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Screening for emphysema via exhaled volatile organic compounds

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Abstract
Chronic obstructive pulmonary disease (COPD)/emphysema risk groups are well defined and screening allows for early identification of disease. The capability of exhaled volatile organic compounds (VOCs) to detect emphysema, as found by computed tomography (CT) in current and former heavy smokers participating in a lung cancer screening trial, was investigated. CT scans, pulmonary function tests and breath sample collections were obtained from 204 subjects. Breath samples were analyzed with a proton-transfer reaction mass spectrometer (PTR-MS) to obtain VOC profiles listed as ions at various mass-to-charge ratios (m/z). Using bootstrapped stepwise forward logistic regression, we identified specific breath profiles as a potential tool for the diagnosis of emphysema, of airflow limitation or gas-exchange impairment. A marker for emphysema was found at m/z 87 (tentatively attributed to 2-methylbutanal). The area under the receiver operating characteristic curve (ROC) of this marker to diagnose emphysema was 0.588 (95% CI 0.453–0.662). Mass-to-charge ratios m/z 52 (most likely chloramine) and m/z 135 (alkyl benzene) were linked to obstructive disease and m/z 122 (most probably alkyl homologs) to an impaired diffusion capacity. ROC areas were 0.646 (95% CI 0.562–0.730) and 0.671 (95% CI 0.524–0.710), respectively. In the screening setting, exhaled VOCs measured by PTR-MS constitute weak markers for emphysema, pulmonary obstruction and impaired diffusion capacity.

S Online supplementary data available from stacks.iop.org/JBR/5/046009/mmedia

1. Introduction

For chronic obstructive pulmonary disease (COPD), the groups at risk are well defined and screening may allow identification of subjects in an early stage of the disease [1]. Emphysema, one of the components of COPD, is diagnosed via computed tomography (CT), by highlighting the areas with an abnormally low density [2]. Tissue density is expressed in Hounsfield units (HU) and the extent of emphysema is expressed as the percentage of the total lung volume below a

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density threshold [3]. The major drawbacks of CT scans are equipment immobility and radiation burden, although recent developments reduced the radiation dose significantly from 5 to 1 millisievert (mSv) [4]. Pulmonary function testing (PFT) is done easily and at lower costs, but the often used parameters from spirometry/flow-volume curves cannot detect emphysema when it is present without airflow limitation [5–7].

In the last decade, there has been an increased interest in exhaled breath analysis because the method is non-invasive, presents minimal risk to patients and allows large numbers of subjects to be investigated [8, 9]. The human breath contains volatile organic compounds (VOCs), which can serve as biomarkers. Proton-transfer reaction mass spectrometry (PTR-MS) is a promising tool for a rapid on-line determination of exhaled VOC profiles [10, 11].

The aim of the study presented in this paper was to explore breath analysis as a non-invasive method for the early detection of emphysema in heavy smokers at risk to develop COPD. The hypothesis is that emphysema is characterized by inflammation, which alters the composition of the exhaled air. The detection of airflow limitation or impaired gas-exchange was a secondary goal.

2. Methods

2.1. Subjects

The subjects included participated in the 'NELSON-project', a Dutch–Belgian multi-center lung cancer screening. Participants were current and former male smokers between 50 and 75 years old, who smoked >15 cigarettes per day during >25 years, or >10 cigarettes per day during >30 years and had <10 years of smoking cessation [12]. No prior diagnosis of either obstruction or emphysema was known. The trial was approved by the Dutch ministry of health (approval number 2000/04WBO). Subjects gave written informed consent. A waiver was received for the current analysis.

2.2. CT Scanning and calculation of emphysema scores

CT scanning was performed by a 16-detector-row scanner (Mx8000 IDT or Brilliance 16P, Philips Medical Systems, Cleveland, OH) at 16 × 0.75 mm collimation. Exposure settings were 30 mAs at 120 kVp for patients ⩽80 kg and 30 mAs at 140 kVp for those >80 kg. Images were reconstructed at 1.0 mm slice thickness, at 0.7 mm increment, at 512 × 512 matrix and using a moderately soft filter (Philips 'B'). All CT scans were analyzed for the presence and extent of emphysema using in-house developed software, imageXplorer (iX), Image Sciences Institute, Utrecht, the Netherlands) [13–15].

Areas with an attenuation < −950 HU (= tissue density <50 gr l^{-1}) were considered to represent emphysema [3]. The emphysema score (ES) is the volume with attenuation below that threshold, expressed as percentage of the total lung volume. An ES ⩾ 1% was considered as abnormal [16]. This threshold represents developing emphysema, as no emphysema should be present in healthy subjects.

2.3. Pulmonary function tests

Pulmonary function tests included the forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), the diffusing capacity for carbon monoxide (Dlco) and alveolar volume (V_A). Tests were conducted according to ERS guidelines, and abnormal pulmonary function values were defined as ⩽ −1.64 residual standard deviations below reference values [17].

On the day of the tests, participants refrained from smoking. They rested for 15 min upon arrival after which spirometry was performed, followed by flow–volume curves. The best of three attempts was selected for analysis. No reversibility testing was done. For descriptive purposes only, subjects were staged according to updated GOLD guidelines [18].

Diffusing capacity measurements were carried out after the spirometry and flow–volume curves. The inhalation mixture contained 0.3% CO and 10% He with balance air. A breath-holding period of 10 s was used. Dlco was not corrected for haemoglobulin [19]. Dlco was corrected for COHb and divided by the alveolar volume (Dlco/V_A).

2.4. Exhaled breath sampling and measurement of VOCs

Exhaled single breath samples were collected in Tedlar bags before PFT, using the method for the measurement of exhaled nitric oxide [20]. The sampling device is described in detail elsewhere [21]. In addition to each breath sample, the inhaled room air was also analyzed and if was found polluted, the corresponding breath sample was discarded.

The analysis of exhaled compounds was performed with a proton transfer reaction mass spectrometer (PTR-MS) [21–23]. Briefly, gases are ionized with H_3O^+ ions, after which the product ions are mass analyzed and detected with a quadrupole mass spectrometer. VOCs are listed as ions at various mass-to-charge ratios (m/z).

The system was calibrated by mixing a variable flow of air free of hydrocarbons (by passing it through a catalyst at 350 °C) with a fixed flow of 0.3 l h^{-1} of a gas mixture of methanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molecular weights ranging from 32 to 106 amu) in concentrations of 1 ppmv (±5%) (Linde, Dieren, the Netherlands). In this way, calibration factors are obtained for these compounds converting ion intensity (in ncps, normalized counts per seconds) to gas mixing ratios (in ppbv, part per billion volume). From these conversion factors, the calibration factors (sensitivity) of other compounds at a specific m/z ratio were calculated, taking into account the kinetic reaction constant (k) for the proton-transfer reaction in the drift tube and transmission efficiency factors of the quadrupole mass spectrometer [11]. For several compounds the reaction constant is known; acetonitrile (m/z 42) has $k = 5.1 \times 10^{-9}$ cm^3 s^{-1}. Concentrations of the compounds which are not identified have been calculated based on a 'standard' reaction constant for protonation of $k = 2.2 \times 10^{-9}$ cm^3 s^{-1}. This allows a first estimate of the respective concentration of an unknown compound (based on the measured count rate).

Table 1. Demographic data (mean ± SD) of subjects by GOLD stage.

Stage	Age (yrs)	Pack years[a]	FEV_1[b]	FEV_1/FVC (%)	Dlco[b]	$Dlco/V_A$[b]
$FEV_1/FVC \geqslant 0.70$	61.3 ± 5.1	38.0 (29.7–46.2)	100.8 ± 14.2	76.3 ± 4.1	95.6 ± 13.1	92.3 ± 15.0
GOLD I	62.7 ± 5.2	38.3 (29.7–46.2)	93.2 ± 9.9	64.6 ± 4.3	82.2 ± 20.0	72.9 ± 16.9
GOLD II	65.4 ± 6.2	46.2 (38.7–49.7)	70.3 ± 7.4	59.2 ± 6.7	78.7 ± 19.5	75.9 ± 18.7
GOLD III	61.8 ± 6.4	38.0 (34.2–43.7)	49.9 ± 3.5	44.0 ± 7.6	61.9 ± 4.3	59.9 ± 3.1

[a] Median (25–75%).
[b] As percentage predicted.

The limit of detection (LOD) is considered from a signal-to-noise ratio (S/N) of 2 accordingly to $LOD = 2 \times SD_{blank}/sensitivity$. The SD_{blank} represents the standard deviation of the background count rates determined by $SD_{blank} = (I_{blank}/\tau)^{1/2}$, where I_{blank} is the intensity of the background signal (counts per second) and τ is the dwell time (1.5 s) [11]. The background signal was measured 2 × 3 cycles with the catalyst in the sampling line (clean air).

The Tedlar bags were tested earlier with respect to suitability for breath sampling and storage [24]. The collection method and the chemical analysis were validated for accuracy, precision, selectivity, limits of detection, sensitivity and reproducibility and last degradation of VOCs in the collection bag [24].

2.5. Statistics

We calculated means, standard deviations and 95% confidence intervals (CI) for normal distributed parameters and medians with the interquartile range for non-normal distributed ones.

Suitable markers from the spectrum were selected using stepwise forward logistic regression [25]. However, the ∼150 mass numbers measured deliver an unfavorable case/variable ratio and the outcome of the logistic regression can be unstable. We approached this problem via bootstrapping, which takes a sample with replacement from the database and the logistic regression is run on that sample [26]. The *p*-value for entry was set at 0.15 and for removal at 0.30 in this initial selection phase. The outcome is a set of mass numbers that discriminate between 'emphysema' and 'no emphysema', and this set is stored. This procedure was repeated 500 times, generating 500 sets of markers. Now, stable and suitable markers will appear often in these 500 sets (ideally 100%): the occurrence of each mass number in the 500 sets was therefore calculated. The markers with a ⩾50% occurrence were selected and subjected to a confirmatory logistic regression.

This confirmatory logistic regression estimated the probability of an individual to belong to the diseased group, and these probabilities were used to calculate the area under the receiver operating characteristic curve (ROC): that area renders the probability to diagnose emphysema or lowered FEV_1/FVC and $Dlco/V_A$ values correctly.

Table 2. Median (25–75% range) emphysema scores broken down by GOLD stage. Emphysema scores are depicted as the percentage lung volume < −950 HU.

	Emphysema score (%)
$FEV_1/FVC \geqslant 0.70$	0.18 (0.06–0.38)
GOLD I	0.51 (0.21–1.15)
GOLD II	1.27 (0.49–2.23)
GOLD III	3.98 (3.61–6.25)

Next to the above, a number of other statistical methods are available [27–29], and in the supplementary data (available at stacks.iop.org/JBR/5/046009/mmedia), we present the outcome of such methods. The advantage of this approach is that results obtained here are validated via alternative methods.

3. Results

A cohort of 204 subjects was recruited for this study; the characteristics are shown in table 1. Fifty-three subjects were classified as GOLD I, 25 as GOLD II and five subjects as GOLD III. No GOLD IV subjects were found. The median ES according to the GOLD stage are given in table 2. Of all subjects, 54.4% (95% CI 47.4–61.3%) still smoked; the smoking/non-smoking ratio in the groups with a lowered/normal FEV_1/FVC was not different ($p = 0.766$), nor was it in the group with/without emphysema ($p = 0.199$).

Acetonitrile, measured with PTR-MS at m/z 42, is a known indicator for smoking, and it indeed discriminated well between ex- and current smokers ($p < 0.001$) (see table 3) [30]. The ROC area was 0.831 (95% CI 0.770–0.891).

Emphysema was diagnosed in 43 subjects (21.1%, 95% CI 16.0–27.2%). The bootstrapping approach delivered m/z 87 as a marker (table 4). The median levels for m/z 87 in emphysema subjects are higher, but the overlap in values is considerable. The ROC area to detect emphysema was 0.558 (95% CI 0.453–0.662).

Table 3. Median (25–75% range) of m/z 42 (acetonitrile) levels broken down by smoking status.

	LOD (ppbv)		Median levels (25–75% range)	
m/z		*p*-value[a]	Current smoker (ppbv)	Ex-smoker (ppbv)
42	0.31	<0.001	726 (358.3–1078)	116 (74.6–188.3)

[a] *p*-values from the confirmatory logistic regression.

Table 4. Suitable m/z to detect emphysema (levels in normalized counts/sec).

	LOD (ppbv)		Median levels (25–75% range)	
m/z		p-value[a]	No emphysema (ppbv)	Emphysema (ppbv)
87	1.31	0.06	16.8 (6.2–34.3)	26.9 (6.1–44.2)

[a] p-values from the confirmatory logistic regression.

Table 5. Suitable m/z to detect an abnormal FEV1/FVC.

	LOD (ppbv)		Median levels (25–75% range)	
m/z		p-value[a]	Normal FEV_1/FVC (ppbv)	Abnormal FEV_1/FVC (ppbv)
52	0.47	0.007	2.6 (1.3–3.2)	2.1 (1.3–3.1)
135	0.82	0.002	1.8 (1.2–2.6)	2.2 (1.4–3.1)

[a] p-values from the confirmatory logistic regression.

Table 6. Suitable m/z to detect an abnormal $Dlco/V_A$.

	LOD (ppbv)		Median levels (25–75% range)	
m/z		p-value[a]	Normal D_{lco}/V_A (ppbv)	Abnormal D_{lco}/V_A (ppbv)
122	0.82	0.046	2 (1.3–2.5)	1.6 (1–2.2)

[a] p-values from the confirmatory logistic regression.

Eighty-three subjects (40.7%, 95% CI 34.2–47.5%) showed a FEV_1/FVC value below the lower limit of normal. The bootstrapping approach delivered m/z 52 and m/z 135 (table 5). The levels of m/z 52 are reduced in obstruction, but m/z 135 is not. The ROC area to detect an abnormal FEV_1/FVC of this combination of markers was 0.646 (95% CI 0.562–0.730).

Seventy-one (34.8%, 95% CI 28.6–41.6%) showed a $Dlco/V_A$ value below the lower limit of normal. The bootstrapping approach delivered m/z 122 as a marker (table 6), which was reduced in diseased subjects. The ROC area to detect an abnormal $Dlco/V_A$ was 0.671 (95% CI 0.524–0.710).

It is known that identification of compounds is notoriously difficult with PTR-MS, as one mass-to-charge ratio may be associated with a parent molecule, fragment of the parent molecules and/or clusters between water and small molecules with strong permanent dipole moments. A list with possible candidates for the four mass-to-charge ratios of interest for this study is given in table 7. When known, the fragmentation ions are also specified.

A possible compound for m/z 52 is chloramine (H_2NCl). For subjects with abnormal FEV_1/FVC, the identity of the ion at $m/z = 52$ as chloramine is confirmed by the presence of the isotopic ion $m/z = 54$ with an intensity of 6.5% which is close to the expected isotopic abundance of 7.2% (correlation coefficient of 0.8).

The ion m/z 87, found as a marker for emphysema, is the parent ion of 2,3 butanedione (diacetyl), 2-or 3-pentanone, 2-or 3-methylbutanal (table 7). Apart from these, several other substances (not listed in the table) are suggested to be present in exhaled breath. In our instrument as well as in other reported studies [31, 32], the main contributions to these compounds are attributed to m/z 87 and 69. For the emphysema subjects, the related percentage contributions for the ion traces at m/z

Table 7. List of possible compounds for the m/z markers indicated in tables 4–6.

Possible compounds	Related ions	References
***m/z* 87**		
Pentanal	**69**, 87, 41	[45, 32, 33]
2,3-butanedione	**87**, 61, 88, 43	[46]
2-methyl-3-buten-2-ol (MBO)	87, **69,** 41	[31, 32]
2-methylbutanal	**87**, 69	[31]
2-pentanone	**87**, 45, 88	[33], [32]
3-methyl-2-butanone	**87**, 88	[32]
3-methyl-2-buten-1-ol	87, **69,** 41	[31]
3-methyl-3-buten-1-ol	87, **69,** 41	[31]
3-methylbutanal	87, **69**	[31]
3-pentanone	87, **69**	[32]
***m/z* 52**		
Propiolonitrile		[37]
Chloramine		[47]
***m/z* 122**		[37]
Benzamide		
2,6-dimethyl-benzenamine		
4-aminobenzenecarbonal		
1-(4-pyridinyl)-ethanone		
1-(3-pyridinyl)-ethanone		
n-ethyl-benzenamine		
Benzeneethanamine		
N,*N*-dimethyl-benzenamine		
2-propyl-pyridine		
2-(1-methylethyl)-pyridine		
Other alkyl homologs		[36]
***m/z* 135**		[32, 37]
sec-butylbenzene		
1,2,3-trimethylbenzene		
1,3,-diethylbenzene	**135**, 79	
1,4,-diethylbenzene		
n-butylbenzene		
1,2,-diethylbenzene		
1,2,3,5-tetramethylbenzene		[48]
1,2,4,5-tetramethylbenzene		

In bold are the major fragments.

87 and 69 are 80% and 20%, respectively, and the linear correlation coefficient between the two ions of 0.8. Hence, we indicate as the most probable candidate 2-methylbutanal and exclude several compounds with m/z 69 as the major fragment, such as pentanal, 2-or 3- methyl-2- or 3-buten-1 or 2-ol, etc.

For ketones such as 2,3 butanedione or 2-pentenone, hardly any fragmentation is reported so far. The protonated molecular ion is the most abundant mass, often followed by the isotopic ion [33]. In the emphysema patients, a weak correlation was found between $m/87$ and m/z 88 (linear correlation coefficient of 0.3). Moreover, the isotopic ion at m/z 88 is highly influenced by the presence of N,N-dimethyl-acetamide, a compound released by the Tedlar bags [24]. Based on these considerations, we cannot assess m/z 87 to any of the two ketones mentioned above.

Peroxyacetyl nitrate (PAN) was reported as a possible source of m/z 122 in selected ion flow tube mass spectrometry (SIFT-MS) [34] using H_3O^+ as primary ions. However, in the PTR-MS m/z 122 undergoes subsequent reaction with water, and therefore, m/z 77 is used instead for PAN detection [35]. Possible compounds for m/z 122 are the alkyl homologs as found in breath analysis with PTR-MS [36, 37].

The ion m/z 135 is attributed to alkyl benzene compounds [32] (table 7).

4. Discussion

Markers in exhaled breath appear to be weakly related to emphysema and pulmonary function outcome in this screening setting. In other settings, more favorable characteristics and/or markers may be found [38].

The challenge in the field of exhaled VOCs is the selection of suitable markers from a wide range, which can be done based on an in-depth knowledge of the inflammatory processes and the VOCs generated. This requires prior definition of markers and often represents an educated guess (candidate marker approach). A scanning approach, like in genetic genome wide scans, can be more fruitful. One of the problems of this approach is that the outcome can be highly sample dependent, due to the unfavorable case/parameter ratio [39]. One way to deal with that problem is to chart the degree of sample dependence via bootstrapping. As said, bootstrapping draws many samples from a dataset and in some the effects of e.g. influencing data points will be present. In such samples, the outcome is vastly different, and therefore we selected markers with a high prevalence in the bootstrap samples. Bootstrapping is also known to lead to a better extrapolation of the results, as the sampling error is reduced. The fact that a well-known smoking marker (m/z 42) discriminated ex- from current smokers is reassuring and validates both the measurement system and the statistical approach [30]. Still, it is possible that this technique is less well suited for the purpose, therefore we decided to use several other methods, described in the supplement; none of these alternatives lead to another conclusion (for details, see the supplementary data available at stacks.iop.org/JBR/5/046009/mmedia).

Confounders (e.g. recent smoking, food, etc) may bias the outcome. However, smokers and ex-smokers are randomized over the emphysema/non-emphysema and over obstructed/non-obstructed groups. The smoking status was obtained before CT-scanning and pulmonary function tests were carried out. Smoking bias is therefore improbable. Further proof of a lack of (recent) smoking bias is that the smoking indicator acetonitrile does not appear as a marker in any of our analysis [30]. Similar arguments can be used to exclude food or other effects. Diseases such as lung cancer or α_1-antitrypsin deficiency are also randomized over the groups as their presence was unknown at the time of sampling or their effects are negligible as these diseases are seldom in the general population ($\sim$1% of all COPD cases are due to α_1-antitrypsin deficiency). All materials in contact with the in-/exhaled air were inert/non-absorbable, and hence they can also not influence the outcome.

As we aimed to detect emphysema within (former) heavy smokers, we did not include healthy (non-smoking) controls. The contrast between the groups studied would increase, favoring marker detection, but at the same time outcome would be difficult to extrapolate to COPD screening in smokers. Furthermore, a possible bias due to differences is smoking habits would have been introduced.

Despite a lack of bias and the sensitive measurements, we must conclude that the one emphysema marker we report is not a strong one. The area under the ROC (AUC) of 0.558 does not constitute a suitable marker: a 0.7 value is often considered as a minimum. For obstruction and gas-exchange problems, the results were slightly better, but still these markers also did not reach the 0.7 threshold. Furthermore, m/z 87 which is most probably assessed to 2-methylbutanal is weakly correlated with m/z 42 ($r = 0.272$), indicating that it is not related to smoking.

There can be several explanations for the low AUCs of the ROC. First of all, the emphysema severity in this non-hospital-based cohort was limited, since severe COPD was an exclusion criterion for participation. Emphysema is a slow-developing disease and the amount of inflammation causing it can be low grade, and so the probability of exhaling distinct fingerprints will be equally low. We could have included subjects with severe emphysema, but as these constitute more advanced disease, we consider that approach as less useful. Emphysema needs to be addressed in an early stage to be able to prevent further deterioration. A recent cross-sectional case-control study with e-Nose reported a good discrimination between asthma and COPD, but not between COPD and non-smoking controls [40]. The COPD subjects in that study were characterized by severe obstruction (post-bronchodilator FEV_1 57% of predicted) and are much more diseased than the subjects included in this study. We therefore propose as an explanation that COPD/emphysema in heavy smokers do not generate sufficiently different exhaled VOC profiles to be used as markers.

The second explanation can be that we used an unbiased selection of heavy smokers. Other exhaled breath studies on e.g. lung cancer, which report suitable markers, often used a case-control design [41, 42]. In such a design, diseased subjects are contrasted to healthy subjects in equal numbers, mostly after matching. Such a design modifies the

characteristics by increasing the prevalence of disease and hence the contrast: it will be easier to find markers in such a high-contrast study. In screening studies, the prevalence of the disease is much lower, and it becomes much more difficult to select a few subjects amongst many non-diseased subjects. The lower the prevalence of a disease, the stronger the marker for disease must be or the less the overlap in marker values between groups to be able to detect the diseased subjects.

The third possible explanation lies in the type of instrument used. PTR-MS is suitable for detecting only a small amount of masses with satisfactorily speed and low detection limit (mass range up to 160 amu). Compounds with higher molecular weight which could be potential candidates are thus not considered. The identification of compounds measured by PTR-MS is always tentative. Alternative solutions to overcome these limitations include the use of ion traps, coupling of PTR-MS with GC, the very recently developed time-of-flight mass spectrometry in combination with PTR-MS and the analytical techniques such as GC-MS or SIFT-MS [43, 44]. However, in this discovery phase, this is not a disadvantage [40].

To summarize, in COPD and/or emphysema screening studies, exhaled VOCs have not provided valuable diagnostic information so far.

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