13.**

The trial was conducted in accordance with the ethics and principles of the deceleration of Helsinki and Good Clinical Practice Guidelines. All patients provided written consent. The National Research Ethics Service Committee East Midlands has approved the study protocol (REC number: 16/LO/1747). Integrated Research Approval System (IRAS) 198921.

Clinical adjudication

A clinical adjudication process was introduced to precisely define and quantify the diagnostic labels in the study, addressing any potential misclassification. A panel of two senior clinical adjudicators (SS & NG) reviewed all available case notes, imaging and determined the primary diagnosis for each case by discussion to reach a concordance. The degree of diagnostic uncertainty was marked on a 100 mm visual analogue scale (VAS scale), blinded to given diagnosis and blood biomarkers.

The process was implemented with emphasis on mirroring an acute triage pathway, where all pathology data required to support the diagnosis e.g. CRP, BNP are not available at the initial clinical review.

The degree of diagnostic uncertainty obtained from the clinical adjudication process was factored into the block randomisation and subjects with higher diagnostic uncertainty (≥upper quartile = 20mm) were assessed separately as previously described **(Figure 3c-d).**

Collection of breath samples

Exhaled breath collection was attempted in all consented participants using a CE marked breath sampling device 'Respiration Collector for *In Vitro* Analysis' RECIVA® (Owlstone Nanotech Ltd), in combination with a dedicated clean air supply unit. The ReCIVA® device aims to standardise the collection of alveolar breath by providing the patient with a VOC-clean air supply; controlling the flow, volume and fraction of breath collected, while directly sampling the exhaled VOCs onto the sorbent tubes. The ReCIVA® settings mode was set to 'lower airways only', the continuous monitoring of the CO_2 and partial pressure allowed targeting the VOC-

enriched alveolar fraction of breath. The collection volume, flow rate and maximum sampling time were set to 1 L, 250 mL min^{-1}, and 900 seconds respectively. Breath sampling was well tolerated by all participants.

At the time of sampling, the room air and air supply were also sampled as environmental controls. This involved attaching a sorbent tube to a handheld personal pump (Escort Elf, Sigma Aldrich, Dorset, UK) and having the sampling end either open to the room air or attached to the ReCIVA® air supply line via a T-piece. 1 L of air was collected in total at a flow rate of 0.5 L min^{-1} for 2 min. Sorbent tubes were immediately capped (brass caps, Markes International Ltd) and placed in a fridge at 4 °C before being dispatched to the laboratory within 72 h. In an attempt to minimise background variation, sample collection was completed, when possible, in the same treatment room attached to the admissions ward. Unwell patients and those requiring supplemental oxygen, however, had their samples collected by their bedside.

Sample storage and preparation

Samples were dry purged on arrival for 2 min using nitrogen (CP grade with inline trap, BOC, Leicester, UK) at a flow rate of 50 mL min^{-1} and then stored in the fridge at 2 °C until analysed. Before analysis, samples were left to reach room temperature before being spiked with a 0.6 µL aliquot of 20 µg mL^{-1} standard solution containing deuterated toluene and octane, into a flow of nitrogen at a flow rate of 100 mL min^{-1} for 2 min, purging the excess solvent.

Analysis of Room Air and Air Supply samples

Two separate elastic net regression models were fitted to peak tables for room air and air supply samples, both peak tables where $log_e(x+1)$ transformed and adjusted for batch effects (collection date) using PEBA. The independent variables were the final set of 101 features and the dependent variable was clinical diagnosis (Acute Asthma, Acute COPD, Pneumonia, Heart Failure or Healthy volunteers). After repeating 10-fold cross validation 100 times for each of the two models, only two features were found to have stable non-zero regression coefficients. These features were for air supply, a component of the pneumonia score and for room air, a component of the healthy score, highlighting the robustness of the selected feature separation models.

Exhaled Breath analysis

TD-GCxGC-FID/MS

Breath samples were analysed by thermal desorption with comprehensive two-dimensional gas chromatography (GCxGC) using flow modulation and coupled to dual flame ionisation detection and mass spectrometry (MS). Dual detection, with the use of MS and flame ionisation detection (FID), utilises the excess flow from the flow-based modulator suited for volatile analyses, providing both quantitative and qualitative results.

Analysis by GC×GC was optimised and conducted using an Agilent 7890A gas chromatogram, fitted with a CFT flow modulator and 5799B mass spectrometer with a high efficiency EI ion source (Agilent Technologies Ltd, Stockport, UK). The instrument was coupled to a TD-100xr thermal desorption auto-sampler (Markes International Ltd, Llantrisant, UK). Samples were analysed in trays; typically six per tray along with a reference mixture containing n-alkanes and aromatics run every tray and a reference indoor air VOC mixture run every four trays. Data was acquired in MassHunter GC-MS Acquisition B.07.04.2260 (Agilent) and processed (i.e. baseline correction, alignment, feature extraction) with a workflow previously developed and optimised, using GC Image™ v2.8 suite (GC Image, LLC. Lincoln, NE, US) and Python. The sorbent tubes used were Tenax/TA with Carbograph 1TD (Hydrophobic, Markes International Ltd) with matching cold trap. Chromatographic features arising from analytical artifacts were removed from the peak table (e.g. ubiquitous siloxanes).

For purposes of quality control, samples were analysed using a detailed sample history, metadata and experimental data were recorded at every stage of the collection and analysis using the open-access LabPipe toolkit.

Chemical speciation of identified breath biomarkers

The chemical nature of volatile metabolites exhaled in breath comprises a diverse mixture of non-novel, low-molecular weight compounds. Thus, for the majority of features, chemical identification involved comparison with an authentic reference compound in accordance with the Metabolomics Standard Initiative (MSI) Level 1 criteria for metabolite identification **(Figure 16).** Identification was based on a minimum of two independent and orthogonal identifiers including primary and secondary retention time, mass spectral similarity match and calculated retention

index. When an authentic reference compound was unavailable, chemical identification was compliant with MSI Level 2 for putative annotations. The highly structured chromatographic data and group-type separation afforded by GCxGC, alongside a well-characterised chromatographic space from analysing an extensive library of authentic compounds, gave increased confidence in the tentative assignments made. The orthogonal separation of GCxGC also meant chemical identification of unknown metabolites could be made, at minimum, in compliance with MSI Level 3 for putative chemical classification.

The diagnostic accuracy of the reported exhaled breath VOCs was tested following the Standards for reporting of Diagnostic Accuracy Studies guidelines; and for multivariate prediction models, Transparent Reporting of multivariate prediction model for Individual Prognosis or Diagnosis (TRIPOD) was followed.

Quality control and quality assurance systems

A number of traceable and verifiable quality control and quality assurance (QC/QA) procedures have been applied throughout the breath sampling and analysis steps. This ensured efficient prevention of any anticipated defects and high deliverable standards. In order to eliminate any samples from the final analysis that were of poor quality four criteria were used to selected for high quality breath samples. These were:

1. ≥800 mL of breath collected from the patient to ensure sufficient pre-concentration of trace VOCs present in breath.
2. The concentration of isoprene and acetone in the air supply were ≤ 3 standard deviations of the mean air supply concentration. This ensured that no breath samples were mis-assigned as air supply samples.
3. The concentration of isoprene and acetone in breath were ≥10 and ≥ 5 standard deviations, respectively, above the levels measured in the patient air supply. This ensured that the samples were not mis-assigned air supply samples, and that breath had been collected onto the sorbent tubes
4. The chromatogram, on visual review, was not distorted by an abundance of exogenous compounds (i.e. overloaded peaks).

The number of breath samples fulfilling all QC/QA criteria is outlined in **(Figure 6).**

Sample analysis QC/QA procedures

For purposes of quality control, samples were analysed in accordance with a previously published workflow and a detailed sample history, metadata and experimental data were recorded at every stage of the collection and analysis using the open-access LabPipe toolkit. The chromatographic method was optimised for peak shape, sensitivity and separation; quality control charts of the internal standards were used to track the stability of the TD-GCxGC-FID/MS analysis, and instrument performance was evaluated following the assessment of the variation of retention times, peak area and shape of VOCs in two standard reference mixtures every six samples. Before being conditioned and sent to clinic, the number of heat cycles and weight for each tube was recorded to monitor tube age and integrity. For each conditioning cycle, all tubes were given a batch number and a batch blank was analysed to monitor contamination from the beginning of the sample preparation process. Furthermore, all batches were given an expiry of two weeks to ensure routine monitoring. To minimise the influence of biological and analytical confounders (e.g. circadian rhythm, sample stability), potential effects due to the operator, date of analysis, time of day collected, storage time before dry purging, sample storage time after dry purging and collection volume were assessed and where necessary accounted for in the batch correction. In addition to the routine analysis of reference standards, used to monitor retention shift and instrument response, the TD-GCxGC analytical system underwent a programmed heat cycle between each sample to reduce potential issues arising from sample carry-over, and a TD-trap blank and empty sorbent tube were analysed every six samples to monitor the instrument baseline signal.

Statistical procedures

Statistical analysis was performed using R (3.6.1 and 4.0.0, R Core Team (2019). This research used the SPECTRE High Performance Computing Facility at the University of Leicester. Baseline data and figures were presented as mean ± (SD), and median (IQ range). Data was analysed using (ANOVA) to assess the differences between groups for normally or approximately normally-distributed variables and Kruskal-Wallis for non-normally distributed variables. Pearson chi-squared and Fisher's exact were used to assess the differences in categorical variables. All *P* values are two sided and significant at the 0.05 level, unless reported otherwise. Study sample size calculations were informed based on sample size estimation for adequate sensitivity and or specificity **(Sample size estimation section).**

Discovery and replication sets

The 277 subjects were randomised *post-hoc* to Discovery and Replication cohorts in a 1:1 ratio through block random assignment. Randomisation was stratified based on (I) adjudicated clinical diagnosis, (II) time to breath-testing from the point of hospital admission, and (III) clinical diagnostic uncertainty score. The R package randomizer was used to perform block random assignment. After block randomisation there were 139 and 138 subjects in the discovery and replication sets respectively.

Examination of topological equivalence in the discovery and replication sets

Topological data analysis is an unsupervised machine-learning tool used for the analysis of large-scale, high-dimensional, complex datasets. It is highly sensitive to patterns that are often overlooked by other data reduction tools like Principal Component Analysis (PCA).

TDA captures the shape of data and provides a meaningful geometric representation where complex relationships within the data points are preserved and jointly considered.

Prior to performing TDA each feature was $log_e(x+1)$ transformed. TDA parameters were set as: number of hypercubes=20, where the number of hypercubes refers to the number of overlapping intervals of the projection. The distance between data points was measured using the Euclidean distance. The first two linear discriminant functions (LD1) and (LD2) were used as the projection. Clustering on the overlapping intervals on the projection was done using agglomerative (bottom up) hierarchical clustering with complete linkage. TDA was performed using Kepler Mapper 1.4.0 with Python 3.5.

Herein, the equivalence between topological data shapes generated using 805 volatile features extracted from the GCxGC-MS peak table was computed, in both the discovery and replication cohorts **(Figure 2).**

Exhaled breath feature selection

Feature selection was implemented via Lasso and Elastic-Net Regularized Generalized Linear Models (GLMNET) using the glmnet package in R. After removing features present in <80% of all samples from the $log_e(x+1)$ transformed discovery GCxGC-

MS peak table, 735 feature matrix was obtained. A multinomial regression model using LASSO regularization was fitted to the 735 feature matrix in the discovery set using 10 fold cross validation, with the dependent variable in the model being clinical diagnosis (Acute Asthma, Acute COPD, Pneumonia, Heart Failure or Healthy volunteers). The 10-fold cross validation was repeated 100 times, features that had a non-zero regression coefficient in more than 80 of the cross validation runs were considered as being stable candidate features predictive of the outcome (clinical diagnosis), and this resulted in 278 stable candidate features.

A multinomial regression model using elastic net regularization was fitted to the 278 features with the dependent variable in the model being clinical diagnosis. Following the chemometric inspection detailed above and the lasso and elastic regression analysis, a final set of 101 exhaled breath volatile compounds was generated **(Figure 7).**

A multinomial regression model using elastic net regularization was fitted to the matrix of 101 breath biomarkers with the 10-fold cross validation repeated 100 times. The R package glmnetUtils was used to determine the optimal value of α the elastic net penalty, the best value for α was 0 (Ridge regression). Linear combinations of the most stable features from the multinomial regression model fitted to the 101 biomarkers formed a set of scores for predicting probability of belonging to the different disease groups (acute Asthma, acute COPD, pneumonia, heart failure or healthy volunteers). Ridge regression with a logit link function (binary logistic regression) was fitted to the 101 breath relevant features, the dependent variable was 'acute disease', as a binary outcome. The linear predictor from the combination of the most stable features was used to as a score to predict acute disease.

Co expression and feature enrichment analysis

It was of interest to investigate if within the final set of 101 features, sets of 'co expressed' features existed, i.e. sets containing features that are correlated. Considering sets of co-expressed features has value in terms of reducing the dimensions of a problem and mitigating the multiple testing problem through the use of enrichment score. Co expression and feature enrichment analysis are described in the **(Supplementary Information).**

Metabolite sets were derived based on Ward hierarchical cluster analysis using the ChemRICH method **(Figure 5A),** and more broader communities were derived from Louvain cluster analysis to help interpret the correlation graphs **(Figure 5B, see Supplementary Information section on co-expression and feature enrichment analysis).** Covariation among metabolites lacks evidential value on its own, therefore, set-level significance was established using the Kolmogorov-Smirnov test (K-S test) using the ChemRICH method, Tanimoto coefficients were calculated to asses intra-set chemical similarity using Metabox, and the frequency of occurrence in the published literature and relevant databases considered (KEGG, ChEBI, Human Metabolome Database, Human Breathomics Database and microbial VOC database). Chemical similarity is of interest because compounds derived from similar pathways may also share common structural features or chemical groups. This combined data-driven and chemistry-driven approach has been shown to improve enrichment analysis and allowed further interpretation core findings herein **(Figure 11)**.

Supplementary Information (SI)

Probability distributions of breath features (biomarkers):

The features in the GCxGC peak table fell into 3 broad categories: (1) constant features (all samples had a value of zero), (2) features that contained a mixture of zero and non-zero values, and (3) features that contained all non-zero values. The zero values have arisen owing to the measurement being below the instrument's lower limit of detection. Constant features were removed prior to fitting the main model.

Graphical distribution of the final 101 features (biomarkers), mainly falling into type 2 and 3 categories is illustrated in **(Figure 8).** For certain features the spike in the 0 values can be clearly seen. Based on these observations a reasonable choice for a theoretical model for the probability distribution of a feature from a GCxGC-MS peak table might be the Zero Modified Log Normal distribution.

Mitigating the adverse impact of batch effects in biomarker pattern detection

Batch effect is a common issue in omics data analysis. The existence of batch effects makes it challenging to compare data collected and analysed at different processing times **(Figures 9 & 10).**

The following factors were investigated as possible contributing batch variation factors:

I. Batch_ID - date of sample collection:

(1) Batch 1 – August 2017 - October 2017

(2) Batch 2 – November 2017 - March 2018

(3) Batch 3 – April 2018 – December 2018

II. Operator: (N: 1-6) – indicating members of the study team operating the RECIVA over the entire course of the sampling program

III. Time of the day sample was collected (circadian rhythm):

(1) 1 = between 9-11am

(2) 2 = between 11am-1pm

(3) 3 = between 1-3pm

(4) 4 = between 3-5pm

IV. Time sample stored wet

(1) 1 = 0-2 days

(2) 2 = 2-5 days

(3) 3 = 5-10 days

(4) 4 = 10-20 days

(5) 5 = 20-42 days

(6) 6 = over 42 days

V. Time stored dry (following dry purging)

(1) 1 = 0-2 days

(2) 2 = 2-5 days

(3) 3 = 5-10 days

(4) 4 = 10-20 days

(5) 5 = 20-42 days

(6) 6 = over 42 days

VI. Volume of breath collected (over 80% threshold):

(1) 1 = 100%

(2) 2 = 90-99%

(3) 3 = 80-89%

Figure 9 is a visualization of the GCxGC-MS peak table comprising all 805 features using t Stochastic Nearest Neighbor Embedding (tSNE). Clustering due to 'date of collection' was seen (top left plot). No obvious clustering seemed to be present for the remaining factors. The effect collection date was adjusted for by applying Parametric Empirical Bayesian Adjustment (PEBA). The ComBat function from the SVA package for Bioconductor was used to perform PEBA. The results of this adjustment are shown in **(Figure 10).** It can be seen that the clustering due to collection date is no longer apparent. The batch effect adjusted peak table was used in all subsequent feature selection models.

Model Accuracy

The overall classification accuracy for the statistical model using all five biomarker scores from the final set of 101 exhaled breath features was assessed by comparing the balanced accuracy of model trained using the true class labels versus the balanced accuracy of the same model tested using randomly shuffled class labels. This process was repeated 1000 times. The overall classification accuracy using all five biomarker scores was 0.722, 95% CI (0.6653 - 0.774) and the results demonstrated in **Figure 14.**

Chemical speciation of identified breath biomarkers

In order to confirm to the chemical identity of the concatenated list of 101 exhaled breath peaks, a standard reference compounds, where available, were purchased and analysed. This included a C8-C20 saturated alkanes certified reference material (Sigma Aldrich, Dorset, UK), an aromatics calibration standard (NJDEP EPH 10/08 Rev.2, Thames Restek, Saunderton, UK), a multi-component indoor air standard (Sigma Aldrich, Dorset, UK), two terpene reference mixtures (Spex Centriprep, Emerald Scientific, San Luis Obispo, US), and individual standards from Sigma Aldrich (Merck Life Sciences), Greyhound Chromatography, Scientific Lab Supplies, Alfa Chemicals and Santa Cruz Biotechnology.

Figure 16 lists the chemical assignment of the selected predictive markers from the regression model detailing chemical name, CAS registry number, KEGG, Human Metabolome Database and ChEBI identifiers and MSI-compliant metabolite identification level, concentration range and fold change (expressed as log_2) between acute and control groups, and compound contribution towards disease-specific biomarker risk scores (†adjusted p-value <0.05).

Sample size estimation

In the study protocol the aim was to recruit 550 subjects, had 550 subjects been recruited then we would be powered to identify sensitive biomarkers (≥ 80%) of acute breathlessness with a maximum marginal error in the estimate for sensitivity not exceeding 5% with 95% confidence. Similarly, we are powered to identify specific biomarkers (≥ 80%) of acute breathlessness with a maximum marginal error in the estimate for specificity not exceeding 5% with 80% confidence, however we have achieved a total sample size of n=277.

Based on a total sample size of n=277 post hoc sample size calculations were performed using a sensitivity of 70% and 80% with ± (10% ,15% and 20% precision) for obtaining a biomarker capable of 'ruling out' an acute disease class. The same targets were applied to specificity. Calculations were performed for using a 95% confidence level.

It was assumed an 80% acute disease prevalence for recruitment and 1:5 patients recruited were non-breathless healthy controls **(Table 2)**. It was acknowledged that the assumption of an 80% acute disease prevalence places a limitation on the validity of the sample size calculations, however the estimate of 80% prevalence is not unreasonable based on clinical expectation.

Table 2*: demonstrates that the sample sizes in discovery (n=139) and replication (n=138) are sufficient to identify sensitive and specific biomarkers (≥ 70%) of acute breathlessness with a maximum marginal error in the estimate for sensitivity not exceeding 20% (95% confidence). Similarly, from Table 2 the sample sizes in discovery and replication are sufficient to identify sensitive and specific biomarkers*

(≥ 80%) of acute breathlessness with a maximum marginal error in the estimate for specificity not exceeding 15% (95% confidence).

	Confidence Level	Marginal Error	Prevalence of Acute Breathlessness	Sample Size Required
Sensitivity (70%)	95%	10%	80%	100
Specificity (70%)	95%	10%	80%	403
Sensitivity (70%)	95%	15%	80%	45
Specificity (70%)	95%	15%	80%	180
Sensitivity (70%)	95%	20%	80%	25
Specificity (70%)	95%	20%	80%	100
Sensitivity (80%	95%	10%	80%	77
Specificity (80%)	95%	10%	80%	307
Sensitivity (80%)	95%	15%	80%	34
Specificity (80%	95%	15%	80%	137
Sensitivity (80%)	95%	20%	80%	19
Specificity (80%)	95%	20%	80%	77

Co expression and feature enrichment analysis

Graph construction and Cluster Analysis

Subjects from both the Discovery and Replication sets were combined into a data matrix $\mathcal{M}_D$ comprising the 101 features that were obtained from previous regression analysis, with healthy subjects excluded. The Spearman rank correlation matrix was calculated for the data matrix $\mathcal{M}_D$.

A scale free graph g was constructed by generating the adjacency matrix$\mathcal{M}_{Adj} = \left|\hat{C}\right|^{\beta}$.

Where $\hat{C}$ is the sample correlation matrix of$\mathcal{M}_D$, and $\beta \geq 1$.

The pickSoftThreshold function from the WGCNA package in R was used to estimateβ. The igraph package in R was used to construct g using$\mathcal{M}_{Adj}$, g is a weighted and unsigned graph. The graph g will be referred to as the "correlation graph".

Louvain clustering was then performed on the correlation graph and 8 feature sets were obtained.

The 8 feature sets obtained from Louvain clustering on correlation graph were used in an enrichment analysis. Instead of considering individual features and how they might distinguish different disease groups, sets of features are considered, the idea being that features in combination may have better discriminatory capability. The bioconductor (version 3.12) packages GSVA and limma were used to perform enrichment analysis. Feature set 3 was found to be enriched in Asthma and HF, feature set 5 was found to be enriched in HF alone, see Tables 3-6. The enriched feature sets 3 and 5 did not demonstrate improved diagnostic accuracy over the scores obtained from regression analysis.

Table 3*: Demonstrates the results of the enrichment analysis performed in the asthma group using the 8 feature sets obtained from the Louvain clustering on the correlation graph (Figure S9)*

	logFC	AveExpr	t	P.Value	adj.P.Val	B
Set 3	0.133890616	0.004263125	2.879346419	0.004319299	0.034554392	-2.142608513
Set 1	-0.120196744	-0.011684816	-1.854952871	0.064743701	0.258974804	-4.369194843
Set 7	0.061793373	0.015703055	1.157435072	0.248165085	0.544802368	-5.347558772
Set 5	-0.060241661	-0.027387588	-0.996694751	0.31984657	0.544802368	-5.50959215
Set 6	-0.044261401	-0.018231244	-0.830155612	0.407218311	0.544802368	-5.652155219
Set 2	0.042555754	0.007308014	0.827706348	0.408601776	0.544802368	-5.65405913
Set	-	0.012747522	-	0.48408697	0.55324225	-5.74507753

4	0.035370385		0.700755726	4	6	
Set 8	0.007185736	0.005059851	0.128594967	0.897778335	0.897778335	-5.967968995

Table 4*: Feature enrichment in COPD using 8 features sets obtained by Louvain clustering on the correlation graph.*

	logFC	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**
Set3	-0.090944102	0.004263125	-1.847462511	0.065824888	0.31930245	-4.015728578
Set5	-0.101063794	-0.027387588	-1.579494626	0.115447869	0.31930245	-4.359177814
Set6	0.081933094	0.018231244	1.451613602	0.147823971	0.31930245	-4.504601143
Set1	0.096742783	0.011684816	1.410314835	0.159651225	0.31930245	-4.548997592
Set4	0.058154903	0.012747522	1.08835486	0.277454107	0.443926572	-4.851845137
Set2	-0.043701593	0.007308014	-0.802920566	0.422759696	0.489518896	-5.055725798
Set7	0.044836139	0.015703055	0.793305124	0.428329034	0.489518896	-5.061530212
Set8	0.002536665	0.005059851	0.042881813	0.965828915	0.965828915	-5.299201629

Table 5*: feature enrichment in heart failure using 8 features sets obtained by Louvain clustering on the correlation graph.*

	logFC	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**
Set3	-0.16062091	0.004263125	-2.841944019	0.004842214	0.032792407	-2.229765975
Set5	0.195734539	-0.027387588	2.664418025	0.008198102	0.032792407	-2.6741005
Set2	0.147456886	0.007308014	2.359677827	0.019036076	0.050762869	-3.37356668
Set1	0.087558967	0.011684816	1.111758525	0.267276909	0.534553819	-5.37643629
Set6	-0.054112112	0.018231244	-0.835023125	0.404477256	0.64716361	-5.628009236
Set7	-0.040272554	0.015703055	-0.620631141	0.535390206	0.711290217	-5.773994793

Set4	0.028289272	0.012747522	0.461124606	0.645097706	0.711290217	-5.85478987
Set8	-0.025165368	0.005059851	-0.370531932	0.711290217	0.711290217	-5.890086764

Table 6*: feature enrichment in Pneumonia using 8 features sets obtained by Louvain clustering on the correlation graph.*

	logFC	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**
Set2	-0.092675555	0.007308014	-1.658089062	0.098514794	0.350018518	-4.257598246
Set3	0.083374576	0.004263125	1.6493092	0.100301314	0.350018518	-4.268325935
Set6	0.082784378	0.018231244	1.428260455	0.154426336	0.350018518	-4.520076707
Set5	-0.08936284	-0.027387588	-1.36002492	0.175009259	0.350018518	-4.590632855
Set8	0.029388432	0.005059851	0.483786536	0.628947737	0.86559581	-5.192394684
Set7	-0.024708996	0.015703055	-0.425730243	0.670659348	0.86559581	-5.212141706
Set1	0.017148026	0.011684816	0.243432731	0.807863636	0.86559581	-5.257780971
Set4	0.009296591	0.012747522	0.169424129	0.86559581	0.86559581	-5.269216827

Example 1 – Overview

Exhaled breath from 277 participants, recruited from acutely breathless hospitalised patients and matched healthy controls, was sampled and analysed to identify dysregulation of metabolic classes in cardio-respiratory disease and investigate whether exhaled VOC profiles could predict acute cardio-respiratory exacerbations despite diagnostic uncertainty, and thus have a potential role in phenotyping acute cardio-respiratory breathlessness.

Participants' mean (SD) age was 60.8 ± (16.8) years, 51% were males, 30 patients required supplemental oxygen on admission and the mean admission modified early warning score (mEWS-2 score) was 2. The cohort was made up of patients presenting with the following exacerbation subtypes; acute severe asthma (n= 65), acute severe

COPD (n= 58), acute severe heart failure (n=44), community acquired pneumonia (n=55), and healthy volunteers (n=55), recruited between May 2017 and December 2018 **(Figure 6)**. Participants' demographic and clinical characteristics are summarised in **Table 7**. Breath samples were collected using a ReCIVA© device, adopting a standardised sampling and gated protocol that enriches alveolar volatiles, and analysed using thermal desorption (TD) coupled to comprehensive two-dimensional gas chromatography (GCxGC) with dual flame ionisation detection (FID) and mass spectrometry (MS) **(Figure 1 and Methods).**

Table 7*: Demographics and clinical characteristics of study participants.*

	Total no	**Healthy controls**	**Acute asthma**	**Acute COPD**	**Pneumonia**	**Heart failure**	**p value**
Total no of participants (n=)	**277**	**55**	**65**	**58**	**55**	**44**	
Demographics							
Age * , years	60.8 ± (16.8)	63.05 ± (11.78)	44.3 ± (17.93)	69.82 ± (8.16)	60.67 ± (16.50)	70.72 ± (11.04)	.124
Gender Male (n=) (%)	143 (51%)	26 (47%)	25 (38%)	33 (56%)	27 (49%)	32 (72%)	**.008 ¥**
Body Mass Index (BMI)*[a]	29.5 ± (7.3)	28.2 ± (4.5)	31.5 ± (9.0)	27.5 ± (7.7)	29.2 ± (6.9)	31.5 ± (6.5)	.767
Smoking Current smoker (n=) (%)	53 (19%)	4 (7%)	13 (20%)	21 (36%)	11 (20%)	4 (9%)	**.001 ¥**
Vital signs							
Temperature (Celsius)*	36.7 ± (0.6)	36.1 ± (0.4)	36.8 ± (0.5)	36.7 ± (0.5)	37.1 ± (0.7)	36.5 ± (0.3)	**.000**
Heart rate (beats/min)*	87.2 ± (18.5)	68.1 ± (9.54)	99.6 ± (17.2)	92.9 ± (15.6)	90.3 ± (15.4)	81.3 ± (15.6)	**.005**
Respiratory rate (breaths/min)*	18.9 ± (4.2)	13.0 ± (1.8)	20.5 ± (3.4)	21 ± (2.5)	20.4 ± (4.6)	19.1 ± (1.8)	**.000**
Oxygen saturations (%)*	95.8 ± (3.0)	97.7 ± (1.3)	96.1 ± (2.5)	94.0 ± (2.9)	94.5 ± (0.5)	96.5 ± (1.9)	**.001**
Systolic Blood Pressure (mmHg)*	131.5 ± (19.2)	134 ± (15.7)	133 ± (17.7)	133 ± (20.5)	126 ± (19.4)	128 ± (22.2)	.515
Total mEWS-2	1 (0-	0 (0-1)	2 (1-	3 (1-5)	2 (1-3)	1 (0-2)	.000

score [^b]	3)		3.5)				
Symptoms assessment							
Breathlessness VAS score (mm)*[c]	58.1 ± (31.6)	6.2 ± (9.3)	76.6 ± (14.2)	71.6 ± (19.2)	67.8 ± (22.1)	67.9 ± (20.0)	**.000****
Cough VAS score (mm) *[c]	43.3 ± (33.2)	8.7 ± (14.3)	64.5 ± (26.7)	57.8 ± (27.0)	53.6 ± (30.6)	24.3 ± (25.2)	**.000****
Wheeze VAS score (mm) *[c]	41.8 ± (34.9)	3.4 ± (6.4)	66.2 ± (24.5)	60.3 ± (29.0)	45.1 ± (34.8)	28.1 ± (28.6)	**.000****
eMRC[d] score (n=) (%)							
1	17 (6%)		1 (1.5%)	8 (13%)	7 (12%)	1 (2%)	**.000¥**
2	6 (2%)		0 (0%)	0 (0%)	5 (9%)	1 (2%)	**.000¥**
3	15 (5%)		6 (10%)	0 (0%)	7 (12%)	2 (4.5%)	**.000¥**
4	50 (18%)		16 (25%)	11 (19%)	6 (11%)	17 (38.5%)	**.000¥**
5a	112 (40%)		38 (51%)	32 (55%)	22 (41%)	20 (46%)	**.000¥**
5b	21 (7%)		3 (4.5%)	7 (13%)	8 (15%)	3 (7%)	**.000¥**
Exposure to antibiotics and steroids within 2 weeks of hospital admission							
Antibiotics (n=) (%)	61	n=0 (0%)	n=24 (36.9%)	n=23 (39.6%)	n=10 (18.2%)	n=4 (9.0%)	**.002¥**
Steroids (n=) (%)	57	n=0 (0%)	n=28 (43.0%)	n=24 (41.3%)	n=3 (5.4%)	n=2 (4.5%)	**.000¥**
Morbidity and mortality measures							
Length of hospital stay	3 (2-6)		2.0 (1.0-	4.0 (2.0-	4.0 (2.0-5.0)	7.0 (4.0-11)	.000**

(days) ^			3.0)	6.0)			
30-60 days hospital readmission (n=)	29		7	9	6	7	.461¥
1 year all-cause mortality	12	0	1	5	1	5	.078¥
Laboratory parameters							
C-reactive protein (CRP) (mg/L)^	11 (5.0-34.2)	5 (5-5)	10.0 (5.0-23.0)	12.0 (5.0-20.7)	108.0 (53.5-245.3)	11.0 (5.0-22.0)	**.000****
Blood Eosinophil count 10^9/L^	0.13 (0.06-0.24)	0.17 (0.09-0.24)	0.18 (0.06-0.42)	0.13 (0.06-0.24)	0.08 (0.04-0.14)	0.13 (0.08-0.23)	**.000****
Troponin T (ng/l)^	3.3 (1.0-11.4)	2.05 (1.0-2.7)	1.55 (1.0-3.4)	3.75 (2.6-10.9)	4.3 (2.18-11.3)	20.2 (13.4-59.6)	**.000****
Brain natriuretic peptide (BNP) (ng/l)^	40.5 (20.6-98.9)	28.40 (17.60-39.88)	20.4 (12.1-40.0)	56.3 (24.3-95.0)	56.3 (27.4-132.1)	611.8 (172.1-1259.1)	**.000****
Questionnaires							
Asthma Quality of Life Questionnaire (AQLQ) total*	65		117.3 ± (37.3)				
COPD Assessment test (CAT) *	58			26.7 ± (7.3)			
COPD Decaf score *	58			1.7 ± (0.8)			
CURB65 score^	55				2 (1-3)		
NYHA score^	44					2 (1-3)	

Continuous variables are presented as mean ± standard deviation. Categorical variables are presented as numbers (%).

[a] The body mass index (BMI) is the weight in kilograms divided by the square of the height in meters.

[b] Modified Early warning score - 2 (MEWS-2) is a guide widely used by medical services to determine the degree of illness of a patient based on their vital signs including respiratory rate, oxygen saturations, temperature, blood pressure, and heart rate. Vital signs collected at the point of admission for acute disease groups.

[c] Participants were asked to determine their degree of breathlessness, cough and wheeze on a 100mm visual analogue scale (VAS) on admission. Higher scores indicate worse symptoms.
[d] Extended Medical research Council (eMRC) scale is a validated measure of perceived respiratory disability, scored from 1 to 5b. Higher scores indicate worse disability.
* Data is expressed as mean (SD) or n (%) ± (SD), ^ Data expressed as median (IQ range), ** Kruskal-Wallis test comparing non-parametric data, ¥ Pearson Chi Squared and Fisher's Exact test.
ANOVA was used to assess the differences between groups for normally distributed continuous variables and kruskal-Wallis for non-parametric continuous variables. Pearson chi-squared and Fisher's exact were used to assess the differences in categorical variables. The results were considered statistically significant at p-values <0.05.

Example 2 - Unbiased discovery using topological data analysis identifies breath markers of acute disease

To achieve an unbiased discovery of exhaled VOCs predictive of the acute disease groups, patients were block randomised *post-hoc* into a discovery cohort of 139 participants (acute asthma n= 33, acute COPD n= 29, acute heart failure n=22, community acquired pneumonia n=28, healthy volunteers n=27), and a replication cohort of 138 participants (acute asthma n= 32, acute COPD n= 29, acute heart failure n=22, community acquired pneumonia n=27, healthy volunteers n=28). Randomisation allowed internal replication of diagnostic breath biomarkers, whilst adjusting for relevant confounders. Details of the randomisation and further clinical characteristics of the cohorts can be found in **Methods and Tables 1 and 8.** Chemometric analysis and quantification of VOCs was performed blinded to clinical diagnosis by two analytical chemists (MW and RC), with bio-statistical analyses linking subject identifier to chemometric biomarkers performed following data lock by an independent statistician (MR).

805 unique chromatographic features (peaks) were detected across the breath sample set using TD-GCxGC-FID/MS. Topological data analysis (TDA) applied to these 805 chromatographic features, yielded topologically distinct networks that distinguished underlying causes of acute breathlessness whilst anchoring to corresponding blood-based biomarkers in both the discovery and replication cohorts **(Figure 2)**. Specifically, healthy volunteers and patients with acute heart failure formed distinct topological groupings in both discovery and replication populations, whilst respiratory admissions due to acute asthma, acute COPD and pneumonia formed a topological continuum albeit within distinct regions of a single network in the replication cohort with similar findings in the discovery cohort, with the except of acute asthma forming a distinct grouping.

Table 8: *Baseline demographics and clinical characteristics of the discovery and replication cohorts. VAS: Visual Analogue Scale (100mm), participants were asked to rate their breathlessness, cough and wheeze on a 100mm VAS on admission. ANOVA was used to assess the differences between groups for normally distributed continuous variables and kruskal-Wallis for non-parametric continuous variables. Pearson chi-squared and Fisher's exact were used to assess the differences in categorical variables. The results were considered statistically significant at p-values <0.05. * Data is expressed as mean (SD) or n (%) ± (SD).*

	Discovery	Replication	p value
Total number (n=)	**139**	**138**	
Acute asthma (n=)	33	32	
Acute COPD (n=)	29	29	
Pneumonia (n=)	28	27	
Heart failure (n=)	22	22	
Healthy volunteers (n=)	27	28	
Demographics			
Age (years) mean ± (SD)	60.6 ± (16.9)	61.0 ± (16.8)	.846
Gender Male (n=) (%)	65 (46%)	78 (56%)	.104¥
Height (meters)*	1.66 ± (0.13)	1.68 ± (0.16)	.215
Weight (kilograms)*	82.5 ± (21.1)	85.7± (25.6)	.260
Body Mass Index (BMI)*	29.5 ± (6.7)	29.6 ± (7.9)	.896
Breathlessness			
Breathlessness VAS score (mm)*	56.1 ± (32.3)	60.2 ± (30.7)	0.292
V1 cough VAS score (mm)*	41.6 ± (33.3)	44.5 ± (33.2)	0.479
V1 wheeze VAS score (mm)*	40.65± (35.1)	43.1± (34.8)	0.558

Laboratory parameters			
C-Reactive Protein (mg/dl)	10.0 (1.0-449.0)	12.0 (1.0-321.0)	0.740
Blood eosinophil count 10^9/L	0.13 (0.01-1.9)	0.13 (0.01-2.15)	0.825
Troponin T (ng/l)	3.4 (1.0-1658.4)	3.15 (1.0-810.1)	0.565
BNP (ng/l)	40.1 (1.0-1576.0)	42.6 (1.0-2631.9)	0.780

Example 3 - Biomarker profiling and risk scores

In order to create a concatenated list of exhaled breath biomarkers suitable for diagnostic application, a threshold of 80% feature-presence per patient group was applied, below which features were removed **(Figure 7)**. This approach was further supported by the unique distribution properties of breath biomarkers **(Figure 8)** and to enable the generation of patient specific multi VOC biomarker risk scores. Further filtering steps using Least Absolute Shrinkage and Selection Operator (LASSO) and Elastic Net regression methods, followed by removal of 38 peaks that were considered to be chemical and material artefacts (e.g. siloxanes), and generated a final panel of 101 exhaled breath volatiles **(Figure 7)**. Therefore, the analysis plan permitted the identification of a rich and chemically diverse response in the VOC profile as opposed to only a handful of individual VOC markers and afforded the generation of biomarker risk scores. The data was examined for batch effects and was adjusted accordingly. Batch effects detected related to major instrument maintenance events (which occurred twice creating three groups, see Supplementary Information section on batch adjustment). No significant contributions were observed based on the ReCIVA device used, operator, time of day, or volume of breath sample collected, most likely nullified by the simultaneous and consecutive recruitment across all cohorts throughout the study to reduce potential biases **(Figure 9-10)**. The value of the generated VOC biomarker risk score was found to be significantly higher in acute cardio-respiratory patients compared to healthy volunteers **(Figure 3a)**. For the discovery cohort (n=139), the VOC biomarker risk score was able to effectively differentiate participants with acute cardio-respiratory exacerbations from age-matched healthy controls with an area under the curve (AUC) of 1.00 (1.00-1.00) $p<0.0001$, sensitivity 1.00 (1.00-1.00), specificity (1.00-1.00), positive predictive value (PPV) 1.00 (1.00-1.00), negative predictive value (NPV) (1.00-1.00). For the replication cohort (n=138), the same VOC biomarker risk score differentiated

participants with acute disease from healthy controls with AUC 0.89 (0.82-0.95) p<0.0001, sensitivity 0.79 (0.71-0.86), specificity AUC 0.85 (0.72-0.98), PPV of 0.95 (0.91-0.99), NPV of 0.51 (0.36-0.65) **(Figure 3b)**.

Following a clinical adjudication process **(Methods),** each patient was assigned a degree of clinical diagnostic uncertainty using a 100mm visual analogue scale (VAS) at the point of clinical triage **(Figure 3c)**. Diagnostic uncertainty was defined as patients with values higher than or equal to the upper quartile of 20mm on the VAS. The acute disease VOC biomarker risk score was able to identify acute disease with an AUC 0.96 (0.92-0.99) p<0.0001, sensitivity 0.90 (0.82-0.97), specificity 0.92 (0.85-0.99), PPV 0.93 (0.86-0.99), NPV 0.89 (0.81-0.97) **(Figure 3d)**.

Further comparative ROC analysis was performed to assess the diagnostic accuracy of asthma biomarker score against predominantly infection-driven respiratory illnesses (Pneumonia and COPD) in the pooled cohort curve AUC: 0.70 (0.62-0.78) p<0.0001, sensitivity 0.72 (0.64-0.83), specificity 0.64 (0.55-0.73), PPV 0.54 (0.43-0.64), NPV 0.80 (0.72-0.88). ROC analysis was performed to assess the diagnostic value of heart failure biomarker score against other acute disease groups AUC: 0.78 (0.70-0.86) p<0.0001, sensitivity 0.77 (0.64-0.89), specificity 0.71 (0.64-0.78), PPV 0.40 (0.29-0.50), NPV 0.92 (0.88-0.97) **(Figure 15).**

Example 4 - Correlation of exhaled breath biomarker scores with blood-based biomarkers and admission observations

As previously described, VOC biomarker risk scores were generated for each of the acute disease subgroups and healthy subjects without cardio-respiratory breathlessness. There was a weak, but statistically significant positive correlation, in the combined discovery and replication cohorts (n=277), between the VOC scores for pneumonia and CRP (n=277, r=0.33, p<0.0001), acute heart failure and BNP (n=277, r=0.33, p<0.0001), in addition to a significant negative correlation between the healthy-state VOC score and CRP and BNP (n=277, r= -0.15, p<0.0001, and -0.21, p<0.0001 respectively) **(Figure 4a).**

Interestingly, significant correlations were also identified between the acute disease VOC score and vital observations carried out during triage **(Figure 4b).**

Example 5 - Chemical classification of predictive markers in disease groups

Chemical identification of the 101 biomarker panel involved comparison with an authentic reference compound in accordance with the Metabolomics Standard Initiative (MSI) Level 1 criteria for metabolite identification **(Figure 16).**

The most common chemical classes associated with acute breathlessness in this study included straight-chain and methyl-branched hydrocarbons (30%), ketones (10%), aldehydes (8%) and terpenes (13%), followed by sulphur-containing VOCs (7%), alcohols (6%), aromatics (5%), esters (3%), nitrogen-containing VOCs (3%), ethers (2%), halogen-compounds(1%), and an assortment of other less prevalent and less relevant classes such as acrylates (12%) **(Figure 16).**

Example 6 - Metabolite Set Enrichment and Chemical Similarity Analysis

Unlike functional indications, which are reliant on mapping metabolites with known well-annotated metabolic pathways, metabolic changes indicative of response can be derived independently. To derive clues of responsive indication, the panel of 101 features was assessed for covarying clusters i.e. metabolite sets **(Figure 5A, and Figure 11).**

Overall twenty metabolite sets were identified, eleven of which were enriched during acute cardio-respiratory exacerbations. The seven metabolite sets that were upregulated consisted of predominantly acyclic and branched hydrocarbons **(sets 3, 5, 7 and 9 in Figure 11).** The results from the analysis herein demonstrate significantly enriched, co-expression of hydrocarbons with high chemical similarity providing primary evidence of exhaled VOCs indicative of disease response measured *in vivo.* This is clearly seen in **(Figure 5a)**, with the metabolite sets (inner tree) labelled by broader chemical classifications (outer ring); C_{5-7}, C_{8-10} and C_{11-16} form clusters based on carbon number also exhibiting the highest change during acute exacerbation.

Example 7 - Diagnostic accuracy of breath biomarker scores in cardio-respiratory disease subgroups

A multinomial regression model using elastic net regularization was fitted to the matrix of 101 breath biomarkers with the 10-fold cross validation repeated 100 times. Linear combinations of the most stable features from the multinomial regression model fitted to the 101 biomarkers formed a set of scores for predicting probability of

belonging to the different disease groups (acute Asthma, acute COPD, pneumonia, heart failure or healthy volunteers). The median values of the exhaled breath VOC scores and their distribution across disease subgroups are detailed in **Figure 12.**

For the pooled cohort (n-277) the overall classification accuracy using all five biomarker scores was 0.722, 95% CI (0.6653 - 0.774) **(Figure 14).** The balanced accuracy for acute asthma was 0.8274, for acute COPD 0.7751, for heart failure 0.7967, for community acquired pneumonia 0.7935, and for healthy controls was 0.9274.

Discussion

In this pragmatic, acute-care study, the validity of breath biomarker profiling in high-acuity patients presenting with acute cardio-respiratory breathlessness was evaluated. Using GCxGC-MS, the inventors observed that robust and validated sampling of alveolar breath coupled with GCxGC-MS biomarker characterisation demonstrated high diagnostic accuracy for acute cardio-respiratory exacerbations. Putative biomarker risk scores from subsets of breath VOC biomarkers that classify cardio-respiratory exacerbation subtypes and warrant validation in replication studies have also been identified. Furthermore, several classes of VOCS that are highly correlated and selectively enriched or supressed in acute disease (including subgroups), compared to health, providing potential insights into broad dysregulation of the metabolome in acute cardio-respiratory exacerbations have been identified.

This study is the first to attempt to characterise exhaled breath VOCs in a large cohort with severe cardio-respiratory exacerbations and the results position this study as a proof-of-concept for the use of breathomics in acute clinical settings.

The analytical methods described herein were underpinned by robust biomarker development protocols using TD-GCxGC-FID/MS, integral to the standardisation and integration of breath analysis in large translational studies. Several potential confounders including batch variation, were addressed in detail **(SI).** Furthermore, biomarker quantification of the 101 VOC modelled followed the recommendations of the Metabolomics Standard Initiative (MSI) with 58 compounds identified against pure and traceable standards (level I), 21 putative identities based on mass spectral and retention index library matches (level 2) and 22 classified on mass spectral data

Figure 16. Markers that appeared to localise to individual cardio-respiratory conditions could be readily visualised **(Figure 5).**

The identification of hydrocarbons and carbonyls as the major chemical classes was consistent with current mechanistic understanding, postulated as chemical endpoints of lipid peroxidation, a result of oxidative stress during inflammation. Aldehydes such as nonanal, decanal and hexanal were predictive for asthma, ketones included 2-pentanone (asthma), cyclohexanone (pneumonia) and 2,3-butanedione (COPD). Individual hydrocarbons such as 2,4- and 2,2-dimethylpentane; 2-methylbutane, 4-methyldecane, 5-methylnonane and isoprene are predictive for pneumonia and heart failure. Sulphur-containing VOCs, such as 3-methylthiophene, allyl methyl sulphide and carbonyl sulphide (found to be predictive of COPD) are associated with bacterial metabolism, postulated to originate from the gut and on occasions as a result of radiation injury. 2,3-butanedione is also predictive of COPD.

Not all the compounds were considered to be endogenous VOCs, with 27 attributed to contamination from personal care products such as cosmetics Figure 16. Eleven of the features predictive of the control group were assigned as either fragrances (e.g. alpha isomethyl ionone) or waxy long-chain chemicals used in cosmetics as emollients and surfactants (e.g. stearyl vinyl ether and isopropyl myristate). These were likely captured in the breath sample because of the proximity of the sorbent tubes to the patients' face.

Co-expression and enrichment analysis of the Louvain clusters on the correlation graph **(Feature enrichment analysis section – Tables 3-6)**, revealed a set of highly correlated metabolites significantly enriched in specific disease groups. Comparison of the Louvain clusters with the metabolite sets identified using the method previously described, demonstrated strong overlap **(Figure 5A and 5B).** The metabolites enriched in heart failure were a cluster of highly correlated C_{5-7} hydrocarbons and C_{3-5} carbonyls with high chemical similarity (based on Tanimoto coefficients as determined in **Methods and Figure 11.** The cluster included 2,4- and 2,2-dimethylpentane; 2-methylbutane, 2-methyl-1,3-butadiene (isoprene), 3-methylpentane, hexane and cyclohexane.

The analysis also revealed a separate set of highly correlated aldehydes (nonanal, decanal, undecanal, and a methyldecanal isomer), lower in acute exacerbations of asthma compared with acute exacerbations of COPD and pneumonia. Depletion of VOCs during *in vitro* experiments has been reported as a consequence of metabolic activity by immune cells, but the association herein is tentative and should be interpreted with caution due to the correlation between inhaled air and exhaled air concentrations of these compounds (median Spearman rank = 0.60), also previously observed.

In conclusion, the inventors have conducted an acute care volatile breath biomarker study using robust clinical and analytical technology and have identified high diagnostic sensitivity and specificity of biomarkers in acute cardio-respiratory disease, alongside robust biomarker identification and mechanistic association warranting further metabolomic phenotyping approached in acute cardio-respiratory exacerbations.

CLAIMS

1. A method of diagnosing a cardiorespiratory disease in a subject, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled breath from the subject,

wherein if one or more of the VOC biomarkers is present in the sample, the subject may have a cardiorespiratory disease.

2. A method of treating a cardiorespiratory disease in a subject, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled air from the subject,

wherein the presence of one or more of the VOC biomarkers in the sample suggests the subject has a cardiorespiratory disease, and

administering a therapeutic agent to the subject, in order to treat the cardiorespiratory disease.

3. A method of treating a cardiorespiratory disease in a subject, the method comprising:

administering a therapeutic agent to the subject, who has been diagnosed with a cardiorespiratory disease using the method according to the invention.

4. A method of selecting a subject for treatment with a therapeutic agent or composition for a cardiorespiratory disease, the method comprising:

detecting the presence of one or more cardiorespiratory disease-VOC biomarkers in a sample of exhaled air from the subject,

wherein the presence of one or more of the VOC biomarkers in the sample suggests the subject has a cardiorespiratory disease, and

selecting the subject for treatment with a therapeutic agent or composition for the cardiorespiratory disease.

5. A method of determining if a therapeutic agent or composition is effectively treating a cardiorespiratory disease in a subject, the method comprising:

determining the concentration of one or more cardiorespiratory disease-VOC biomarkers in a test sample that has been exhaled by the subject, and

comparing the concentration of the at least one or more VOCs in the test sample with the concentration in a reference sample,

wherein if the concentration of the one or more VOC biomarkers in the test sample is lower compared to the concentration in a reference sample, it is indicative that the therapeutic agent or composition is effectively treating the cardiorespiratory disease in the subject.

6. The method according to claim 5, wherein the concentration of the VOC biomarker in the test sample is lower by (or reduced by at) least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% compared to the concentration in the reference sample.

7. The method according to any one of the preceding claims, wherein the subject is experiencing breathlessness.

8. The method according to any one of the preceding claims, wherein a two-dimensional gas chromatography coupled with mass spectrometry is used to detect the presence of the one or more VOC biomarkers in the sample.

9. The method according to any one of the preceding claims, wherein the cardiorespiratory disease is one or more diseases selected from the group comprising: asthma, COPD, heart failure and pneumonia.

10. The method according to any one of the preceding claims, wherein the one or more cardiorespiratory disease-VOC biomarkers is one or more selected from Figure 16.

11. The method according to any one of the preceding claims, wherein the one or more cardiorespiratory disease-VOC biomarkers is a selection of one or more of the following: hexane; octane; tetradecane,2,3-butanedione; hexanal; 2-methyl-2-propenal; 1-hexadecanol; 2-methyl-1,3-dioxolane; limonene; eucalyptol; menthone; p-mentha-1,4/8-diene; 3-carene; beta phellandrene; sesquiterpenoid; xylene; 2,3-dimethylnapthalene; carbonyl sulphide; 4-cyanocylohexene; methenamine; dichloromethane; N,N-dimethyl-1-nonanamine; and a alkenyl hexanoic acid ester.

12. The method according to any one of the preceding claims, wherein the one or more cardiorespiratory disease-VOC biomarker is one or more asthma-VOC biomarkers; one or more COPD-VOC biomarkers; one or more heart failure-VOC biomarkers; and/or one or more pneumonia-VOC biomarkers.

13. The method according to claim 12, wherein the one or more asthma-VOC biomarkers is a selection of one or more of the following: 3-methylpentane; 2-methylnonane; decane; 1-nonene; methyldecanal isomer; undecanal; 3-methylbenzaldehyde; 2-ethylhexanol; tetrahydrofuran; 1,4-dioxane; beta-bisabolene; and N,N-dimethyl-1-dodecanamine.

14. The method according to claim 12, wherein the one or more COPD-VOC biomarkers is a selection of one or more of the following: nonane; 4-methylundecane; 1-decanol; menthol; camphene; galaxolide; 3-methyl thiophene; and N,N-dimethyl-1-dodecanamine.

15. The method according to claim 12, wherein the one or more heart failure-VOC biomarkers is a selection of one or more of the following: undecane; cyclohexene; butanal; 2-methyl-2-propenal; tridecanal; ethyl acetate; 1,3-dioxolane; beta myrcene; ethylbenzene; and decyl isobutyl ether.

16. The method according to claim 12, wherein the one or more pneumonia-VOC biomarkers is a selection of one or more of the following: 2,6-dimethyloctane; diemthylundecane isomer; 1-decene; 3-buten-2-one (methyl vinyl ketone); 1-(methylthio)-1-propene; 1-methylthio-propane; and dodecylacryalte.

Intellectual Property Office

Application No:	GB2110365.0	**Examiner:**	Mr Gareth Prothero
Claims searched:	1 to 13 in part	**Date of search:**	5 May 2022

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1 to 13	Molecules, Vol. 26 (6), 2021, Monedeiro F. et al., "Needle Trap Device-GC-MS for Characterization of Lung Diseases Based on Breath VOC Profiles". See in particular Table 1 and Section 4 "Conclusions". Available online at https://www.mdpi.com/1420-3049/26/6/1789 [date accessed 3/5/22].
X	1 to 13	Paediatric Asthma, Vol. 42, 2013, Robroeks C.M. et al., "Exhaled volatile organic compounds predicts exacerbations of childhood asthma in a 1-year prospective study", pp. 98-106. See in particular Table 5.
A	-	ERJ Open Research, Vol. 8 (2), 2021, Ibrahim W. et al., "A systematic review of the diagnostic accuracy of volatile organic compounds in airways diseases and their relation to markers of type-2 inflammation". See in particular Table C. Available online at https://openres.ersjournals.com/content/erjor/early/2021/04/01/23120541.00030-2021.full.pdf [accessed on 3/5/22]

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

G01N

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, MEDLINE, BIOSIS, Patent Fulltext, CAS ONLINE

International Classification:

Subclass	Subgroup	Valid From
G01N	0033/497	01/01/2006