FAECAL VOLATILE ORGANIC COMPOUNDS ANALYSIS USING FIELD ASYMMETRIC ION MOBILITY SPECTROMETRY: NON-INVASIVE DIAGNOSTICS IN PAEDIATRIC INFLAMMATORY BOWEL DISEASE

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Non-standard abbreviations

VOC = volatile organic compound
FAIMS = field asymmetric ion mobility spectrometry
eNose = electronic nose
GC-MS = gas chromatography–mass spectrometry

Abstract

Background and Aims

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), remains challenging to diagnose. Diagnostic work up carries a high burden, especially in paediatric patients, due to invasive endoscopic procedures. IBD is associated with alterations in intestinal microbiota composition. Faecal volatile organic compounds (VOCs) reflect gut microbiota composition. Aim of this study was to assess the diagnostic accuracy of faecal VOC profiling as non-invasive diagnostic biomarker for paediatric IBD.

Methods

In this diagnostic accuracy study performed in two tertiary centres in the Netherlands, faecal VOC profiles of 36 de novo, treatment-naïve paediatric IBD patients (23 CD, 13 UC), and 24 healthy, matched controls were measured by field asymmetric ion mobility spectrometry (Owlstone Ltd, Lonestar ®, UK).

Results

Faecal VOC profiles of de novo paediatric IBD patients could be differentiated from healthy controls; (AUC ± 95% CI, p-value, sensitivity, specificity; 0.76 ±0.14, $p<0.001$, 79%, 78%). This discrimination from controls was observed in both CD (0.90 ±0.10, $p<0.0001$, 83%, 83%) and UC (0.74 ±0.19, p= 0.02, 77%, 75%). VOC profiles from UC could not be discriminated from CD (0.67 ±0.19, p= 0.0996, 65%, 62%).

Conclusion

Field asymmetric ion mobility spectrometry allowed for discrimination between faecal VOC profiles of de novo paediatric IBD patients and healthy controls, conforming the potential of faecal VOC analysis as a non-invasive diagnostic biomarker for paediatric IBD. This method may serve as a

complementary, non-invasive technique in the diagnosis of IBD, possibly limiting the needed number of endoscopies in children suspected for IBD.

Keywords

Volatile organic compounds; electronic nose; ion mobility spectrometry; inflammatory bowel disease

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Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing disorder of the gastrointestinal tract, which presents itself in two major forms, Crohn's disease (CD) and ulcerative colitis (UC). Paediatric patients, with CD or UC, mostly present with classical symptoms such as abdominal pain, diarrhoea, rectal bleeding and weight loss.[1] Currently, the diagnosis of IBD is based on a combination of clinical symptoms, laboratory markers, radiologic findings and endoscopy of the upper and lower gastrointestinal tract, with histologic examination of mucosal biopsies. [2] These endoscopic procedures remain essential in both initial work-up and in following up of the disease activity, but carry a high burden on patients, especially in children. Typically, this group requires hospitalisation for intensive bowel preparation by nasogastric tube, and general anaesthesia to perform the endoscopy. This emphasizes the need to develop new, non-invasive, cost-effective tests with high accuracy for diagnosing and monitoring disease activity of paediatric IBD.

Current biomarkers in the diagnosis and follow-up of IBD disease activity include C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), faecal calprotectin (FC) and lactoferrin, but these biomarkers are characterized by relatively low specificity, especially in children. [3] The intestinal microbiota has increasingly been recognized as a relevant disease factor in IBD. Previous studies have described a decrease in bacterial diversity and an alteration in the abundance of specific bacterial communities, compared to healthy controls.[4-9] Although microbiome-based diagnostics can currently not replace standard diagnostic techniques, it has been considered to have potential as a complementary, non-invasive technique in the diagnosis of IBD. However, microbiota-based diagnostic algorithms are not yet available, microbiota analysis is expensive and application in daily practice is limited by the need for intensively trained personnel to perform the complex, time-consuming statistical analyses.

The colonic microbiota produces a characteristic metabolic profile by fermentation of non-starch polysaccharides, composed of gaseous carbon-based molecules (including volatile organic compounds (VOCs)) [9]. VOCs also originate from human physiological metabolic processes and

pathophysiological processes such as oxidative stress and inflammation, and are excreted as waste products through all conceivable bodily excrements.[10] Therefore, changes in the faecal VOC fingerprint are considered to reflect alterations of both gut microbiota and human metabolism.[9] Assessment of VOCs using sophisticated analytical techniques has led to identification of potential disease-specific biomarkers for a variety of gastro-intestinal diseases, including malignancies, infections and inflammatory diseases. [11-14]

More recently, a technology that has found use in medical diagnostics is Field asymmetric ion mobility spectrometry (FAIMS). Used extensively in military/security applications it is now increasingly being used for the detection of gas phase biomarkers from human waste. Compared with traditional gas chromatography-mass spectrometry (GC-MS) and electronic noses it has higher sensitivity, compact form factor, uses air as the carrier gas and has minimal drift. It achieves separation by measuring the mobility of ionised molecules in high-electric fields. Furthermore, faecal samples do not require specialized preparations or solutions prior to analysis. Thus, with low drift and high sensitivity, it should be feasible in a clinical setting to monitor changes of VOC pattern over time. In the present study we have aimed to measure faecal VOCs by FAIMS to discriminate paediatric IBD patients from healthy controls.

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Materials and methods

Subjects

Between December 2013 and October 2015 we included all eligible children aged 4 to 17 years suspected for IBD in this two-centre study (VU university medical centre and Academic Medical Centre, both located in Amsterdam, the Netherlands). The diagnosis of IBD was made according to the revised diagnostic Porto-criteria for paediatric IBD, including endoscopic and histologic and radiologic findings.[15] Localization and behaviour of disease were classified according to the Paris Classification.[16] Disease activity was assessed by Physician Global Assessment (PGA-score).[17] C-reactive protein (CRP), leucocytes and faecal calprotectin (FC) levels were determined at diagnosis. Exclusion criteria were diagnosis of unclassified type of IBD, use of antibiotics or immune modulating agents within the last six months prior to the study, culture-proven infectious gastroenteritis in the last six months prior to inclusion, history of surgery of the gastrointestinal tract (except appendectomy), previous diagnosis of chronic gastrointestinal disease (such as inflammatory bowel disease, celiac disease, functional constipation or short bowel syndrome).

The control group consisted of asymptomatic healthy volunteers in the age range of 4-17 years, attending primary and secondary schools in similar regions of the Netherlands (Noord-Holland, Zuid-Holland, Flevoland). An identical protocol was used for collection, storage, transport, handling and VOC analysis of these faecal samples. The study was approved by the University's Ethics Committee of both participating centres (2015.393).

Samples

Paediatric patients undergoing diagnostic ileocolonoscopy and esophagogastroduodenoscopy under suspicion of IBD, were instructed to collect a faecal sample prior to bowel preparation. The faecal samples were collected in a sterile container, at home stored preferably at -20°C, within 2 hours of collection, and after delivery to the hospital stored at -20°C until analysis by FAIMS. Protocol on collection and storage of the faecal samples of the control groups was similar to the study group.

Field Asymmetric Ion Mobility Spectroscopy (FAIMS)

A commercial setup was used for FAIMS analysis (Lonestar® with ATLAS sampling system, Owlstone Ltd, UK). This instrument uses a NI-63 radiation source to ionize VOCs after entering the instrument. In the FAIMS process, an increasing electric field is applied to the ionised molecules as they pass between two plates. To one of these plates a compensation voltage is added, which removes the effect of the molecular movement brought about by the application of the electric field. Thus, only molecules with specific mobilities exit the plates and are detected. By scanning through a series of compensation voltages and field strengths (described as the dispersion field) we are able to create a 3D VOC map of a complex mixture of chemicals in a faecal headspace (Figure 1). Further details of this analysis has been described previously.[18]

VOC profiling

Faecal VOC profiling using FAIMS took place after a mean sample storage period of 23 months in IBD (CD 25, UC 21) and 39 in healthy controls. Faecal samples were thawed to room temperature (20°C) one hour prior to VOC analysis. A mixture of 0.5 g faecal sample with 10mL tap water was manually shaken to homogenize the sample. [14] To move the sample headspace into the FAIMS instrument, the sample was first placed in the ATLAS sample system. Here room air was compressed (0.1MPa) and cleaned before being pushed over the top of the sample and into the FAIMS machine at a flow rate of 2L/min. The temperatures were set at 35°C for the sample/bottle holder, 70°C for the lid and 100°C for the filter region.**(Fig. 1)** [14] The air in the FAIMS was refreshed between samples by analysing the headspace of a clean jar. The dispersion field (DF) passed through 51 equal settings between 0% and 100%. The compensation voltage (CV) was set between +6V and -6V in 512 steps for each dispersion field, to produce 26,112 data points per sample. Measuring both positive and negative ion counts a total of 52.224 data points were generated. To preclude environmental effects, each faecal sample was analysed three times sequentially, producing three matrices in 540s, for analysis we used only the second and third matrix.

Statistical methods

The FAIMS data was processed using a well-established pipeline, which has been developed specifically for these types of studies and has previously been reported.[19,23,24] In brief, first a pre-processing step was applied to each run in the form of a 2D wavelet transform (using Daubechies D4 wavelets). This performs two tasks, first as a data compression step and secondly as it can aid in the selection of chemical species by extracting 'peaks', which results in concentrating the chemical information into a small number of wavelet coefficients. This has the effect of improving and simplifying subsequent analysis steps. A threshold is then applied to remove data with little or no discriminatory power (known from previous work).

This was followed by a 5-fold cross-validation, using 80% of the data as a training set, and the remaining 20% as a test set. Within each fold, important features were identified using a Wilcoxon rank sum test from the training set. The two most statistically important features were then used to predict the result of the test set. Four different classifiers were used for prediction, specifically:, Random Forest, Gaussian Process Classifier, XGB (a boosting algorithm) and Sparse Logistic Regression. Of these, the following generated the best classification results: Healthy vs Disease (Random Forest), CD vs Healthy (XGB), CU vs Healthy (sparse logistic regression), CD vs CU (sparse logistic regression). We note that in this paper we are focusing on the best classifier in each case (which could be considered a source of overfitting) and therefore all the results are shown in the supplementary information. However, we note that the results were generally consistent across multiple classifiers in each case, suggesting that a range of classifiers can be effective for this task. This is also our experience with FAIMS data in other contexts.

Ethical Considerations

The study is approved by the Medical Ethical Review Committee (METc) of VU University Medical Center, registered with the US Office for Human Research Protections (OHRP) as IRB00002991. Written informed consent was obtained from of all paediatric IBD patients, healthy children and their parents.

Results

Thirty-six children with de novo, treatment-naïve IBD were included (13 UC, 23 CD). The control group consisted of 24 asymptomatic healthy children. All controls were age matched. Subject characteristics of the IBD patients and the control group are described in Table 1. Besides a female gender predominance in the UC group compared to the other subgroups, no statistically significant differences in subject characteristics were present between CD, UC and controls.

The results of the FAIMS data comparing CD, UC and controls are displayed in Table 2. Faecal VOCs of IBD patients could be discriminated from the control group (AUC ± 95%CI, p-value, sensitivity, specificity; 0.76 ±0.14, $p<0.001$, 79%, 78%).

Faecal VOC profiles of CD patients differed from the healthy control group (0.90 ±0.10, $p<0.001$, 83%, 83%). Furthermore, patients with UC could be discriminated from the healthy control group (0.74 ±0.19, $p<0.02$, 77%, 75%). VOC profiles could not distinguish UC from CD (0.67 ±0.19, $p= 0.0996$, 65%, 62%). This data is shown a box plot of probability in figure 2 and ROCs in figure 3.

Discussion

In the present study, we have compared faecal VOC patterns of de novo, treatment-naïve paediatric IBD patients with active disease to healthy controls by means of Field Asymmetric Ion Mobility Spectrometry (FAIMS). We observed that faecal VOC profiles of children diagnosed with active CD and UC could be discriminated from healthy controls with modest accuracy. Our results are in line with a previous study on the potential of faecal gas analysis to detect biomarkers of disease activity in paediatric IBD, using an electronic nose device (Cyranose®). In that study, faecal VOC profiles of 45 children with de novo IBD (26 UC, 29 CD) could be discriminated from 28 healthy controls, during exacerbation and upon achieving clinical remission (AUC ± 95%CI, p-value, sensitivity, specificity; Table 3). A similar distinction was observed between UC versus CD, both during exacerbation and remission .[19]

Recent studies using FAIMS technology have shown that VOCs derived from breath and urine can be used to discriminate adult patients with de novo IBD from healthy controls. [9] However, there is a lack of data involving VOC analysis in paediatric patients using FAIMS.

It could be hypothesized that in the diagnostic work-up of gastrointestinal diseases, analysis of faecal VOCs is more appropriate compared to VOCs deriving from other excreta. Human faeces contains the end-product of digestive, excretory processes, diet and the bacterial metabolism of the colon. [20] Since IBD is characterized by mucosal inflammation of the intestines and associated with intestinal microbial shifts, analysis of faecal VOCs could possibly offer a more direct and integral view on disease activity compared to, for example breath and urine.

In a previous study of our research group using a Cyranose® eNose, we observed that paediatric IBD patients could be discriminated from controls by faecal VOC profiling, both during active disease and upon achieving remission. [19] This finding was confirmed in a recent study on VOC profiling in exhaled breath from adult IBD patients, using FAIMS technique (0.70 ±0.10, p<0.001, 67%, 67%).[19, 21]. This observation is in concordance with observations of several studies on microbiota profiling in IBD,

describing significant differences in gut microbiota composition between adult CD, UC and healthy controls. [22]

Furthermore, in children microbial diversity and richness of specific bacterial communities seemed to differ between de novo CD and UC, although described results in studies on microbiota in IBD are not consistent [5, 6, 28] In contrast to our previous study on faecal VOC analysis in paediatric IBD, in the present study we did not observe a significant difference in VOC profiles between UC and CD. A possible explanation for this apparent discrepancy may be caused by differences in examined cohorts. However, IBD cohorts in both studies were comparable, with similar participating hospitals and comparable patient characteristics. It seems more likely that observed differences are due to the way VOCs were detected. By eNose (Cyranose®), VOC groups present in the gaseous mixture of interest interacts with one or more eNose sensor, creating VOC patterns based on a change in electronic resistance of each sensor. By FAIMS a smell print is obtained based on ionized VOCs' mobility over an electric field, which is a completely different mechanism. Hypothetically, minor differences in VOC profiles as measured by FAIMS may induce significant differences in VOC outcome as measured by a traditional eNose and vice versa. Notably, eNoses in particular employ chemical active sensors are prone to batch variation, fouling and ageing effects. This results in the eNose requiring re-training on a weekly and potentially daily basis. This is not the same for FAIMS, which is a physical measurement and thus suffers from minimal drift. Future studies comparing both techniques, and using similar samples, are needed to obtain detailed insight on this aspect which may affect VOC outcome. These studies should preferably include a subgroup of controls with gastro-intestinal symptoms as diarrhoea and abdominal pain, allowing us to compare faecal VOC profiles of IBD with an intention-to-diagnose cohort. Furthermore, it has been shown that differences in sampling conditions and characteristics, like sample mass, fecal sample temperature, water content, duration of storage at room temperature, all affect VOC outcome (Berkhout 2016). Optimal conditions have not yet been defined, but these observations underline the need for standardization of study study protocols.

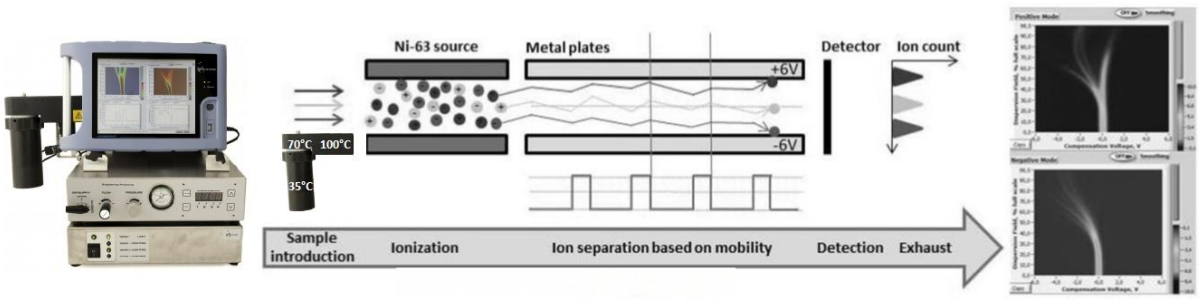
Strength of this study technique is the exclusion of bias by medication since all patients were treatment-naïve prior to collection of the faecal sample. We used a standard methodology guideline on sampling collection, storing and preparing for comparability to future studies and easy application for medical practice. Furthermore, we used a well-defined, matched control group.

One of the limitations of this study is the relatively small sample size. This prevented us to assess the potential influence of exogenous VOCs from environmental factors, previous use of medication and diet. Possibly, parents of children suffering from IBD may have altered their normal diet in an attempt to control symptoms. In case of systematic dietary alterations, this could have resulted in a type I error (false positive outcome). The paediatric patients and the children from the control group are derived from a relatively limited geographic area with a more or less common culture and diet. However, detailed daily dietary information would be valuable to investigate a possible correlation with measured faecal VOC's. Patient characteristics were similar in the three subgroups, except for sex, with a predominance for female in the UC subgroup, however, previous studies have shown that gender does not affect VOC composition. [23-25]

In conclusion, we observed that faecal VOC analysis by FAIMS could discriminate paediatric de novo IBD patients from healthy controls, with modest accuracy. The apparently high specificity of faecal VOCs compared to faecal calprotectin underlines the potential of this method to serve as a complementary, non-invasive technique in the diagnosis of paediatric IBD, possibly limiting the needed number of endoscopies in a subset of children suspected for IBD.

Figure 1. Field Asymmetric Ion Mobility Spectrometer (FAIMS)[14] FAIMS device Lonestar®, Owlstone, UK..The faecal sample was placed in a glass bottle holder, which is connected with the FAIMS unit. The faecal VOCs were transported to this unit using a carrier gas (dry air., Here, the VOCs were ionised (using a Ni-63 source), leading to a composition of various sizes and types of ions. These ionised molecules enter an electric field waveform and pass between two metal plates. The applied voltage of this created field, also known as dispersion field (DF), varies with a proportionate effect on

an ion's mobility. Application of a high positive voltage followed by a longer period of a low negative voltage creates an asymmetric electric field waveform. The integral of this voltage over a time period is zero. A "zigzag" path is formed on the way through the plates toward the sensor, when ions have the same mobility in high and low electric fields. An ion exits the plates when it contacts the plates and loses its charge, leading it undetected. Therefore, a counteracting and balancing voltage is applied, which is called the 'compensation voltage'(CV). This CV can be set whereby the drift from a specific ion is compensated for and the ion will be detected by the sensor. A complex mixture of gasses can be separated by their differences in mobility in high and low electric fields by ranging through dispersion fields and compensation voltages. Variations in the strength of the DF and CV generates a data-rich chemical fingerprint, a 'smell print'.[26].



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