Original article

Exhaled volatile organic compounds identify patients with colorectal cancer

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Background: An effective screening tool for colorectal cancer is still lacking. Analysis of the volatile organic compounds (VOCs) linked to cancer is a new frontier in cancer screening, as tumour growth involves several metabolic changes leading to the production of specific compounds that can be detected in exhaled breath. This study investigated whether patients with colorectal cancer have a specific VOC pattern compared with the healthy population.

Methods: Exhaled breath was collected in an inert bag (Tedlar®) from patients with colorectal cancer and healthy controls (negative at colonoscopy), and processed offline by thermal-desorber gas chromatography–mass spectrometry to evaluate the VOC profile. During the trial phase VOCs of interest were identified and selected, and VOC patterns able to discriminate patients from controls were set up; in the validation phase their discriminant performance was tested on blinded samples. A probabilistic neural network (PNN) validated by the leave-one-out method was used to identify the pattern of VOCs that better discriminated between the two groups.

Results: Some 37 patients and 41 controls were included in the trial phase. Application of a PNN to a pattern of 15 compounds showed a discriminant performance with a sensitivity of 86 per cent, a specificity of 83 per cent and an accuracy of 85 per cent (area under the receiver operating characteristic (ROC) curve 0·852). The accuracy of PNN analysis was confirmed in the validation phase on a further 25 subjects; the model correctly assigned 19 patients, giving an overall accuracy of 76 per cent.

Conclusion: The pattern of VOCs in patients with colorectal cancer was different from that in healthy controls. The PNN in this study was able to discriminate patients with colorectal cancer with an accuracy of over 75 per cent. Breath VOC analysis appears to have potential clinical application in colorectal cancer screening, although further studies are required to confirm its reliability in heterogeneous clinical settings.

Surgical relevance

Cancer may cause metabolic derangement, resulting in altered volatile organic compounds (VOCs) present in exhaled breath. Patients with colorectal cancer show a characteristic pattern of VOCs in their breath. The VOC pattern can distinguish between patients with colorectal cancer and normal subjects. This VOC test may have potential clinical application as a non-invasive and reliable screening tool.

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Introduction

The development of systems biology has revolutionized several aspects of cancer research including screening, offering new opportunities for the identification of novel biomarkers in early diagnosis[1]. Metabolomics is the most recently developed component of 'omics' research, examining the endpoints of cellular metabolism by using a range of techniques including high-throughput technologies such as nuclear magnetic resonance spectroscopy, high-performance liquid chromatography, and gas chromatography linked to mass spectrometry (GC-MS). These systems are capable of simultaneously

analysing many types of micromolecule reflecting the status of cellular metabolism. Currently, between 2000 and 3000 small molecules (metabolites) constitute the metabolome[2], representing the downstream output of global cellular networking as a manifestation of the cellular phenotype fingerprint. It is postulated that different diseases are characterized by specific metabolomic profiles[3]. A metabolome may be identified in several types of biological sample, including faeces, urine, serum, sputum and breath. In this regard, breath analysis could be considered the favoured option for medical diagnostic purposes, mostly because of its non-invasive nature, low cost and ready patient compliance[4].

Volatile organic compounds (VOCs) in exhaled breath were first isolated by Pauling and colleagues[5] in 1971. Alteration in VOC production in patients with cancer has been postulated to relate to (per)oxygenation of cell membrane-based polyunsaturated fatty acids resulting from genetic and/or protein mutations within tumour cells and the increased relative prevalence of reactive oxygen species within cancer cells[6,7]. Those VOCs, wherever produced in the body, can reach the pulmonary alveoli where they are exhaled, permitting an objective method of measurement. VOCs consist largely of benzene, alkanes and aldehydes (or their derivatives), and several studies have demonstrated that various cancers, including lung and breast cancer, melanoma, mesothelioma and hepatocellular carcinoma, are associated with specific VOC profiles that differ from normal[8–12].

Given that colorectal cancer is the second leading cause of cancer-related death in Europe and the third in the USA, the quest continues for novel non-invasive screening systems with the potential for high patient compliance and low cost that have an equivalent sensitivity/specificity to colonoscopy, but that will improve on the diagnostic accuracy of faecal occult blood or stool DNA testing or virtual colonoscopy for early detection of colorectal cancer and precursor adenomatous polyps[13–15]. VOC profile analysis in the diagnosis of colorectal cancer has not yet been reported. This pilot study aimed to investigate whether patients with colorectal cancer have a specific pattern of VOCs compared with healthy controls.

Methods

This prospective observational study was designed in two phases. The aim of the initial trial phase was to identify and select VOCs of interest, and to set up a VOC pattern potentially capable of discriminating between patients with colorectal cancer and normal controls using an appropriate statistical model. The aim of the subsequent validation phase was prospectively to validate the model in a blinded fashion on a further series of patients and healthy controls; these subjects were not included in the previous phase. The study was approved by the local ethics committee, and all the participants provided written informed consent for breath analysis and testing.

The patients with colorectal cancer had histologically proven disease and were admitted to the surgical department between June and December 2011. Patients underwent breath collection in the afternoon before surgery and before starting mechanical bowel preparation. Patients receiving neoadjuvant radio/chemotherapy were excluded because of possible unknown effects on cancer metabolism, as were those with asthma or severe chronic obstructive pulmonary disease because of possible difficulties in the collection of breath samples. Also excluded were patients with unstable diabetes because of the potential for ketoacidotic breath and those with a previous diagnosis of another malignancy. After surgery, patients were staged according to the International Union Against Cancer tumour node metastasis staging system for colorectal cancer[16]. Healthy controls were chosen from patients undergoing screening colonoscopy and found to be disease-free. Patients with inflammatory bowel disease or diverticulitis were excluded from the study in order to avoid possible influences of the inflammatory state on VOC production. These subjects had breath samples collected at least 7 days after a negative colonoscopy. Any use of drugs and any coexisting morbidities were recorded in both groups.

Exhaled breath collection and sampling

Exhaled breath collection was standardized for all subjects and was carried out in the same room of the surgical department under similar conditions. Subjects refrained from eating and drinking for 3 h before the test, remaining in the same room for at least 10 min before breath collection so that an equilibrium was created between the lung and ambient air. Breath sampling was based on a validated method described previously[17]. In an earlier study the intraobserver variability of the breath analysis was evaluated in ten subjects by comparing two breath samples for each subject. This showed good reproducibility of the data, so a single breath analysis was carried out for each subject recruited in the present study. Patients breathed tidally for 5 min through a mouthpiece connected to a three-way non-rebreathing valve with an inspiratory VOC filter (A2; North Safety, Middelburg, The Netherlands) in order to exclude exogenous VOCs. Following a single deep inspiration, patients exhaled a single vital capacity

volume (about 2 litres) into a 3-litre Tedlar® bag (Sigma-Aldrich, St Gallen, Switzerland) made of an inert material (polyvinyl fluoride) connected to the expiratory port[11]. All bags were returned to the department of chemistry for analysis immediately following breath collection.

Analysis of volatile organic compounds

Analysis of VOCs in exhaled breath consisted of three principal steps; namely, the adsorption of VOCs on to sorbent cartridges, thermal desorption and analysis by GC-MS[11,17]. The sorbent cartridge was composed of a cylindrical stainless steel net (100 mesh) with an external diameter of 4·8 mm containing Carboxen™ 1003, Carbopack™ B and Carbopack™ Y as an adsorbent bed (Sigma Aldrich, Milan, Italy). The cartridge was connected on one side to the sampled Tedlar® bag and on the other to a low-flow sampling pump (Pocket Pump; SKC, Houston, Texas, USA). In the sampling step the flow was set to 25 ml/min for 30 min; at this setting, a volume of 0·75 litres per bag was drawn. Sampled cartridges were then desorbed thermally (Markes International, Llantrisant, UK) with samples being analysed by GC-MS (GC-6890 PLUS, MS-5973 N; Agilent Technologies, Santa Clara, California, USA). Performance characteristics and reproducibility of sampling, and the analytical method have been reported previously[11,17], including the optimal choice of adsorbent material, flow rate, sampling time, sampling volume and measurement artefact of Tedlar® bags. The entire process took approximately 2 h from collection to final result.

Statistical analysis

A probabilistic neural network (PNN) was applied to determine the best discrimination between cancer and normal samples. PNN is an implementation of a statistical algorithm called kernel (K)[18] discriminant analysis, in which operations are organized into a multilayered feed-forward network. The input layer contains N nodes (one for each of the N input aspects of a featured vector). There are fan-out nodes that branch at each featured input node to all nodes in the hidden layer so that each hidden node receives the complete input featured vector x. The hidden nodes are collected into groups. Each hidden node in the group for class K corresponds to a Gaussian function centred on its associated featured vector in the Kth class.

All of these Gaussian functions in a class group feed their functional values to the same output layer node for that class, so there are K output nodes. An internal validation of each of the statistical methods used was carried out by the leave-one-out method[19]. For each method, N models are built, where N is the number of rows in the training data set so that (N – 1) rows are used to build each model, with the Nth remaining row being used to test the model. Finally, the statistical model that best discriminates between patients and controls was tried on unknown data and blinded to the operators as part of the second phase of the study.

Results

Seventy-eight consecutive subjects participated in the trial phase of the study: 37 patients with histologically proven colorectal cancer and 41 healthy volunteers (*Fig. 1*). The colorectal cancer group comprised 20 men and 17 women, with a mean(s.d.) age of 63(10) years. The cancer was located in the right colon in eight patients, the left colon in 20 and the rectum in nine. Nineteen patients had stage I/II and 18 stage III/IV disease. The control group included 13 men and 28 women, with a mean age of 47(12) years. The two groups were matched for age ($P = 0·210$) and sex ($P = 0·181$). The second phase of the study included 15 patients with colorectal cancer and ten healthy controls. There were seven men and eight women with colorectal cancer, with a mean age of 67(11) years. Tumours were stage I/II in six and stage III/IV in nine patients. The control group comprised six men and four women of mean age 56(10) years.

In the trial phase, initial analysis conducted on breath samples from both groups identified 58 VOCs for consideration; there was no discernible single absolute marker of disease. To discriminate between healthy controls and patients with colorectal cancer, selected variables were used as inputs in a multivariable model.

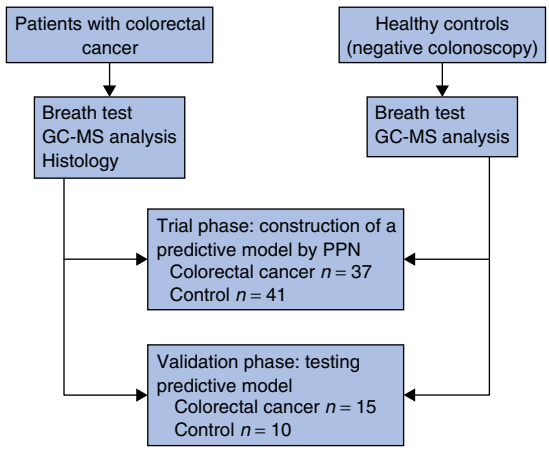

Fig. 1 Design of the study. GC-MS, gas chromatography–mass spectrometry; PNN, probabilistic neural network

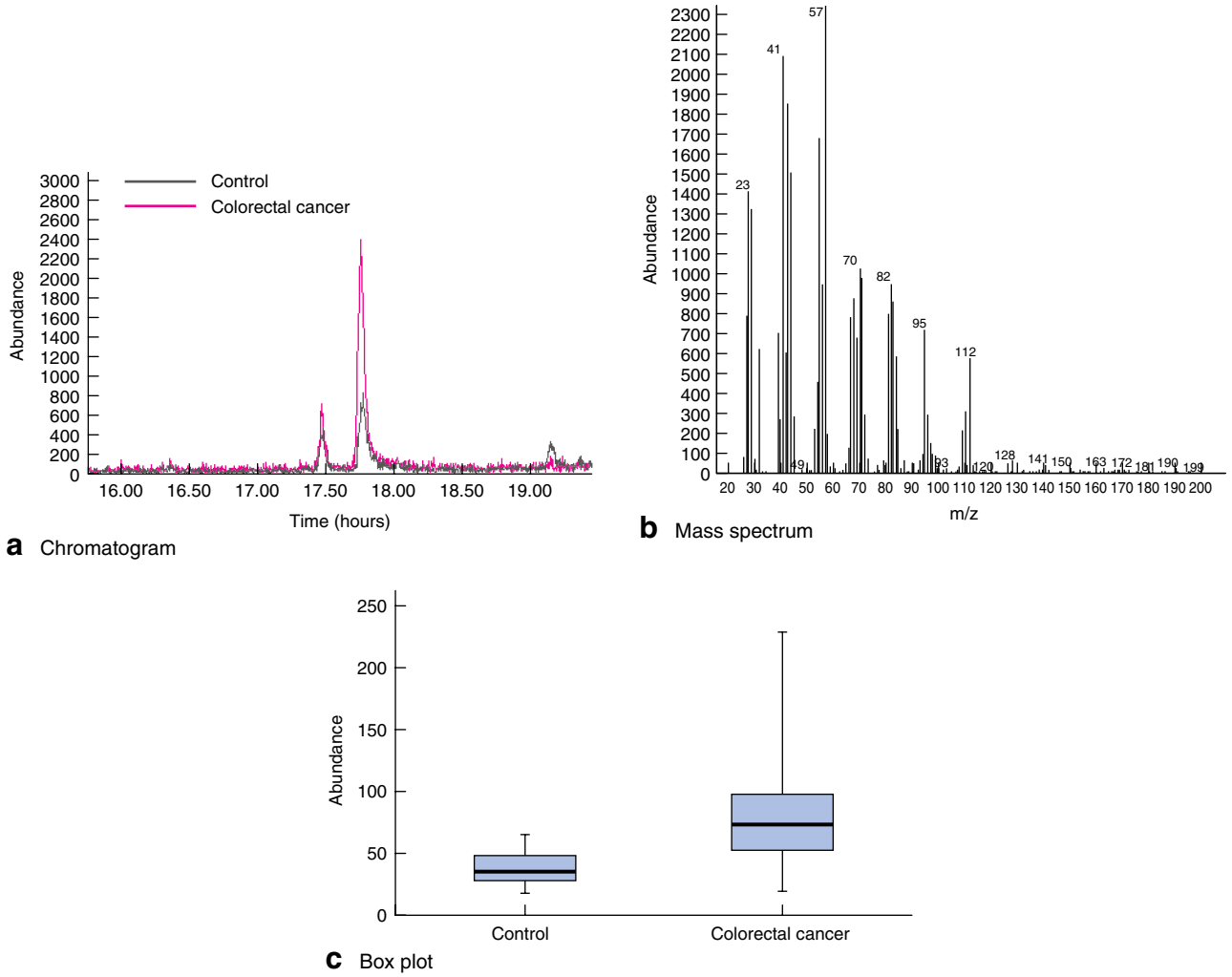

Fig. 2 Detection of the volatile organic compound decanal in breath samples. **a** Comparison between gas chromatograms from patients with colorectal cancer and healthy volunteers; there was a clear difference in decanal concentration between groups. **b** Mass spectrum identifying decanal. m/z, Mass-to-charge ratio. **c** Box plot showing abundance of decanal in samples from patients with colorectal cancer and healthy volunteers. Horizontal line within box, box and error bars represent median, interquartile range and range respectively. There was high discriminant power between groups (Wilcoxon rank sum test $P = 0{\cdot}052$)

Background VOC concentrations in a clean Tedlar® bag were monitored to determine the base level of contamination in new or cleaned bags; analysis of bags filled with clean and humidified (50 per cent) air showed that *N*,*N*-dimethylacetamide (DMCA) and phenol were routinely detectable as normal bag emissions. Both of these compounds are present in the production process of Tedlar® itself, and other studies have reported relatively high background concentrations of both DMCA and phenol from Tedlar® bags[20,21]. Following exclusion of infrequent compounds, a box plot of the remaining variables was constructed; variables not significantly different between patients and controls were removed, leaving 15 with potentially high discriminant power in the operating conditions of this study. Data for one example, decanal (an aldehyde compound), with such discriminant power are shown in *Fig. 2*. The 15 variables were identified from an international library (NIST MS search 2.0; http://chemdata.nist.gov/mass-spc/ms-search) and are shown in *Table 1*; variables A (4-methylundecane; retention time (RT) = 11·3) and B (trimethyldecane; RT = 13·2) are compounds that are currently not well identified because of their low recognition capability.

Table 1 Discriminating variable compounds considered in the statistical analysis

	Recognition capability (%)
Nonanal	80
4-Methyl-2-pentanone	88
Decanal	92
2-Methylbutane	87
1,2-Pentadiene	95
2-Methylpentane	87
3-Methylpentane	87
Methylcyclopentane	80
Cyclohexane	94
Methylcyclohexane	92
1,3-Dimethylbenzene	96
4-Methyloctane	80
1,4-Dimethylbenzene	80
A (4-methylundecane, RT = 11·3)	59
B (trimethyldecane, RT = 13·2)	72

RT, retention time.

Data analysis

A PNN model was applied to the pattern of compounds selected; the network was optimized using DTREG software (Phillip H. Sherrod, Brentwood, Tennesee, USA) and by removing unnecessary neurones after model construction. The confusion matrix for the leave-one-out validation set and the performance of the PNN model resulted in a predictive accuracy of 85 per cent, a sensitivity of 86 per cent and a specificity of 83 per cent. The network misclassified five of 37 patients with colorectal cancer and seven of 41 controls, but provided good predictive ability. The area under the receiver operating characteristic (ROC) curve for this PNN model was 0·852 (*Fig. 3*).

The PNN set up in the trial phase was tested on a further series of 25 breath samples, from 15 patients with colorectal cancer and ten healthy controls. When these samples were tested in a blinded analysis, the output of the model correctly assigned 19 subjects, whereas three controls and three patients with colorectal cancer were allocated incorrectly; the overall accuracy was 76 per cent. No differences were found in VOC patterns among patients with colorectal cancer stages I/II and III/IV, nor was there any effect of sex on VOC profiles.

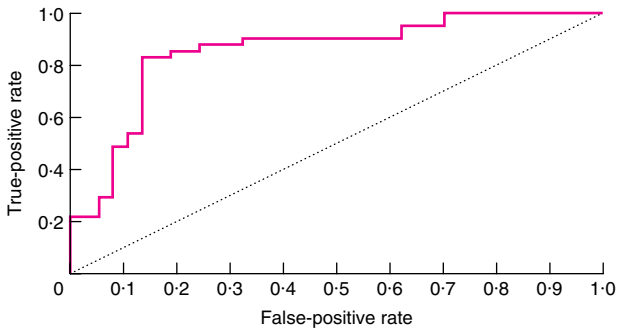

Fig. 3 Receiver operating characteristic (ROC) curve for the probabilistic neural network model. Area under ROC curve is 0·852

Discussion

The availability of an effective and reliable colorectal cancer screening tool is of paramount importance in the health service plans of Western countries to permit early diagnosis and/or identification of precursor polyps. Improved tests are required that are able consistently to show high sensitivity and specificity for these diagnoses, and that are easy to perform and capable of engendering high patient compliance. Recent advances in molecular biology in colorectal cancer have focused on several compounds[22–24], although some have displayed inadequate sensitivity and specificity in the discrimination between normal controls and patients with either established cancer or precursor lesions. The application of metabolomics for such screening purposes is designed to define specific chemical fingerprints of cellular function that act as a snapshot of active cellular physiology, reflecting its metabolic profile, but that are not defined by conventional assessments of RNA expression or proteomic cellular analyses. The exact interplay between this system biology and its functional genomics, integrating proteomic and genetic with end-product metabolic information as an early cancer screening tool, is at present not completely known. In recent years several studies have assessed the capacity of breath analysis to diagnose lung cancer[7], asbestos exposure[11], breast cancer[9], malignant melanoma[10], aerodigestive squamous cell carcinoma[25] and hepatocellular carcinoma[12].

The present study has shown that patients with colorectal cancer have a different selective VOC pattern compared with healthy controls, based on analysis of 15 of 58 specific compounds in exhaled breath samples. Analytical statistical methodology using neural networking allowed discrimination between the two groups, with a relatively high accuracy of 76 per cent in a blinded evaluation. The possibility of using breath analysis as a diagnostic tool for colorectal cancer can be considered as an expansion of its more common use, for example in the diagnosis of benign gastrointestinal disorders, such as lactose intolerance and *Helicobacter pylori* infection, gastrointestinal transit disorders and cystic fibrosis[26–28].

Each disease appears to have a specific VOC profile, suggestive of several different derangements in metabolic

pathways. A range of different analytical methods have been used for breath analysis, including cross-reactive species nanosensor array technology linked to GC-MS[29], different solid-phase absorbents for marker microextraction[12], ion mobility spectrometric techniques designed to increase the detection threshold of VOCs[30], colorimetric analysis of chemically sensitive compounds impregnated into disposable cartridges[31], and various techniques to detect non-polar molecules within the exhaled breath condensate[32].

Further differences may be detected as variations exist between the ratio of exogenous VOCs, which are adsorbed from the environment, to endogenous VOCs generated by cellular biochemical processes in the body[5]; the biochemical pathways of some compounds appearing in exhaled breath are not completely understood at present. Most studies have focused on single VOC agents or collation of a few VOCs as specific biomarkers, although some have adopted a discriminant function analytical model of multiple compound clusters derived from available spectral libraries to predict membership within a particular disease group[9,11]. The present findings are in agreement with those of Peng and colleagues[33], who assessed a mixture of tumours including lung, breast, colorectal and prostate lesions, and measured exhaled condensates with nanosensors. In this latter study, there was accurate discrimination between normal and 'cancerous' breath, and between the breath analyses of some cancer types, irrespective of age or sex.

Levels of some specific VOCs such as 1,3-dimethylbenzene, 1,2-pentadiene, cyclohexane, methylcyclohexane and 4-methyloctane were higher in patients with colorectal cancer than in controls (average concentrations for patients with colorectal were about double). These other VOCs showed variable profiles in different cancers and variable correlation with one another, suggesting that it is the pattern of VOCs rather than a single VOC that is more likely to be representative of the metabolic derangements evident in colorectal cancer[34]. To keep the samples as homogeneous as possible, patients with severe chronic obstructive pulmonary disease, inflammatory bowel disease, decompensated diabetes or a previous history of other malignancies were excluded from this preliminary study.

Currently there is no accepted working protocol describing the monitoring procedure, combinations of VOCs to be assessed and the best statistical method for group discrimination. Future work on VOCs will assess the predictive value of different compound patterns in polyp detection, investigate breath profile assessment after colorectal cancer resection as a potential means of monitoring disease recurrence, and compare VOC profiles in colorectal cancer with those in inflammatory bowel diseases and other gastrointestinal tract cancers.

The present findings further support the value of breath testing as a screening tool. The next step will be to increase the number of subjects involved in order to provide a simpler algebraic formula that will make this evaluation easier, to identify a diagnostic marker and to improve the performance of the statistical method when applied to samples in a blinded fashion. The methodology could also be improved by the use of an electronic nose[17].

Disclosure

The authors declare no conflict of interest.

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Commentary

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The development of accurate, mass-screening tools for the detection of colorectal cancer is paramount, given the social burden imposed and the recognized polyp–cancer sequence in most cases. There are non-invasive techniques designed for detection of cancer by-products, including stool mutated DNA, micro-RNA analysis and tumour-associated antigens/antibodies. Another approach is measurement of the by-products of cellular metabolism, exploiting 'metabolomic' differences between cancer- and non-cancer cells using gas chromatography and mass spectrometry. The detection of volatile organic compounds (VOCs) in exhaled breath reflects abnormal peroxygenation of cell membrane-based polyunsaturates from cancer cells, although its discriminatory capacity and statistical methodology are not sufficiently developed to provide a consistent diagnostic performance capable of separating diseased from non-diseased states.

Today, the breath 'concept' has expanded to the monitoring of asthma, defining heart transplant rejection, testing for *Helicobacter pylori* infection, lactose intolerance and bacterial overgrowth, routinely monitoring ventilation during anaesthesia and, of course, catching transgressors driving under the influence of alcohol.

This preliminary study by Altomare and colleagues shows that patients with colorectal cancer have different VOC breath patterns compared with healthy controls, and a sophisticated statistical methodology discriminates between groups with relatively high accuracy. There are, however, a number of issues related to this technique that remain unclear. In lung cancer, exhaled VOC biofingerprints ('breathprints') vary between tumour histologies. How do we cater for exogenous cross-contamination within the measurement system or from ambient air? What do we know about the influence of endogenous VOC production by the gut and how to standardize for its effect? Can we correct for exhaled VOCs by subtracting inspiratory levels, calculating alveolar gradients or discarding dead space to result in measurements that are reproducible? In the future, we will need to integrate novel digital and nanosensors with these 'artificial electronic noses', allocating them the task of discriminating disease-specific recognition patterns[1].

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Disclosure

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