

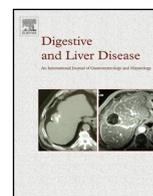


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Alimentary Tract

Non-invasive exhaled volatile organic biomarker analysis to detect inflammatory bowel disease (IBD)

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ABSTRACT

Introduction: Early inflammatory bowel disease (IBD) diagnosis remains a clinical challenge. Volatile organic compounds (VOCs) have shown distinct patterns in Crohn's disease (CD) and ulcerative colitis (UC). VOC production, reflecting gut fermentome metabolites, is perturbed in IBD. VOC sampling is non-invasive, with various compounds identified from faecal, breath and urine samples. This study aimed to determine if FAIMS (field asymmetric ion mobility spectroscopy) analysis of exhaled VOCs could distinguish IBD from controls.

Methods: Seventy-six subjects were recruited, 54 established IBD (25 CD, 29 UC) and 22 healthy controls. End expiratory breath was captured using a Warwick device and analysed by FAIMS. Data were pre-processed using wavelet transformation, and classification performed in a 10-fold cross-validation. Feature selection was performed using Wilcoxon rank sum test, and sparse logistic regression gave class predictions, to calculate sensitivity and specificity.

Results: FAIMS breath VOC analysis showed clear separation of IBD from controls, sensitivity: 0.74 (0.65–0.82), specificity: 0.75 (0.53–0.90), AUROC: 0.82 (0.74–0.89), p -value 6.2×10^{-7} . IBD subgroup analysis distinguished UC from CD: sensitivity of 0.67 (0.54–0.79), specificity: 0.67 (0.54–0.79), AUROC: 0.70 (0.60–0.80), p -value 9.23×10^{-4} .

Conclusion: This confirms the utility of exhaled VOC analysis to distinguish IBD from healthy controls, and UC from CD. It conforms to other studies using different technology, whilst affirming exhaled VOCs as biomarkers for diagnosing IBD.

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1. Introduction

Inflammatory bowel disease (IBD) is a common condition affecting the Western world with approximately 115,000 people in the UK suffering from Crohn's disease (CD) and 146,000 with ulcerative colitis (UC) [1,2]. The pathogenesis of IBD is not entirely understood, but widely believed to be the result of complex interactions between an individual's genetic susceptibility, environmental

triggers (e.g. diet, life style, etc.) and the influence of an individual's gastrointestinal bacterial colonies [3]. Bacterial diversity is problematic to study, as <50% of organisms can be successfully cultured and modern genomic techniques, though far more successful, are too expensive and impractical for every day clinical use [3].

Current diagnostic tools for IBD include a thorough history, faecal inflammatory markers, endoscopic investigations with histological examination, capsule endoscopy and imaging. Despite the diverse array of investigations available, the diagnosis of CD or UC can often be difficult when disease is limited to the colon, and may rely on histology. Even so, the accepted standard of histology can often fail to distinguish between CD from UC.

The detection of patterns of volatile organic compounds (VOCs) and their utility as disease specific gas phase biomarkers has been a

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Table 1
Demographic data of inflammatory bowel disease (IBD) patients and healthy controls.

Disease	Crohn's disease (n = 25)	Ulcerative colitis (n = 29)	Controls (n = 22)
Mean age in years (SD)	43.7 (14.9)	47.9 (15.5)	41.2 (13.5)
Gender ratio (M:F)	11:14	9:20	2:20
Smoking – mean cigarettes/day (SD)	2.8 (5.6)	0.8 (2.9)	1.3 (2.6)
Alcohol – mean units/week (SD)	3.3 (5.0)	3.6 (5.2)	6.0 (7.2)
Mean BMI (SD)	27.7 (6.5)	25.8 (5.4)	25.6 (4.0)
Medications	5 ASA: 15 AZA/MP: 6 Anti-TNF α : 3 Oral steroids: 1 PPI: 2 Salbutamol inhaled: 3 Inhaled steroid: 2	5 ASA: 23 AZA/6MP: 5 Anti-TNF α : 2 Oral steroids: 1 PPI: 1 Salbutamol inhaled: 1 Inhaled steroid: 1	Salbutamol (inhaled): 3 Levothyroxine: 2
Mean SCAI/HBI score (range; SD)	5.0 (0–14; 4.7)	1.9 (0–7; 1.9)	NA
Disease extent	Ileal disease: 8 Colonic: 9 Ileo-colonic: 5 Unclear records: 2	Left sided: 0 Subtotal/pan colonic: 29	NA
CRP in mg/L (SD)	4.7 (2.8)	3.8 (2.6)	

5-ASA – aminosalicylate acid, Aza – azathioprine, MP – mercaptopurine, PPI – proton pump inhibitor, Anti-TNF α – anti tumour necrosis factor alpha.

rapidly developing area within several medical domains [4]. Analysis of patients breath using GC–MS (gas chromatography and mass spectrometry) has revealed VOC patterns which have distinguished not just cancer from non-cancer patients but also multiple cancer subtypes including lung, breast, prostate and colorectal cancer [5]. Urinary VOC patterns have been shown to distinguish between not only inflammatory bowel disease (IBD) and healthy control patients but between active and quiescent disease for both IBD subtypes using E-nose and FAIMS [3]. Several other GI conditions have been demonstrated to be distinguishable by analysis of VOCs including pelvic radiotherapy induced side effects; bile acid diarrhoea (BAD); coeliac disease; colorectal cancer (CRC) and non-alcoholic fatty liver diseases (NAFLD), including the distinction of cirrhotics from non-cirrhotics [4,6–8].

VOCs have been identified as being perturbed in many physiological and pathological states, including a variety of diets and numerous diseases. The exact mechanisms by which VOCs are generated are the subject of on-going research, but their production in the gastrointestinal tract is hypothesised to be the direct result of fermentation of dietary non-starch polysaccharides. This being the case, they represent the complex interaction of colonocytes, human gut microflora and invading pathogens [10]. The resultant fermentation products, ‘the fermentome’ [9–12], can exist in the gaseous phase and are present in exhaled breath, sweat, urine and faeces [4]. Their presence in bodily secretions from sites distant to the GI tract (sweat, exhaled air and urine) is believed to be possible due to the alteration of gut permeability afforded by certain disease states, including IBD [13]. We believe that VOCs represent a bio-signature specific to patients, which can be affected by a variety of factors including, genetics, disease state and environmental factors, such as diet.

The aim of our study was to determine whether exhaled VOCs could be used to distinguish IBD from healthy controls, and also UC from CD, using field asymmetric ion mobility spectroscopy (FAIMS) technology.

2. Materials and methods

2.1. Subjects

A total of 76 subjects were recruited prospectively for this study. Fifty-four patients had histologically confirmed IBD (29 UC and 25 CD) as well as 22 healthy controls. IBD patients were recruited prospectively from dedicated IBD clinics at University Hospital

Coventry & Warwickshire. Details of medication and symptoms were recorded and simple colitis activity index (SCAI) for UC and Harvey Bradshaw index (HBI) calculated at time of recruitment. Along with serological markers of inflammation, healthy controls were volunteers who did not report any overt gastrointestinal symptoms and were not on routine oral medication. The mean age of the IBD cohort was 47 years (standard deviation 15) and there were 22 males and 34 females. The demographics of the subjects are shown in Table 1, including disease extent.

2.2. Study design

This was a case control study, with recruitment of sequential patients attending an IBD outpatient clinic at University Hospital Coventry & Warwickshire, UK. Demographic data and disease activity score indices were collected from the patients.

2.3. Breath sampling

Morning breath samples were taken from both patients and controls after a minimum of a 2 h fast and absence of cigarette smoking. The specimens were collected using a 3 L Tedlar[®] bag (Thames Restek, UK) which had a ¼" OD nozzle tube and threaded valve for sample capture. A custom made breath capture device was constructed to allow sampling. The breath capture device allows patients to breathe naturally through a mouth piece into the attached Tedlar[®] bag. The device comprises of a filter, to ensure controlled environmental standards for sampling, to which is connected to a 2 way valve. The two way valve allows the patient to inhale clean air (which first passes through the filter) and then exhale into the Tedlar[®] bag. These valves also had custom made PTFE adaptors attached to allow for connection to disposable mouthpieces, whilst the other adaptors connect to an additional three way tap between the exhaled breath path and the Tedlar[®] bag. This tap is then attached to the breath bag via disposable tubing and allows for the isolation of end-tidal breath in the bags, which has higher VOC content. All samples were obtained from patients directly and placed in a freezer (–20 °C) as soon as possible. The samples were then shipped to the University of Warwick with a total of 1 h to reach room temperature before analysis (including the transport time). Once defrosted the sample bags were attached to the FAIMS via the custom unit as described in the next section. All samples were tested on the same day as capture.

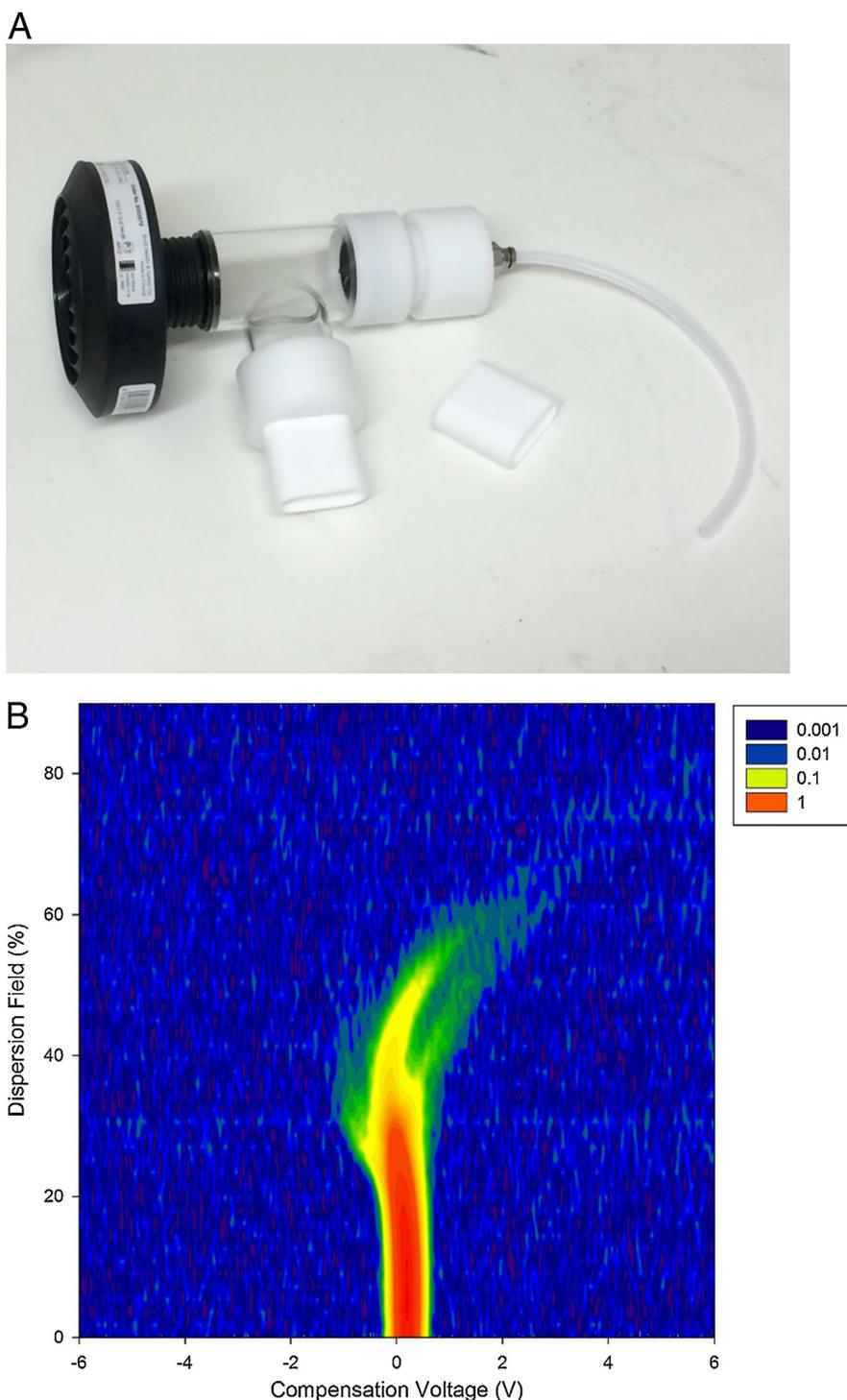


Fig. 1. (a) The experimental equipment used to capture the sample and (b) a typical breath output from a Crohn's disease patient.

2.3.1. FAIMS (*field asymmetric ion mobility spectroscopy*)

Here, a commercial setup was utilised (Lonestar, Owlstone, UK). This system achieves separation of chemical components on the basis of differences in molecular mobilities in a high electric field. FAIMS allows gas molecules to be separated and analysed at atmospheric pressure and room temperature. Once the sample is ionised, the resulting ions are passed between two metal plates and then an asynchronous high voltage waveform is applied to these plates, subjecting the ionised molecules to high electric fields. The difference in movement of these ions within this high electric field can be measured, thus resulting in a separation of the complex mixture.

The Lonestar was modified with a custom set up designed specifically for breath sampling. This custom made unit comprises an inlet port for the Tedlar® bags, compressed air running through a mass-flow controller (MFC) and an external pump on the instrument exhaust. The use of a MFC allowed for greater control of the air:sample ratio to optimise the analysis conditions. The flow rate of the sample was set by controlling the difference between the pump (set to 1.8 L/min) and the compressed air line (1.5 L/min) this results in a vacuum and the sample inlet then draws 300 mL/min of the breath sample into the machine for analysis. Each sample was run to collect 2 matrices of data, using approximately 2.3 L of

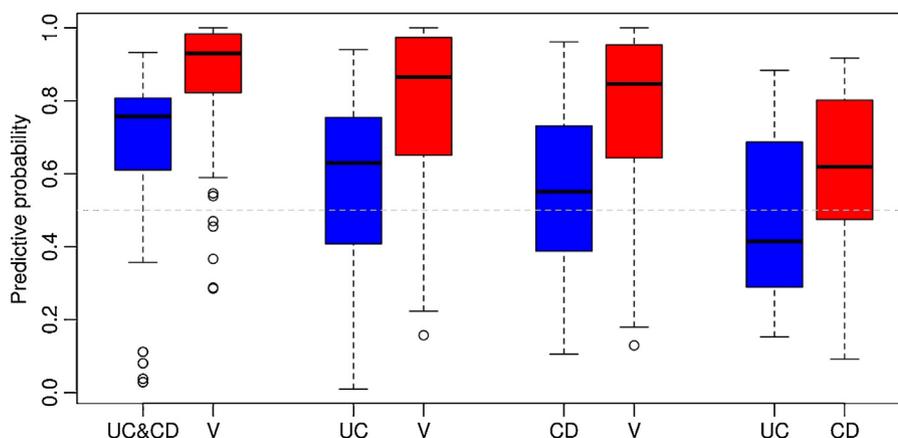


Fig. 2. Predictions of classifier to different combinations of diseases and controls (UC – ulcerative colitis, CD – Crohn's disease, V – volunteer).

sample. Fig. 1a shows a photo of the capture setup and Fig. 1b an example output of a breath sample from a Crohn's patient.

2.4. Statistical methods

Data from the FAIMS equipment were processed using a previously developed pipeline which has performed well on FAIMS data in the past [6–8]. The data were pre-processed in an unsupervised manner by performing a 2D wavelet transformation and using the wavelet coefficients as the new representation of the data. Features with small standard deviations (<0.002) were discarded. Supervised feature selection and class prediction was performed within a 10-fold cross-validation, where feature selection and classifier training was performed on the training set, and class predictions produced for the test set. A Wilcoxon rank sum test was used to calculate p -values for each feature having significantly different distributions between classes, and all but the two most significantly differing features were discarded. A sparse logistic regression classifier was used to produce the class predictions which are used for sensitivity and specificity calculation.

2.5. Ethics

Scientific and ethical approval was obtained from the Warwickshire Research & Development Department and Warwickshire Ethics Committee 09/H1211/38. Written informed consent was obtained from all patients who participated in the study.

3. Results

The demographic data of the IBD patient, both UC and CD, and healthy control patients are described in Table 1. There was a female gender predominance overall and this was more marked in the control group. Previous studies have not shown any effect on VOC profiling by age or gender [4,6–8]. The IBD patients were mainly in remission (SCAI and HBI scores of 5 and 2 respectively).

We studied the predictive performance of the data from the FAIMS analysis of exhaled breath using a pipeline consisting of wavelet transformation, feature selection and a sparse logistic regression classifier. This was used to classify samples and calculate sensitivities and specificities as part of a 10-fold cross-validation. Fig. 2 shows the predictive power of IBD (UC & CD) vs controls, UC vs controls, CD vs controls and finally UC vs CD.

The analysis showed that exhaled VOCs were able to distinguish IBD from control patients using breath samples with a sensitivity of 0.74 (95% confidence intervals (CI): 0.65–0.82) and specificity of

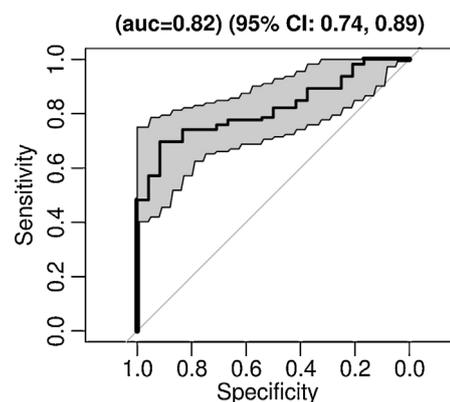


Fig. 3. Receiver operator curve (ROC) plots of IBD (UC and CD) vs healthy controls.

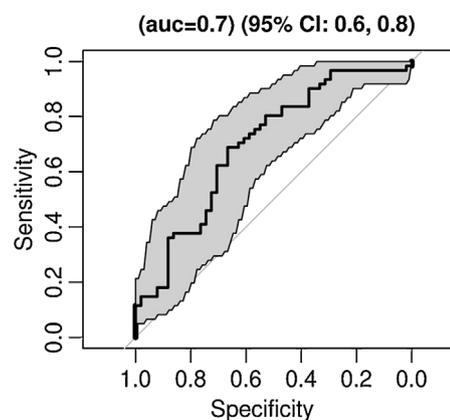


Fig. 4. Receiver operator curve (ROC) of CD vs UC.

0.75 (95% CI: 0.53–0.90), p -value 6.2×10^{-7} . The AUROC was 0.82 (95% CI: 0.74–0.89) – Fig. 3.

When analysing the IBD cohort, FAIMS could distinguish those with UC from those with CD with a sensitivity of 0.67 (95% CI: 0.54–0.79) and specificity of 0.67 (95% CI: 0.54–0.79), p -value 9.23×10^{-4} . The AUROC was 0.70 (95% CI: 0.60–0.80) – Fig. 4. Table 2 highlights the full set of sensitivities and specificities and AUROC numbers with 95% confidence intervals.

4. Discussion

Our study provides further evidence of the potential utility of *breathomics* as a non-invasive and inexpensive means of

Table 2
Sensitivities and specificities of FAIMS techniques for distinguishing the IBD patients from the healthy control subjects and UC from CD.

Comparison	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)
UC & CD vs controls	0.74 (0.65–0.82)	0.75 (0.53–0.90)	0.82 (0.74–0.89)
UC vs controls	0.61 (0.47–0.73)	0.62 (0.41–0.81)	0.70 (0.59–0.81)
CD vs controls	0.69 (0.54–0.81)	0.67 (0.45–0.84)	0.77 (0.66–0.88)
UC vs CD	0.67 (0.54–0.79)	0.67 (0.54–0.79)	0.70 (0.60–0.80)

distinguishing between patients with IBD and healthy controls by VOC analysis. It also shows some early potential to possibly distinguish CD from UC, although the sensitivity and specificity of 0.67 is currently lower compared to colonoscopy, is still an improvement from clinical acumen to distinguish the two conditions in real time. It expands on previous findings in breath (using different technology) as well as studies showing the validity of urinary VOC smell prints in those with gastrointestinal and liver diseases [3,4,6–8].

Exhaled VOCs have been demonstrated to be able to distinguish patients with IBD from healthy controls in paediatric populations. Patel et al., demonstrated that exhaled VOCs could distinguish IBD from healthy controls with AUC of 0.96 (95% CI: 0.93–0.99). This was undertaken using selective ion flow tube mass spectroscopy (SIFT-MS), with no discernible differences between UC and CD patients, nor was there a correlation with disease activity [14,15]. They found that 3 specific VOCs were implicated (1-octene, 1-decene and (E)-2-nonene). SIFT-MS analysis was also used to distinguish between CD and active UC, with systemic pentane production found to be predominant. This is thought to reflect cellular lipid peroxidation, which is a consequence of mucosal inflammation [1,16]. Other studies have found that mean breath pentane, ethane, propane, 1-cotene, 3-methylhexane, 1-decene and nitric oxide levels were elevated and mean breath (E)-2-nonene, hydrogen sulphide and methane were decreased in IBD compared with healthy controls [10]. It has also been shown that condensed cytokines in breath are higher in IBD compared with healthy controls. Levels of exhaled pentane, ethane, propane, isoprene and NO levels have been shown to correlate with IBD activity, whilst breath condensate interleukin-1B levels were inversely related to clinical disease activity [17]. More recently exhaled VOC analysis using SIFT-MS has been shown to distinguish CD and UC from healthy controls in adult patients (AUROCs 0.86 and 0.74 respectively), and CD from UC (AUROC 0.83). This ROC analysis was conducted using combinations of statistically significant VOCs (dimethyl sulphide, hydrogen sulphide, hydrogen cyanide, ammonia, butanal, and nonanal) [18].

VOCs are believed to be produced by colonic fermentation and represent the result of complex interactions between the colonocyte cells, human faecal flora, mucosal integrity and invading pathogens [10]. The VOCs pass into bodily fluids and as a result, VOCs found in urine, faeces and breath have huge potential as biomarkers to aid in the assessment of many gastrointestinal diseases. Any changes found in the pattern of VOCs are reflective of changes and variations within the gastrointestinal environment.

This study to our knowledge is the first utilising portable FAIMS technology for breath VOC detection in IBD. One of the limitations of this study is its sample size which was relatively small and we have not controlled for age or sex but in previous studies (including breath studies) neither of these variables seemed to affect VOC profiling [4,6–8]. In our protocol we used a two hour fast which seemed appropriate in the absence of any evidence or agreed consensus as to optimal fasting period prior to breath analysis in IBD.

It should be noted that for FAIMS technology, unlike SIFT-MS, it is challenging to identify specific chemicals. Currently, understanding the reactive ion path of specific chemicals is still in its infancy and therefore alternative “smell print” approaches provide the best means of extracting relevant chemical information from the instrument output. Though in some studies SIFT-MS outperforms the diagnostic power of FAIMS, importantly FAIMS is a fraction of the price of SIFT-MS (10–20% of the cost), is small, portable and uses air as the carrier gas [19]. Thus, the value of FAIMS is the potential for real time separation of VOC profiles utilising non-invasive technology (which is portable, breath analysis can be carried out at the bedside) to aid in the detection of IBD and distinction of UC from CD. Though, in this study we do not undertaken direct breath sampling, developments are in place to facilitate this. Importantly, our results were also comparable [18].

The nature of the biological samples required for the process is more likely to be acceptable to patients than other invasive methods although likely to serve as an adjunct to current methods. Further validation studies would be required, to confirm the accuracy of the technique and determine if it can distinguish active from quiescent disease, as urine has been demonstrated to be able to do.

Conflict of interest

None declared.

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