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

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5

6 **Non-standard abbreviations**

7 VOC = volatile organic compound

8 FAIMS = field asymmetric ion mobility spectrometry

9 eNose = electronic nose

10 GC-MS = gas chromatography–mass spectrometry

11

## 1 **Abstract**

### 2 **Background and Aims**

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

### 9 **Methods**

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

### 14 **Results**

15 Faecal VOC profiles of de novo paediatric IBD patients could be differentiated from healthy controls;  
16 (AUC  $\pm$  95% CI, p-value, sensitivity, specificity;  $0.76 \pm 0.14$ ,  $p < 0.001$ , 79%, 78%). This discrimination  
17 from controls was observed in both CD ( $0.90 \pm 0.10$ ,  $p < 0.0001$ , 83%, 83%) and UC ( $0.74 \pm 0.19$ ,  $p =$   
18  $0.02$ , 77%, 75%). VOC profiles from UC could not be discriminated from CD ( $0.67 \pm 0.19$ ,  $p = 0.0996$ ,  
19 65%, 62%).

### 20 **Conclusion**

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

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

3 **Keywords**

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

5 **Financial disclosure:** NONE

6 **Grand support:** NONE

7 **Conflict of interest:** NONE

8 **Declaration of funding interest:** NONE

9

## 1 Introduction

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

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

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

1 pathophysiological processes such as oxidative stress and inflammation, and are excreted as waste  
2 products through all conceivable bodily excrements.<sup>(10)</sup> Therefore, changes in the faecal VOC  
3 fingerprint are considered to reflect alterations of both gut microbiota and human metabolism.<sup>(9)</sup>  
4 Assessment of VOCs using sophisticated analytical techniques has led to identification of potential  
5 disease-specific biomarkers for a variety of gastro-intestinal diseases, including malignancies,  
6 infections and inflammatory diseases.<sup>(11-14)</sup>  
7 More recently, a technology that has found use in medical diagnostics is Field asymmetric ion  
8 mobility spectrometry (FAIMS). Used extensively in military/security applications it is now  
9 increasingly being used for the detection of gas phase biomarkers from human waste. Compared  
10 with traditional gas chromatography-mass spectrometry (GC-MS) and electronic noses it has higher  
11 sensitivity, compact form factor, uses air as the carrier gas and has minimal drift. It achieves  
12 separation by measuring the mobility of ionised molecules in high-electric fields. Furthermore, faecal  
13 samples do not require specialized preparations or solutions prior to analysis. Thus, with low drift  
14 and high sensitivity, it should be feasible in a clinical setting to monitor changes of VOC pattern over  
15 time. In the present study we have aimed to measure faecal VOCs by FAIMS to discriminate  
16 paediatric IBD patients from healthy controls.

17

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## 1 **Materials and methods**

### 2 **Subjects**

3 Between December 2013 and October 2015 we included all eligible children aged 4 to 17 years  
4 suspected for IBD in this two-centre study (VU university medical centre and Academic Medical  
5 Centre, both located in Amsterdam, the Netherlands). The diagnosis of IBD was made according to  
6 the revised diagnostic Porto-criteria for paediatric IBD, including endoscopic and histologic and  
7 radiologic findings.<sup>(15)</sup> Localization and behaviour of disease were classified according to the Paris  
8 Classification.<sup>(16)</sup> Disease activity was assessed by Physician Global Assessment (PGA-score).<sup>(17)</sup> C-  
9 reactive protein (CRP), leucocytes and faecal calprotectin (FC) levels were determined at diagnosis.  
10 Exclusion criteria were diagnosis of unclassified type of IBD, use of antibiotics or immune modulating  
11 agents within the last six months prior to the study, culture-proven infectious gastroenteritis in the  
12 last six months prior to inclusion, history of surgery of the gastrointestinal tract (except  
13 appendectomy), previous diagnosis of chronic gastrointestinal disease (such as inflammatory bowel  
14 disease, celiac disease, functional constipation or short bowel syndrome).  
15 The control group consisted of asymptomatic healthy volunteers in the age range of 4-17 years,  
16 attending primary and secondary schools in similar regions of the Netherlands (Noord-Holland, Zuid-  
17 Holland, Flevoland). An identical protocol was used for collection, storage, transport, handling and  
18 VOC analysis of these faecal samples. The study was approved by the University's Ethics Committee  
19 of both participating centres (2015.393).

20

### 21 **Samples**

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

## 1 Field Asymmetric Ion Mobility Spectroscopy (FAIMS)

2 A commercial setup was used for FAIMS analysis (Lonestar<sup>®</sup> with ATLAS sampling system, Owlstone  
3 Ltd, UK). This instrument uses a NI-63 radiation source to ionize VOCs after entering the instrument.  
4 In the FAIMS process, an increasing electric field is applied to the ionised molecules as they pass  
5 between two plates. To one of these plates a compensation voltage is added, which removes the  
6 effect of the molecular movement brought about by the application of the electric field. Thus, only  
7 molecules with specific mobilities exit the plates and are detected. By scanning through a series of  
8 compensation voltages and field strengths (described as the dispersion field) we are able to create a  
9 3D VOC map of a complex mixture of chemicals in a faecal headspace (Figure 1). Further details of  
10 this analysis has been described previously.<sup>(18)</sup>

11

## 12 VOC profiling

13 Faecal VOC profiling using FAIMS took place after a mean sample storage period of 23 months in IBD  
14 (CD 25, UC 21) and 39 in healthy controls. Faecal samples were thawed to room temperature (20°C)  
15 one hour prior to VOC analysis. A mixture of 0.5 g faecal sample with 10mL tap water was manually  
16 shaken to homogenize the sample.<sup>(14)</sup> To move the sample headspace into the FAIMS instrument,  
17 the sample was first placed in the ATLAS sample system. Here room air was compressed (0.1MPa)  
18 and cleaned before being pushed over the top of the sample and into the FAIMS machine at a flow  
19 rate of 2L/min. The temperatures were set at 35°C for the sample/bottle holder, 70°C for the lid and  
20 100°C for the filter region.**(Fig. 1)**<sup>(14)</sup> The air in the FAIMS was refreshed between samples by  
21 analysing the headspace of a clean jar. The dispersion field (DF) passed through 51 equal settings  
22 between 0% and 100%. The compensation voltage (CV) was set between +6V and -6V in 512 steps  
23 for each dispersion field, to produce 26,112 data points per sample. Measuring both positive and  
24 negative ion counts a total of 52,224 data points were generated. To preclude environmental effects,  
25 each faecal sample was analysed three times sequentially, producing three matrices in 540s, for  
26 analysis we used only the second and third matrix.

## 1 Statistical methods

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

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

1 **Ethical Considerations**

2 The study is approved by the Medical Ethical Review Committee (METc) of VU University Medical

3 Center, registered with the US Office for Human Research Protections (OHRP) as IRB00002991.

4 Written informed consent was obtained from of all paediatric IBD patients, healthy children and their  
5 parents.

6

## 1 **Results**

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

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

10 Faecal VOC profiles of CD patients differed from the healthy control group ( $0.90 \pm 0.10$ ,  $p < 0.001$ , 83%,  
11 83%). Furthermore, patients with UC could be discriminated from the healthy control group ( $0.74$   
12  $\pm 0.19$ ,  $p < 0.02$ , 77%, 75%). VOC profiles could not distinguish UC from CD ( $0.67 \pm 0.19$ ,  $p = 0.0996$ , 65%,  
13 62%). This data is shown a box plot of probability in figure 2 and ROCs in figure 3.

14

## 1 Discussion

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

12 Recent studies using FAIMS technology have shown that VOCs derived from breath and urine can be  
13 used to discriminate adult patients with de novo IBD from healthy controls.<sup>(9)</sup> However, there is a  
14 lack of data involving VOC analysis in paediatric patients using FAIMS.

15 It could be hypothesized that in the diagnostic work-up of gastrointestinal diseases, analysis of faecal  
16 VOCs is more appropriate compared to VOCs deriving from other excreta. Human faeces contains the  
17 end-product of digestive, excretory processes, diet and the bacterial metabolism of the colon.<sup>(20)</sup>

18 Since IBD is characterized by mucosal inflammation of the intestines and associated with intestinal  
19 microbial shifts, analysis of faecal VOCs could possibly offer a more direct and integral view on  
20 disease activity compared to, for example breath and urine.

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

1 describing significant differences in gut microbiota composition between adult CD, UC and healthy  
2 controls. <sup>(22)</sup>

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

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

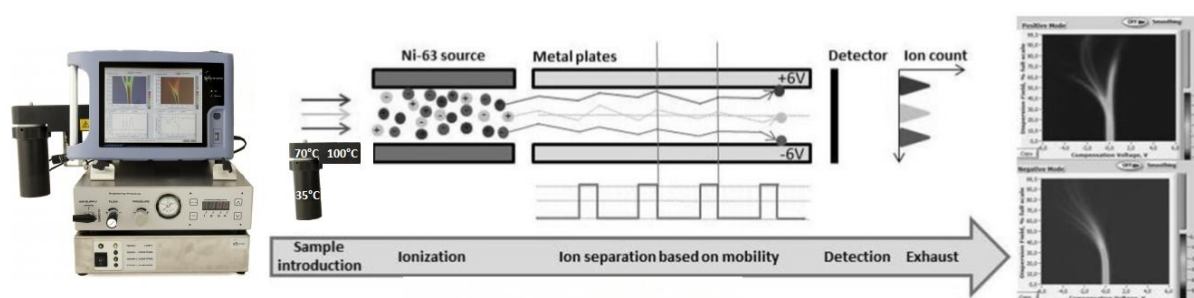
5 One of the limitations of this study is the relatively small sample size. This prevented us to assess the  
6 potential influence of exogenous VOCs from environmental factors, previous use of medication and  
7 diet. Possibly, parents of children suffering from IBD may have altered their normal diet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

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

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



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



11

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