Original article

Profile of exhaled-breath volatile organic compounds to diagnose pancreatic cancer

S. R. Markar, B. Brodie, S.-T. Chin, A. Romano, D. Spalding and G. B. Hanna

Department of Surgery and Cancer, Imperial College London, London, UK

Correspondence to: Professor G. B. Hanna, Division of Surgery, Department of Surgery and Cancer, Imperial College London, Tenth Floor QEQM Building, St Mary's Hospital, South Wharf Road, London W2 1NY, UK (e-mail: g.hanna@imperial.ac.uk)

Background: **Pancreatic cancer has a very poor prognosis as most patients are diagnosed at an advanced stage when curative treatments are not possible. Breath volatile organic compounds (VOCs) have shown potential as novel biomarkers to detect cancer. The aim of the study was to quantify differences in exhaled breath VOCs of patients with pancreatic cancers compared with cohorts without cancer.**

Methods: **Patients were recruited to an initial development cohort and a second validation cohort. The cancer group included patients with localized and metastatic cancers, whereas the control group included patients with benign pancreatic disease or normal pancreas. The reference test for comparison was radiological imaging using abdominal CT, ultrasound imaging or endoscopic ultrasonography, confirmed by histopathological examination as appropriate. Breath was collected from the development cohort with steel bags, and from the validation cohort using the ReCIVA™ system. Analysis was performed using gas chromatography–mass spectrometry.**

Results: **A total of 68 patients were recruited to the development cohort (25 with cancer, 43 no cancer) and 64 to the validation cohort (32 with cancer, 32 no cancer). Of 66 VOCs identified, 12 were significantly different between groups in the development cohort on univariable analysis. Receiver operating characteristic (ROC) curve analysis using significant volatile compounds and the validation cohort produced an area under the curve of 0·736 (sensitivity 81 per cent, specificity 58 per cent) for differentiating cancer from no cancer, and 0·744 (sensitivity 70 per cent, specificity 74 per cent) for differentiating adenocarcinoma from no cancer.**

Conclusion: **Breath VOCs may distinguish patients with pancreatic cancer from those without cancer.**

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Introduction

Pancreatic cancers are estimated to cause over 40 000 deaths annually in the USA and were estimated to be the fourth largest contributor to overall cancer deaths in 2017[1]. Only 15–20 per cent of patients have potentially curable disease at the time of diagnosis[2,3]. Referrals for investigation of suspected pancreatic cancer from primary care depend on symptom recognition. Large primary care database studies and patient surveys indicate that patients with pancreatic cancer visit their primary care physician frequently in the months and years before diagnosis[4]. However, almost half of patients are still diagnosed as a result of an emergency presentation to hospital[5]. Currently the majority of European referral guidelines for the assessment of pancreatic cancer are focused on patient demographics and clinical presentation, and commonly include people aged 60 years and over with weight loss and other symptoms[6]. Early symptoms are intermittent and overlap with those of other common benign conditions. The difficulty in symptom recognition is compounded by a lack of effective objective diagnostic methods that could be employed in general practice. The vast majority of biomarker studies have targeted high-risk groups, such as those with hereditary pancreatitis, familial pancreatic cancer and intraductal papillary mucinous neoplasms. To date, translation of biomarkers into clinical use has failed for a variety of reasons, including failure to include appropriate controls, such as patients with chronic pancreatitis, and failure to account for confounding factors such as biliary obstruction and diabetes[7,8].

The role of volatile organic compounds (VOCs) in exhaled breath as biomarkers has been investigated in cancers of the breast, oesophagus, stomach, colon, rectum

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and lung[9–14]. The authors[15,16] have previously developed and validated a breath test for the diagnosis of oesophagogastric and colorectal adenocarcinomas. The primary objective of the present study was to investigate changes in exhaled-breath VOCs from patients with primary pancreatic cancers compared with people who have benign pancreatic disease or normal pancreas.

Methods

All enrolled patients were recruited from the Imperial College NHS Trust from March 2016 to December 2016. Regional ethical approval was granted (14/LO/1136). Details of the study were explained to all eligible patients, and fully informed and written consent was obtained before enrolment. Demographic and clinical information was collected.

Study design and patient recruitment

A development cohort and a validation cohort were studied. In the development cohort, exhaled breath was collected and analysed to identify VOCs that differed in concentration between patients with cancer and controls without cancer. The compounds were used to develop the diagnostic model for pancreatic cancer and validated using a second independent cohort.

In both the development and validation cohorts, patients with pancreatic cancer were compared with a control (no cancer) group that included patients with benign pancreatic diseases. For the pancreatic cancer group, patients with localized pancreatic cancers provided breath samples before operation on surgical wards or in the endoscopy unit before undergoing endoscopic ultrasonography. Patients with non-resected metastatic pancreatic cancers were recruited from oncology clinics. For the control group, patients were recruited with a diagnosis of other pancreatic conditions, including intraductal papillary mucinous neoplasm, cysts, pseudocysts and chronic pancreatitis. Patients scheduled for abdominal ultrasound examination with a normal-appearing pancreas on imaging were also recruited to the control group.

Reference test

All diagnoses were confirmed with a standard reference test. Pancreatic cancer was confirmed by abdominal CT or endoscopic ultrasonography and histologically by fine-needle aspiration biopsy. Abdominal CT or ultrasonography was used to examine the pancreas of patients in the control group.

Exhaled breath collection

Exhaled breath was collected using previously validated methodology[11] that was informed by investigations regarding the influence of breath manoeuvres and hospital environment on VOC measurements[17,18]. All patients were fasted and ceased smoking for a minimum of 4 h before breath sampling to minimize the risk of oral contamination or dietary intake acting as a confounder. Atmospheric air from sample collection rooms and the laboratory was also analysed to investigate the effects of background VOCs on collected breath samples.

The method of breath sampling was changed from use of inert steel bags (GastroCH$_4$ECK®; Bedfont Scientific, Maidstone, UK) in the initial profiling study to use of the ReCIVA™ breath sample system (Owlstone Medical, Cambridge, UK) in the validation study. The ReCIVA™ is a reproducible system that allows direct collection of breath on to thermal desorption (TD) tubes. The system will be used in a planned multicentre study and so was tested in the present validation cohort.

Breath was collected using secure 500-ml steel breath bags (GastroCH$_4$ECK®), which were washed through with synthetic air before sampling. Patients were asked to perform deep nasal inhalation followed by complete exhalation through the mouth. Alveolar air was collected preferentially over dead-space air by capturing end-expiratory breath. VOCs from breath bags were then preconcentrated on to TD tubes by transferring 250 ml of breath at 50 ml/min across the tubes by use of 10-mm diameter tubing and hand-held air pumps (210-1002MTX; SKC, Blandford Forum, UK) (*Fig. 1*).

With the ReCIVA™ system, breath sampling remains completely non-invasive and involves placing a disposable face mask around the nose and mouth, and instructing the patient to perform normal tidal breathing. A constant supply of clean air is supplied to the patient's mask by means of a pump connected to an active charcoal scrubber (CASPER system; Owlstone Medical). The ReCIVA™ system uses an internal carbon dioxide monitor and pressure sensors to capture alveolar breath preferentially and transfer it directly on to TD tubes. As with bag collection, a total of 250 ml of alveolar breath was transferred on to the TD tubes.

Mass spectrometric analysis

All samples were analysed within 48 h of collection. Data from degradation studies have shown that volatiles remain stable within breath bags or when stored on TD tubes for 48 h[19]. TD–gas chromatography (GC)–mass spectrometry (MS) is an analytical method used for the identification and quantification of volatile and semivolatile

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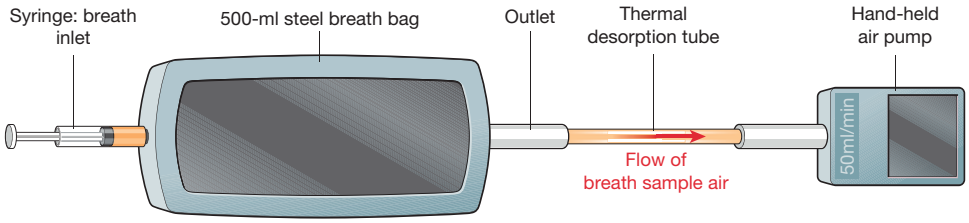

Fig. 1 Process of concentrating volatile organic compounds from steel breath bags on to thermal desorption tubes. The arrow indicates the direction of flow of the breath sample

compounds. VOCs entering the GC inlet travel through a chromatography column (Zebron ZB-624; Phenomenex, Macclesfield, UK), are separated according to their affinity with the stationary phase, and exit the column at a specific retention time. The VOCs then enter a mass spectrometer (5977A MSD; Agilent Technologies, Stockport, UK), where they are ionized, separated based on their mass-to-charge (m/z) ratios and eventually detected. The combination of both GC and MS allows improved compound identification compared with use of either component individually.

VOCs were concentrated before GC–MS analysis by fixing them to adsorbent materials that line the inside of the TD tube. All TD tubes (Tenax® TA/Carbograph™ 5TD; Markes International, Llantrisant, UK) were conditioned (TC-20™ tube conditioning station; Markes International) at 300°C for 80 min. The tubes were loaded on to carousels, checked for leakage, and dry purged for 3 min to remove excess moisture in order to ensure that VOCs were not oxidized upon heating. Using an automated TD system (TD-100™; Markes International), the tube was then heated at 280°C, with the breath sample transferred on to a 10°C cold trap for 10 min of desorption (nitrogen flow 50 ml/min). Subsequently, the cold trap was heated rapidly to 290°C, transferring the VOCs to the chromatography column. In an attempt to minimize fixation of background VOCs to the tubes, the time from tube conditioning to preconcentration never exceeded 1 h. Further details of the GC–MS methodology can be found in *Appendix S1* (supporting information).

Data extraction

Chromatograms and mass spectral data were extracted using MassHunter Qualitative software (Agilent Technologies, Santa Clara, California, USA). The chemical identity of every peak, with retention times between 3 and 47 min, was confirmed by reference to the National Institute of Standards and Technology (NIST) MS library version 2.0. Each identified compound was then semiquantified through a representative mass ion. In a first step, peaks were extracted in automated fashion using Agilent MassHunter Quantitative software, which ran the analysis across all chromatograms. In a further step, data were revised manually with the aim of correcting errors due to misidentifications, random variation in retention times and possible co-eluting chromatographic peaks. A researcher blinded to the clinical disease state of the patient undertook the data extraction.

Statistical analysis

Tumour disease status and confounding factors were considered independent variables and VOC abundance was considered the dependent variable. A Shapiro–Wilk statistical test was performed to check the data distribution. Significant differences in the abundance of VOCs between cancer and no-cancer groups in the development cohort were assessed using univariable Mann–Whitney *U* tests, as the data were not normally distributed. VOCs found to be significant on univariable analysis were included in a logistic regression analysis to form the basis of a diagnostic model for use in the validation cohort. Receiver operating characteristic (ROC) curves were produced by plotting the true-positive rate (sensitivity) against the false-positive rate (1 – specificity). ROC plots were constructed for comparison of cancer *versus* no cancer and adenocarcinoma *versus* no cancer. An additional subset ROC analysis was undertaken for localized pancreatic adenocarcinoma *versus* no cancer. The area under the curve (AUC) was used to assess the predictive power of the model and its ability to distinguish between cancer and no cancer. Sensitivity and specificity values were extracted from the coordinates of the ROC plots. The cancer group included all subgroups of pancreatic cancer, whereas the no-cancer group included both positive control (other pancreatic non-cancer disease) and normal pancreas groups.

Statistical analysis was also performed to identify significant differences between the groups in age, ethnicity, sex, gastro-oesophageal reflux disease, pancreatitis, gastric ulcers, hepatitis, diabetes mellitus, smoking status and

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Table 1 Demographic and clinical data in development and validation cohorts

	Development cohort			Validation cohort		
	Cancer ($n = 25$)	No cancer ($n = 43$)	P†	Cancer ($n = 32$)	No cancer ($n = 32$)	P†
Age (years)*	70 (62–77)	60 (44–72)	0·170‡	68 (61–72)	58 (49–74)	0·108‡
Sex ratio (M : F)	15 : 10	21 : 22	0·374	21 : 11	18 : 14	0·442
Caucasian	19	23	0·065	24	21	0·412
Gastro-oesophageal reflux	10	13	0·412	7	8	0·873
Pancreatitis	3	10	0·098§	4	12	0·021§
Liver impairment	6	14	0·455†	9	3	0·055§
Previous malignancy	5	4	0·218§	2	3	0·999§
Gastric ulcer	1	2	0·999§	0	0	0·999§
Viral hepatitis	2	7	0·060§	1	0	0·999§
Diabetes mellitus	11	9	0·060†	8	4	0·246§
Smoking status			0·061¶			0·594¶
Current	1	10		8	6	
Ex-smoker	13	12		12	10	
Never	11	21		12	16	
Alcohol intake			0·468¶			0·688¶
Within guidance	23	36		29	28	
Excessive	2	4		3	4	
Missing	0	3		0	0	
Cancers						
Localized adenocarcinoma	7	–		14	–	
Localized NET	4	–		2	–	
Metastatic adenocarcinoma	10	–		14	–	
Metastatic NET	4	–		2	–	

*Values are median (i.q.r.). Steel bags were used for breath sampling in the development cohort and the ReCIVA™ breath sample system in the validation cohort. NET, neuroendocrine tumour. †χ^2 test, except. ‡Kruskal–Wallis test, §Fisher's exact test and ¶likelihood ratio test.

alcohol intake. Kruskal–Wallis test was used for analysis of age as a continuous variable, whereas all other nominal potential confounder data were assessed using the χ^2 test, Fisher's exact test or the likelihood ratio test depending on the expected count numbers and the number of variables tested. All confounders were subsequently tested against VOC abundance by means of linear regression. $P < 0{\cdot}050$ was considered significant, and all statistical tests were two-sided. All statistical analysis was carried out using SPSS® version 24 (IBM, Armonk, New York, USA).

Results

A total of 68 patients were recruited to the model development cohort (*Table 1*). Twenty-five patients were assigned to the cancer group and 43 to the no-cancer group. Cancers included localized adenocarcinoma (7; pancreatic ductal adenocarcinoma in 6), localized neuroendocrine tumour (NET) (4), metastatic adenocarcinoma (10) and metastatic NET (4). The no-cancer group included 20 positive controls and 23 patients with normal pancreas.

A further 64 patients were recruited to the validation cohort, 32 with and 32 without cancer. Cancers included local adenocarcinoma (14; pancreatic ductal adenocarcinoma in 12), local NET (2), metastatic adenocarcinoma (14) and metastatic NET (2). The no-cancer group comprised 24 positive controls and eight patients with normal pancreas. There were no significant differences in patient demographics or co-morbidities between the cancer and no-cancer groups (*Table 1*).

Analysis of volatile organic compounds

Qualitative analysis of chromatograms yielded 66 VOCs that were identifiable from the NIST database. Twenty-two of these were excluded from further analysis as they were either found to be at high concentrations in background air or considered unlikely to be produced endogenously. The identity of the remaining 44 VOCs, as well as their retention times and characteristic m/z ratio, were subsequently used to establish VOC relative abundance.

Ten VOCs had a significantly altered abundance in cancers in the development cohort (*Table 2*). Further analysis also revealed 12 VOCs with a significantly altered abundance in a comparison of adenocarcinoma *versus* no cancer. Of these, the abundance of five was found to be raised in cancer (formaldehyde, acetone, acetoin,

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Table 2 Significant volatile organic compounds in comparisons of samples from patients with cancer and those without cancer

	Cancer *versus* no cancer			Adenocarcinoma *versus* no cancer		
	Bags	ReCIVA™	*P**	Bags	ReCIVA™	*P**
Formaldehyde	↑	↑	0·011	↑	↑	0·038
Pentane	↓	↓	0·002	↓	↓	0·007
Acetone	↑	↑	0·049	↑	↑	0·019
Isopropyl alcohol	↑	↑	0·002	↑	↑	0·001
n-Hexane	↓	↓	< 0·001	↓	↓	0·001
1-(Methylthio)-propane	↓	↓	0·025	↓	↓	0·013
Acetoin	↑	↑	0·003	↑	↑	< 0·001
Benzaldehyde	↓	↓	0·002	↓	↓	0·003
Undecane	↑	↑	0·049	↑	↑	0·044
Tetradecane	↓	↓	0·019	↓	↓	0·035
Amylene hydrate			0·457	↓	↓	< 0·001
1-Butanol			0·407	↓	↓	0·005

Steel bags were used for breath sampling in the development cohort and the ReCIVA™ breath sample system in the validation cohort. Arrows indicate the direction of change for the cancer cohort compared with the cohort without cancer. Arrows are omitted for non-significant volatile organic compounds. *Mann–Whitney *U* test for bag data.

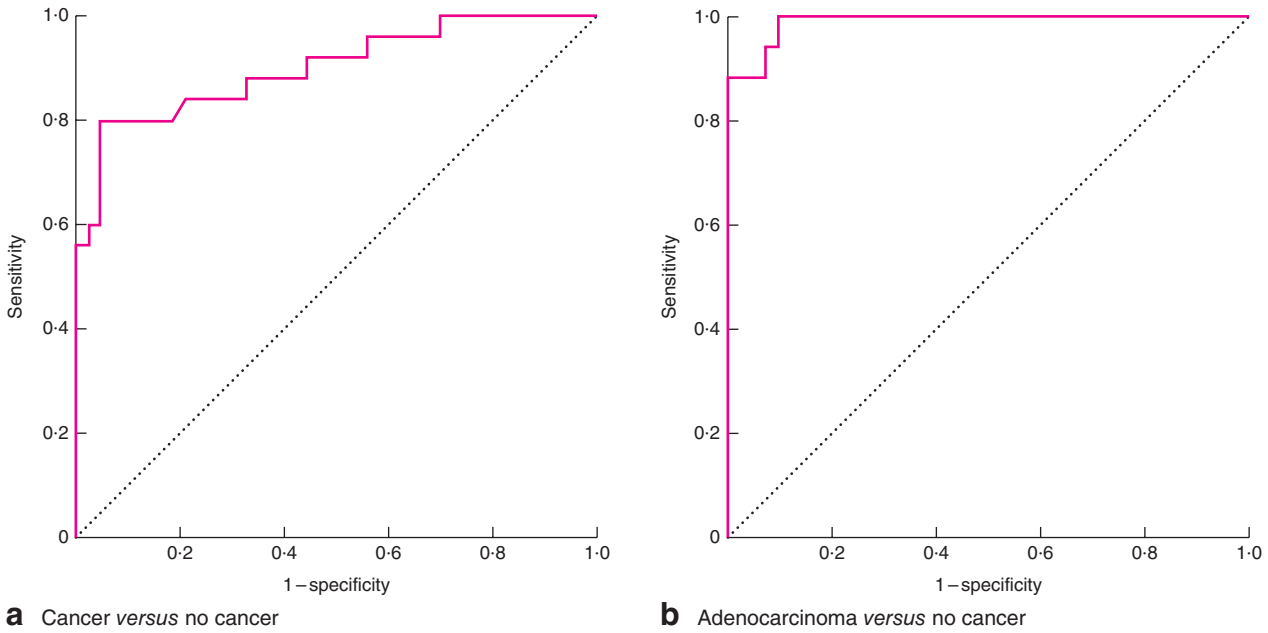

Fig. 2 Receiver operating characteristic (ROC) plots for **a** cancer *versus* no cancer and **b** adenocarcinoma *versus* no cancer using data from the development cohort. Steel breath bags were used for breath sampling

undecane, isopropyl alcohol), and the remaining seven were reduced in breath from patients with cancer (pentane, *n*-hexane, 1-butanol, 1-(methylthio)-propane, benzaldehyde, tetradecane, amylene hydrate). This direction of change was found to be the same for all significant VOCs in data from both the development and validation cohorts.

Linear regression analysis revealed that pancreatic cancer disease status was the strongest predictor of all significant differences in VOC abundance (*Tables S1* and *S2*, supporting information). No confounders were found to be independent predictors of abundance of any of the significantly dysregulated VOCs.

Receiver operating characteristic (ROC) curve analysis

ROC plots were constructed for both cohorts using only VOCs that were significantly dysregulated in breath from patients with cancer in the development cohort. For the model development study, the ROC plot had an AUC of

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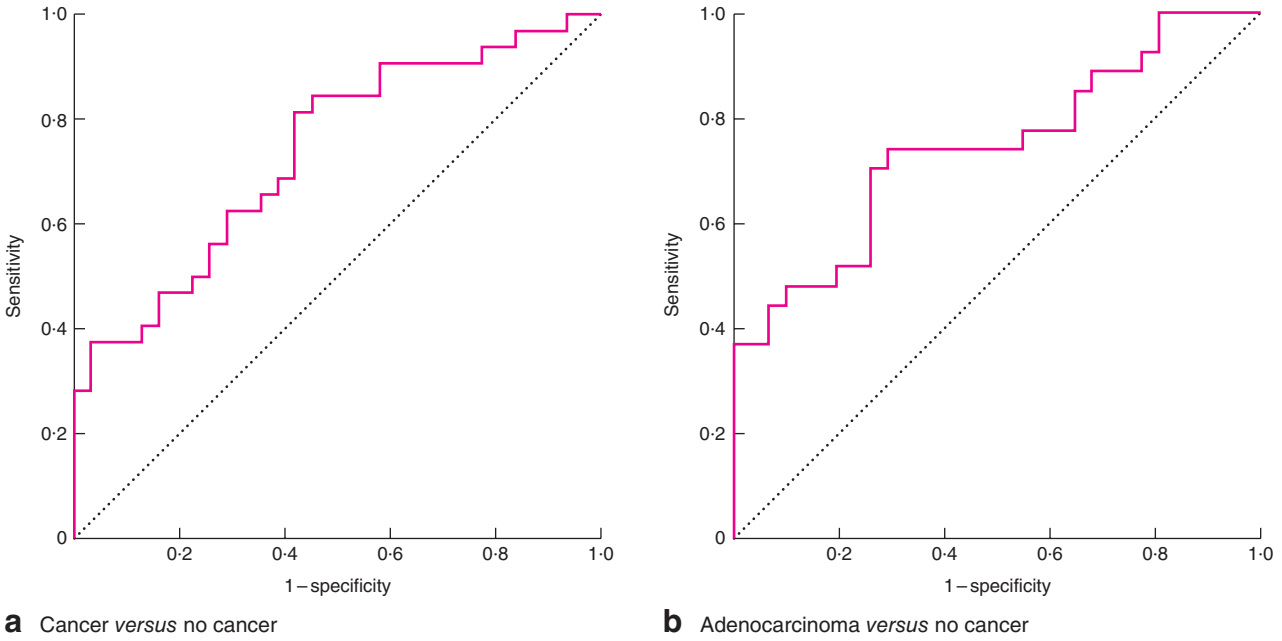

Fig. 3 Receiver operating characteristic (ROC) plots for **a** cancer *versus* no cancer and **b** adenocarcinoma *versus* no cancer using data from the validation cohort. The ReCIVA™ breath sample system was used for breath sampling

0·901 (95 per cent c.i. 0·819 to 0·982) for distinguishing cancer from no cancer, with a sensitivity and specificity of 80 and 95 per cent respectively (*Fig. 2a*). The AUC for the analysis of adenocarcinoma *versus* no cancer was 0·990 (0·973 to 1·00), with a sensitivity and specificity of 94 and 91 per cent respectively (*Fig. 2b*). For localized adenocarcinoma *versus* no cancer, the AUC was 1·000, with a sensitivity and specificity of 100 per cent, as all cases of localized adenocarcinoma were correctly distinguished from non-cancer cases.

For the model validation study, the AUC for distinguishing cancer from no cancer was 0·736 (0·614 to 0·858), producing a sensitivity of 81 per cent and specificity of 58 per cent (*Fig. 3a*). The AUC for distinguishing adenocarcinoma from no cancer was 0·744 (0·615 to 0·873) with a sensitivity of 70 per cent and specificity of 74 per cent (*Fig. 3b*). In the validation study, the AUC for localized adenocarcinoma *versus* no cancer was 0·855 (0·732 to 0·914), with a sensitivity of 79 per cent and specificity of 81 per cent.

Discussion

Analysis of VOCs in exhaled breath identified a total of 12 compounds that were significantly dysregulated in patients with pancreatic cancer. The significant VOCs were from three main chemical groups, namely aldehydes, alkanes and alcohols. All ROC models showed good discrimination, with AUC values over 0·700. Discrimination was stronger in the models distinguishing adenocarcinoma from no cancer.

The chemical group with the largest number of significantly dysregulated breath VOCs in pancreatic cancer was the aldehyde group. Other studies[9,13,20] have also demonstrated changes in breath aldehyde in patients with cancer, including oesophagogastric, colorectal and lung tumours. The specific aldehydes of interest were different depending on the cancer site. There are few data available on the mechanisms underlying breath aldehyde changes in pancreatic cancer. One possible explanation is altered activity of enzymes, such as aldehyde dehydrogenase isoform 1 (ALDH1), as demonstrated in an *in vitro* study[21]. ALDH1 causes the irreversible breakdown of aldehydes to their corresponding carboxylic acid or alcohols, and for this reason is thought to be crucial for the survival of cancer stem cells[22]. By comparing a range of normal and cancerous epithelial tissues, Deng and colleagues[23] were able to demonstrate that pancreatic cancers showed the most extensive expression and activity of ALDH1. This increased activity and expression of ALDH1 in pancreatic cancers may explain the decreased levels of benzaldehyde and the altered levels of alcohols observed in the present study.

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Currently, carbohydrate antigen (CA) 19-9 is the most commonly used tumour marker for pancreatic cancer. However, it shows a non-specific increase in a number of benign and malignant conditions, including pancreatitis, cirrhosis, acute cholangitis and colorectal cancer[3]. Furthermore, is not expressed in 5–10 per cent of the Caucasian population due to a Lewis a^-/b^- genotype[24]. Overall, only 65 per cent of patients with surgically resectable pancreatic cancer have a raised level of CA19-9[3]. As breath testing in pancreatic cancer was still in the early validation phase in the present study, it was considered inadvisable to make firm comparisons between breath VOC and CA19-9 testing.

The strength of the study lies in its design, with the inclusion of a positive control group, a reference test for each patient, and an independent cohort of patients to validate volatile biomarkers employing a different breath collection method. The method used in the validation study lends itself to multicentre clinical investigations, as ReCIVA™ provides a reproducible breath collection method and TD tubes offer a robust transport system that keeps volatile compounds stable for approximately 4 weeks. The results provide the foundation for a planned, large multicentre study that could further establish the potential of breath VOC testing as a diagnostic tool for pancreatic cancers. The identification of VOC cancer biomarkers permitted cross-platform mass spectrometric validation and mechanistic studies of VOC production in cancer states, thus increasing the scientific rigour of the breath VOC diagnostic field. Other research groups have used sensor-based technology such as an electronic nose to identify the presence or absence of a disease state[25]. A recent study[26] used ion-mobility MS to diagnose pancreatic cancer from urinary VOCs, with an impressive diagnostic accuracy (91 per cent sensitivity and 83 per cent specificity). Nevertheless, this study requires external validation.

The study has several limitations. The results of this single-centre investigation must be externally validated. Importantly, confounders not included in the present analysis such as weight or BMI may influence the concentration of VOCs. The method of breath sampling was changed between the development and validation cohorts, which may have influenced the VOC concentration and recovery of certain VOCs. However, in the validation study, the identity of compounds dysregulated in pancreatic cancer was confirmed and not the specific levels of each compound. The performance of the test should be examined in early pancreatic cancer as an ultimate goal for the breath test that could influence disease survival. The present study included patients with locally advanced and metastatic disease as this group represents the majority of patients with pancreatic cancer in clinical practice and should not be missed by the diagnostic model. However, the diagnostic accuracies for localized pancreatic adenocarcinoma in the development and validation cohorts were 100 and 86 per cent respectively, suggesting the potential for early diagnosis that requires more robust specific investigation in a large-scale multicentre study.

Breath VOC sampling is a completely non-invasive test with high acceptability among patients and clinicians[11,17–19]. The authors envisage using exhaled breath testing as a triage investigation to establish the risk of pancreatic cancer in patients presenting with non-specific symptoms to guide referral for CT. Another application is screening for high-risk groups such as those with hereditary pancreatitis, familial pancreatic cancer, resent-onset diabetes and intraductal papillary mucinous neoplasms. The final application of breath testing in the patient care pathway will depend on test sensitivity and specificity in large multicentre clinical trials, and its performance in early pancreatic cancer and high-risk groups.

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Supporting information

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