The fecal scent of inflammatory bowel disease: detection and monitoring of IBD based on volatile organic compound analysis

Sofie Bosch [1], Dion Wintjens [2], Alfian Wicaksono [3], Johan Kuijvenhoven [4], René van der Hulst [4], Pieter Stokkers [5], Emma Daulton [3], Marieke Pierik [2], James A Covington [3], Tim GJ de Meij [6] and Nanne KH de Boer [1]

[1] Amsterdam UMC, Vrije Universiteit Amsterdam, Department of Gastroenterology and Hepatology, AG&M research institute, Amsterdam, The Netherlands

[2] MUMC+, Maastricht University, Department of Gastroenterology and Hepatology, Maastricht, The Netherlands

[3] University of Warwick, School of Engineering, Coventry, United Kingdom

[4] Spaarne Gasthuis, Department of Gastroenterology and Hepatology, Hoofddorp and Haarlem, The Netherlands

[5] OLVG West, Department of Gastroenterology and hepatology, Amsterdam, The Netherlands

[6] Amsterdam UMC, Vrije Universiteit Amsterdam, Department of Pediatric Gastroenterology, AG&M research institute, Amsterdam, The Netherlands

Corresponding Author

Sofie Bosch, MD

Amsterdam UMC, Vrije Universiteit Amsterdam

Department of Gastroenterology and Hepatology

De Boelelaan 1117, 1081HZ

Amsterdam, the Netherlands

T: 0031-20-4440613

F: 0031-20-4440554

E: S.Bosch1@vumc.nl

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Author contributions

de Boer, de Meij and Bosch designed the study protocol and experiment. Wintjens, Bosch, van der Hulst, Kuijvenhoven and Stokkers collected the samples. Wintjens and Bosch prepared the samples for analyses. Daulton and Bosch run the sample measurements. Daulton and Covington developed the sample test method. Wicaksono and Covington analyzed the data. Bosch drafted a first version of the manuscript. Wintjens, Wicaksono, Kuijvenhoven, van der Hulst, Stokkers, Daulton, Pierik, Covington, de Meij and de Boer critically reviewed the manuscript.

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Abstract (words: 380)

Background The gold standard to diagnose and monitor inflammatory bowel disease (IBD) remains endoscopic assessment, which is invasive and costly. Fecal calprotectin (FCP) is the most commonly used non-invasive biomarker to assess IBD but has limited specificity. The aim of the current study was to evaluate the potential of fecal volatile organic compounds (VOC) analysis to diagnose IBD and to identify disease exacerbation.

Methods IBD patients who visited the outpatient clinic of one of the two tertiary hospitals were eligible to participate, independently of disease activity. All patients collected fecal samples prior to their scheduled consult. Healthy controls (HC) were patients without mucosal abnormalities observed during colonoscopy. Active disease was defined as FCP ≥250 mg/g, remission as FCP <100 mg/g combined with Harvey Bradshaw Index <4 points for Crohn's disease (CD) or Simple Clinical Colitis Activity Index <3 points for ulcerative colitis (UC). VOCs were measured from the headspace of fecal samples using gas chromatography-ion mobility spectrometry. Data were split into two sets, 70% for training and validation and 30% as a test set. A Wilcoxon rank-sum test was used to find e th20, 50 and 100 most discriminatory features. Random Forest, Support Vector Machine, Gaussian Process and Neural Net classification were used to provide statistical results.

Results A total of 280 IBD patients and 227 HC provided 292 CD, 197 UC and 227 HC fecal samples. Of IBD samples, 107 and 84 were CD active (CDa) and remission (CDr) samples, and 80 and 63 were UC active (UCa) and remission (UCr) samples, respectively. Based on VOC profiles, UC and CD could be discriminated from HC with high accuracy (AUC (95%CI), p-values: UCa vs HC 0.96(0.94-.99), p<0.0001; UCr vs HC 0.95(0.93-0.98), p<0.0001; CDa vs HC 0.96(0.94-0.99), p<0.0001; CDr vs HC 0.95(0.93-0.98), p<0.0001). A small difference was observed between fecal VOC profiles of UC and CD (0.55(0.50-0.6), p=0.03). There was no significant difference between active disease state and remission (UCa vs UCr 0.63(0.44-0.82), p=0.082; CDa vs CDr 0.52(0.39-0.65), p=0.64).

Conclusion Based on fecal VOC analysis, IBD would be discriminated from HC both during active disease state and remission, though there was no difference between active disease and remission. These characteristics imply that fecal VOC analysis seems to hold potential as non-invasive biomarkers for IBD disease detection, but not to monitor disease activity in patients with established diagnosis of IBD.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic gastrointestinal diseases characterized by periods of relapse and remission, and are together referred to as inflammatory bowel disease (IBD). Over the past few decades, a worldwide increase in incidence has been observed with mean annual rates ranging from 14.0 per 100.000 persons in Western Europe to 21.3 per 100.000 persons in Australia in 2011[1, 2]. The gold standard to diagnose and monitor mucosal inflammation in IBD patients is ileocolonoscopy substantiated by histological assessment of biopsy specimens and/or radiology. This diagnostic workup is invasive, expensive and carries risks of complications. The use of non-invasive biomarkers for IBD evaluation is therefore preferred. Fecal calprotectin (FCP) is the most commonly used non-invasive biomarker to assess and monitor IBD. This test is characterized by a high sensitivity for mucosal inflammation (0.98, 95%CI 0.95-0.99), but limited specificity for IBD (0.81-0.91 for adults, and 0.68 for children) [3]. For example, FCP elevations have been observed in patients with infectious diarrhea, celiac disease, rheumatoid arthritis and gastrointestinal malignancies[4-7]. Therefore, the search for a more specific non-invasive IBD biomarker remains warranted.

Volatile organic compounds (VOCs) are gaseous carbon-bound chemicals that include hydrocarbons, alcohols, aldehydes, ketones, esters and organic acids. Fecal VOCs are thought to represent both metabolic processes in the human body and the interaction between gut microbiota and host[8]. These molecular end-products can be found in all bodily excrements dependent on their volatility and sample temperature. Great potential of VOC profiles as noninvasive biomarkers have been described for various gastrointestinal diseases[9-11]. The detection of pediatric IBD using fecal VOC patterns has also been subject of various studies, with promising results, but the literature on its potential in adults is limited[12-14]. The aim of the current study is to validate the potential of fecal VOC patterns to detect IBD and to assess their potential to identify disease exacerbation in adults.

Methods

Study design

This study was performed at the outpatient clinics of the gastroenterology and hepatology department in two tertiary referral hospitals (Amsterdam UMC, location VUmc, Amsterdam and Maastricht University Medical Center (MUMC+) in Maastricht), and two district hospitals (OLVG West in Amsterdam and Spaarne Gasthuis (SG), location Hoofddorp and Haarlem) all located in The Netherlands. This study was approved by the Medical Ethical Review Committee (METc) of the Amsterdam UMC, location VUmc under file number 2016.135, by the METc of the MUMC+ under filenumer NL24572.018.08, and by the local medical ethical committee of the OLVG West and Spaarne Gasthuis. Written informed consent was obtained from all study participants. Once sample collection was complete, all samples were shipped to the School of Engineering, University of Warwick (Coventry, UK) for VOC analysis.

Study participants

Inflammatory bowel disease patients

All patients aged 18 years or older with an established diagnosis of IBD based on clinical, endoscopic, histological and/or radiological criteria and with a scheduled consult at the outpatient clinic of one of the two tertiary referral hospitals, and independently on disease activity, were asked to participate in this study[15]. Patients were asked to collect a fecal sample and to complete a questionnaire on the same day, which included information on age, gender, BMI, smoking status, abdominal symptoms, medication use, dietary habits, comorbidity and questions on clinical disease activity based on the Harvey Bradshaw Index (HBI) for CD patients and the Simple Clinical Colitis Activity Index (SCCAI) for UC patients [16, 17]. Active disease was defined as an FCP level of ≥250 mg/g. Remission was defined as FCP <100 mg/g combined with a HBI <4 points or SCCAI <3 points. All IBD patients were included in the primary statistical analysis assessing the diagnostic potential of fecal VOCs to differentiate between IBD and HC. Only IBD patients with clearly defined disease activity based on FCP and HBI/SCCAI levels were included in the secondary analyses aiming to assess the difference in fecal VOC pattern between active disease and remission. Demographic and clinical data (including Montreal classification and history of bowel surgery) were obtained from electronic patient files[18].

Healthy controls

All patients aged 18 years and older with a scheduled colonoscopy at the Amsterdam UMC, OLVG West and Spaarne Gasthuis were asked to participate in this study regardless of their endoscopy indication. They were also asked to complete a questionnaire on age, gender, BMI, smoking status, abdominal symptoms, bowel movements, dietary intake, comorbidity and medication use. Patients

without endoscopic abnormalities observed during endoscopy were included in this study as healthy controls (except asymptomatic external haemorrhoids, asymptomatic diverticula and/or small anal fibromas). In case of mucosal biopsies to exclude microscopic alterations, histologic reports were checked and subjects were only included as HC if no histologic abnormalities were detected by the pathologist. Exclusion criteria to be included as HC were mucosal abnormalities observed during endoscopy, a history of bowel diseases (e.g. celiac disease, IBD, CRC), failure to perform a complete colonoscopy because of various reasons (e.g. inadequate bowel cleansing, pain) and/or the collection of insufficient fecal sample mass prior to endoscopy to perform VOC analysis.

Sample collection

Inflammatory bowel disease

Amsterdam University Medical Centers

Between February 2015 and November 2017, IBD patients were asked to collect two fecal samples (Stuhlgefäß 10ml, Frickenhausen, Germany) from the same bowel movement prior to the consult: one for FCP levels and one for VOC analysis. The sample for FCP measurement was sent to the hospital by mail directly after collection. The sample for VOC analysis was stored in their own freezer within one hour following collection and transported to the hospital in cooled condition using ice packs and/or ice cubes on the day of their consultation. The samples were stored at -24°C directly upon arrival at the hospital until further handling.

Maastricht University Medical Center

Between October 2009 and December 2010 patients were asked to collect stool from one bowel movement on the day of their consult and bring it fresh to the hospital. This stool sample was stored in the fridge (4 °C) directly upon arrival at the hospital. From this bowel movement, two samples were prepared on the day of arrival. One for fecal calprotectin measurements (using ELISA) and one for research purposes. The second sample was stored in the freezer at -80°C on the day of delivery until further handling.

Healthy controls

Between February 2015 and November 2017, patients from the Amsterdam UMC, OLVG West and SG collected a fecal sample in a container (Stuhlgefäß 10ml, Frickenhausen, Germany) prior to bowel cleansing and endoscopic assessment. They were asked to store their sample in their own freezer within one hour after collection. These samples were transported to the hospital in cooled condition using ice packs and/or ice cubes on the day of their endoscopy. The samples were stored at -24°C directly upon arrival at the hospital.

Sample preparation and analyses of volatile organic compounds

The samples were then shipped to the University of Warwick on dri-ice for preparation. From the original samples, one subsample of 0.5 g per participant was weighted on a calibrated scale (Mettler Toledo, AT 261 Delta Range, Ohio, United States), transferred into a gass vial (20ml headspace vial, Thames Restek, Saunderton, UK), labelled and re-stored in a -24°C freezer until further handling. The amount of sample was chosen to provide an optimum ratio of VOCs to the sample headspace, as validated by our research team in a previous sampling method study for VOC pattern-recognition using field asymmetric ion mobility spectrometry (FAIMS)[19].

Fecal volatile organic compound analysis

Fecal samples were analysed using gas chromatography coupled to an ion mobility spectrometer (GC-IMS, FlavourSpec®, G.A.S., Dortmund, Germany). The instrument is formed on a GC column, coupled with a drift tube IMS. The GC provides pre-separation of the complex mixture of chemicals found in the headspace, before detection by IMS. Within the IMS, volatile organic compounds are ionized, in this case, by means of soft chemical-ionization initiated by a low-radiation tritium (H3) source, creating reactant ions with the gas atmosphere. The ionized VOCs travel at atmospheric pressure against the flow of an inert drift gas. In general, larger molecule are struck more times than smaller molecules, losing momentum and thus, taking longer to travel along the tube. The drift time of each substance is therefore determined by the ions mass and geometrical structure. The resulting ion current is measured by an electrometer as a function of time[20]. During this study, GC-IMS was connected to an automatic sampling system with chiller allowing processing of a batch of 32 samples kept in cooled condition (4 °C) until start of the analyses. The samples were heated to 80 °C during the 8 minutes prior to analyses. Then, a syringe transports the headspace from the vial into the injector port of the instrument and into the GC column. The experiments were performed at 40 °C using nitrogen 99.9% (3.5 bar) as carrier gas and the IMS was performed at 45 °C using nitrogen as drift gas. Flow rates were set at 150ml/min (0.364 kPa) (IMS), and at 20 ml/minute (34.175 kPa) for 6 minutes (GC). A schematic overview of the setup is depicted in Figure 1.

Statistical analysis

Prior to the statistical analyses, the data was pre-processed to only crop areas that contain chemical information, then a threshold is applied to remove back group noise and finally corrected for instrumental disturbances by baseline correction. This reduces the data points per sample from around 11 million to a more management 100,000. Data were split into three sets, 70% for training and validation and 30% as test set. Wilcoxon rank-sum test was used to find the 20, 50 and 100 most discriminatory features and Sparse Logistic Regression, Random Forest, Gaussian Process, Support

Vector Machine and Neural Net classification were used to provide statistical results from the 30% test set based on the 70% training set.

Results

Baseline characteristics

A total of 280 IBD patients (164 CD patients, 112 UC patients, 4 IBD-undetermined) were included in this study. Sample collection of IBD patients is depicted in *Figure 2.* In total, 495 fecal IBD samples (292 CD, 197 UC, 6 IBD-U) were collected during the follow-up period of this study. Of these, 107 were active CD, 84 were CD in remission, 80 were active UC and 63 were UC in remission according to the previously mentioned criteria. The number of samples collected per individual varied as 159 patients provided one sample, 65 patients were sampled twice, 34 patients collected three samples, 10 patients collected four samples, 10 patients collected five samples, and two participants provided six and eight samples. Samples of these IBD patients were compared to 227 HCs who all collected a single sample. Baseline demographics of all study participants are given in *Table 1.* There was no significant difference in gender between CD and UC patients compared to HC. Mean age of the IBD group was 46.1 (±29.8) compared to 60.6 (±11.8) for HC. Mean FCP levels for active disease and remission were 664.6 mg/g and 29.9 mg/g for CD and 1108.5 mg/g and 39.5 mg/g for UC, respectively *(Table 2)*.

Fecal volatile organic compound analysis

The results of the VOC analysis by means of GC-IMS are shown in *Table 3.* For every comparison, the results from the Sparse Logistic Regression classification based on the 100 most discriminative features are presented. A complete overview of the data generated using all five classifiers based on the 20, 50 and 100 most discriminative features are given in Supplemental Table 1-3.

Inflammatory bowel disease versus healthy controls

IBD patients could be discriminated from HC with a high diagnostic accuracy (AUC ± 95%CI, sensitivity, specificity, PPV, NPV, P-values; 0.96 (0.92 – 0.99), 0.97, 0.92, 0.98, 0.87, <0.0001)(*Table 3, Supplementary table 1-3)*. Likewise, high diagnostic accuracy was found for the detection of CD during active state and remission (0.96 (0.94 – 0.99), 1, 0.92, 0.74, 1, <0.0001 for active CD; 0.95 (0.93 – 0.98), 1, 0.90, 0.67, 1, <0.0001 for CD in remission) (*Table 3, Supplementary tables 1-3)*. This was similar for the detection of UC both during active state and remission (0.96 (0.94 – 0.99), 1, 0.92, 0.74, 1, <0.0001 for UCa; 0.95 (0.93 – 0.98), 1, 0.88, 0.52, 1, <0.0001 for UCr) (*Table 3, Supplementary tables 1-3)*. Corresponding Receiver Operating Characteristic (ROC) curves are visualized in *Figure 3a-d.*

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Crohn's disease versus ulcerative colitis

Fecal VOC patterns of CD and UC differed statistically significant, though the diagnostic accuracy was very low (0.55 (0.50-0.60), 0.17, 0.96, 0.90, 0.36, 0.03) *(Table 3, Figure 3h)*. Furthermore, there was no difference between UC and CD when comparing active disease and remission subgroups separately (*Table 3).*

Active disease versus remission

There was a slight significant difference in fecal VOC patterns between active IBD (UC and CD combined) and remission (0.59 (0.51-0.67), 0.21, 0.96, 0.90, 0.39, 0.019) (*Figure 5)*. However, comparing active and remission state of CD and UC subgroups separately, this significance was not found (CD active vs CD in remission 0.52 (0.39-0.65), 0.72, 0.43, 0.71, 0.45, 0.645; UC active vs UC in remission 0.63(0.44-0.82), 0.67, 0.57, 0.79, 0.42, 0.08) (*Table 3, Figure 3i).*

Discussion

We demonstrated high diagnostic accuracies for the detection of adults with IBD, UC and CD based on fecal VOC profiles using GC-IMS, both during active disease state and remission. Furthermore, VOC profiles of the phenotypes CD and UC, and of IBD in active disease state and remission, differed statistically, but not clinically significant.

The presented results are comparable to the reported sensitivity of the currently used non-invasive biomarker FCP (0.98, 95%CI 0.95-0.99) to discriminate between IBD and HC[3]. Additionally, in the present study, the specificity of fecal VOC patterns for IBD both during active disease and remission was higher compared to reported values for AUC (0.81-0.91) for FCP in adults. It is known that FCP is a biomarker for mucosal inflammation and is therefore sensitive to detect active IBD, whereas fecal VOCs seem highly sensitive and specific for both active disease state and remission. This underlines its diagnostic potential in clinical practice.

The potential of fecal VOC profiles to discriminate CD from HC is in concordance with the existing literature. To the best of our knowledge, in only one study this potential has previously been assessed in an adult population, including fecal samples of 117 CD, 100 UC and 109 HC analyzed by gas chromatography – mass spectrometry (GC-MS). Although AUC values were not provided in that article, active CD and HC could be separated excellently based on three unique metabolites; Differences between active CD and HC were (amongst several others) mainly based on increased levels of alcohols, ketones and aldehydes. Though, in contrast to our findings, study results of Ahmed et. al did not allow to discriminate between UC patients compared to HC. In addition, they did find separation of UC and CD profiles both during active disease and remission. Other studies have been performed to assess the diagnostic potential of fecal VOCs for IBD using pattern-based techniques in pediatric cohorts, such as an electronic nose (eNose) instruments (CD 29, UC 26, HC 28) and field asymmetric ion mobility spectrometry (FAIMS) (23 CD, 13 UC, 24 HC)([12, 13]. The study using eNose technology demonstrated similar accuracies to the current study for the detection of IBD and was also able to distinguish UC from CD, whereas the study using FAIMS was only able to separate CD from HC with high accuracy. Comparing UC to HC and CD, only moderate separation was found (AUC of 0.74 and 0.67, respectively). In the current study, active IBD was discriminated from remission with a very weak accuracy and there was no difference between the active and inactive subgroups of CD as well as UC. The existing literature on the differentiation between active and inactive IBD based on VOC profiles is both scarce and contradictory. Only one study so far has assessed IBD activity based on fecal VOC profiles [21]. In that study, active and inactive CD could be separated significantly based on fecal VOC profiles, though with some overlap, whereas active and inactive UC did not differ.

The separation of CD patients was mainly based on aldehyde levels (e.g. heptanal, propanal, benzeneactealdehyde). This accuracy was similar to a study on VOC profiles comparing 135 breath samples of CD in remission with 140 breath samples active CD using GC-MS, where an AUC of 0.98 was measured [22]. Though, this accuracy was based on different metabolite alterations (i.e. elevation of 2,2,4-trimethylpentane, 1-butoxy-2-propanol, heptadecane and decrease of isoprene and acetone). In addition, a high diagnostic accuracy to differentiate between 62 active and 70 inactive UC breath VOC profiles was found by the same research group (AUC 0.94), based on increased levels of 2,4-dimethylpentane and methylcyclopentane and decreased levels of octane, acetic acid and m-cymene[23]. Urinary VOC profiles have also been found to discriminate between active IBD (24 CD, 24 UC) and remission (4 CD, 4 UC) using the pattern-recognition technique FAIMS, with moderate accuracies (AUC CD 0.66, UC 0.74)[24].

Alterations in the fecal VOC patterns may be explained by alterations in metabolic processes, like the secretion of inflammatory end products in the colon or alterations in dietary intake, by microbial dysbiosis or a combination of all the above. In a recent study on canine olfaction, in vitro breast cancer and colon cancer were grown and it was observed that dogs were able to differentiate between the metabolic waste retrieved from these cancer cells and from benign cells, but not between the cell waste of breast and colon cancer, implying that both cancers share a common smell sprint[25]. The same might apply for inflammatory diseases like Crohn's disease and ulcerative colitis of which it may be hypothesized that the VOC patterns of CD and UC patients are based on a shared (metabolic) reaction, explaining the similarities in VOC patterns observed in the current study. This might also mean that fecal VOC patterns may not be sufficiently specific to differentiate between IBD and other causes of mucosal inflammation (such as infectious colitis).

The discrimination between IBD and HC as well as inability to discriminate between active and quiescent disease in the current study may partly be explained by one of the main sources of fecal VOCs: the gut microbiota. The fecal microbiota of a healthy individual consists of over 400 different species which play an important role in the defense against invading organisms. There is a large inter-individual diversity in the fecal microbiota composition of healthy individuals [26]. Nonetheless, the microbiome of an individual is remarkably stable, suggesting the presence of a core microbial community, which is dependent of host factors [27, 28]. In multiple studies, this microbial stability has been found greater in healthy individuals compared to IBD patients of which the microbial composition is defined by more deviations over time and a decrease in diversity, specifically in abundance of *Firmicutes* in CD patients and a decrease in butyrate-producing bacteria in UC patients (*F Prausnitzii, R Hominis)* [29-34]. This may well explain the high diagnostic accuracy to discriminate between IBD patients and HC based on fecal VOC profiles in the current study. Remarkably, the

variability of microbial composition in IBD patients does not well correlate with disease activity. Inflammation has not been found directly associated with significant deviations from the healthy microbial core and fluctuations in microbiota composition have been observed during clinical remission as well, which has hampered the identification of microbial changes related to the presence of flare-ups [29, 30, 35]. These findings may explain the inability to discriminate between active disease state and remission based on fecal VOCs as observed in the current study. Another explanation for the inability to discriminate between active IBD disease state and remission is the medication use in both groups that may have masked differences in VOC profiles.

The dissimilarities with other studies considering differentiation between active and remission disease state may also be due to the use of different techniques to analyze VOC profiles. In the current study, we made use of pattern-recognition, since this is a fast and relatively cheap manner to analyze fecal VOCs and is therefore highly adequate for clinical implementation. A downside of this technique is the inability to identify specific metabolites. Differences between IBD and HC based on fecal VOCs have previously been demonstrated due to an altered composition of esters, short chain fatty acids (SCFAs) and cyclohexanecarboxylic acid, of which the first group is believed to be associated with bacterial dysbiosis[36]. The differences between active disease state and remission in fecal as well as breath VOC profiles originate from a different group of metabolites, mainly aldehydes and ketones [22, 23, 37]. These metabolites play a role in inflammatory processes as they are the metabolic products of tissue damage and oxidative stress, and may therefore be the result of a more general host-response to inflammation rather than an IBD specific metabolic alteration. It is possible that the GC-IMS column we used has been sensitive to the range of metabolites differentiating IBD from HC, but not to the metabolites produced in inflammatory processes.

Strength of this study was the large sample size, which allowed for the creation of a training set for the machine learning classifiers, an internal and an external validation set. In addition, this was a prospective multicenter cohort which made use of endoscopy controlled healthy individuals. The large scale of the cohort has contributed to the generalizability and the endoscopy controlled HC group excluded bias by other colonic abnormalities. A limitation of this study was the use of clinical activity indices and FCP for defining disease activity in the current study, instead of using the gold standard: endoscopic assessment. Because of its invasiveness, it was not ethically feasible to ask of study participants to undergo this investigation without immediate clinical indication. Although we used the second best marker next to clinical activity scores, FCP has a low specificity, which might have led to the inclusion of IBD patients with non-IBD mucosal inflammation in the active disease state group. Furthermore, sample age might have been of influence on study outcomes, especially on the IBD samples collected in the period of 2009-2010[19]. However, our results from a post hoc

analyses comparing IBD samples to HC samples collected in a similar time period did not influence diagnostic accuracy found in this study and we therefore believe that this has not influenced our results significantly. Last, although we have designed this study using pattern-recognition because of its suitability for clinical implementation, the inability to detect specific metabolites complicated the comparison to other literature.

Future research should focus on the validation of specific metabolites within the fecal VOC pattern allowing to differentiate between IBD and HC, preferably including subgroups of patients with non-IBD induced mucosal inflammation for a reliable assessment of its specificity. Using these specific metabolites, an easy to use, disease-specific sensor may be built into a so called 'smart toilet', enabling fast and accurate IBD detection. Additionally, to assess the potential of fecal VOCs for IBD monitoring, the potential differences in VOC patterns between active IBD and remission should be further studied in an endoscopy-controlled cohort with standardized follow-up moments, ensuring the sole inclusion of patients with active disease and remission, based on mucosal appearance and histology findings. Third, it would be interesting to compare the fecal metabolite composition and microbiota in IBD simultaneously in a multi-omics approach, exploring the origin of the fecal VOC pattern alterations.

In conclusion, our results suggest that fecal VOC pattern analysis is a promising technique for non-invasive diagnosis of IBD. Because of its high specificity, this new technique may be beneficial to both patients and health care costs by lowering the number of (unnecessary) invasive endoscopies currently needed to diagnose IBD in patients with a high FCP value. Since fecal VOC patterns did not allow for differentiation between disease activity state, its potential for monitoring intra-individual course of IBD may be hampered and should be assessed in a future study enrolling an endoscopy controlled cohort.

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