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Title: Mass spectrometry analysis of mixed breath, isolated bronchial breath and gastric endoluminal volatile fatty acids in oesophagogastric cancer

Mina E Adam[1*], Matyas Fehervari[1*], Piers R Boshier[1], Sung-Tong Chin[1], Geng-Ping Lin[1,], Andrea Romano[1], Sacheen Kumar[1,2], George B Hanna[1]

[1]*Department of Surgery & Cancer, Imperial College London, United Kingdom*

[2]*Department of Upper Gastrointestinal Surgery, The Royal Marsden Hospital, United Kingdom*

[*]These authors contributed equally to experimental work and preparation of the manuscript

Corresponding Author

Professor George Hanna,

Department of Surgery and Cancer,

Imperial College London,

10th Floor QEQM Building,

St Mary's Hospital,

London, W2 1NY UK

E-mail: g.hanna@imperial.ac.uk

Telephone No: +44 (0)207 886 2125

Fax No: +44 (0)207 8862125

Abstract

A non-invasive breath test has the potential to improve survival from oesophagogastric cancer by facilitating earlier detection. This study aimed to investigate production of target volatile fatty acids (VFAs) in oesophagogastric cancer through analysis of *ex vivo* headspace above un-derivatised tissues and *in vivo* analysis within defined anatomical compartments including mixed breath, isolated bronchial breath and gastric endoluminal air. VFAs were measured by PTR-ToF-MS and GC-MS. Levels of VFAs (acetic-, butyric-, pentanoic- and hexanoic-acid) and acetone were elevated in *ex-vivo* experiments in the headspace above oesophagogastric cancer compared to samples from control subjects with morphologically normal and benign conditions of the upper gastrointestinal tract. In 25 patients with oesophagogastric cancer and 20 control subjects, receiver operating characteristic analysis for cancer specific VFAs butyric acid (P<0.001) and pentatonic acid (P=0.005) within *in-vivo* gastric endoluminal air gave an area under the curve of 0.80 (95% CI 0.65 to 0.93; P=0.01). Compared to mixed and bronchial breath samples, all examined VFAs were found in highest concentrations within oesophagogastric gastric endoluminal air. In addition, VFAs were higher in all samples derived from cancer patients compared to controls. Equivalence of VFA levels within the mixed and bronchial breath of cancer patients suggests that their origin within breath is principally derived from the lungs and by inference the systemic circulation as opposed to direct passage from the upper gastrointestinal tract. These findings highlight the potential to utilise VFAs for endoluminal gas biopsy and non-invasive mixed exhaled breath testing for oesophagogastric cancer detection.

Keywords: Breath analysis; real-time monitoring; volatile organic compounds; fatty acid; endoluminal

Introduction

The chemical analysis of volatile organic compounds (VOCs) in humans is a rapidly evolving field that has the potential to contribute to the non-invasive detection of multiple disease states. A recent systematic review on the diagnostic accuracy of VOC-based exhaled breath tests showed their potential for non-invasive cancer detection.[1]

Previous studies have reported higher concentrations of specific VOCs, including volatile fatty acids (VFAs), within the exhaled breath, gastric content and urine of patients with oesophagogastric cancer.[2-7] Whilst several studies have suggested a role for these VFAs in important regulatory processes in oesophagogastric cancer,[8,9,10] many of the biochemical pathways relating to their origin in humans are as yet unknown. Notwithstanding it is postulated that deregulated production of specific VFAs occurs directly from cancer tissues. These VFAs may pass in to the systemic circulation with subsequent partition across the alveolar-capillary barrier. Alternatively, VFAs may be released directly by the mucosa of the aerodigestive tract.[11,12] Hence targeted quantification of these compounds within the headspace of oesophagogastric tissue and isolated body compartments may prove helpful in determining the origin and mechanisms of release of these compounds.

The ability to interpret VOCs measurements from complex and dynamic biological matrices remains challenging. Technological advances in gas phase analytical techniques permit measurement of VOCs emitted from the headspace of bio fluids and histological specimens at levels of parts-per-trillion by volume (*pptv*). In particular, mass spectrometry techniques including Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) and Gas Chromatography Mass Spectrometry (GC-MS) have been widely utilised for VOC detection in human studies.[13-16] PTR-ToF-MS in notable for its ability to perform real-time analysis of a full mass spectrum within a fraction of a second and with separation and identification of isobaric ions.

The purpose of this study was to investigate production of targeted VFAs in oesophagogastric cancer through analysis of *ex vivo* headspace above tissues and *in vivo* within different anatomical compartments including mixed breath, isolated bronchial breath and gastric endoluminal air. Determining the relative abundance of VFAs within these compartments may provide a clearer understanding of their source of origin and association with oesophagogastric cancer. Endoluminal gas sampling provides a true gas biopsy for the detection of oesophagogastric cancer.

Methodology

Study Population

Subjects were recruited from St Mary's Hospital, Imperial College Healthcare NHS Trust between 2015 and 2017. Comparative analysis of tissue headspace VFAs was performed in patients with: (i) biopsy proven oesophagogastric cancer; benign disease of the upper gastrointestinal tract (e.g., esophagitis, gastritis and peptic ulcer disease), and; morphologically normal upper gastrointestinal tract with a negative rapid urease test for *Helicobacter Pylori* on endoscopy. All patients were required to be fasted and to refrain from smoking for a minimum of six hours prior to testing. Patients were excluded if they had known liver disease, small bowel/colonic conditions or a synchronous cancer at another site. Local ethics committee approval through the NHS Health Research Authority was granted (Ref: 5/LO/1140 and 12/WA/0196) and written informed consent was obtained from all patients prior to enrolment in this study.

Targeted analysis of volatile fatty acids within tissue headspace ex vivo

In the initial phase of this study VFA emissions from *ex vivo* tissue biopsies were investigated. Tissue samples were taken using 2.8mm cold biopsy forceps (EndoJaw, Olympus, UK) during upper gastrointestinal endoscopy. Samples were retrieved from the diseased area. In patients with a normal upper gastrointestinal tract, tissue samples were taken from the body of the stomach. Tissue samples were extracted from the cold biopsy forceps in a sterile manner and immediately snap frozen in liquid nitrogen. All samples were stored at -80^{o}C prior to analysis.

Preliminary experiments were performed to evaluate the effect of leaving biopsies to thaw at room temperature for 0.5, 1.0 and 2.0 hours in a sealed vial prior to headspace analysis. These initial experiments determined no benefit in allowing tissue biopsy headspace to develop for longer than 30 minutes. Accordingly, this time point was chosen for all subsequent analyses.

Biopsies were placed in a 20ml screw-capped vial (Thermo Scientific, Hemel Hempstead UK), which were allowed to equilibrate at room temperature for 30

minutes. Selected VFAs (acetic-, butyric-, pentanoic- and hexanoic acid) and acetone were analysed using the H_3O^+ precursor ion of PTR-ToF-MS (PTR-ToF 1000, Ionicon Analytik GmbH, Innsbruck, Austria) (Table 1). Analysis of acetone was performed, as it is both a breakdown product of fatty acid oxidation and important intermediary of global energy metabolism. Drift tube conditions were: temperature 110°C, pressure 2.30 mbar and voltage 350V, resulting in an E/N of 84 Td (1 Townsend = 10^{-17} V cm^2). Sampling was carried out by means of a heated (110°C) PEEK tubing and the inlet flow was set at 130sccm. Headspace analysis was performed by piercing the septum of the vial with a sterile needle attached to the PTR-ToF-MS sample inlet. A second perforation in the septum was created to allow clean air to be flushed in to the vial at a rate of 40ml/min (Figure 1a).

Table 1. Summary of analytical information for compounds detected and quantified by PTR-ToF-MS using the H_3O^+ precursor ion

Compounds	Molecular formula	*m/z*	Characteristic product ions
Acetone	C_3H_6O	59.049	$C_3H_6OH^+$
Acetic acid	$C_2H_4O_2$	61.028	$C_2H_4O_2\ H^+$
Butyric acid	$C_4H_8O_2$	89.060	$C_4H_8O_2H^+$
Pentanoic acid	$C_5H_{10}O_2$	103.075	$C_5H_{10}O_2H^+$
Hexanoic acid	$C_6H_{12}O_2$	117.091	$C_6H_{12}O_2H^+$

In a single patient, direct headspace analysis of a gastric tumour and adjacent 'normal' mucosa was performed immediately after resection of the whole stomach. A sterile polystyrene sample container (60 mL) was modified to permit the passage of the PTR-TOF-MS sample line through its base and was placed over the tumour and the headspace was analysed for 60 seconds (Figure 1b). Headspace above adjacent gastric mucosa that was macroscopically uninvolved by tumour was subsequently analysed.

Analysis by PTR-ToF-MS

A PTR-ToF 1000 mass spectrometer equipped with a commercial SRI feature

(Ionicon Analytik GmbH, Innsbruck, Austria) was used for the analysis. Detailed system setup has been described previously.[17] During the current experiments, a series of quality checks were conducted on the PTR-ToF-MS daily. Quantitative accuracy was within ±10% of a certified standard, represented by a Trace Source™ benzene permeation tube (Kin-Tek Analytical Inc., La Marque TX). When H_3O^+ was used as the primary ion, O_2^+ impurities were <2%. Repeatability of fragmentation patterns with H_3O^+ was assessed by measuring the ratio between peaks *m/z* 89 and 71 which was used to represent the quasi-molecular and the most representative fragment for butyric acid, as obtained from a permeation tube standard. The values measured on the different days were within ±2% of the mean. When required, the voltage of the microchannel plate and the mass resolution (>1,500 m/δm) was optimised using *m/z* 89 (butyric acid with H_3O^+) as reference peak. Data were first extracted using PTRMS viewer version 3.2.2.2 (Ionicon Analytik) and subjected to further analysis using in-house generated scripts written using R-programming language. Target analysis was performed for compounds presented in Table 1.

Targeted analysis of volatile fatty acids within separate in vivo compartments

The second phase of this study focused on validating the findings of *ex vivo* tissue experiments, *in vivo,* through the targeted analysis of VFAs within three anatomical compartments: (i) mixed breath; (ii) isolated bronchial breath, and; (iii) gastric endoluminal air.

Analysis of 'mixed breath': prior to either upper gastrointestinal endoscopy and/or elective surgery a 500 mL mixed (containing dead space and alveolar air) breath sample was collected using the ReCIVA breath sampler (Owlstone, Cambridge, UK) in accordance with an established methodology (Figure 2a).[18] Briefly patients were asked to breathe tidally into the device through a single use facemask. Exhaled breath was pumped on to four thermal desorption (TD) tubes (Markes International, Llantrisant, UK) pre-packed with 200 mg of Tenax and 100 mg of Carbograph 5 to a total volume of 500ml per tube.

Analysis of isolated bronchial breath: in cancer patients undergoing surgery (staging laparoscopy and upper gastrointestinal endoscopy), a sample of isolated bronchial breath was obtained shortly after induction of general anaesthesia and endotracheal intubation. Bronchial breath (500ml) was sampled directly onto TD tubes using a handheld precision 210-1002MTX pump (SKC Ltd, Dorset, UK). Breath was sampled from the capnography port of the ventilator circuit throughout the respiratory cycle (Figure 2b). The following standardised ventilatory settings were applied 5mins prior to and for the duration of sampling: fraction of inspired oxygen 100%; respiratory rate 10 breaths per minute, and; 5mmHg positive end expiratory pressure. All traces of volatile anaesthetic gases were removed from the anaesthetic circuit prior to bronchial sampling to avoid their potential influence on breath gas analysis. Total intravenous anaesthesia was induced and maintained using alfentanil and propofol.

In vivo analysis of gastric endoluminal air: a dedicated method was developed to sample gastrointestinal endoluminal air through the operating channel of a flexible endoscope. After inflation of the stomach with medical air during upper gastrointestinal endoscopy a 2mm wide sample line (V-green, Vygon, Paris, France) was advanced in to the gastric lumen. The proximal end of the sampling line was connected to a TD tube and a sample of 500ml luminal air was obtained at a rate of 250 ml/min using a handheld precision 210-1002MTX pump (Figure 2c).

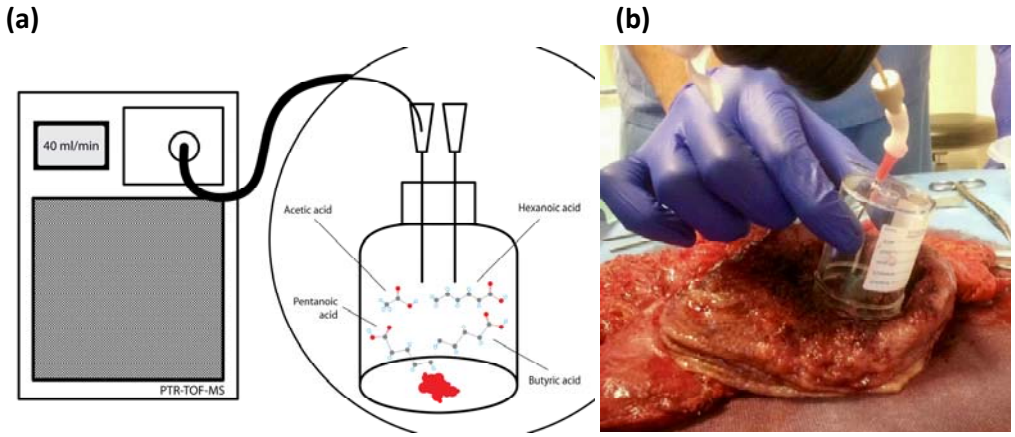

Figure 1. (a) *Ex vivo* headspace analysis with PTR-ToF-MS. (b) Direct PTR-ToF-MS analysis of the headspace of cancer and healthy tissue regions of a surgically resected stomach

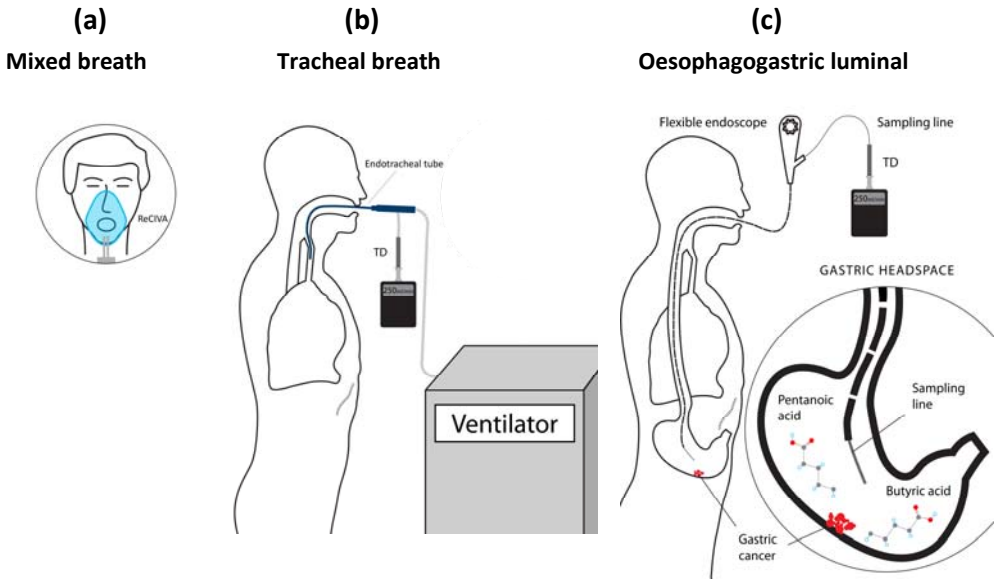

Figure 2. (a) Pre-procedure mixed breath samples collection with the ReCIVA device and (b) intra-operative sampling of the isolated bronchial breath via the endotracheal tube. (c) Sampling of the gastric endoluminal headspace via a suction channel of a standard endoscope with a custom made catheter directly adjacent to the tumour. (TD, thermal desorption tube).

Analysis by Thermal Desorption GC-MS

Samples were analysed using an Agilent 7890B GC with 5977A MSD (Agilent Technologies, Cheshire, UK), coupled to a Markes TD-100 device (Markes International, Llantrisant, UK). Prior to sample collection TD tubes were conditioned at 325°C for 40 minutes in a stream of nitrogen passed through a hydrocarbon trap (Supelco, US) using a Markes International TC-20 tube conditioner (Markes International, Llantrisant, UK). Details of the conditions of analysis using TD-GC-MS have been published elsewhere.[19] Briefly, TD tube samples were pre-purged for 1 min at 50 mL/min constant helium flow rate prior to 280°C for 10 min. Following secondary desorption by heating the cold trap (U-T12ME-2S) from 10 °C to 290°C at 99°C/min and held for 4 min. The GC flow path was heated constantly at 140°C. VOC separation was performed on a ZB-624 capillary column (60 m × 0.25 mm ID × 1.40 µm df; Phenomenex Inc., Torrance, USA) programmed at 1.0 mL/min constant Helium carrier flow. Oven temperature profile was set at 40°C initially for 4 min, ramp to 100°C (5°C/min with 1 min hold), ramp to 110°C (5 °C/min with 1 min hold), ramp to 200°C (5°C/min with 1 min hold), finally ramp to 240°C at 10 °C/min with 4 min hold. The MS transfer line was maintained at 240°C whilst 70 eV electron impact at 230°C was set while the quadruple was held at 150°C. MS analyser was set to acquire over the range of 20 to 250 m/z with data acquisition approximated to 6 scans/sec. GC-MS data was then processed using MassHunter software version B.07 SP1 (Agilent Technologies, Cheshire, UK) while MS data of the separated VOC component was compared with NIST Mass Spectral Library version 2.0 for identification of target compounds.[20]

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics 22 (SPSS Inc., Chicago, IL) and Prism (Ver. 7.0d, GraphPad Software, San Diego, CA). VOC data (not normally distributed) is presented as median and interquartile range. The Kruskal-Wallis test was used to compare the measured concentrations of VOCs between both the three patient groups and three in vivo compartments. The Mann-Whitney U test was use for pairwise comparisons. Correlation between variables was assess using Pearson correlation coefficient For data from gastric endoluminal experiments

receiver operating characteristic (ROC) analysis was performed for VFAs found to be significant on univariate analysis after determining their test probabilities using binominal logistic regression. Unsupervised Principal Component Analysis (PCA) and supervised orthogonal partial least square analysis (OPLS) was performed with MetaboAnalyst 4.0 software (McGill University, Canada). A p-value ≤0.05 was taken as the level of statistical significance.

Results and discussion

Targeted analysis of volatile fatty acids within tissue headspace ex vivo

Target VFAs were significantly increased above oesophagogastric cancer tissue biopsies compared to healthy controls (Table 2). Importantly, the same VFAs were also increased, albeit to a lesser extent, in the headspace of upper gastrointestinal mucosa affected by benign inflammatory conditions. Average biopsy weight was 5.5±4.3mg and did not correlate with any measured VOC concentration (R^2<0.010, P>0.086).

Table 2. Headspace concentrations (*ppbv*) of VOCs significantly increased in oesophagogastric cancer tissue

	Cancer N=45	Benign disease N=19	Healthy controls N=64	*P*[1]
Acetone	42.4 (10.8-365.1)	22.7 (10.1-42.9)	12.7 (6.0-26.2)	<0.001
Acetic acid	10.7 (4.7-26.0)	6.9 (5.3-16.6)	4.6 (0.1-8.5)	0.001
Butyric acid	2.2 (0.7-5.9)	1.1 (0.3-1.8)	0.7 (0.4-1.6)	0.004
Pentanoic acid	0.5 (0.2-0.7)	0.4 (0.3-0.6]	0.3 (0.2-0.5)	0.029
Hexanoic acid	0.6 (0.2-2.8)	0.6 (0.3-1.0)	0.2 (0.1-0.7)	0.033

Values are presented as median and interquartile range. [1]Kruskal–Wallis test

Direct sampling of the headspace of a gastric cancer immediately following surgical resection of the whole stomach was performed in a single patient using PTR-ToF-MS (Figure 3). Acetone (795.3 vs. 388.8*ppbv*), acetic acid (29.0 vs. 18.1*ppbv*), butyric acid (2.8 vs 1.8*ppbv*), pentanoic acid (1.1 vs 0.8*ppbv*) and hexanoic acid (1.7 vs 1.0*ppbv*) were observed at higher concentrations within the *in-situ* headspace above the tumour compared to macroscopically normal adjacent gastric mucosa. VFAs concentrations measured through direct sample analysis were appreciably higher than in tissue biopsy experiments potentially reflecting either the significantly greater tissue biomass above which measurements were taken or the analysis of 'fresh' tissue immediately after resection.

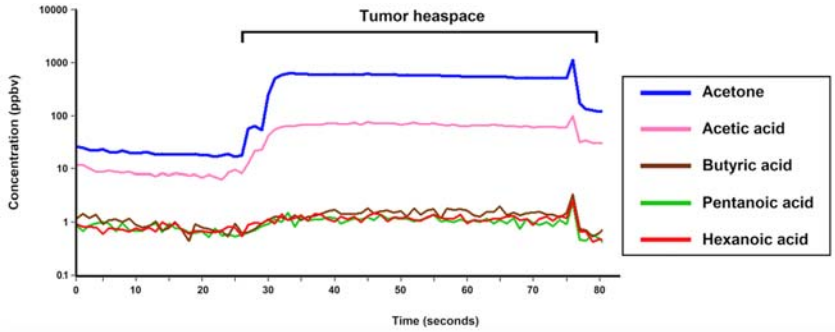

Figure 3. PTR-ToF mass spectrum of direct sampling from a fresh gastric cancer specimen.

Targeted analysis of volatile fatty acids within isolated in vivo compartments

In total 25 patients with oesophagogastric cancer (17 male, 74±14yrs) and 20 control subjects (10 male, 57±17yrs) were recruited. Baseline sampling of mixed breath using the ReCIVA device was completed in all patients and an additional isolated bronchial breath sample was collected in all cancer patients. In two patients, intraluminal gastric headspace sampling was abandoned due to contamination of the sampling line with gastric secretions. The median peak areas of the different VFAs in these compartments are presented in Table 3. ROC analysis for butyric and pentatonic acid within the endoluminal gastric air gave an area under the curve of 0.80 (95% CI 0.65 to 0.93; *P*=0.01)(Figure 4). Unsupervised PCA and supervised OPLS analysis demonstrated that the examined VFAs contribute to the clustering and discrimination of gastric endoluminal air between cancer and control subjects (Figure 5).

Table 3. Median values of peak areas (counts rate x10^3) of volatile fatty acids in different aerodigestive compartments between patients and controls

	Cancer mixed breath N=23	**Control mixed breath N=20**	***P*[1]**	**Cancer bronchial breath N=25**	***P*[2]**	**Cancer gastric endoluminal N=23**	**Control gastric endoluminal N=20**	***P*[3]**
Acetic acid	364 (280-567)	125 (7-425)	0.006	304 (208-439)	0.170	750 (319-1020)	747 (297-1076)	0.344
Buytric acid	16 (7-35)	4 (1-24)	0.047	16 (8-24)	0.653	157 (109-192)	49 (20-104)	<0.001
Pentanoic acid	8 (3-11)	4 (0-11)	0.026	8 (4-13)	0.644	144 (36-152)	31 (75-80)	0.005
Hexanoic acid	6 (2-12)	7 (2-30)	0.620	12 (5-17)	0.082	149 (52-217)	64 (24-252)	0.511

P^1 = mixed breath, cancer vs. control; P^2 = bronchial breath vs. mixed breath in patients with cancer; P^3= endoluminal air, cancer vs. control

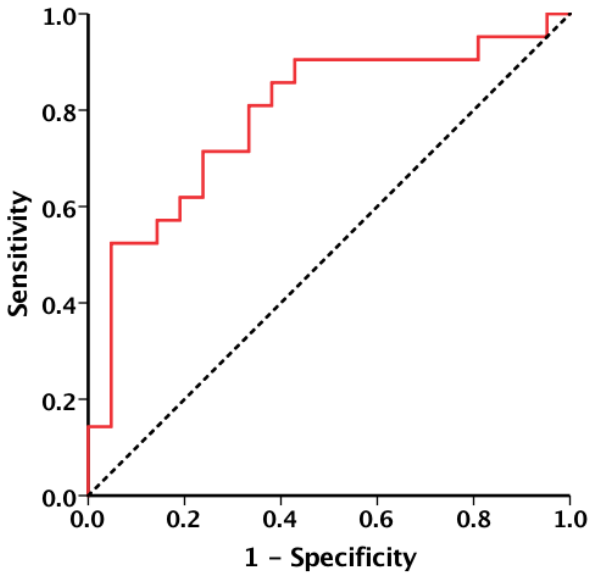

Figure 4. Receiver operating characteristic curve for gastric endoluminal volatile fatty acids significant on univariate analysis; butyric acid and pentatonic acid area under the curve of 0.80 (95% CI 0.65 to 0.93; *P*=0.01)

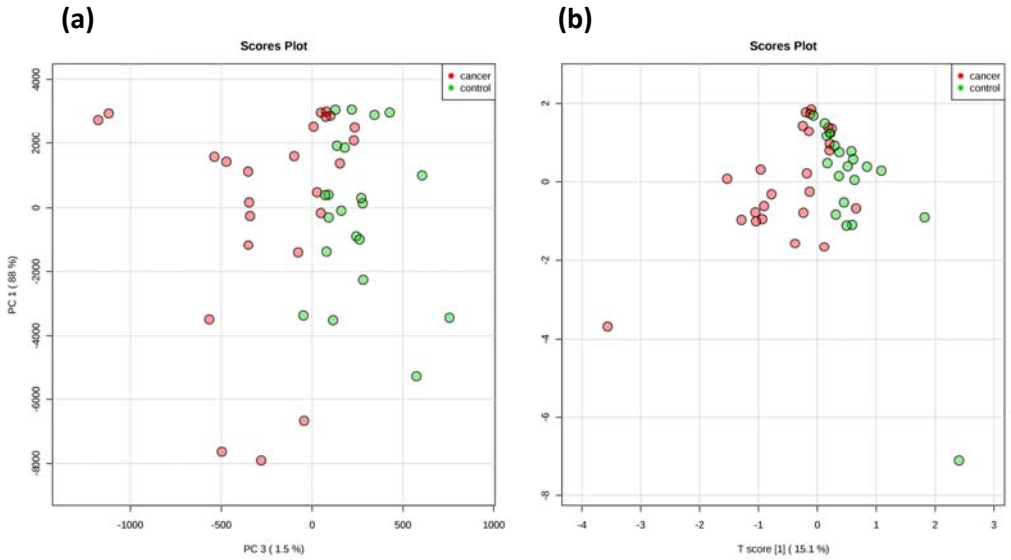

Figure 5. Principal Component Analysis (A) and Orthogonal Partial Least Square (B) of gastric endoluminal volatile fatty acids in cancer and control patients.

Compared to mixed and bronchial breath samples, all examined VFAs were found at their highest concentrations within gastric endoluminal air. In addition, VFAs tended to be higher in all samples derived from cancer patients compared to controls. Butyric acid and pentanoic acid were found to be significantly elevated in the mixed breath and gastric endoluminal air of cancer patients compared to controls, with endoluminal levels being approximately ten times greater than found in mixed breath. Equivalence of VFA levels within mixed and bronchial breath samples from cancer patients suggests that their origin within breath is principally derived from the lungs and by inference the systemic circulation as opposed to direct passage from the upper gastrointestinal tract. It is noteworthy that whilst acetic acid levels were significantly elevated in the mixed breath of cancer patients, equivalent enriched levels were found in gastric endoluminal air of both cancer and control subjects. This could suggest that the raised levels of acetic acid found within the exhaled breath of patients with oesophagogastric cancer may be influenced by other, as yet undetermined, systemic sources.

Taken together our findings appear to support an association between cancer and dysregulated VFA metabolism.[21,22] Fatty acids are absorbed within the small and large bowel and play an important role in many cellular functions.[22] Fatty acids may contribute to carcinogenesis through cell membrane production, energy metabolism, cell signalling and prevention of apoptosis.[23] In human malignancies, including gastric cancer, over expression of *fatty acid synthase* leads to increased *de novo* synthesis of fatty acids and is associated with poor prognosis.[23-25]

Acetic acid is a metabolic intermediate within the pathway of acetyl-CoA synthesis. Previous studies investigating VOC release from gastric content and urine, observed higher concentrations of acetic acid in oesophagogastric cancer patients compared to healthy controls.[7,8] Zhang *et al.* performed NMR spectroscopy of blood samples from patients with oesophageal adenocarcinoma and reported that changes in the trichloroacetic acid cycle were dominant factors in the biochemistry of this cancer.[26] Hasim *et al.* also reported increased levels of acetate in the NMR profile of urine in patients with oesophageal cancer compared to healthy controls.[27]

In a recent multicenter validation study investigating exhaled breath analysis for oesophagogastric cancer, butyric acid was identified as a key discriminatory VOC.[2] Shi *et al.* also reported that 4-phenybutyric acid promotes gastric cancer cell migration via histone deacetylase mediated HER3/HER4 upregulation.[28] Butyric acid can also be produced from periodontopathic bacteria as an extracellular metabolite and it has been implicated in the development of oral cancer.[18]

Pentanoic acid is an aliphatic fatty acid that has an important role in tumorgenesis.[10] Moreover, both exhaled pentanoic and hexanoic acid were identified as principal VOCs in published diagnostic prediction models for oesophagogastric cancer.[6,28] Using TD-GCxGC-ToF-MS, Stadler *et al.* identified hexanoic acid as a potential marker of tissue necrosis and decomposition in cadavers.[29] Accordingly hexanoic acid may be released in higher amounts within regions of necrosis in oesophagogastric tumours. Hexanoic acid has also been reported to be significantly increased in the plasma of patients with high-grade dysplastic colonic adenomas compared to controls.

Acetone and other ketone bodies are thought to permit sustained abnormal tumour growth by acting as an alternative energy sources.[30-32] Acetone is produced through lipolysis or from acetyl-CoA as a breakdown product of fatty acid oxidation. Higher concentrations of acetone were previously observed within the gastric content and urine of oesophagogastric cancer patients compared to controls.[6,7] Hasim *et al.* have reported significantly increased blood plasma acetone concentrations in patients with poorly differentiated oesophageal cancer.[28] Ketones may function as chemo-attractants and stimulate the migration of epithelial cancer cells promoting primary tumour growth.[32]

In the face of growing evidence for the association between VFAs and deregulated tumour metabolism the mechanism whereby they are released in to exhaled breath remains relevant but incompletely understood. There are thought to be two main pathways by which VOCs may partition between the body and exhaled breath,

through passage from the systemic circulation across the alveolar capillary barrier or via direct release from the upper airways and digestive tract.[13] Importantly, this study was able to measure isolated bronchial breath in intubated cancer patients. Whilst acknowledging inconsistencies in the methods used to assess breath from patients who were intubated or breathing spontaneously, general consistency in the levels of exhaled VFAs within these compartments has two principal implications. Firstly, whilst these VFAs may be concurrently found in relative abundance within the upper gastrointestinal endoluminal air, this does not appear to be a source of significant contamination of exhaled breath. Secondly, if the tumour is indeed the source of these VFAs in exhaled breath, the process whereby they are transported to the lung within the systemic circulation before being partitioned across the alveolar capillary barrier leads to a significant attenuation in their detectable levels.

The current study has limitations. Firstly, due to the size constraints of endoscopic biopsy techniques and to ensure a universal tissue retrieval technique, it was not feasible to obtain larger tissue samples. Although VFA concentrations did not correlate with biopsy weight, the *in-situ* experiment suggests that larger tissue volume may generate comparably higher concentrations of target VFAs. Secondly, this is the first study to have attempted to analyse VFAs from three defined body compartments: mixed breath, bronchial breath and gastric endoluminal air. Whilst it was intended that a 500ml gas sample were taken from each compartment variation in the conditions of sampling mean that caution should be taken when trying to infer direct correlations between the relative abundance of VFAs. Finally, the current study does not attempt to define the precise cellar origin of detected VFAs.

There are diagnostic clinical implications of these studies. The marked difference in VFA levels in the gastric endoluminal air of cancer compared to control patients offers the opportunity for an endoluminal gas biopsy for cancer detection. Secondly, the non-significant difference between exhaled and isolated bronchial breath supports the use of mixed exhaled breath for non-invasive cancer detection without the need for complex devices for alveolar sampling.

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Conflicts of interest disclosure

The authors have no conflict of interest to declare.

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