Catching wind of non-invasive biomarkers for inflammatory bowel disease and colorectal cancer

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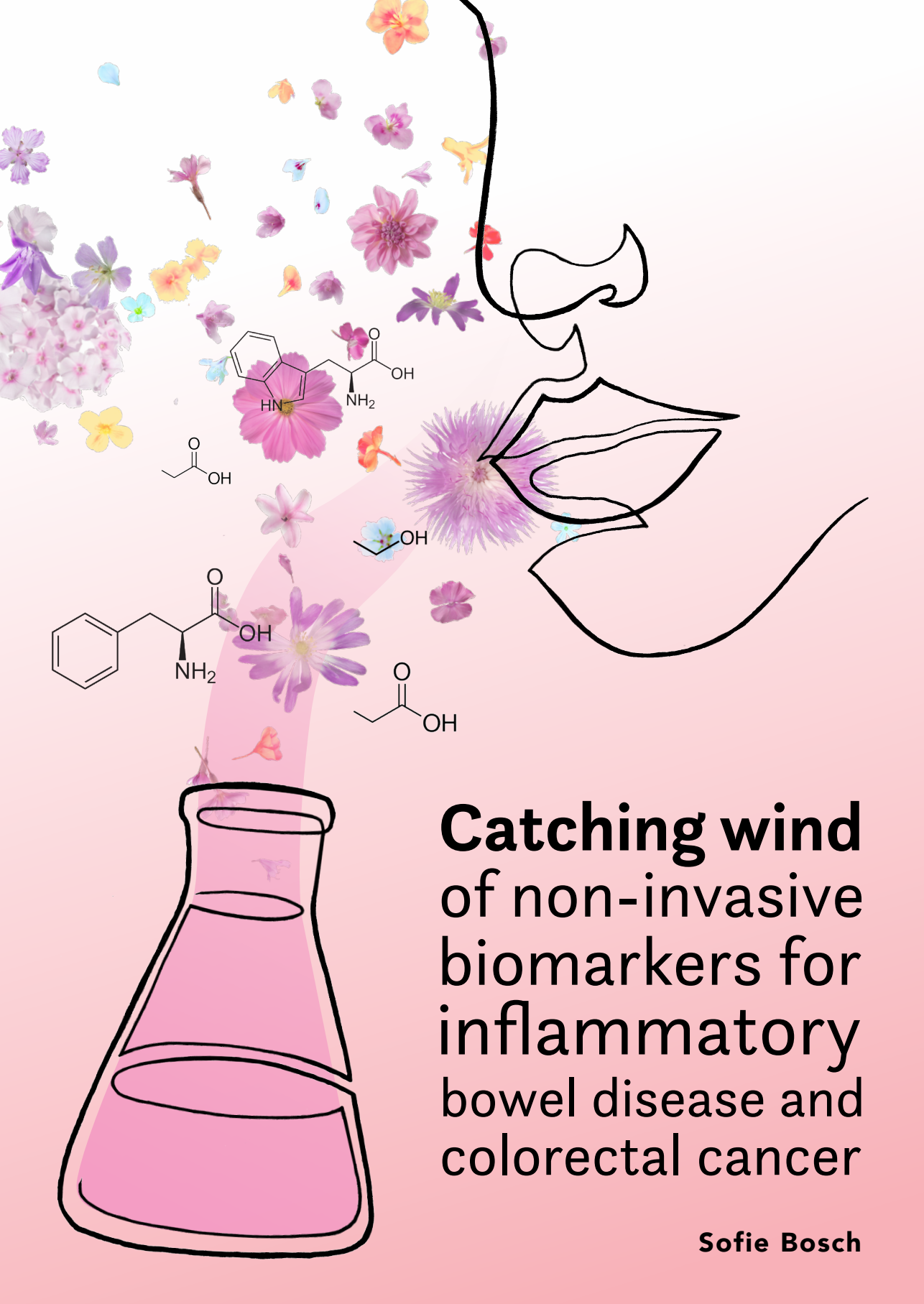

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Sofie Bosch

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CATCHING WIND OF NON-INVASIVE BIOMARKERS FOR INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER

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geboren te Eindhoven

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CHAPTER 1

General introduction and outline of the thesis

GENERAL INTRODUCTION

Inflammatory bowel disease and the need for non-invasive clinical tests

The course of inflammatory bowel disease (IBD) is characterised by a chronic pattern of relapse and remission of gastro-intestinal inflammation. IBD usually develops during the teenage years or young adulthood and consists of two phenotypes: Crohn's disease (CD), which is characterised by segmental inflammation of the entire gastro-intestinal tract, and ulcerative colitis (UC) in which inflammation exclusively affects the colorectal area. Chronic (subclinical) mucosal inflammation is associated with a variety of severe complications and irreversible bowel damage leading to the need for bowel surgery and a lower quality of life[1]. Therefore, deep remission and continuous tight monitoring of patients is required.

In clinical practice, the diagnostic work up and follow-up of IBD patients includes endoscopic assessment, which is an invasive and costly procedure [2]. The burden is especially high in children, who usually need hospitalization for administration of laxatives by nasogastric tube prior to ileocolonoscopy, which is performed under general anaesthesia. As such, non-invasive biomarkers have been proposed for the purpose of IBD detection and follow-up. Faecal calprotectin is the most commonly used non-invasive biomarker to diagnose and monitor of IBD. This marker is characterised by a high sensitivity for mucosal inflammation (0.98, 95% CI 0.95–0.99) but lacks specificity for IBD (0.68, 95% CI 0.50–0.86), consequently leading to the performance of unnecessary endoscopies[3]. In addition, early prediction of changes in disease state adds to the timely management and treatment adjustment for IBD patients, which improves disease outcome and prevents drug-related side effects[4]. No non-invasive biomarkers have yet been validated for purpose of disease course prediction.

Colorectal neoplasia: opportunities and pitfalls of screening and surveillance programs

Colorectal cancer (CRC) ranks second in terms of cancer mortality world-wide[5, 6]. Its overall 5-year survival rate is 64.4% for colon cancer and 66.6% for rectal cancer, depending on the cancer stage at diagnosis. Early detection and treatment are critical factors in the course and prognosis of CRC, as the survival rate decreases with disease progression[7]. Most of the CRC lesions develop from adenomas in the so-called adenoma-carcinoma sequence, in which it is thought that a series of mutations result in carcinogenic development. Identification and removal of high-risk adenomas (advanced adenomas) has been described to decrease CRC incidence and mortality [8, 9].

In the US, the CRC screening program is mainly performed by 10-yearly colonoscopy in which all adenomas are required to be removed, whereas in Europe, guidelines recommend a more cost-effective approach, using faecal immunochemical tests (FIT) to select high-risk individuals for endoscopic screening[10, 11]. However, based on FIT, CRC is missed in 1-47% of the cases, advanced adenomas in 43-61% of the cases and for non-advanced adenomas, this number is even higher[12]. In addition, specificity is suboptimal, as approximately 7% of the performed tests provide false-positive results leading to the performance of unneeded colonoscopies. After the first screening endoscopy, surveillance is recommended at set intervals, dependent on the characteristics and number of adenomas removes, as these patients remain at risk of recurrent adenoma growth[13]. The yield of pathology in this

surveillance program is low, while interval-cancer still occurs (for example, 1.8% CRC yield vs 0.6% interval cancers)[14, 15]. No non-invasive markers have yet been validated to improve the currently used, FIT-based, CRC screening program and timing of the surveillance endoscopies.

The potential of omics platforms for disease detection and follow-up

Omics data analysis is a biological system approach aimed to characterize a medium (e.g. mucosal biopsy, urine, breath, stool) by selection and/or quantification of important biological features. This is mostly done based on statistical machine learning methods using high-throughput data after deep phenotyping of the material on these specific features. The biological features may exist of various omics branches, such as the genome (DNA), transcriptome (RNA), proteome (protein spectra), metabolome (metabolite composition) and microbiome (bacterial composition). By analysing and combining these omics platforms, new associations can be revealed between different layers of the human metabolism, leading to new insights into pathophysiological pathways and host-pathogen interaction. In addition, this type of 'multi-omics' analysis often allows for the selection of relevant biomarkers for disease detection and follow-up. This leads to a more personalised medical approach compared to current widely used screening markers like FIT and FCP, which hold less disease-specific characteristics.

A relatively new technique within the omics spectrum is the analysis of volatile organic compounds (VOC), the so-called volatolome. These gaseous carbon-based molecules originate from both physiological and pathophysiological processes in the human body and are considered to reflect the human metabolism, the gut microbiota, its function and interaction with the host [16, 17]. VOCs can be captured non-invasively from all conceivable bodily excrements, including urine, breath, blood and faeces. The use of VOC profiles, or so called 'smell prints', is increasingly considered to have potential as biomarker in the diagnostic work-up and monitoring of various gastrointestinal diseases. In Figure 1, an example of such a 'smell print' obtained by an advanced 'electronic nose (eNose)' is presented.

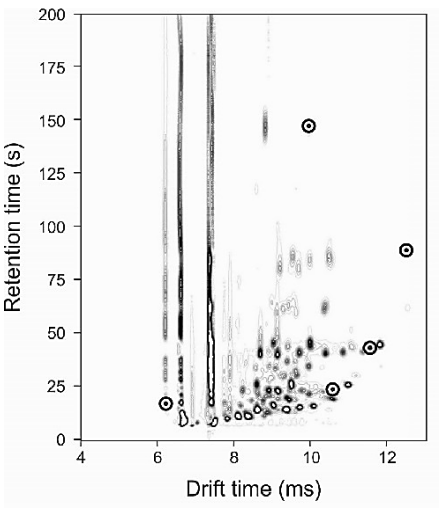

Figure 1. This figure originates from a study in which we aim to predict the disease course of inflammatory bowel disease, presented in chapter eight. Depicted is an example output of the gas chromatography – ion mobility spectrometry instrument. The Y-axis represents retention time from the gas chromatography column, the X-axis represents drift time through the ion mobility spectrometry column. Darkness intensity depicts the level of measured metabolite. In this figure, bullets mark the locations in the VOC profiles that discriminate cases from controls.

The majority of studies on faecal VOCs have been performed using gas chromatography-mass spectrometry (GC-MS), allowing for identification of individual VOCs on molecular level. This technique is expensive, time-consuming and requires specialised personnel[18]. Pattern-recognition based eNose techniques, such as conducting polymers, field asymmetric ion mobility spectrometry (FAIMS), and gas chromatography – ion mobility spectrometry (GC-IMS) are examples of instruments that are lower in expense and allow for fast measurements, underlining their potential as non-invasive biomarkers for clinical practice. Though, there is little knowledge on robustness of these measurements.

This thesis focuses on the potential of eNose measurements for clinical practice. In addition, this thesis describes the exploration of non-invasive biomarkers for the detection and follow-up of inflammatory bowel disease and colorectal neoplasia using various 'omics' platforms.

OUTLINE OF THE THESIS

This thesis is divided in four parts. In part I, we investigate factors of influence on faecal VOC composition and propose multiple methods to correct for these factors. In part II, we assess the diagnostic potential of faecal VOC compounds for the detection and follow-up of paediatric and adult IBD. In part III, we investigate whether the liquid faecal metabolome may serve as non-invasive diagnostic biomarker for paediatric IBD. In part IV, the potential of multiple omics platforms for the detection and surveillance of colorectal neoplastic laesions are described.

The **first part** consists of three chapters, in which important confounding factors and effect-modificating variables for faecal VOC analysis are described. In the second chapter, the effects of various sampling protocols on diagnostic accuracy of VOC analysis are assessed. We define a standard operating procedure which ensures maximum accuracy for disease detection. In the third chapter, we describe the influence of various demographical and lifestyle factors, such as age, body-mass index, gender, smoking habits, co-morbidity, medication use and diet. Correction methods for these variations are proposed. In chapter four, we evaluate the influence of sensor drift on diagnostic accuracy of faecal VOC profiles for the detection of IBD. In this chapter, the importance of correction for this drift is visualised and correction methods are proposed.

The **second part** of this thesis consists of four chapters all focussing on detection of IBD based on faecal VOC profiles. In chapter five, we describe the diagnostic potential of the faecal smell patterns to differentiate between paediatric patients with IBD, symptomatic patients without IBD and asymptomatic healthy controls. In chapter six, we compare diagnostic accuracy of faecal and urinary VOC profiles for the detection of inflammatory bowel disease in a paediatric cohort. In chapter seven, the potential of faecal VOC profiles to detect IBD and IBD activity is presented in a large multi-centre cohort of adult patients. In chapter eight, a prospective follow-up of IBD patients is described in which we aim to predict IBD disease course based on faecal VOC profiles.

In the **third part,** we assess the potential of the liquid faecal amino acid profiles (aminogram) as diagnostic biomarker for paediatric IBD patients. In chapter nine, we describe the potential of faecal aminograms for the detection of *de novo* treatment-naïve IBD patients. In chapter ten, we hypothesize on the pathophysiology underlying the observed increase in faecal aminogram profiles in paediatric IBD patients. In chapter eleven, our previous study outcomes are validated by comparing both faecal and serum aminograms in an intention-to-diagnose setting comparing paediatric IBD patients and patients presenting with IBD-like symptoms, in whom IBD was excluded.

The **fourth part** of this thesis consist of four chapters evaluating the potential of multiple omics platforms for the detection and surveillance of colonic neoplasia. The twelfth chapter provides an overview of the available literature on the evidence of faecal VOCs as biomarker for CRC and adenoma detection. In the thirteenth chapter, the potential of faecal scent patterns for the discrimination between patients with colorectal cancer, advanced adenomas, large adenomas, small adenomas and controls is assessed in a large multi-centre cohort. In chapter fourteen, we integrate data on the faecal microbiota, human faecal proteome and faecal amino acid profiles in a multi-centre cohort of patients with CRC, adenomas and controls. We present novel biomarker panels outperforming accuracy of the currently used CRC screening test for both CRC and adenomas and integrate these omics platforms to reveal associated pathologic pathways. In chapter fifteen, the regulation of adenoma-associated gut microbiota and faecal aminograms is assessed in a prospective cohort of adenoma patients, pre- and post-polypectomy, and control patients, pre- and-post colonoscopy, to evaluate whether these techniques may serve as guidance for the timing of surveillance endoscopy.

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PART I

Optimising volatile organic compound analysis

CHAPTER 2

Optimised sampling conditions for faecal volatile organic compound analysis by means of field asymmetric ion mobility spectrometry

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ABSTRACT

Background

Faecal volatile organic compounds (VOCs) are increasingly considered as potential non-invasive, diagnostic biomarkers for various gastrointestinal diseases. Knowledge of influence of sampling conditions on VOC outcomes is limited. We aimed to evaluate effects of sampling conditions on faecal VOC profiles and to assess under which conditions an optimal diagnostic accuracy in the discrimination between paediatric inflammatory bowel disease (IBD) and controls could be obtained.

Methods

Faecal samples from de novo treatment-naïve paediatric IBD patients and healthy controls (HC) were used to assess effects of sampling conditions compared to the standard operating procedure (reference standard), defined as 500mg of sample mass, diluted with 10mL tap water, using field asymmetric ion mobility spectrometry (FAIMS).

Results

A total of 17 IBD (15CD and 2 UC) and 25 HC were included. IBD and HC could be discriminated with high accuracy (accuracy=0.93, AUC=0.99, $p<0.0001$). Smaller faecal sample mass resulted in a decreased diagnostic accuracy (300mg accuracy=0.77; AUC=0.69, p=0.02; 100mg accuracy=0.70, AUC=0.74, p=0.003). A loss of diagnostic accuracy was seen towards increased numbers of thaw-freeze cycles (one cycle: accuracy=0.61, AUC=0.80, p=0.0004, two cycles: accuracy=0.64, AUC=0.56, p=0.753, three cycles: accuracy=0.57, AUC=0.50, p=0.5101) and when samples were kept at room temperature for 180 minutes prior to analysis (accuracy=0.60, AUC=0.51, p=0.46). Diagnostic accuracy of VOC profiles was not significantly influenced by storage duration differences of 20 months.

Conclusion

Application of 500mg sample mass analysed after one thaw-freeze cycle, showed best discriminative accuracy for differentiation of IBD and HC. VOC profiles and diagnostic accuracy were significantly affected by sampling conditions, underlining the need for implementation of standardised protocols in faecal VOC analysis.

INTRODUCTION

Analysis of volatile organic compounds (VOC) is a relatively new technique within the field of metabolomics. VOCs are carbon-based chemicals originating from both physiological and pathophysiological processes in the human body. Faecal VOCs are considered to reflect microbiota composition, function and interaction with the host [1, 2]. They are increasingly considered to have potential as biomarker in the diagnostic work-up and monitoring of various gastrointestinal diseases, e.g. inflammatory bowel disease (IBD), colorectal cancer and even sepsis [3-11]. Various studies have demonstrated the diagnostic potential of VOCs in both paediatric and adult IBD populations, by analyzing VOCs deriving from urine, exhaled breath and faecal [6, 12-14]. The majority of studies on faecal VOCs have been performed using Gas Chromatography/Mass Spectrometry (GC/MS), allowing for identification of individual VOCs on molecular level. This technique is expensive, time-consuming and requires specialised personnel and is therefore not suitable for utilization in a clinical setting [15]. Pattern recognition based techniques, like electronic noses (eNose) and field asymmetric ion mobility spectrometry (FAIMS), are examples of instruments that are lower in expense and faster, allowing for their application as a non-invasive biomarker in clinical practice. However, traditional eNoses contain sensors that are notorious for batch-to-batch variation, fouling and ageing effects and sensors drift [14, 16]. Novel measurement of VOCs using physical techniques, coupled with pattern recognition, like FAIMS, have a higher sensitivity and minimal drift. It achieves separation by measuring the differences in mobility of ionised molecules in high-electric fields.

Data on the potential influence of sampling and storage methods on faecal VOC profiles are scarce. We aimed to evaluate effects of environmental factors and sampling conditions on faecal VOC profiles, using FAIMS. In addition, we aimed to assess under which conditions an optimal diagnostic accuracy could be obtained in the differentiation between paediatric IBD and controls. This may lead to the development of rationale-based standardization protocols on faecal VOC analysis, paving the way towards reliable comparisons between different study outcomes, and implementation of VOC-based diagnostics in clinical practice.

METHODS

Study design

This case-control study was performed at the outpatient clinic of the paediatric gastroenterology departments in two tertiary referral hospitals, the VU University medical centre (VUmc) and the Emma Children's Hospital, Academic Medical Centre (AMC), both located in Amsterdam, The Netherlands.

Study participants

Inflammatory bowel disease

IBD subjects were selected from an existing cohort of de novo treatment-naïve paediatric patients, consisting of 125 subjects (78CD, 47UC), aged 4 to 17 years, recruited between October 2013 and July 2017 at the VU University Medical Centre (VUmc) and Academic Medical Centre (AMC). Diagnosis of IBD was based on endoscopic, histologic and

radiologic findings, according to the revised Porto-criteria[17]. Localisation and behaviour of IBD were classified during endoscopy, based on the Paris Classification[18]. Physician Global Assessment (PGA) combined with levels of C-reactive protein (CRP) and faecal calprotectin (FCP) were used as an index of the clinical disease activity [19, 20]. All IBD patients were asked to collect a faecal sample prior to endoscopy and bowel preparation [14]. Inclusion criteria also included sufficient faecal material for VOC analysis (3.4 grams per subject). Exclusion criteria were use of antibiotics, probiotics or immunosuppressive therapy in the three months prior to inclusion, a concomitant diagnosis of a gastrointestinal disease or immunocompromised disease (i.e. HIV, leukemia) and abdominal surgery (except for appendectomy). In addition, children with proven infectious colitis (parasites in stools, or positive stool culture for Salmonella spp., Shigella spp.,Yersinia spp., Campylobacter spp., or toxigenic Clostridium spp.) were excluded.

Healthy controls

Healthy controls (HC) were children aged 4 to 17 years selected from elementary and high schools in North-Holland, the Netherlands between June 2016 and December 2016. All participants were asked to collect a faecal sample, and complete a questionnaire on abdominal symptoms, bowel habits, including consistency of stool using the Bristol stool chart, medication use and medical history[21]. Exclusion criteria for HC were similar to IBD, with the addition of diagnosis of IBD and/or a functional gastrointestinal disorder according to the Rome IV criteria based on the questionnaires.

Matching procedure

From the original cohort of 125 IBD patients, 106 were not eligible for this study due to insufficient quantities of the faecal samples. A total of 17 IBD patients (15CD, 2UC) could be matched on age at sample collection and gender with 25 participants in the HC group.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre (VUmc) under file number 2016.393, and by the local medical ethical committee of the Emma Children's Hospital (AMC). Written informed consent was obtained from all parents, and from the children in case of age over 12 years.

Sample collection IBD and controls

All study participants collected fresh faecal samples in a stool container (Stuhlgefäß 10ml, Frickenhausen, Germany). Patients with IBD collected their faecal sample prior to endoscopy and bowel lavage. Participants were instructed to store the faecal samples in the freezer at home directly after collection. The samples were transported to the hospital in cooled condition, using cooling elements or ice cubes. Directly upon arrival in the hospital the samples were stored in the freezer (-24 °C) until analysis.

Sample preparation IBD and controls

The influence of faecal samp le mass, number of thaw-freeze cycles, duration of storage in room temperature, were assessed by comparing VOC-profiles derived from subsamples taken from the original faecal sample of each HC and IBD subject. The subsamples were weighted on a calibrated scale (Mettler Toledo, AT 261 Delta Range, Ohio, United States),

labelled and re-stored in a -24°C freezer until further handling. We compared the variables of interest with our standard operating procedure (reference standard), defined as a mixture of 500mg of faecal, diluted with 10mL tap water and kept in room temperature for 10 minutes prior to analysis. These reference standard settings were chosen since they were used in several previous studies on faecal VOC profiling in a range of gastroenterology diseases and have provided us with positive results [14, 22].

Variables of interest

Effect of faecal sample mass on diagnostic accuracy of faecal VOC profiles was assessed by comparing subsamples weighing 100mg and 300mg with the reference standard mass of 500mg.

The influence of the number of thaw-freeze cycles on the diagnostic accuracy was analysed by comparing the reference standard to subsamples, which underwent one, two and three additional thaw-freeze cycles. For every additional cycle, the sample was kept at room temperature for 10 minutes and subsequently kept on dry ice until the sample was frozen. In order to assess the effect of duration of storage at room temperature on the diagnostic accuracy, VOC from samples kept at room temperature (18 degrees) for 180 minutes were compared to the reference standard. Variables of interest are presented in Table 1.

As described above, the effect of every variable on the diagnostic accuracy of faecal VOCs were assessed by comparing IBD subjects with HC. In addition, we assessed the influence of the variables on the VOC pattern. By combining the HC and IBD subjects, we were able to compare the variables to the reference standard.

Table 1. Variables of interest

Variables of interest	Faecal sample mass (mg)	Thaw-freeze cycles (N)	Time out of freezer (min)
Reference standard	500	0	10
Mass variable 1	300	0	10
Mass variable 2	100	0	10
Thaw-freeze variable 1	500	1	10
Thaw-freeze variable 2	500	2	10
Thaw-freeze variable 3	500	3	10
180 minutes out of freezer	500	0	180
Storage time 1	500	0	10
Storage time 2	500	0	10

FAIMS analysis

For this study a commercially available FAIMS instrument (Lonestar®, Owlstone, Cambridge, UK) was used. Prior to the analyses, the FAIMS instrument was checked for contamination using air and water blanks. The faecal samples were thawed to room temperature for ten minutes prior to VOC analysis, and manually homogenised after diluting the faecal sample

with 10mL tap water by using a micropipette of 5000µl. The Lonestar® was setup as used in previous studies[14, 22, 23]. To transport the sample headspace into the Lonestar®, compressed air (0.1MPa) was used as the carrier gas. This air meets the European Pharmacopoeia criteria for medical air and its composition, pressure, temperature and water density are checked for continuality regularly. When entering the Lonestar, this carrier gas is filtered by a Carbon filter (Restek, Bellefote, VS). The flow rate was set on 2.0L/min, the temperature for the sample holder was set at 35°C, for the lid at 70°C and 100°C for the filter region. After every sample run, the Lonestar® was refreshed using 5mL of tap water. Furthermore, the dispersion field was set between 0% and 100% (in the ratio of the high electric field to low electric field) and passed through 51 equal settings. The compensation voltage was set between +6V and -6V in 512 steps for each dispersion field. All samples were analysed randomly. Each faecal sample was analysed three times subsequently, resulting in three matrices, taking 540s to perform. In order to preclude environmental effects, the first matrix was excluded from analyses since this measurement includes the heaspace gas generated from both the sample and the environment (e.g. air in tubes). For the statistical analysis, only the second matrix was used for optimal diagnostic potential. The third measurement was made as a back-up file, but was not used in this study. The raw data output was analysed at the School of Engineering, University of Warwick, United Kingdom[15].

Statistical analysis

The demographic data of each group (IBD patients and healthy controls) were compared using the Man-Whitney-U test for non-parametric continues data, and the Fisher's exact test for dichotomous data using SPSS Statistics (version 22, IBM, NY, USA). As previously reported, the FAIMS produces high dimensional data in terms of the number of features and covariates measured per sample. Therefore, a data compression was performed before feature identification and classification. Each FAIMS data (sample) consists of 52224 data points in a 2D matrix. Data compression was undertaken by applying a 2D discrete wavelet transformation. For the variables of interest in which the accuracy to discriminate between IBD and HC was assessed, feature selection and classifier training were performed to 90% of data (training set) and class predictions were produced from 10% of the data set (test set), in a 10-fold cross validation. The Wilcoxon-rank-sum test was used to calculate p-values in the training sets to identify which features best for disease prediction. From this, 4 statistically important features were used. Four classification algorithms were applied, Sparse Logistic Regression, Random Forrest, Gaussian Process and Support Vector Machine. A receiver operator characteristic (ROC) curve was created to predict the area under the curve (AUC), p-values, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy. For the influence of sampling method on VOC composition, in which IBD and HC samples were combined and measurements of the same subjects samples were repeated, data were analysed using SPSS statistics 22 (IMB). The raw sensor data was recombined with feature selection using the Wilcoxon rank sum tests. Paired t-tests were performed to assess the potential of the features to discriminate between sample handling methods. Scatterplots for the discrimination between samples were created for each variable of interest. Axes depict the recombination of the raw sensor data by means of features. Individual VOC profiles are illustrated as marked points. The intersection of the lines deriving from the invidual VOC profiles demonstrates the mean VOC profile of this specific variable of interest.

Post hoc analyses

Our main target of this study was to assess the optimum sampling method to discriminate between IBD and healthy controls based on VOC analyses by means of FAIMS. We found there is a gap of knowledge on the effects of sample storage duration on VOC integrity. Therefore, the effect of duration of storage in the freezer was analysed by repeating measurements from a previous study, conducted by van Gaal et al. in which faecal VOC profiles of 36 de novo IBD patients were compared to 24 HC[14]. Based on the availability of faecal samples from this study, 10 IBD (all CD) subjects and 10 HC could be included for reassessment of VOC profiles. Storage time differed 20 months between the measurements, with a median storage time in the freezer of 43 months for the first and 63 months for the second measurements. Baseline characteristics and disease specifics of these study subjects are described in Table 3. For both these analyses, the reference standard was used. Diagnostic accuracy to detect IBD as well as the difference in VOC profile were assessed using the statistical analyses described above.

RESULTS

Baseline characteristics

Seventeen de novo, treatment-naïve paediatric IBD patients (15CD, 2UC) were selected from the original cohort and were matched to 25 HC. Patient characteristics are shown in table 2. There were no significant differences in age, sex and sample age between IBD and HC. The range of the sample age was, however, larger in the IBD group compared to HC.

Table 2. Baseline characteristics

	Inflammatory bowel disease			
	Crohn's disease (n=15)	Ulcerative colitis (n=2)	Healthy controls (n=25)	p-value
Sex, male (n, [%])	10[66.7]	0[0]	14[56]	0.858
Age, yr (median[IQR])	13.0[11-15]	[10-16]*	12.0[4.0]	0.614
Sample age, mos (median [IQR])	11.0[2-16]	[11-26]*	11.0[1.0]	0.376
Physician's global assessment				
Quiescent	1	0	NA	
Mild	9	2	NA	
Moderate	5	0	NA	
Severe	0	0	NA	
Faecal calprotectin (µg/g) (median[IQR])	1936[1006-2390]	[1800-2734]*	NA	
CRP (mg/l) (median[IQR])	24.3[2.5-42]	2.5**	NA	

Table continues

	Inflammatory bowel disease		Healthy controls (n=25)	p-value
	Crohn's disease (n=15)	Ulcerative colitis (n=2)		
1Crohn's disease localisation				
Ileal (L1)	1	NA	NA	
Colonic (L2)	5	NA	NA	
Ileocolonic (L3)	6	NA	NA	
Proximal disease (L4)	1	NA	NA	
1Crohn's disease behaviour				
B1 (NSNP)	14	NA	NA	
B1p (NSNP+p)	0	NA	NA	
B2 (S)	1	NA	NA	
B2p (S + p)	0	NA	NA	
B3 (P)	0	NA	NA	
B3p (P + p)	1	NA	NA	
1Ulcerative colitis localisation				
Proctitis (E1)	NA	1	NA	
Left-sided (E2)	NA	1	NA	
Extensive (E3)	NA	0	NA	

All values were obtained at study inclusion. Localisation was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and magnetic resonance enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. 1Based on Paris classification for inflammatory bowel disease (24) *min-max values ** one missing value

For the assessment of the influence of sample age on diagnostic accuracy, faecal samples of 10 IBD patients (CD only) and 10 HC were selected from the previous study and re-measured[14]. Patients characteristics for this variable are described in Table 3.

Table 3. Demographics sample analysis of the influence of duration time on VOC profiles

	Crohn's disease (N=10)	Healthy controls (N=10)	p-value
Sex, male (n, [%])	5[50]	2[20]	0.350
Age, yr (median [IQR])	14.1[3.38]	7.8[3.72]	0.007
Sample age first measurement, mos (median[IQR])	23.4[21-31]	52.2[51-52.4]*	0.000
Sample age second measurement, mos (median[IQR])	43.2[41-51]	71[70-72]*	0.000
Physician's global assessment			
Quiescent	0	NA	

Table continues

	Crohn's disease (N=10)	Healthy controls (N=10)	p-value
Mild	0	NA	
Moderate	3	NA	
Severe	7	NA	
Faecal calprotectin (µg/g) (median[IQR])	1067[1218]	NA	
CRP (mg/l) (median[IQR])	29[29]	NA	
[1]Crohn's disease localisation			
Ileal (L1)	0	NA	
Colonic (L2)	3	NA	
Ileocolonic (L3)	7	NA	
Proximal disease (L4)	5	NA	
[1]Crohn's disease behaviour			
B1 (NSNP)	8	NA	
B1p (NSNP+p)	0	NA	
B2 (S)	0	NA	
B2p (S + p)	0	NA	
B3 (P)	1	NA	
B3p (P + p)	1	NA	

All values were obtained at study inclusion. Localisation was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and magnetic resonance enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. 1Based on Paris classification for inflammatory bowel disease (24) *one value missing.

Faecal VOC profiles per variable of interest

The results of the VOC analysis displayed per variable of interest are shown in Table 4. For each analysis, the outcome of the Sparse Logistic Regression is noted. A complete overview of the data generated by the four different classification models is given in supplemental Table 1a-1d.

Standard operating procedure

A typical FAIMS pattern (flame) of both the IBD samples and control samples is depicted in Figure 1. By application of the reference standardsettings, IBD and HC could be differentiated with high accuracy (Accuracy, AUC (95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.93, 0.99 (0.96 – 1), 0.94, 0.96, 0.94, 0.96, 1.178e-10)(Table 4, Supp table 1a-1d, Figure 2).

Sample mass

IBD could be differentiated from HC using a lower sample mass, but diagnostic accuracy decreased compared to reference standard for both 300 mg per sample (Accuracy, AUC

(95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.77, 0.69 (0.52 – 0.86), 0.88, 0.44, 0.52, 0.85, 0.02101) and 100 mg per samples (Accuracy, AUC (95% CI), Sensitivity, Specificity, PPV, NPV, P values; 0.70, 0.74 (0.59 – 0.90), 0.76, 0.72, 0.65, 0.82, 0.00364)(Table 4, Supp table 1a-1d, Figure 1).

Thaw-freeze cycles

After adding one extra thaw-freeze cycle to reference standard, a decrease in diagnostic accuracy was observed (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.61, 0.80(0.65-0.94), 0.76, 0.80, 0.72, 0.83) (Table 4, Supp table 1-4, Figure 1). After addition of a second and third thaw-freeze cycle, differences in VOC profiles between IBD and HC dissolved (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.64, 0.56 (0.38 – 0.74), 0.76, 0.48, 0.50, 0.75, 0.7534 and 0.57, 0.50 (0.32 – 0.69), 0.47, 0.72, 0.53, 0.67, 0.5101 respectively)(Table 3, Supp table 1a-1d).

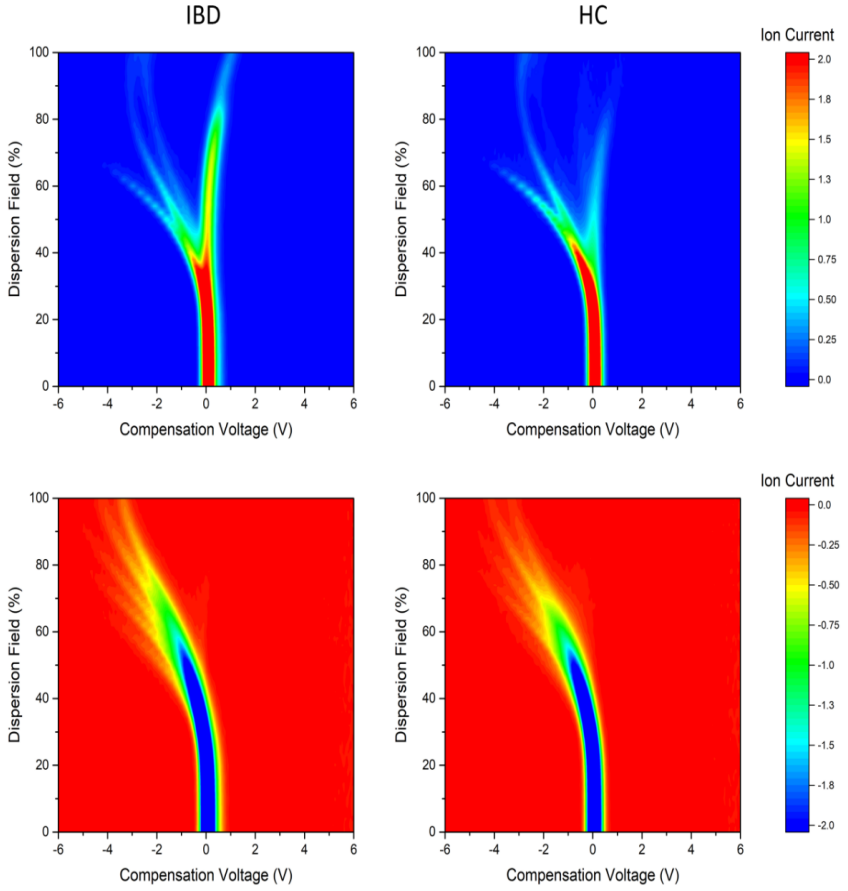

Figure 1. Typical FAIMS pattern of patients with inflammatory bowel disease and healthy controls
Depicted with a blue background are the positive ion currents. Depicted with a red background are the negative ion currents.

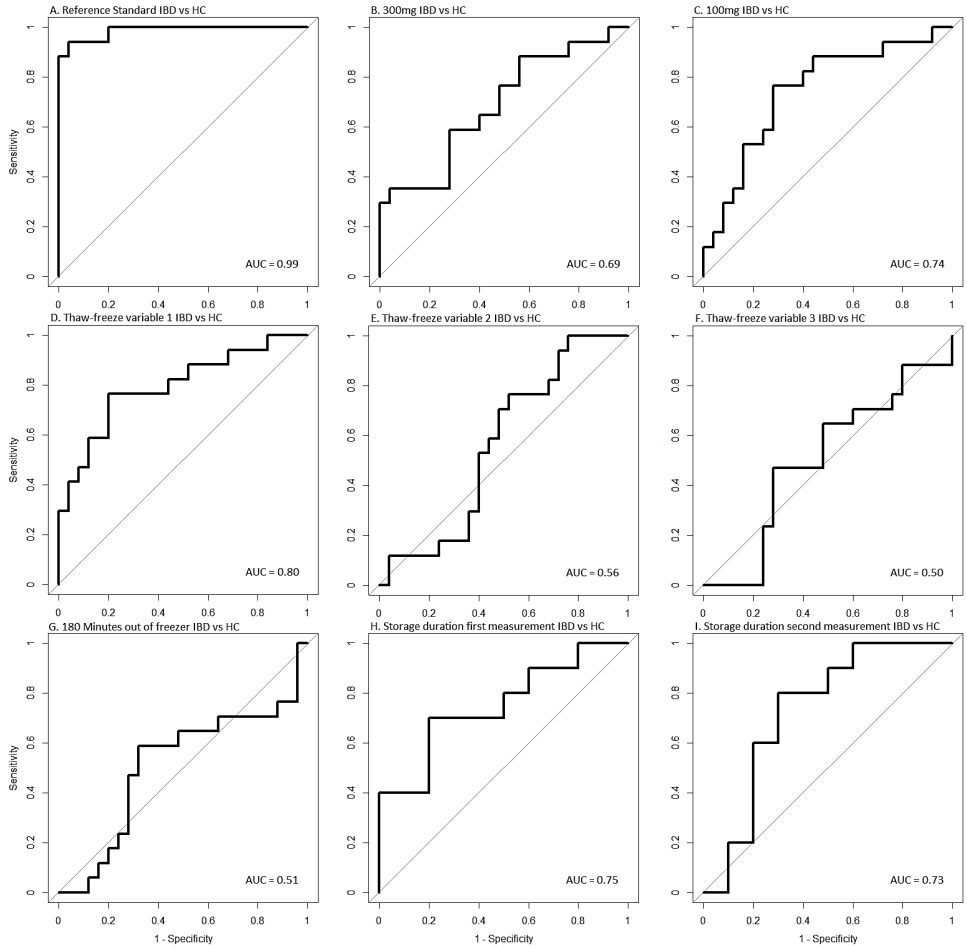

Figure 2. Receiver operating characteristics for each variable of interest for the differentiation between inflammatory bowel disease and healthy state

All receiver operating characteristic curves are obtained by Sparse Logistic Regression analyses. Abbreviations: AUC, area under the curve; IBD: Inflammatory bowel disease; HC: Healthy controls.

Duration of storage at room temperature

After keeping the samples at room temperature for 180 minutes prior to VOC analysis, differences in VOC outcome between IBD and HC dissolved (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.60, 0.51 (0.32 – 0.70), 0.59, 0.68, 0.56, 0.71, 0.4596)(Table 4, Supp table 1a-1d).

Influence of sampling method on overall VOC composition

In order to assess the influence of sampling conditions on the detected VOC patterns, HC and IBD subjects were combined to form one single study group. The comparisons between the four features are shown in Table 5. Differences in VOC pattern between sampling methods are depicted in Figure 3. Both faecal samples weighing 300mg and 100mg demonstrated

a significantly different VOC profile compared to the standard reference mass of 500mg (Feature 1,2 and 4: p-value <0.001 for and feature 3: p-value=0.027 for 300mg, feature 1-4: p-value<0.0001 for 100mg). All of the variables in thaw-freeze cycles differed to a similar extent from the reference standard (feature 1-4: p-value <0.0001 for all variables). A similar difference as with the previous variables was seen when comparing the VOC profiles of the reference standard to the VOC profiles of samples kept at room temperature for 180 minutes prior to VOC analysis (feature 1-4: p-value<0.0001).

Post hoc analyses: Duration of storage in freezer

The diagnostic accuracy to discriminate IBD from controls was not influenced by differences in duration of storage time prior to VOC analysis (43 versus 63 months) (Accuracy, AUC (95%CI), sens, spec, PPV, NPV, P values; 0.75, 0.75 (0.53 – 0.97), 0.70, 0.80, 0.78, 0.73, 0.0262 versus 0.75, 0.73 (0.49 – 0.97), 0.80, 0.70, 0.73, 0.78, 0.0376) (Table 4, Supp table 1a-1d, Figure 1). The VOC composition of the two variables showed a significant difference in three features (feature 1, 2 and 3 with p-values of <0.0001, <0.0001 and 0.021, respectively) (Table 5, Figure 3).

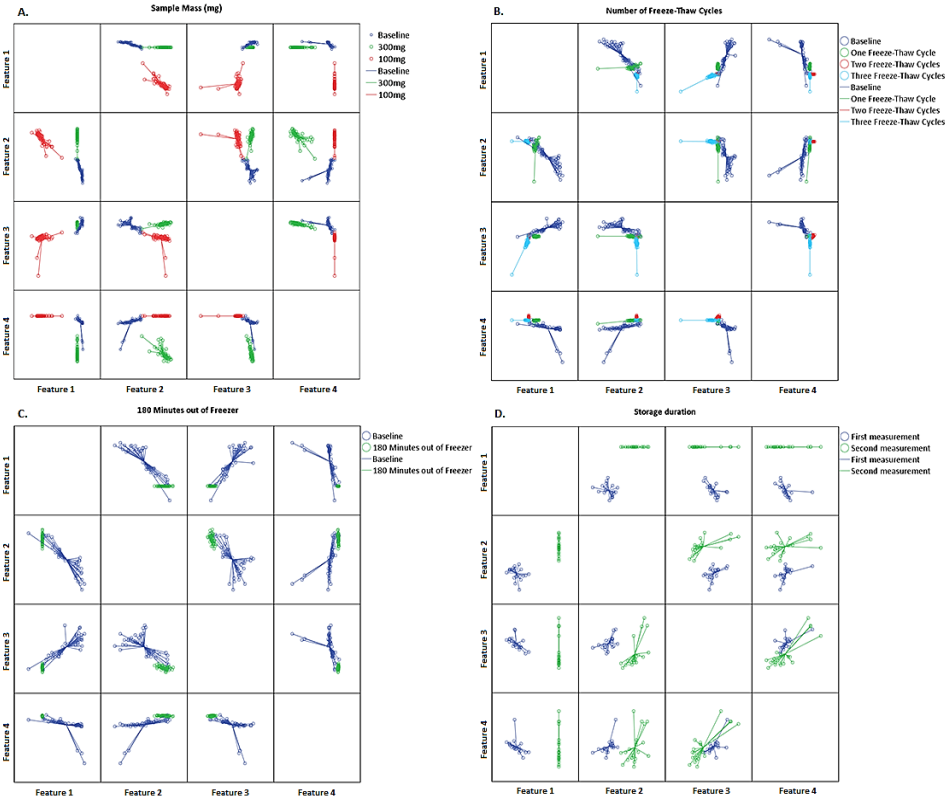

Figure 3. Scatterplot for the differentiation between sampling methods measured by field asymmetric ion mobility spectrometry

Scatterplot for the differentiation between sampling methods measured by field asymmetric ion

mobility spectrometry, including (a) sample mass; (b) number of freeze-thaw cycles; (c) 180 minutes out of freezer; (d) storage duration. Axes depicted are recombinations of the raw sensor data by means of feature selection using Wilcoxon rank sum analyses, creating four features per measurement. The marked points are the individual VOC signals. The intersection of the lines deriving from the individual signals are the mean VOC profile of that specific variable.

Table 4. Performance characteristics for the differentiation between IBD and Healthy for all of the variables of interest by faecal VOC analysis.

Analysis	p-value	Accuracy	AUC (± 95% CI)	Cut-off	Sensitivity (± 95% CI)	Specificity (± 95% CI)	PPV	NPV
Reference standard (17IBD, 25HC)	1.178e-10	0.93	0.99 (0.96 - 1)	0.0014	0.94 (0.71 - 1)	0.96 (0.8 - 1)	0.94	0.96
Mass variable 1 (17IBD, 25HC)	0.02101	0.77	0.69 (0.52 - 0.86)	0.47	0.88 (0.64 - 0.99)	0.44 (0.24 - 0.65)	0.52	0.85
Mass variable 2 (17IBD, 25HC)	0.003642	0.70	0.74 (0.59 - 0.9)	0.44	0.76 (0.5 - 0.93)	0.72 (0.51 - 0.88)	0.65	0.82
Thaw-freeze variable 1 (17IBD, 25HC)	0.0004713	0.61	0.8 (0.65 - 0.94)	0.49	0.76 (0.5 - 0.93)	0.8 (0.59 - 0.93)	0.72	0.83
Thaw-freeze variable 2 (17IBD, 25HC)	0.7534	0.64	0.56 (0.38 - 0.74)	0.66	0.76 (0.5 - 0.93)	0.48 (0.28 - 0.69)	0.5	0.75
Thaw-freeze variable 3 (17IBD, 25HC)	0.5101	0.57	0.5 (0.32 - 0.69)	0.063	0.47 (0.23 - 0.72)	0.72 (0.51 - 0.88)	0.53	0.67
180 minutes out of freezer (17IBD, 25HC)	0.4596	0.60	0.51 (0.32 - 0.7)	0.13	0.59 (0.33 - 0.82)	0.68 (0.46 - 0.85)	0.56	0.71
Storage duration, first measurement (10CD vs 10 HC)	0.0262	0.75	0.75 (0.53 - 0.97)	0.47	0.7 (0.35 - 0.93)	0.8 (0.44 - 0.97)	0.78	0.73
Storage duration, second measurement (10CD vs 10 HC)	0.0376	0.75	0.73 (0.49 - 0.97)	0.58	0.8 (0.44 - 0.97)	0.7 (0.35 - 0.93)	0.73	0.78

For each analysis, the best Sparse Logistic Regression outcome is shown. Sensitivities, specificities, p-values and AUCs are reported for the respective optimum cut-points.. Abbreviations: AUC, area under the curve; PPV: positive predictive value; NPV: negative predictive value. *Reference standard is defined as 500mg sample, diluted in 10mL water, thawed 10 minutes to room temperature.

Table 5. Paired feature analyses per variable of interest with corresponding p-values

Variables of interest	Feature 1 (p-value)	Feature 2 (p-value)	Feature 3 (p-value)	Feature 4 (p-value)
Sample mass (mg)				
500 vs 300	<0.0001	<0.0001	0.027	<0.0001
500 vs 100	<0.0001	<0.0001	<0.0001	<0.0001
Number of freeze-thaw cycles				
Measured directly vs one cycle	<0.0001	<0.0001	<0.0001	<0.0001
Measured directly vs two cycle	<0.0001	<0.0001	<0.0001	<0.0001
Measured directly vs three cycle	<0.0001	<0.0001	<0.0001	<0.0001
Kept at room temperature				
180 Minutes	<0.0001	<0.0001	<0.0001	<0.0001
Storage time				
First vs second measurement	<0.0001	<0.0001	0.021	0.825

P-value < 0.05 is considered significant.

DISCUSSION

In the present study, VOC profiles and diagnostic accuracy were influenced significantly by altering sampling conditions. Application of 500mg faecal sample mass diluted with 10mL, thawed for 10 minutes prior to analysis after a single thaw-freeze cycle, showed the best discriminative accuracy for differentiation of paediatric IBD and HC.

To our knowledge, this is the first published study to assess under which sampling conditions an optimal accuracy can be obtained in the differentiation between paediatric IBD and healthy state, by analyzing the faecal volatile metabolome using FAIMS. Studies assessing optimization of sampling methods for faecal metabolome analyses have mainly focused on gas chromatography – mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR-spectroscopy) and liquid chromatography – mass spectrometry (LC-MS), which are targeted and untargeted methods for identification of specific metabolites. These studies may hypothetically provide guidance to standardization for the pattern-based FAIMS technique. The results of this study will be discussed and compared to the available literature in the following sections. Regarding sample mass, similar results to eNose, GC-MS, NMR-spectroscopy and LC-MS studies on the faecal metabolome, in both humans and rats, were found in our study, showing a difference between the use of 500mg from that of lower masses [16, 24, 25]. Deda and colleagues have shown that the sample weight to volume ratio has a major effect on the number and signal intensity of features detected in faecal samples with GC-MS. This also applied for the spectral signal intensity when using NMR-spectroscopy, and for the peak area intensity when using LC-MS[24]. The increased accuracy to differentiate between IBD and HC when using a larger faecal mass, as observed in our study, may be explained by this increase of richness in number and intensity of VOCs.

Observed differences in VOC profiles between faecal samples enduring one versus multiple thaw-freeze cycles, are in line with previous research on VOC patterns using different eNose devices [16, 26]. It could be hypothesised that these effects are caused by changes in microbiota composition or function, although in a previous study no differences were found in microbiota composition between analyses of fresh samples versus samples frozen at minus 80 degrees and subsequently thawed prior to analysis [27]. A recent study suggested a release of microbial intracellular contents following thaw-freeze cycles, possibly explaining the effects of thaw-freeze cycles on VOC outcome[28]. In our study, it was shown that the diagnostic accuracy decreased with the addition of one extra thaw-freeze cycle, and that IBD could not be differentiated from HC after addition of multiple thaw-freeze cycles. Consequently, future studies on faecal VOC should limit the number of thaw-freeze cycles prior to analysis to a maximum of two.

Consistent with the results from a previous study on faecal VOCs using an eNose device, we measured significant differences between faecal VOC profiles measured directly after thawing (as used in the reference standard) and after 180 minutes stored at room temperature with an accuracy of 0.84 [16]. Furthermore, it was demonstrated that the diagnostic accuracy decreased when samples were kept at room temperature for 180 minutes (AUC= 0.53). These results are in line with a previous study on the impact of storage conditions on crude faecal samples measured by NMR-spectroscopy, showed that metabolic variation was influenced by storage at room temperature and 4 °C[28]. The metabolic profiles of faecal samples did not change after keeping the samples at room temperature for 1 hour. However, samples stored for a longer time prior to the analyses gradually shifted. The overall changes that were seen included decreased levels of fumarate, succinate, glutamate and increased levels of methanol, phenylalanine and alanine and short chain fatty acids like acetate, butyrate, propionate and valerate. To a lesser extent, the same shifts were seen in samples kept at 4 °C, which indicates that the lower temperature slows down the impact on sample integrity, resulting in less alterations in the metabolic profile. In another study comparing VOC profiles of faecal samples kept at 1°C for 14 hours prior to GCMS analysis, there were no significant changes in VOC profiles before and after 14 hours [25]. Since the differences between IBD and HC in this study were analysed by means of pattern-recognition, specific metabolic alterations cannot be elucidated in this study. However, it could be hypothesised that the unstable VOC composition when keeping the samples at room temperature, is caused by ongoing fermentation by the faecal microbiota. Since fermentative processes have shown to be reduced at lower temperature, this could explain why VOC integrity remained stable when samples were kept at 1 and 4 degrees in previous studies[28]. Another explanation is the emission of volatiles in the sample, and contamination with background volatiles. Fermentation, emission and contamination could be avoided by measuring the sample directly after collection. However, clinical implementation of VOC analyses would then become a logistic challenge.

Literature on the influence of storage time of faecal VOCs is scarce. In a study assessing VOC profiles of urine using a similar FAIMS method to the current study, a nine-month shelf-life for urine samples was suggested after it was shown that chemical information was lost over time, regarding both diversity and concentration of gas emission[29]. In addition, in a previous study assessing the effect of sample age on serum VOCs measured

by GCMS a significant difference in metabolite composition was already seen after storage of three weeks in the freezer [30]. In the current study, faecal VOC profiles for IBD, seem less influenced by storage time compared to the previous studies on urine and serum, keeping a similar (high) diagnostic accuracy after storage of 43 months and 63 months. Interestingly, the samples chosen for this comparison, were used in a larger study by van Gaal et al. where an area under the curve of 0.76 was found after a mean storage time of 23 months for the IBD group (25CD, 21UC) and 39 months for the HC group[14]. The increase in the AUC of this sub analysis, although analysed at the same moment, can be explained by the fact that the remaining samples only consisted of CD patients and HC. In the previous study, the AUC for the differentiation between CD and HC was 0.90. There is, however, an important consideration to this post hoc analyses. The diagnostic accuracy was only assessed after a median storage duration of 43 and 63 months. Since there are no previous measurements, it could be possible that massive changes in VOC composition have influenced diagnostic accuracy in the initial months after collection. We cannot exclude this influence based on this study.

The main strength of this study is that we used an IBD group and an HC group to assess not only differences in VOC patterns between sampling methods, but also to assess the influence on the diagnostic accuracy for disease detection. In addition, we used the same subjects' samples for each of the analysis, accounting for various confounding factors of influence on faecal VOCS (e.g. smoking habits, medication use, diet). During each experiment, the remaining variables of interest were kept the same, ensuring optimal comparison based on the variable of interest. Our study also has several limitations. Most importantly, for the influence of storage time on diagnostic accuracy for IBD, we have made use of raw data of a previous study and have re-assessed samples with sufficient sample mass. For this analyses we were only able to include CD patients, and no UC. In addition, sample age differed between groups, which could have influenced diagnostic accuracy by the influence of metabolic degradation on VOC profiles at both measurements. Second, we have made use of unfiltered and unsterilized tap water for sample dilution, and compressed medical air as carrier gas. This protocol was chosen since it has been found a reliable sampling method for the differentiation between various diseases and healthy controls based on faecal VOCs[31-34]. To avoid VOC profile contamination, we have run air and water blanks which were checked on contamination peaks, and met the cleanliness criteria. In addition, we have analysed the samples in a random order, and have excluded the first matrix of every sample analyses to avoid air contamination. However, we cannot fully guarantee exclusion of VOC contamination by differences in tap water composition between measurements. Third, we have not explored the difference between the diagnostic accuracy when using fresh versus frozen samples. As previously described, this seems of important influence on urine and serum VOCs. However, since a diagnostic accuracy of 0.99 was found in this study, we believe that freezing our samples has not significantly influenced our study outcomes. Last, it is possible that optimized faecal sampling conditions are disease specific, and faecal VOC biomarkers to diagnose IBD might have different sensitivity to variations in sampling method compared to faecal VOC biomarkers for other gastrointestinal diseases. We did, however, find significant differences in VOC profiles between sampling methods, emphasizing the importance of the use of one standardised sampling method. Furthermore, it is important to point out that we made use of pattern-recognition in this study, which complicates the

assessment of the influence of specific metabolites. We have chosen to validate specifically the FAIMS method since this device is an easy-to-use tool which could be suitable for clinical implementation [35].

This study highlights the need for one standardised methodology, in both research setting and when using VOCs analysis as a (future) clinical tool. Based on this and previous study results, we would like to suggest to use a standardised protocol with preferably faecal sample masses of 500mg, no more than one thaw session prior to VOC analysis, and analyzation of samples directly after thawing or, if impossible, keeping the samples frozen until further analyses. Future studies should assess the difference in diagnostic accuracy between fresh samples and frozen samples, and the influence of storage duration using multiple measurement moments after sample collection.

In conclusion, this study showed a high discriminative accuracy to differentiate between IBD and HC when using the standard operating procedure. It was shown that the use of less than 500mg, multiple thaw-freeze cycles, storage at room temperature and storage in freezer all influence the diagnostic accuracy. We therefore suggest to use one standardised protocol when performing faecal VOC analysis. In addition, further studies should focus on finding IBD specific VOCs to allow for targeted pattern-recognition.

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CHAPTER 3

The influence of lifestyle factors on faecal volatile organic compound composition as measured by an electronic nose

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ABSTRACT

Background

Faecal volatile organic compounds (VOCs) are gaseous metabolic products which are increasingly considered potential non-invasive biomarkers for the detection of various (gastrointestinal) diseases. The influence of lifestyle factors on faecal VOC patterns remains unexplored but is of importance prior to implementation of VOC analysis as a diagnostic tool. The aim of this study was to investigate the effects of age, gender, body mass index, smoking status, dietary preferences, medication use and co-morbidity on faecal VOC patterns.

Methods

For this study, faecal samples of patients undergoing a colonoscopy were collected prior to endoscopy. All participants completed a questionnaire on lifestyle factors, co-morbidity and medication use. Patients without colonic abnormalities were included in this study. Faecal VOC patterns were analysed by means of an electronic nose (eNose) device (Cyranose® 320).

Results

From the 1039 participants willing to participate in the initial study, 211 were eligible as controls. All unique lifestyle variables investigated in this study affected the faecal VOC composition. The strongest influences were caused by low BMI, a vegetarian diet and an active smoking status, whereas the least influence was found for the variables gender, age > 55 years and previous smokers.

Conclusion

Age, gender, BMI, smoking habits, dietary preferences, co-morbidity and medication use all have unique effects on faecal VOC composition. Future studies should carefully consider this influence on VOC outcome when defining VOC signatures as biomarker for diagnostic purposes.

INTRODUCTION

Over the past few decades, the usefulness of non-invasive biomarkers for the detection and follow-up of gastrointestinal diseases has been recognised. Non-invasive biomarkers may contribute to a refined selection of high-risk individuals in both clinical settings and population based screening programs, and for monitoring of disease courses. This may lead to earlier disease detection, increased curation rates and may lower the need for invasive diagnostic testing and disease treatment. Examples of biomarkers currently used in gastroenterology are faecal calprotectin (FCP) for the detection and follow-up of inflammatory bowel disease (IBD), and the faecal immunochemical test (FIT) for the detection of colorectal cancer (CRC) and adenomas in population based screening. These tests are amongst the first non-invasive biomarkers used, however, accuracies of both biomarkers are hampered by either a limited sensitivity or specificity [1, 2]. Therefore, the search for more accurate non-invasive biomarkers remains warranted.

The analysis of volatile organic compounds (VOCs) is a relatively new technique in the field of biomarker exploration. These carbon-based gaseous chemicals can be measured in the headspace of fluids (e.g. urine and faecal) or breath and are considered the end-products of metabolic processes in the human body. Faecal VOCs are also thought to reflect the interaction between intestinal microbiota and host[3]. The VOC composition may be analysed with chemical analytical techniques, of which gas chromatography-mass spectrometry (GC-MS) is the golden standard. Although these methods allow for stable and reliable measurements, they usually require specialised personnel, are time-consuming and costly. Another way to analyse the VOC composition is by means of pattern-recognition measured with an electronic nose (eNose). Using this technique, volatiles cannot be identified to a molecular level. Instead, diagnosis is done on the complete VOC pattern, created by all the present volatiles taken together. Advantages of eNose techniques are their ability for easy-to-use, fast and relatively inexpensive measurements, which underline their potential for use in clinical practice[4]. Several studies have demonstrated the potential of faecal VOC patterns to detect gastrointestinal diseases[5-8]. These are mainly case-control studies based on the analysis of a broad range of VOCs. The next step in developing cost-effective VOC pattern-based tests is the construction of sensor arrays which allow for the measurement of disease specific VOCs. To reach an optimal diagnostic accuracy, it is of importance that the sensors within the array are not influenced by other factors than the disease state of interest. Especially those factors notorious for altering metabolism and gut microbiota composition are likely to affect the VOC pattern, however, information on the extent of their influence is limited[9-11]. Previous studies have reported effects of gender and BMI on specific faecal volatiles using GC-MS, but data on their influence on the total faecal VOC patterns is lacking[12, 13]. In addition, an influence of dietary habits on VOC patterns may be expected, as a previous study has shown effects of a gluten free diet on the VOC composition of breath samples, though again, using GC-MS[14]. Regarding the use of pattern-recognition, there is some data available revealing an influence of smoking on faecal VOC patterns as measured by an eNose (Cyranose 320 ®)[15]. We aimed to evaluate the extent of the effects of gender, age, BMI, smoking habits, diet, co-morbidity and medication use on faecal VOC patterns as measured by an eNose.

METHODS

Study design

This proof of concept study was performed between February 2015 and November 2017 at the outpatient clinic of the Gastroenterology and Hepatology departments in one tertiary referral hospital (Amsterdam UMC, location VUmc) and two general hospitals (OLVG West in Amsterdam and Spaarne Gasthuis, location Hoofddorp and Haarlem), all located in The Netherlands.

Study participants

All patients aged 18 years and older who were scheduled to undergo a colonoscopy at one of the three contributing hospitals were asked to participate in this study. To ensure that VOC outcomes would not be influenced by mucosal abnormalities, patients were only included if no colonic abnormalities were observed during colonoscopy (with the exception of haemorrhoids, diverticula and small fibroma). Histology reports were checked in the case of mucosal biopsies and patients were excluded if histologic abnormalities were reported by the pathologist (e.g. reactive mucosal alteration, infiltration, dysplasia). Other exclusion criteria were other confirmed bowel diseases (e.g. celiac disease, IBD), inadequate bowel cleansing, failure to perform a complete colonoscopy (defined as intubation up to the caecum), a missing questionnaire and/or the collection of insufficient faecal sample mass to perform this study. In addition, participants were excluded when they had not enough knowledge of the Dutch language to complete the questionnaire. Study design is depicted in Figure 1.

Sample collection

All patients were provided a stool container (Stuhlgefäß 10ml, Frickenhausen, Germany) and asked to collect a faecal sample prior to bowel preparation and colonoscopy and to store the sample in their own freezer (-20 C) within one hour following bowel movements. In addition, participants were asked to complete an online or paper questionnaire which included questions on age, gender, BMI, smoking status, dietary intake, abdominal symptoms, bowel habits, patient history, family history and medication use. The samples were transported to the hospital by the participant on the day of their scheduled colonoscopy using ice packs and/or ice cubes. Samples were stored at -24°C directly upon arrival at the hospital

Variables of interest

We aimed to assess the effect of the variables gender, age, BMI, smoking status, dietary preferences, co-morbidity and medication use on VOC composition. All of the variables were based on the reported outcomes of the questionnaire. All of the variables were categorical. To exclude bias of the effects of hormonal treatment on faecal VOC samples of transgender patients, the variable gender consisted of three options: male, female and other. Age, BMI and smoking status were all divided into four categories: 18-35; 36-50; 51-65 and >65 years old for the age variable, <18.5; 18.5-25; 25-30 and >30 kg/m^2 for BMI, and smokers; former smokers (quit <5 years ago); former smokers (quit >5 years ago) and non-smokers for the smoking status variable. Diet was categorised as 'no adjustments made' and 'adjustments made', with the addition of a vegetarian subgroup. Furthermore, the influence of co-morbidity with non-colonic diseases and the influence of medication use

on faecal VOC profiles were assessed. Co-morbidity was divided into a 'no co-morbidity reported' and 'one or more comorbidities reported' group. Subgroups of patients with diabetes mellitus (type 1 and 2 combined) and subgroups of patients with hypertension were also compared to patients with no reported comorbidities. Medication use was described as 'no medication use' or 'use of one or more drugs in the past three months', with subsets of participants using/having used proton pump inhibitors (PPIs) and antibiotics (AB) three months prior to analyses because of their specific effect on the gastrointestinal tract. These subsets were compared to the participants with no reported medication use.

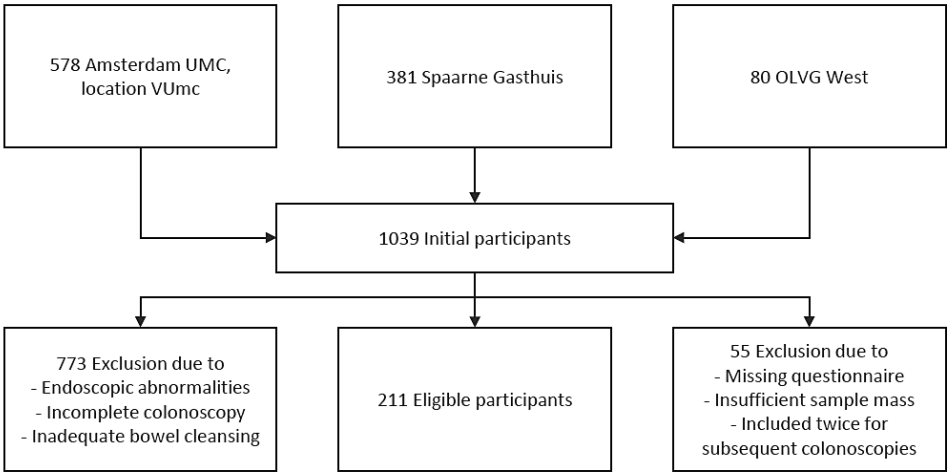

Figure 1. Study design

Sample 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 3 mL sealed vacutainer (BD vacutainer, Franklin Lakes, NJ, USA), labelled and re-stored in a -20°C freezer until further handling. The amount of sample was chosen to provide an optimum ratio of VOCs to the sample headspace, as validated in a previous sampling method study for VOC pattern-recognition using field asymmetric ion mobility spectrometry (FAIMS)[16]. Samples were thawed to room temperature, 30 minutes prior to VOC analysis allowing faecal VOCs to fill the headspace of the sample. These reference standard settings are in line with several previous studies on faecal VOC profiling in a range of diseases which allowed for the discrimination between cases and healthy controls[17].

Cyranose 320®

Faecal volatile organic compounds were measured by means of a Cyranose 320® eNose (Smiths Detections, Pasadena, CA, USA), a handheld chemical gas sensing apparatus, designed to identify VOC patterns of chemical mixtures. The Cyranose 320® consists of a NoseChip®, an array of 32 polymer nanocomposite sensors, each having a specific interaction with distinct VOCs based on a difference in sensor material. The interaction with

VOCs causes the sensormatrix to swell and this change in volume subsequently increases their electrical resistance signal. The sensors response is measured as a relative resistance change specific for each sensor ((maximum resistance – baseline resistance)/baseline resistance). The combination of relative resistance alterations form a so called 'smellprint', specific for unique vapors. The Cyranose® utilizes a software (PCNose) and advanced pattern-recognition algorithms which enables the user to create a library of VOC patterns for single samples and sets of samples.

Faecal volatile organic compound analysis

Samples were analysed in random order in 5 subsequent days. The vacutainer was connected to the Cyranose320® in an air-tight loop system, piercing two needles (BD Blunt fill needle 1.2 x 40 mm, Franklin Lakes, NJ, USA) attached to two tubes (Argyle Covidien tube 3mm, Mansfield, MA, USA) into the cap of the vacutainer on the needle side, and into the snout and outlet of the Cyranose320® on the tube side. Three-way valves (stopcock connecta plus, McFarlane Medical, Melbourne, Australia) were included in the system to control airflow direction and a syringe waterfilter (Nalgene™ 25mm Syringe Filter, Thermo Scientific™, Massachusetts, USA) was inserted to prevent contamination of the eNose by condensation. A baseline reference signal was obtained for 30 seconds prior to the actual VOC analysis. This was done by linking a VOC-filter (A1, North Safety, Middelburg, The Netherlands) to the inlet of the eNose. The actual faecal sample analysis was then performed for 60 seconds and subsequently the sensors were purged for 60 seconds with VOC-filtered air to wash the faecal VOCs off the sensors and create a new baseline reference for the next sample analysis. In case the Cyranose 320® was not used for 30 minutes in between faecal sample measurements, purging of the sensors was again done for 30 seconds. After this, an empty sample was measured to prevent the so called 'first-sniff effect'. The signal of this sample was then used to calculate cross-validation, to ensure comparability of new sample measurements to previously analysed samples. All needles, tubes, connectors and three-way valves were replaced between each sample analysis to prevent contamination of VOCs from previous samples[17].

Statistical analysis

Baseline characteristics for all study participants were computed using SPSS Statistics (version 22, IMB, NY, USA). The eNose data analyses were performed in R version 3.4.4 and consisted of a total of 76 variables: a total of 42 demographic and lifestyle variables andthe outcomes of the 32 sensors. Unscaled and scaled heatmaps of the sensor data were created to visualize the effect of the VOCs on sensor outcomes (Suppl Figure 1 and Figure 2). Spearman correlation was used to assess the correlation between sensor outcomes, to visualize variability in sensor response (Suppl Figure 2). Spearman correlation was also used to calculate correlation coefficients of lifestyle variables, to discover presence of possible confounding factors. Spearman correlation is a non-parametric method to assess the relationship between two variables. Then, factor analysis was used to represent the 32 sensors by a smaller number of variables. Factor analysis is a widely used method for the reduction of multidimensional data. Advantage of this method compared to other data reduction methods, as for example principal component analysis (PCA), is that this type of analysis accounts of random errors of the data, thus, creating less loss of information and therefore a more reliable data reduction. The generated scree plot was used to choose the

number of factors to be extracted (Supp Figure 4). Then, per variable of interest, the sum of the relative electrical resistance change is calculated per factor (the sum of the mean scores per sensor within that factor*the weight of the sensor within that factor). To visualize the effects of the variables on the factors, the scores representing these variables were then plotted in a chart, per category of interest of each variable, with one factor on the X-axis and one factor on the Y-axis. This was chart was then used to interpret effects of the variables on the VOC pattern.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre under file number 2014.404, and by the local medical ethical committee of the OLVG West and Spaarne Gasthuis. Written informed consent was obtained from all study participants.

RESULTS

Characteristics of study population

A total of 1039 participants (578 Amsterdam UMC, 381 Spaarne Gasthuis, 80 OLVG West), collected a faecal sample prior to colonoscopy of which 266 participants had no mucosal abnormalities during endoscopy (*Figure 1).* Of these, 55 participants were excluded, 26 due to missing/incomplete questionnaires, 17 due to insufficient sample mass and 12 due to the fact that they were included twice because of subsequent colonoscopies. Baseline characteristics are reported in *Table 1.*

Table 1. Baseline characteristics of all study participants (n=211)

Age (mean, min-max)	60.5 (26-83)
Age groups (n [%])	
18-35	9 [3.8]
36-50 (n=37)	31 [14.7]
51-65 (n=104);	93 [44.1]
>65 (n=84)	78 [37.0]
Gender (n [%])	
Female	123 [58.3]
Male	87 [41.2]
Other	1 [0.5]
BMI (mean, min-max)*	26.0 [16.2-61.3]
BMI groups (n [%])*	
<18.5	6 [2.8]
18.5-25	80 [37.9]
25-30	78 [37.0]
>30	36 [17.1]

Table continues

Smoking status (n [%]) **		
Non-smoker		99 [46.9]
Smoker		31 [14.7]
Quit smoking		81 [38.4]
	≤5 years	13 [6.2]
	> 5 years	68 [32.2]
Stool consistency (BSC) † (n [%])		
BSC1		17 [8.1]
BSC2		36 [17.1]
BSC3		52 [24.6]
BSC4		59 [28.0]
BSC5		24 [10.0]
BSC6		37 [17.5]
BSC7		12 [5.1]
Diet groups (n [%])		
Regular diet		182 [86.3]
Vegetarian diet		7 [3.3]
Other dietary preferences††		22 [10.4]
Co-morbidity (n [%])		
No co-morbidity reported		126 [59.7]
Co-morbidity reported		85 [40.3]
	DM	13 [6.2]
	HTS	39 [18.5]
	Other‡	48 [22.7]
Medication use (n [%])		
No medication use		50 [23.7]
Use of one or more drugs		161 [76.3]
	PPI	45 [21.3]
	OAB	28 [13.3]
Reason for endoscopic assessment (n [%])		
Positive FIT		20 [9.5]
Rectal blood loss		26 [12.3]
Change in bowel habits		33 [15.6]
Surveillance		48 [22.7]
Abdominal pain		32 [15.2]
Diarrhea		12 [5.7]
Weight loss		4 [1.9]

Table continues

Anaemia	12 [5.7]
Constipation	18 [8.5]
Family history of CRC	17 [8.1]
Monitoring after diverticulitis	2 [0.9]
Monitoring after abscess	3 [1.4]
Monitoring after CRC surgery or EMR of TVA	6 [2.8]
Other†††	5 [2.4]

*12 missing values; **1 missing value; †43 missing values ; ††other consisted of various diets (e.g. glutenfree (5), lactosefree (3), vegan, less chicken, protein rich, low in carbs, FODMAP, less sugar and salt); ‡consists of pulmonal diseases (15) including COPD, asthmatic bronchitis and lung fibroses and 38 other comorbidities, including thyroid disease (8), rheumatoid arthritis (4), heart disease (3), irritable bowel syndrome (2), high cholesterol (2); abbreviations BSC: Bristol stool chart (stool consistency scale comprising seven options with 1 being the most watery and 7 the least), PPI: proton-pump inhibitor, OAB: oral antibiotics, DM: Diabetes Mellitus, HTS: Hypertension; ††† Other consisted of bloating (1), soiling (1), low iron levels (1), rectal slime loss (1), follow-up of aphtous lesion. Abbreviations: CRC, colorectal cancer; EMR, endoscopic mucosa removal; TVA, tubulovillous adenoma.

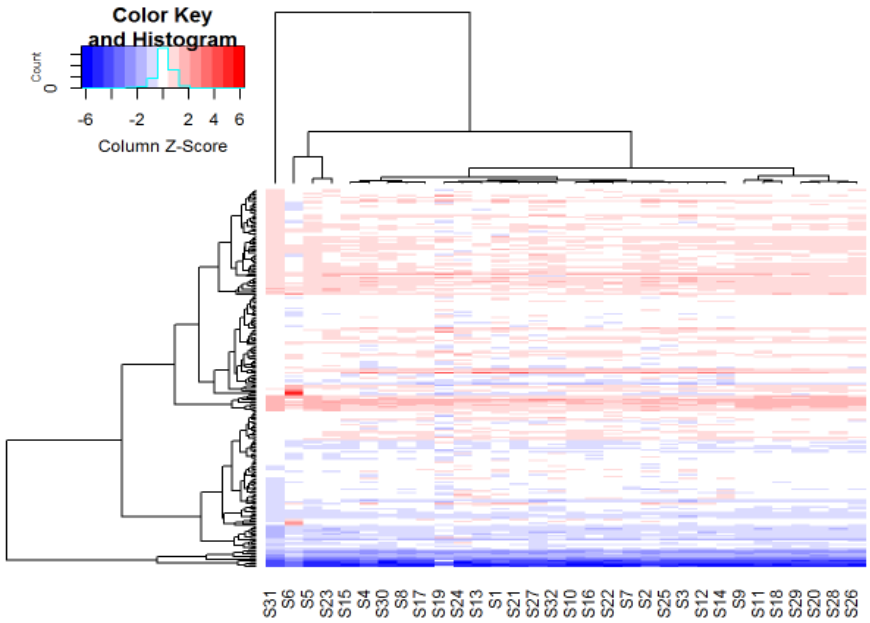

Figure 2. Scaled heatmap of sensor data outcomes split per sample and per sensor. Sensor outcomes are depicted on the X-as, samples are depicted on the Y-as. Per sensor, the mean and standard deviation are computed across all samples. Then, each measurement is subtracted from the mean, dividing it by its standard deviation per sensor to scale all measurements across all sensors.

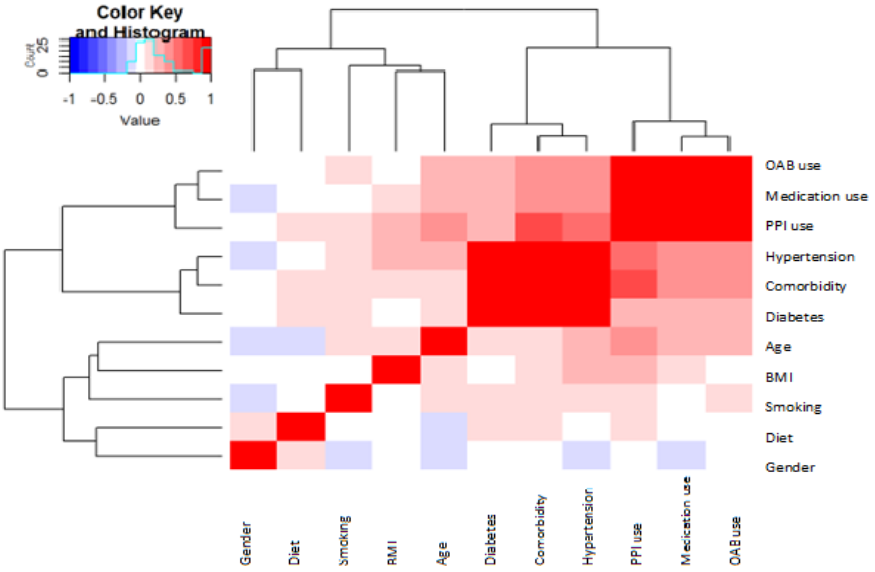

Figure 3. Spearman's correlation coefficients between lifestyle variables. A strong positive correlation was set as 0.7-1.0, moderate positive correlation 0.5-0.7, weak positive correlation 0.3-0.5, no correlation -0.3-0.3, weak negative correlation -0.3 to -0.5, moderate negative correlation -0.5 to -0.7, strong negative correlation -0.7 to 1.0. Abbreviations: OAB, oral antibiotic use; PPI, proton pump inhibitor use; HTS, hypertension; DM, diabetes mellitus; BMI, body mass index. A moderate positive correlations are seen between PPI use and HTS, PPI use and co-morbidity. Weak correlations are found between medication use and HTS, OAB use and HTS, medication use and co-morbidity, age and PPI use.

Sensor resistance analysis

Density plots of the sensor outcomes demonstrated a normal distribution for all sensors. Heatmaps of the sensor outcomes were obtained using pre-defined breaks (*supplementary figure 1*). Then, the sensor data was scaled across all sensors. *Figure 2* depicts the heatmap of these scaled sensor outcomes per sample per sensor. It is found that the sensor outcomes of a singular faecal sample have a common tendency to either have outcomes above, on, or below all mean sensor outcomes. Based on the Spearman coefficient test, strong correlations were found between most of the sensor outcomes(*supplementary fig 2*).

Lifestyle variable analysis

Variable frequencies are reported in *Table 1* and their distribution amongst faecal samples is visualised in *Supplementary figure 3.* Spearman coefficient test was used to assess correlations between variables and is visualised in *Figure 3.* A moderate positive correlation was found between PPI use and HTS, and between PPI use and co-morbidity (correlation coefficient between 0.5 and 0.7). In addition, a weak correlation was found between the following variables: medication use and HTS, OAB use and HTS, medication use and co-morbidity, age and PPI use (correlation coefficient between 0.3 and 0.5). There was no linear relationship between the rest of the lifestyle variables (correlation coefficients between -0.3 and +0.3).

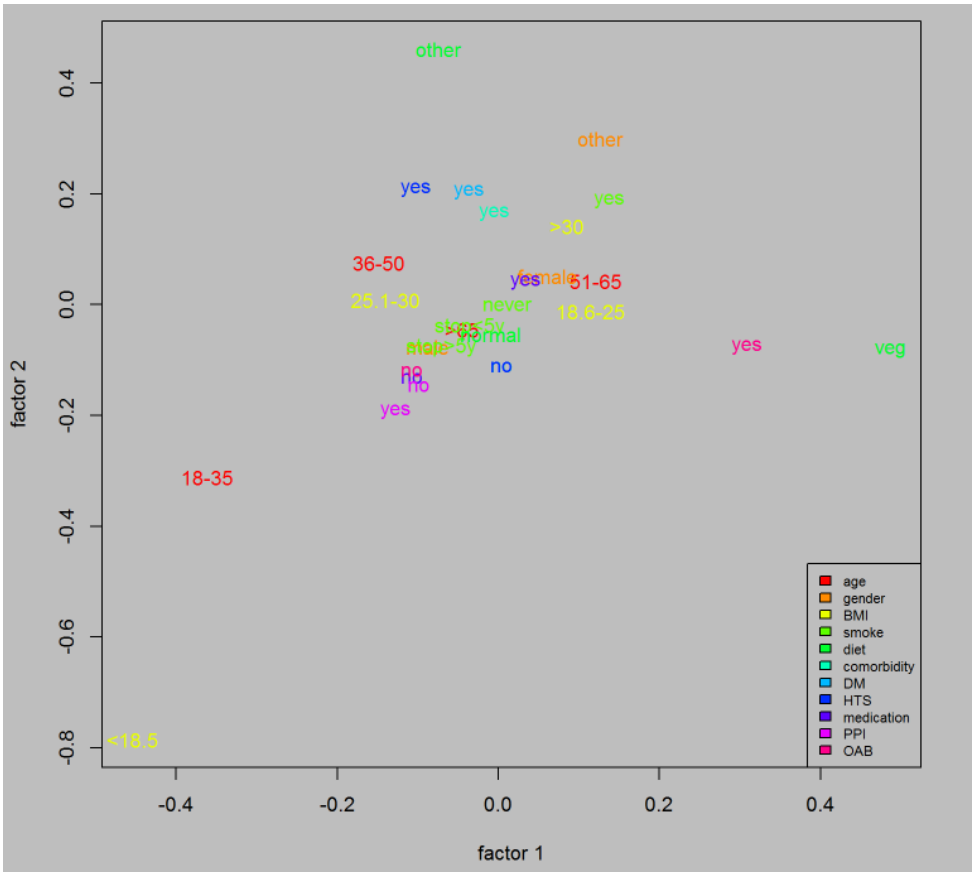

Figure 4: Factor analyses for all lifestyle variables of interest combined. Outcomes for factor 1 are scaled on the X-axis and outcomes for factor 2 are scaled on the Y-axis. For both factors, the further from zero the variable is depicted (either negative or positive), the larger the sum of the relative electrical resistance change is for that factor, or in other words, the further from zero the variable is depicted the larger the influence on all sensors within that factor taken together. For example, large influence on sensors taken together here are BMI<18.5, vegetarian diet and 'other diet', gender specified as other (n=1), an active smoking status, an age between 18-35 years, the use of antibiotics and the presence of co-morbidity. Lowest effect on factors is seen for gender, ex-smokers, age > 55 years.

The influence of lifestyle variables on volatile organic compound patterns

For the factor analysis, the two first factors were chosen based on the Scree Plot (*Supplementary Figure 4).* The first two factors accounted for 0.515 and 0.403 of the total variance, meaning they explained 91.7% of the total variance taken together. The sensor representations within the two factors are described in *Supplementary Table 1 and supplementary Figure 5.* The factor analyses per variable are combined and depicted in *Figure 4.* Outcomes of the factor analysis per lifestyle variable of interest are listed in *Supplementary Figure 6.* For both factors, the further from zero the variable is depicted (either negative or positive), the larger the sum of the relative electrical resistance change is

for that factor, or in other words, the larger the effect of that variable on the sensors taken altogether.

For the variable age, the lowest age group (18-35 years) has the lowest scores for both factor 1 and 2. The age group 36-50 has a low score on factor 1 and a high score on factor 2. The remaining age groups (51-65, >65 years) both have values of approximately 0 on both the factors. For the variable gender, males and females have small opposite values being negative on both factors for males, and positive for females. Gender reported as 'other' is an outlier in this measurement, however, it needs to be noted that this was only one participant. The body mass index group <18.5 is the most outstanding subgroup within this variable with very low scores on both factor 1 and 2, whereas the subgroups 18.5-25, 25-30 and >30 all have values of approximately 0 on factor 2. Non-smokers and previous smokers (either longer and shorter than 5 years) all have values of approximately 0 on both the factors, whereas smokers have high values on both. A regular diet scores approximately 0 on both factors, but a vegetarian diet has high values on factor 1, and other diets taken together have a high value on factor 2. The variables co-morbidity, hypertension and diabetes all show high values on factor 2. The overall use of one or more drugs has a score near 0 for both factors, whereas the use of OAB three months prior to inclusion has high values on factor 1, and the use of PPI has low scores on factor 2.

DISCUSSION

In the present study, we have assessed the influence of lifestyle factors of adults without colonic abnormalities on the faecal VOC patterns as measured by an electronic nose. All of the lifestyle variations investigated in this study were shown to affect the VOC factor analyses. This means that these variables should be taken into account when interpreting study outcomes and when performing faecal VOC analysis either by matching on these variables or by calculating correction factors for the intended device.

The potential of VOC patterns to differentiate between colonic diseases and healthy state has been demonstrated in various studies using VOC profiles originated from faecal, breath and urine (e.g. colorectal cancer, inflammatory bowel disease)[7, 8, 18-20]. These studies have all shown the presence of disease-specific VOC patterns, which may be used for the development of disease-specific sensors. To our knowledge, this is the first published study to explore and compare faecal VOC patterns of a wide spectrum of lifestyle variations in controls that may be of influence on disease specific VOC patterns. Studies assessing the effects of lifestyle on VOC patterns are scarce and have mainly focused on one specific variable. These studies may provide guidance to the standardization of a correction factor for lifestyle variations. The results of the current study will be discussed and compared to the available literature in the following sections.

Gender affects the (hormonal) metabolism and the gut microbiota, and therefore differences could be expected in the faecal VOC composition[21]. Previously, VOC composition differences between 521 male and 822 female healthy adults have been investigated in breath using gas chromatography-mass spectrometry (GC-MS). In line with our study results,

a difference in VOC composition was found, but this was not sufficient to discriminate males from females[12].

Over the past few decades, BMI has been shown to have a correlation with the abundance and variety of bacterial strains of the gut microbiota[21]. The effects of BMI on faecal VOC composition have been subject of a study in which samples of 30 obese patients with a BMI of >30 and suspected non-alcoholic fatty liver disease (NAFLD) were compared to 30 healthy controls using the chemical analytical technique GC-MS[13]. They found 12 different faecal VOCs (e.g. aldehydes, ketones) significantly reduced and 18 faecal VOCs (e.g. propanoic, butanoic and pentanoic esters) significantly increased in obese patients compared to healthy controls. These differences are expected to appear in the VOC pattern as well, however, in the current study similar outcomes were found for participants with BMIs ranging between 18.6 and >30. This difference could be explained by a varying methodology and patient populations such as the inclusion of solely NAFLD patients, no correction for co-morbidity and no information on dietary habits. To our knowledge, there are no studies published in which the effects of a low BMI on VOC composition have been studied. Therefore it is important to note that in the current study, particularly the values of the subgroup BMI<18.5 were different compared to the other BMI groups.

Smoking has previously been subject of a study in which faecal VOC patterns of 11 smokers, 21 non-smokers and 24 former smokers were compared using an eNose (Cyranose 320®) [15]. In line with this study, discrimination of smokers and non-smokers was possible. Former smokers could not be distinguished from both non-smokers and smokers based on the faecal VOC pattern, however, they noted a wide divergence in abstinence of smoking time (6 months – 43 years). In the current study, abstinence of smoking time was divided into subgroups of >5 years and <5 years, which both had outcomes on the factor analyses similar to non-smokers. Based on our study results, only the effect of active smoking should be taken into consideration when performing faecal VOC analysis. Studies on dietary influences on VOC patterns are scarce, yet, in one previous study in which breath samples from 20 HC were compared to the same 20 HC while on a gluten-free diet were measured by GC-MS, differences in VOC compositions were observed[14]. In another study, differences were found in the faecal VOC patterns of 15 breast fed premature infants compared to 15 formula fed premature infants using an eNose[22]. In addition, long term dietary habits have been shown to affect the microbial composition and colonic fermentation, especially in plant based diets and in restricted calorie intake which may also be reflected in the faecal VOC composition[23, 24]. The influence of age on the gut microbiota has been subject of various studies and it has become clear that amongst adults the microbiota diversity varies widely and may be influenced by multiple factors as puberty, the ovarian cycle, pregnancy and menopause . In the current study, especially the age groups 18-35 and 36-50 years old demonstrated different outcomes in the factor analyses, which are the age groups most involved in these events.

Whether non-gastrointestinal co-morbidity influences the faecal microbiota via changes in the metabolism or vice versa has not yet been unraveled, however, associations between the gut microbiota and various metabolic diseases have been observed (e.g. hypertension, diabetes mellitus, atherosclerosis, liver cirrhosis)[25-27]. In the current study it was

demonstrated that the presence of co-morbidity, as well as the presence of HTS and DM had let to different outcomes compared to participants who reported no comorbidities.

It is well known that the use of antibiotics influences the abundance of the microbiota in taxonomic richness and causes a change in community[28] [29]. In patients using PPIs, an overrepresentation of oral bacteria has been found[30, 31]. These microbial alterations may be represented in the faecal VOC composition. In the current study, small opposite outcomes were found for the use of no drugs compared to the 'use of one or more drug' and 'use of PPI', whereas OAB use outcomes differed substantially. This corresponds with the literature on the faecal gut microbiota and this suggests that these drugs should be taken into account when studying potential VOC biomarkers.

In this study we have investigated the change in electrical resistance per sample per sensor by means of an eNose, which led to the insight that the sensor outcomes of a single faecal sample assessment had a common tendency to either have outcomes above, on, or below all mean sensor outcomes. This was also demonstrated by strong correlations between the sensor outcomes. In general, it would be preferred to use VOC sensors that are sensitive to disease specific VOCs and less to lifestyle variances. The current study outcomes, however, suggest that the VOCs within the sample had a similar effect on most of the sensors. This may be due to a similar sensitivity of the sensors to VOCs in general, or samples in general may differ in the level of VOCs they hold. Should the first be the case, correction factors per lifestyle variable may be calculated for the change in electrical resistance per samples. Should the latter be the case, a general stable sample VOC outcome would be necessary to calculate these correction factors for the samples specifically.

The main strength of this study is that we used a large prospective cohort of an endoscopy controlled group, to decrease the chance of colonic abnormalities influencing faecal VOC patterns. In addition, we combined all the lifestyle variables into one factor analysis which allowed for a reliable comparison of the influence the variables had on faecal VOC patterns. Another strength of this study was the standardised protocol of faecal sample collection, storage and VOC analysis. Our study also has several limitations. Most importantly, although this cohort was relatively large, some subgroups were small which hampered the possibilities to perform reliable statistics. Especially the subgroups age 18-35 years (n=9), BMI <18.5 (n=6) and vegetarian diet (n=7) were small, but notably, they accounted for the subgroups of largest influence on VOC patterns. Nevertheless, since the clinical variables were not used to yield the factors, these results are independent, and albeit the fact that these subgroups are small, they have yielded enough variability on the sensors and are responsible to these large variance components.

In addition, it is important to note that we did not include a subgroup of participants with colon diseases. It may be possible that the influence of the lifestyle variables is of different significance on patients with colon diseases since we have not investigated the amount of change in electrical resistance these diseases establish. For example, should colon cancer cause a change in electrical resistance ten times as large as BMI<18.5, the influence of BMI<18.5 would become negligible. Last, it is important to note that the analyses performed with the eNose are all based on pattern-recognition and thus, the specific VOCs

accounting for the change in electrical resistance cannot be identified by this technique. We have chosen to explore the influence of lifestyle variables on faecal VOC patterns using the Cyranose 320®, since pattern-recognition based techniques usually are easy-to-use devices which allow for fast and inexpensive measurements, and the Cyranose 320® has previously been shown to hold potential for the differentiation between various diseases and healthy state[5, 7, 8, 17]. This underlines its potential for implementation in clinical practice.

In conclusion, this prospective study highlights the influence of the variables age, gender, BMI, smoking habits, dietary preferences, co-morbidity and medication use on faecal VOC patterns, and therefore underlines the need for the correction for these lifestyle variables when performing faecal VOC analysis studies. It should be noted that, albeit faeces was used as a medium in this study, VOCs may be found in all bodily excrements and therefore the influences of lifestyle factors found in the current study may also apply for other mediums used (i.e. urine, plasma and/or breath). Future studies investigating disease specific VOC-profiles should therefore investigate whether the range of change in electrical resistance of these variables influences the disease associated VOC pattern, or, should preventively account for these influences in their study design either by matching on these lifestyle variables or by calculating correction factors for the intended device. In addition, it is important to investigate the VOC patterns of multiple colonic diseases in one study. This way, reference values can be calculated per disease and sensitivity and specificity for these disease specific VOC patterns can be investigated accordingly.

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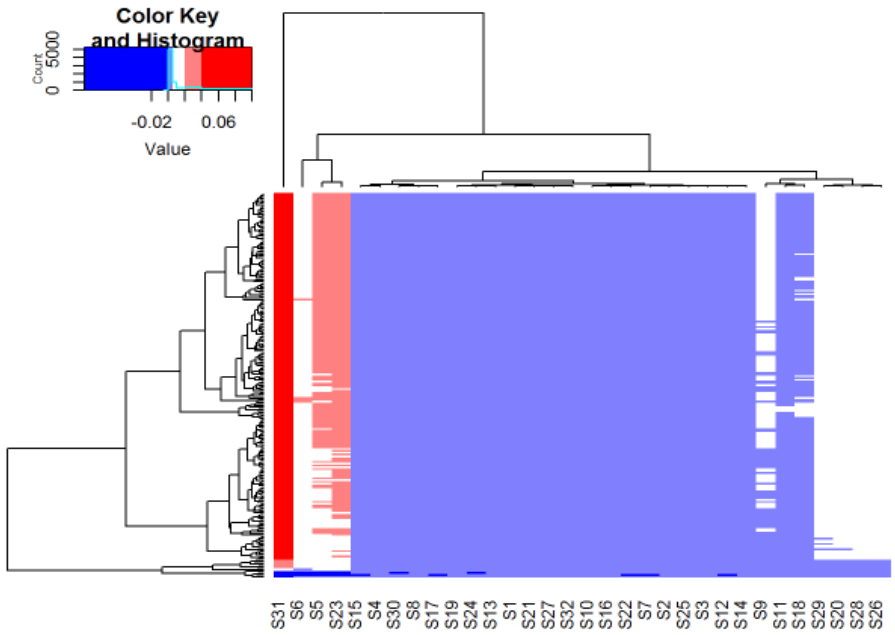
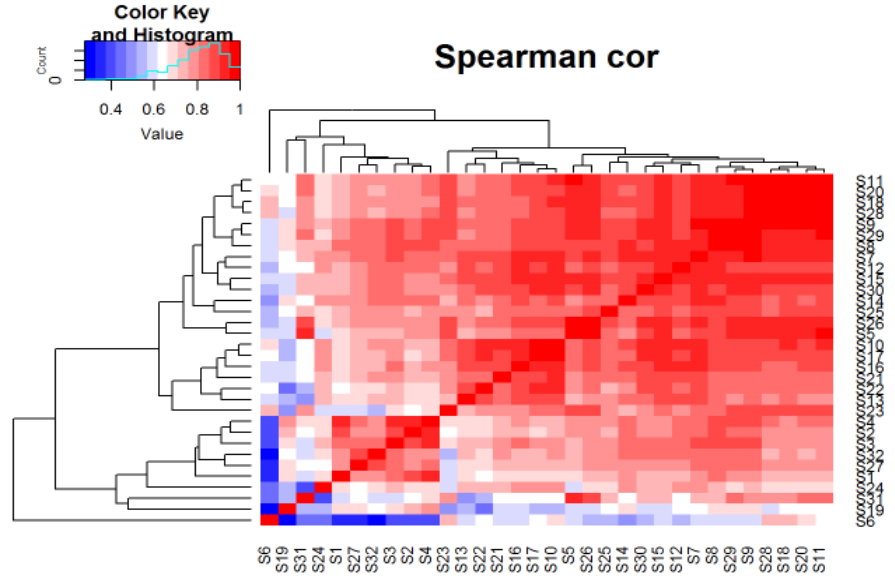
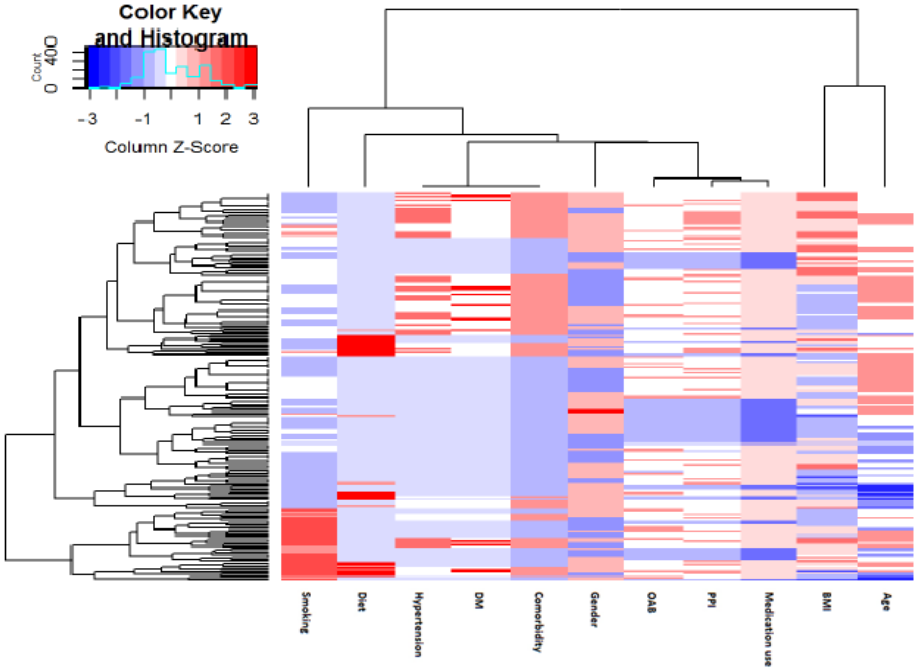


Supplementary figure 1. Heatmap of sensor data distribution. In this heatmap the absolute sensor outcomes are depicted per sensor per sample. Colors for sample outcomes are based on pre-defined breaks set as 0, 0.005, 0.01, 0.02, 0.04, 0.1. Sensors are depicted on the X-axis and samples are depicted on the Y-axis.

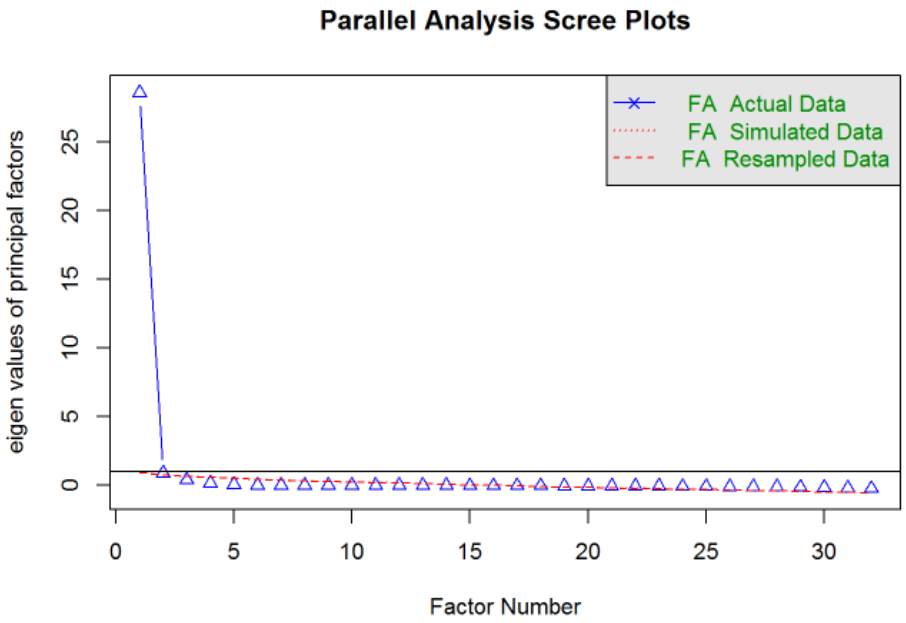


Supplementary Figure 2. Spearman correlation of sensor variables. Sensors are depicted on both

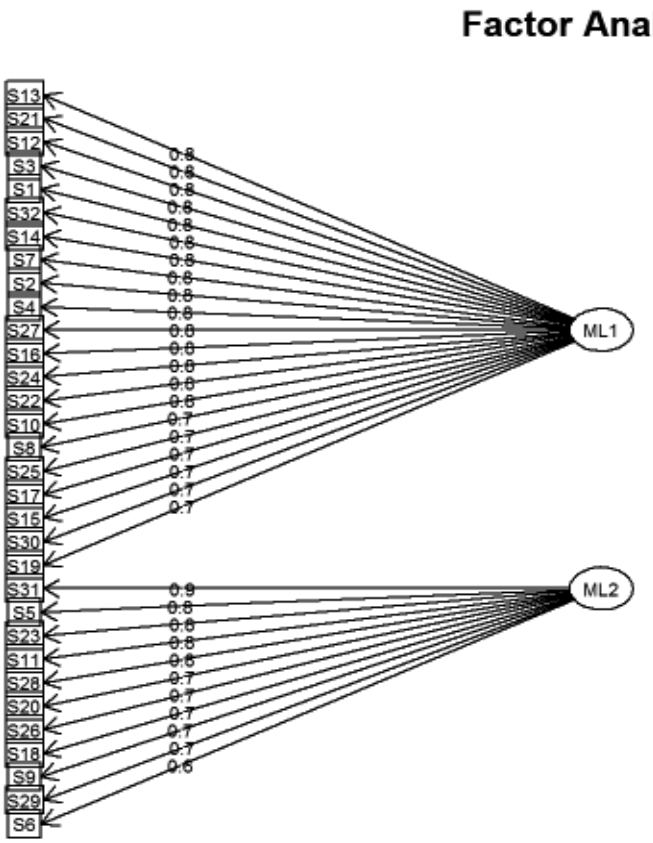
the X-axis and Y-axis. A strong positive correlation was set as 0.7-1.0 (deeply red), moderate positive correlation 0.5-0.7 (red), weak positive correlation 0.3-0.5 (light red), no correlation -0.3-0.3 (white), weak negative correlation -0.3 to -0.5 (light blue), moderate negative correlation -0.5 to -0.7 (blue), strong negative correlation -0.7 to 1.0 (deeply blue).



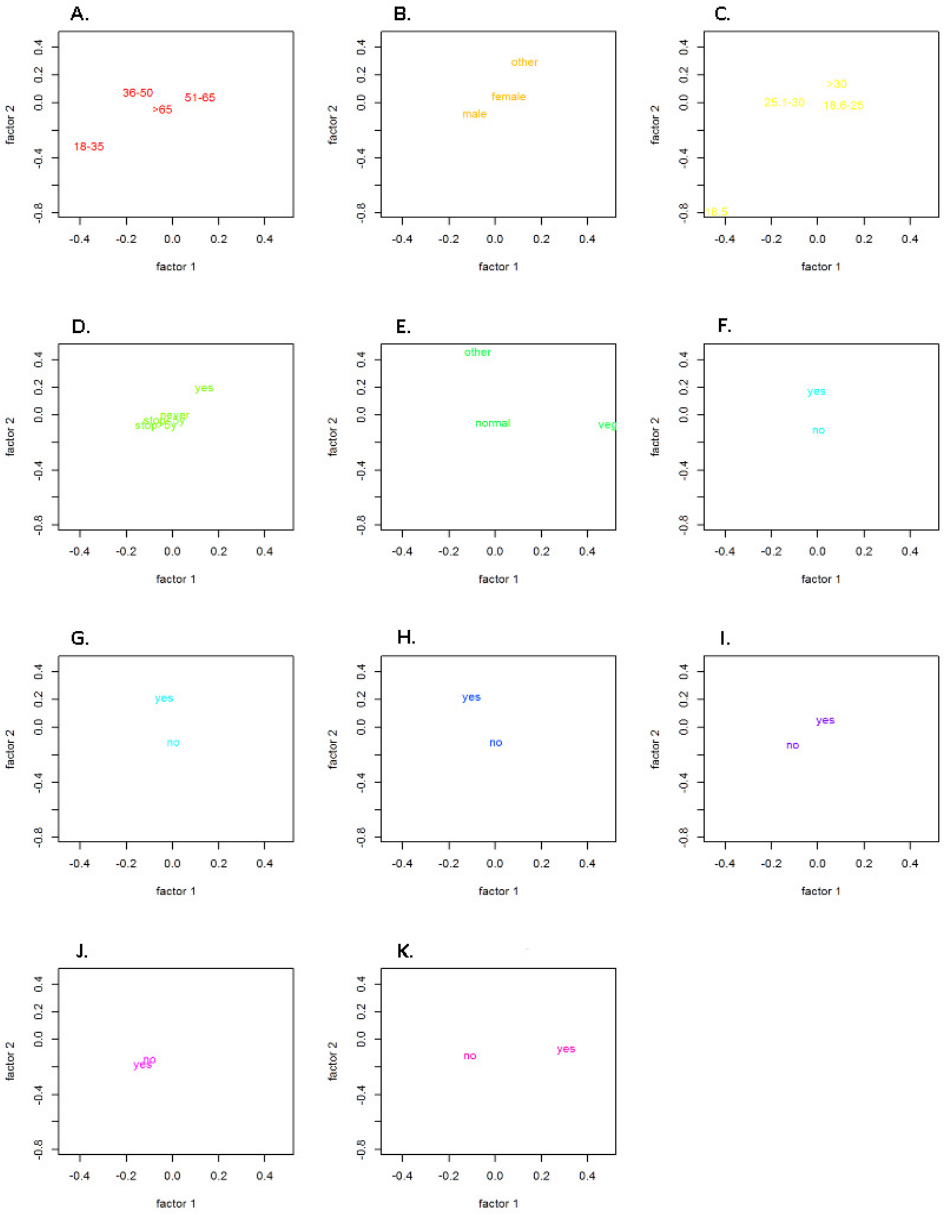
Supplementary Figure 3. Scaled heatmap of variable distribution per sample per lifestyle variable of interest. Variables are depicted on the X-as, samples are depicted on the Y-as. Per variable, the mean and standard deviation are computed across all samples. Then, each measurement is subtracted from the mean, dividing it by its standard deviation per sensor to scale all measurements across all sensors.



Supplementary Figure 4. Scree plot of parallel analyses. The first two factors account for 0.52 and 0.40 of the total sensor outcome variance, which means that together they explain 0.92 of the total variance. Therefore the first two factors are chosen for the factor analyses.



Supplementary Figure 5. Visualization of sensor representation in each two of the factors. The first two factors account for 0.515 and 0.403 of the total sensor outcome variance, which means that together they explain 0.92 of the total variance. Therefore the first two factors are chosen for the factor analyses.



Supplementary Figure 6: Scaled factor analyses per lifestyle variable of interest. Outcomes for factor 1 are scaled on the X-axis and outcomes for factor 2 are scaled on the Y-axis. For both factors, the further from zero the variable is depicted, the larger the sum (either negative or positive) of the relative electrical resistance change for that factor. A: age per group 18-35; 36-50; 51-65; >65, B: Gender, C: Body Mass Index per group <18.5; 18.6-25; 25.1-30; >30, D: Smoking status per group non-smokers, quit >5years ago, quit <5years ago, smokers, E: diet in groups no dietary adjustments made; vegetarian diet; other diet, F: one or more comorbidities reported, G: Diabetes reported, H: Hypertension reported, I: Use of one or more drugs, J: Use of proton pump inhibitors, K: Use of oral antibiotics.

Supplementary Table 1: Loadings of sensors in each of the two factors

Sensors	ML1	ML2
S1	0.803	0.476
S2	0.785	0.497
S3	0.811	0.513
S4	0.784	0.521
S5	0.563	0.816
S6	0.281	0.626
S7	0.793	0.600
S8	0.747	0.653
S9	0.694	0.713
S10	0.756	0.628
S11	0.639	0.762
S12	0.813	0.561
S13	0.835	0.491
S14	0.794	0.571
S15	0.724	0.673
S16	0.770	0.601
S17	0.743	0.636
S18	0.673	0.729
S19	0.672	0.289
S20	0.652	0.750
S21	0.819	0.514
S22	0.762	0.593
S23	0.533	0.811
S24	0.770	0.510
S25	0.743	0.618
S26	0.650	0.746
S27	0.773	0.521
S28	0.647	0.752
S29	0.695	0.710
S30	0.719	0.662
S31	0.390	0.881
S32	0.798	0.491
	ML1	ML2
Sensor loadings	16.475	12.882
Proportion Var	0.515	0.403
Cumulative Var	0.515	0.917

Loadings of sensors in each of the two factors. The loading per factor is shown for each sensor. The first two factors account for 0.515 and 0.403 of the total sensor outcome variance, which means that together they explain 0.917 of the total variance. Therefore the first two factors are chosen for the factor analyses.

CHAPTER 4

The influence of short-term sensor drift in a real-life clinical cohort: Limitations of electronic nose analysis hidden in a black box

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Submitted

PART II

Volatile organic compound profiles as biomarkers for inflammatory bowel disease

CHAPTER 5

Differentiation between paediatric irritable bowel syndrome and inflammatory bowel disease based on faecal scent: proof of principle study

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ABSTRACT

Background

The diagnostic work-up of paediatric irritable bowel syndrome (IBS) and functional abdominal pain – not otherwise specified (FAP-NOS), commonly includes invasive tests for discrimination from inflammatory bowel disease (IBD). Since this carries a high burden on patients, an ongoing need exists for development of non-invasive diagnostic biomarkers for IBS and FAP-NOS. Several studies have shown microbiota alterations in IBS/FAP, which are considered to be reflected by faecal volatile organic compounds (VOC). The aim of this study was to evaluate whether paediatric IBS/FAP-NOS could be discriminated from IBD and healthy controls by faecal VOC analysis.

Methods

IBS/FAP-NOS was diagnosed according to the ROME IV criteria, and *de novo* IBD patients and healthy controls (HC) aged 4 to 17 years were matched on age- and sex. Faecal VOCs were analysed by means of field asymmetric ion mobility spectrometry (FAIMS).

Results

Faecal VOCs of 15 IBS/FAP-NOS, 30 IBD (15 ulcerative colitis, 15 Crohn's disease) patients and 30 HC were analysed and compared. Differentiation between IBS/FAP-NOS and IBD was feasible with high accuracy (AUC (95%CI), P-values; 0.94 (0.88-1), <0.00001). IBS/FAP-NOS profiles could not be differentiated from HC (0.59 (0.41-0.77), 0.167), whereas IBD profiles could with high accuracy (0.96 (0.93 – 1), <0.00001).

Conclusion

Paediatric IBS/FAP-NOS could be differentiated from IBD by faecal VOC analysis with high accuracy, but not from healthy controls. The latter finding limits the potential of faecal VOCs to serve as diagnostic biomarker for IBS/FAP-NOS. However, VOC could possibly serve as additional non-invasive biomarker to differentiate IBS/FAP-NOS from IBD.

INTRODUCTION

Irritable bowel syndrome (IBS) and functional abdominal pain – not otherwise specified (FAP-NOS) are functional gastrointestinal disorders in children, with a worldwide prevalence of about 13%, often lasting for over five years after the diagnosis has been established [1]. Since biochemical diagnostic biomarkers are yet not available, the diagnosis relies on the symptom-based ROME IV criteria [2]. To fulfil one of the different ROME IV criteria, the symptoms must not be explained by another medical condition after appropriate evaluation. Differentiation between IBS and somatic disorders like inflammatory bowel disease (IBD) can be difficult. To exclude somatic diseases, the diagnostic work-up may include colonoscopy, which carries a high burden on patients and leads to high costs and risk of complications [3, 4]. Currently, faecal calprotectin (FCP) is the most commonly used non-invasive diagnostic biomarker to discriminate between IBS/FAP-NOS and IBD, which is characterised by a high sensitivity for mucosal inflammation (0.98, 95%CI 0.95-0.99), but limited specificity (0.68, 95% CI 0.50-0.86) [5]. Therefore, the search for an accurate, non-invasive biomarker to differentiate between functional gastrointestinal disorders and IBD remains warranted.

Alterations of the intestinal microbiota have been described in IBS/FAP-NOS patients[6]. However, described results are contradictory and a specific microbial signature has not yet been defined. Furthermore, microbiota analysis is not easily applicable as non-invasive biomarker in clinical practice, since the analysis is complex, time-consuming and expensive[7]. Assessment of volatile organic compound (VOC) composition, which is considered to reflect microbiota composition and function, is a novel field in metabolomics(8). VOC analysis has shown potential to serve as a diagnostic biomarker for a broad range of gastrointestinal diseases, in particular those linked to microbial dysbiosis, e.g. *Clostridium difficile* infection, IBD, colorectal cancer and necrotizing enterocolitis [8-11]. Field asymmetric ion mobility spectrometry (FAIMS) is an easy-to-use, pattern based technique to assess VOC profiles, characterised by high reproducibility and relatively low costs, and therefore holds the potential as a point of care tool[12].

We hypothesised that paediatric IBS/FAP-NOS and IBD could be differentiated based on differences in faecal VOC profiles. The aim of this study was to investigate whether faecal VOC patterns, analysed by FAIMS, could serve as biomarker to differentiate IBS/FAP from IBD and from healthy controls, in a paediatric population.

METHODS

Study design

This case-control study was performed at the outpatient clinics of the paediatric (gastroenterology) departments of two tertiary centres VU university medical centre, Emma Children's Hospital, Academic Medical Centre (AMC), and one general hospital, OLVG Oost (all centres located in Amsterdam, the Netherlands).The study was performed between December 2013 and December 2016.

Study participants

IBS and FAP-NOS

Children aged 4 to 17 years visiting the outpatient clinic in one of the three hospitals between August 2016 and December 2016, and fulfilling the ROME IV criteria for IBS or FAP-NOS were eligible to participate[2]. During clinical appointment, patients were asked to participate in this study. Patients of whom informed consent was obtained, were provided a stool container and a questionnaire on abdominal symptoms, defecation pattern, including consistency of stool using the Bristol stool chart, medication use and medical history. Exclusion criteria were the use of anti-/probiotics or immunosuppressive therapy three months prior to inclusion, immunocompromised disease (i.e. leukemia, human immunodeficiency virus), diagnosis of a gastrointestinal disease, proven infectious colitis in the month before presentation (determined by positive stool culture for *Salmonella spp., Shigella spp., Yersinia spp. Campylobacter spp.*, *Clostridium spp.* toxins, or parasites in stools) and a history of gastrointestinal surgery (except appendectomy). From all IBS and FAP-NOS patients included in this study, faecal calprotectin levels were assessed to exclude IBD.

Inflammatory bowel disease

Participants aged 4 to 17 years were extracted from an existing cohort consisting of *de novo* treatment-naïve paediatric IBD patients (59 CD, 40 UC), included at the VU University medical centre and the Emma Children's Hospital (AMC) between December 2013 and October 2015 for a study on diagnostic faecal biomarkers. All participants were instructed to collect a faecal sample prior to bowel cleansing, ileocolonoscopy and esophagogastroduodenoscopy. The diagnosis of IBD was made according to the revised diagnostic Porto-criteria for paediatric IBD, including endoscopic, histologic and radiologic findings by means of MR enteroclysis [13]. Localisation and behaviour of disease were classified according to the Paris Classification[14]. Clinical activity was determined at study inclusion based on the Physician Global Assessment (PGA-score), levels of faecal calprotectin (FCP >250ug/g was considered active disease) and C-reactive protein (CRP). Exclusion criteria were similar to the IBS/FAP-NOS group, except for exclusion when diagnosed with IBD.

Healthy controls

Children aged 4 to 17 years attending elementary and high schools in the province North-Holland, The Netherlands, were instructed to collect a faecal sample. Similar to the IBS/FAP-NOS group, all participants completed a questionnaire containing similar items. Exclusion criteria were functional gastrointestinal disorders according to the ROME IV criteria, diagnosis with a gastrointestinal or immunocompromised disease, history of gastrointestinal surgery (except appendectomy), or the use of pro- or antibiotics three months prior to inclusion.

Matching procedure

A total of 15 IBS/FAP-NOS patients (9 IBS, 6 FAP-NOS) were strictly matched to 15UC, 15CD and 30HC, based on age and gender. For this, the following procedure was performed. First, from the 99 IBD patients (59 CD, 40 UC) of the existing cohort, all of the eligible subjects were strictly matched to IBS/FAP-NOS patients. Then, IBD patients were randomly included from the matched groups in a 1:1:1 ratio (IBS/FAP-NOS to UC to CD). After this, 30 HC recruited for this study were matched to the IBS/FAP-NOS group in a 2:1 ratio.

Sample collection

Patients were instructed to collect a fresh faecal sample in a stool container (Stuhlgefäß 10ml, Frickenhausen, Germany) and instructed to store the sample in the refrigerator at home directly following bowel movement. The samples were transported to the hospital by one of the researchers, using cool elements and a cool bag. Here, samples were directly stored at -20 °C until further handling.

Sample analysis

Faecal volatile organic compounds analysis was performed using FAIMS (Lonestar,Owlstone, Ltd.), according to the protocol as described in an earlier study by Bomers et.al. [9]. In short, faecal samples were thawed to room temperature ten minutes prior to VOC analysis. A mixture of 0.5g faecal sample and 3.5mL tap water was manually shaken to homogenize the sample. Compressed air (0.1MPa) was used as carrier gas to transfer the sample headspace into the FAIMS device. The Lonestar was set up in a pressurised configuration with a flow rate of 2L/min. The temperatures were set at 35°C for the sample holder, 70°C for the lid and 100°C for the filter region. After the procedure the air in the Lonestar was refreshed by analyzing the headspace of 10mL tap water[15]. The dispersion field passed through 51 equal settings between 0% and 100% (in the ratio of the high electric field to low electric field). The compensation voltage was set between +6V and -6V in 512 steps for each dispersion field[9]. Each faecal sample was analysed three times sequentially, producing three matrices in 540s. For the statistical analysis, only the third matrix was used for optimal diagnostic potential[12].

Statistical analysis

The demographic data of each group (IBS/FAP-NOS, UC, CD and HC) was compared using the Kruskal-Wallis-H test with addition of the Wilcoxon-rank-sum test for continuous data. The Fisher's exact tests was performed for dichotomous data using IBM SPSS version 22. Each FAIMS data consists of the 52224 data point in a 2D matrix. A pre-processing method was first performed to each data by applying 2D discrete wavelet transform. This step aims to decompose the data and extract subtle chemical signals hidden within a much larger signal. A 10 fold cross validation was then applied, where feature selection and classifier training was performed to 90% of data (training set) and class predictions produced from 10% of data (test set). A Wilcoxon rank sum test as feature selection was used to calculate p-values in training set to identify which features best for disease prediction. From this, 44 statistically important features were used. Four classification algorithms were applied, Sparse Logistic Regression, Random Forest, Gaussian Process, and Support Vector Machine. A receiver operator characteristic curve was created to predict area under curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and p-values.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre under file number 2015.393, and by the local medical ethical committees of the other two participating centres. Written informed consent was obtained from all parents, and from the child in case of age over twelve years.

RESULTS

Baseline characteristics

Baseline characteristics and disease specifics of the study subjects are displayed in Table 1. There were no significant differences in age, sex and BMI between the IBS/FAP-NOS, IBD and HC subgroups. Levels of FCP were below 250ug/g in the IBS/FAP-NOS group, with the exception of one patient (476 ug/g) in which it normalised after repeating the measurement, while the IBD group had a median FCP level of 1237 ug/g (IQR [580 – 1885]). At study inclusion, the majority of IBS/FAP-NOS patients had experienced abdominal symptoms for over a year, with frequencies varying from once a week to daily. All of the children in the HC group were asymptomatic. Faecal frequency was higher in the IBS/FAP-NOS group compared to the HC group, although this was not significant. In addition, no differences in faecal consistency based on the Bristol Stool Chart and way of delivery were found between IBS/FAP-NOS and HC.

Table 1. Baseline characteristics

	Crohn's disease (n=15)	Ulcerative colitis (n=15)	IBS/FAP-NOS (n=15 [9/6])	Control (n=30)
Sex, male (n [%])	9 [60]	8 [53]	8 [53]	15 [50]
Age (median [IQR]), years (minimum-maximum)	12.8 [5.0] (5.9 – 17.9)	11.8 [7.8] (3.2 – 17.8)	12.9 [8.4] (4.4 – 18.1)	12.7 [8.1] (4.1 – 17.9)
Storage time, median [IQR]), months (minimum-maximum)	31.7 [25.3]¥ (8.2 – 54.5)	45.1 [36.2]¥ (15.0 – 59.4)	0.6 [0.6]¥ (0.2 – 2.9)	1.4 [0.3]¥ (0.5 – 4.5)
BMI (median [IQR])	NA	NA	16.7 [5]	17.0 [3]
Bristol stool chart (n [%])	NA	NA		
Type 2			2 [14]*	4 [14]*
Type 3			5 [36]	19 [66]
Type 4			4 [29]	5 [17]
Type 5			3 [21]	1 [3]
Stool frequency (n [%])	NA	NA		
2 times a week or less			2 [14]*	1 [4]*
3-6 times a week			1 [7]	9 [33]
Once a day			5 [36]	14 [44]
2-3 times a day			5 [36]	4 [15]
4 times a day or more			1 [7]	1 [4]
Way of delivery	NA	NA		
Caesarean section (n [%])			3 [23]**	2 [7]*
Natural (n [%])			10 [77]	27 [93]
Frequency of symptoms (IBS/FAP) (n [%])	NA	NA		
None			0 [0]	30 [100]
Once a week			4 [27]	0 [0]
2 to 4 times a week			10 [66]	0 [0]
Every day			1 [7]	0 [0]
Duration of symptoms ((n [%])				NA
Over a year	0 [0]*	1 [7]	10 [67]	
2 to 12 months	11 [73]	7 [47]	3 [20]	
≤2 months	3 [13]	7 [47]	2 [13]	

Table continues

	Crohn's disease (n=15)	Ulcerative colitis (n=15)	IBS/FAP-NOS (n=15 [9/6])	Control (n=30)
Physician Global Assessment				
Quiescent	1	0	NA	NA
Mild	0	3	NA	NA
Moderate	5	5	NA	NA
Severe	9	7	NA	NA
Faecal calprotectin (µg/g) (median[IQR])	1214 [627-1860]	1260 [401-1950]	22 [4.8 – 133]	NA
CRP (mg/l) (median[IQR])	21 [7-68]	4 [<2.5 – 7]	NA	NA
Crohn's disease localisation[1]				
Ileal (L1)	0	NA	NA	NA
Colonic (L2)	6	NA	NA	NA
Ileocolonic (L3)	9	NA	NA	NA
Proximal disease (L4)	5	NA	NA	NA
Crohn's disease behaviour[1]				
B1 (NSNP)	11	NA	NA	NA
B1p (NSNP+p)	2	NA	NA	NA
B2 (S)	0	NA	NA	NA
B2p (S + p)	0	NA	NA	NA
B3 (P)	0	NA	NA	NA
B3p (P + p)	2	NA	NA	NA
Ulcerative Colitis[1]				
Proctitis (E1)	NA	3	NA	NA
Left-sided (E2)	NA	2	NA	NA
Extensive (E3)	NA	10	NA	NA

All values were obtained at study inclusion. Localisation of IBD was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and MR enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. [1]Based on Paris classification for inflammatory bowel disease(14). * Missing data from one subject. ** Missing data from two subjects. ¥ Significant differences between all subgroups p<0.001, analysed using Wilcoxon-rank-sum tests.

IBS/FAP-NOS versus IBD

The results of the VOC analysis by FAIMS technique are shown in *Table 2*. For each analysis, the best performing of the four different applied classification models is shown. A complete overview of the data generated by the four classification models is given in supplemental Table 1-4. Faecal VOCs of IBS/FAP-NOS patients differed from IBD patients (AUC ± 95%CI, sensitivity, specificity, PPV, NPV, P-values; 0.94 (0.88-1), 1, 0.87, 0.79, 1, 0.00000002613). Corresponding Receiver Operating Characteristic (ROC)-curves are visualised in *Figure 1*. An overview of the complete outcome of the four performed classifiers is displayed in *supplementary tables 1-2*. In addition, there were significant differences between VOC

profiles of IBS/FAP-NOS patients and both UC and CD subgroups (*table 2, Supp table 1-4).* A complete overview of the data generated by the four classification models is given in supplemental Table 1-4.

IBS/FAP-NOS versus HC

Children diagnosed with IBS/FAP could not be discriminated from HC (AUC ± 95%CI, sensitivity, specificity, PPV, NPV, P-values; (0.59 (0.41-0.77), 0.6, 0.63, 0.45, 0.76, 0.1667) (*Table 2, Supp table 1-4, Figure 1*).

IBD versus HC

Patients with IBD could be distinguished from HC (AUC ± 95%CI, sensitivity, specificity, PPV, NVP, P-values; 0.96 (0.9-1), 0.93, 0.97, 0.97, 0.94, 0.0000000003962) (*Table 2, Supp table 1-4, Figure 1*). Both IBD subtypes UC and CD could each be differentiated from HC (*Table 2, Supp table 1-4).* Differentiation between CD and UC was not possible based on faecal VOC profiles (AUC ± 95%CI, sensitivity, specificity, PPV, NPV, P-values; (0.67 (0.47-0.88), 0.6, 0.8, 0.75, 0.67, 0.05799) (*Table 2, Supp table 1-4).*

IBS versus FAP

Patient with IBS could not be discriminated from patients with FAP-NOS (AUC ± 95%CI, sensitivity, specificity, PPV, NPV, P-values; (0.76 (0.44-1), 1, 0.6, 0.83, 1, 0.9504) (*Table 2, Supp table 1-4).*

Table 2. Performance characteristics for the discrimination of irritable bowel syndrome, functional abdominal pain-not otherwise specified, inflammatory bowel disease and healthy controls by faecal VOC analysis.

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	P
IBS/FAP-NOS vs IBD	0.94 (0.88 - 1)	1	0.87	0.79	1	0.00000002613
IBS/FAP-NOS vs CD	0.87 (0.73 – 0.1)	0.93	0.82	0.82	0.92	0.0001617
IBS/FAP-NOS vs UC	0.96 (0.91 – 1)	1	0.8	0.83	1	0.000007501
IBS/FAP-NOS vs HC	0.59 (0.41 - 0.77)	0.6	0.63	0.45	0.76	0.1667
IBS vs FAP-NOS	0.76 (0.44 – 1)	1	0.6	0.83	1	0.9504
IBD vs HC	0.96 (0.93 – 1)	0.93	0.97	0.97	0.94	0.0000000003982
UC vs HC	0.98 (0.94 – 1)	0.93	0.97	0.93	0.97	0.0000000005654
CD vs HC	0.95 (0.88 – 1)	0.93	0.93	0.88	0.97	0.0000001636
CD vs UC	0.67 (0.47 – 0.88)	0.6	0.8	0.75	0.67	0.05799

Sensitivities, specificities, p-values and AUCs are reported for the respective optimum cut-points.. Abbreviations: AUC, area under the curve; PPV: positive predictive value; NPV: negative predictive value; IBS: Irritable bowel syndrome; FAP-NOS: functional abdominal pain-not otherwise specified; IBD: Inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn's disease; HC: Healthy controls.

Duration of sample storage

Duration of storage of the collected faecal samples did not differ between IBS/FAP-NOS and HC. IBD samples were stored for a significantly longer period compared to both other subgroups (medium in months; CD 31.7; UC 45.1; IBS/FAP 0.6; HC 1.4, P<0.001).

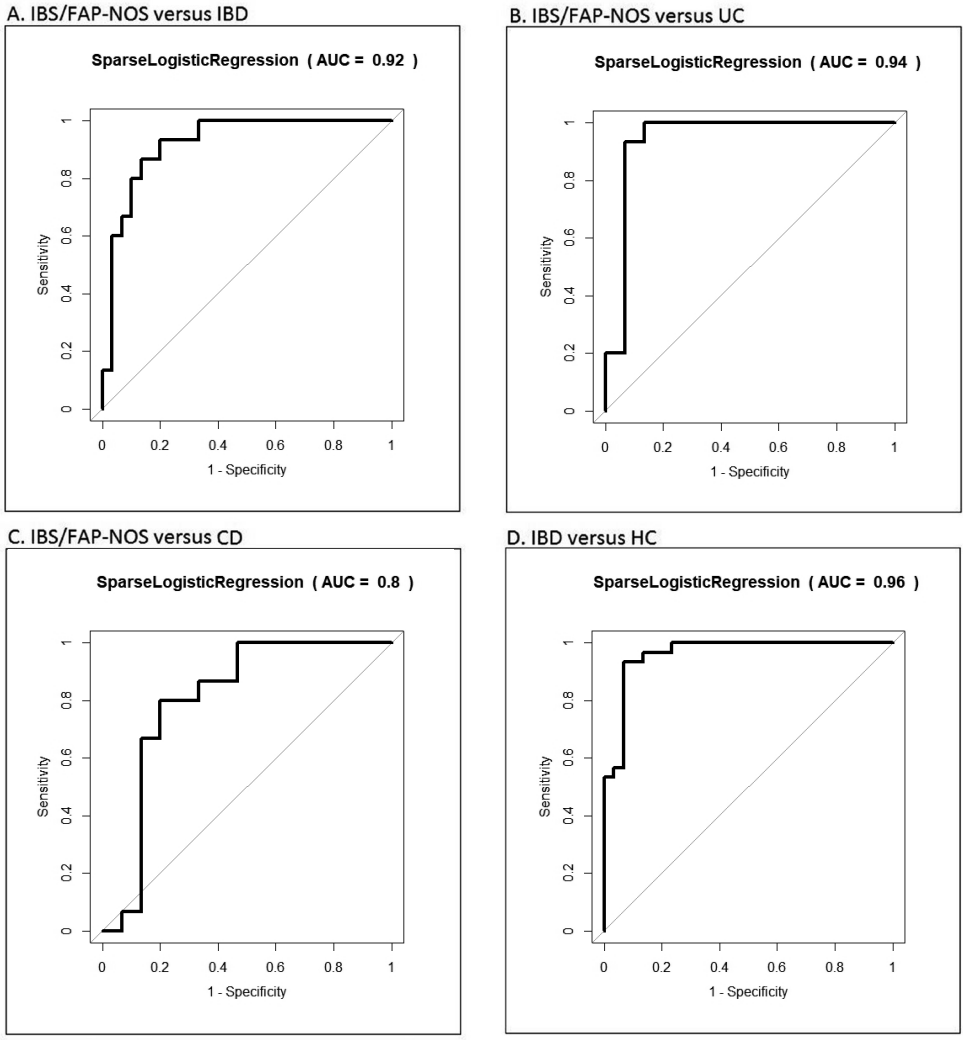

Figure 1. Receiver operating characteristics for irritable bowel syndrome/functional abdominal pain-not otherwise specified versus inflammatory bowel disease, ulcerative colitis and Crohn's disease and IBD versus healthy controls.

AUCs are reported for the Sparse logistic regression analyses. Abbreviations: AUC, area under the curve; IBS: Irritable bowel syndrome; FAP-NOS: functional abdominal pain-not otherwise specified; IBD: Inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn's disease; HC: Healthy controls.

DISCUSSION

In this multicentre case-control study, we observed that faecal VOC profiles could differentiate between paediatric IBS/FAP-NOS patients and children with new onset, treatment naïve IBD with high accuracy, but not from HC. Furthermore, we have validated previous study results indicating that IBD and HC could be discriminated by VOC composition with high accuracy.

Studies on the potential of faecal VOC profiling to discriminate paediatric IBS/FAP-NOS from IBD have not yet been performed. Ahmed et. al. compared faecal VOC profiles of 30 adult diarrhea-predominant IBS (IBS-D) patients, with 62 active CD, 48 active UC and 109 healthy subjects using gas chromatography-mass spectrometry (GC-MS)(16). In that study, IBS-D could be discriminated from IBD based on 44 significantly different levels of metabolites. Specifically, increased levels of 35 metabolites, mostly consisting of esters from short chain fatty acids and (derivates of) cyclohexanecarboxylic acid, were seen in the IBS-D group, whereas only 6 metabolites (aldehydes and ketones) were increased in CD, and three (1-propanol,2-methyl, undecane, methoxy-phenyl oxine) in UC. All of these metabolites were used to construct a discriminatory model with high diagnostic accuracy (AUC IBS-D vs CD 0.97; IBS-D vs UC 0.96; p=0.001). This diagnostic accuracy is comparable to that observed in our study. In addition, in the study by Ahmed and colleagues, significantly increased levels of 48 faecal metabolites were identified in adult IBS-D patients compared to HC (28 increased in IBS-D of which 22 were esters, 20 increased in HC with no specific pattern and all weak associations) and were used for a discriminatory model as well (AUC 0.92; $p<0.05$). In the present study, however, VOC profiles of IBS/FAP-NOS were not significantly different compared to VOC profiles of HC. This difference could possibly be explained by our relatively small sample size. Another explanation could be our heterogeneous IBS/FAP-NOS group in which subjects experienced a variety of symptoms (diarrhea, abdominal pain, bloating, constipation), whereas Ahmed. et. al. solely included patients with diarrhea-predominant IBS type. However, we observed no significant differences in VOC profiles between the two subgroups IBS and FAP-NOS. In addition, the diagnostic accuracy could differ due to the fact that GC-MS and FAIMS analyse metabolite signals based on different techniques[17]. However, since the diagnostic accuracy to differentiate between IBS/FAP-NOS and IBD is highly similar between these studies, we believe this had minimal influence on our study outcomes.

In a study performed by Walton et. al., differences in faecal VOC composition between adult IBS (n=26), active CD (n=22), active UC (n=20) and HC (n=19) were assessed by means of GC-MS. Increased levels of metabolites (especially propanoic and butanoic acids and products from amino acid fermentation) were found in all disease groups, but were only significantly elevated in CD patients[18]. Unfortunately, no AUC values were provided, which complicates comparison with our study. The authors did report considerable overlap of volatile compound levels between the different subgroups, and a wide dynamic range in all groups including the controls.

Volatile organic compounds are considered to reflect (changes in) microbiota composition and function(8). In a recent study, gut microbiota composition of patients with IBS (n=30)

and IBD (60 UC, 50 CD) were compared to HC (n=50) using DNA sequencing[19]. Here, progressive increase in abundance of species belonging to the phyla *Proteobacteria* and *Firmicutes* were detected from HC to IBS to IBD, whereas *Bacteriodetes* representation was gradually reduced along this spectrum. The fact that differences in the microbiota composition between IBS and HC were shown in this study, whereas we did not find these differences based on VOC pattern, contradicts the above-mentioned hypothesis. However, not all microbial changes might reflect in corresponding alterations of VOC composition. Furthermore, VOC composition is not only influenced by the gut microbiota but also by systemic metabolic processes and exogenous VOCs from diet and medication [20]. Despite these facts, our results are in line with the finding that microbial differences between IBD and HC are more pronounced than between IBS and HC.

Until now, paediatric studies on faecal VOCs as non-invasive biomarker for IBD have focused on the discrimination between IBD patients and healthy subjects, showing high accuracy to discriminate between the two groups[10, 21]. This high diagnostic value was found again in the current study. In the previous studies, however, children with abdominal symptoms were not included, limiting to reliable explore the specificity of VOC analysis to discriminate IBD from an intention to diagnose population. Since differentiation between IBS/FAP-NOS and active IBD is often challenging in daily practice, a strength of this study was that a paediatric IBS/FAP-NOS group was included. In addition, potential bias by colonic lavage, colonoscopy and medication on VOC composition was circumvented in IBD patients, since we only included *de novo* treatment-naïve IBD patients. Another strength is the participation of three medical centres, two tertiary hospitals and one general hospital. Furthermore, the performance characteristics of VOC analysis were assessed using supervised learning models, which are suitable for high-dimensional, complex datasets as they allow for reduction of dimensionality. These classifiers have previously been shown effective in studies involving human microbiota[22]. We have provided a complete overview of results of all applied learning models applied, as it is not known yet which model is most useful for faecal VOC analysis.

There were also several limitations. First, the researchers were not blinded for both VOC and data analysis Second, the IBS/FAP-NOS group represents an heterogeneous population, although no significant differences in VOC profiles were observed between these two subgroups. We therefore believe that the heterogeneity of this group has not significantly influenced study outcomes. Another limitation was that we have not taken potential influence of medication and diet on faecal VOC outcome into account, which could possibly have influenced outcome[23, 24]. In addition, it is known that faecal calprotectine has a lower accuracy in CD patients with isolated proximal or ileal CD. It may be possible that these patients can be distinguished based on VOC patterns, since microbiota changes may differ compared to HC and colonic CD and UC, and VOCs are considered to reflect microbiota composition and function. Based on this study, it is hard to draw conclusions about the application of VOC pattern-based diagnostics for specific groupgroups since we did not include CD patients with isolated proximal or ileal disease. Lastly, the potential influence of sample storage time on metabolic degradation of VOCs has not yet been studied. It could be hypothesised that storage duration influences VOC outcome by metabolic degradation, even in frozen state. Since storage time of the IBD

samples differed from that of the HC/IBS/FAP-NOS samples, this may possibly have affected outcome. However, the diagnostic accuracy to differentiate between IBD and HC is similar to our earlier studies, in which samples with comparable storage duration were used [10]. We therefore believe that metabolite degradation has had no substantial influence on presented results. Furthermore, it is important to point out that we used a pattern-recognition method in this study, rather than identification of individual volatiles. We have chosen to use specifically the FAIMS method since this device is an easy-to-use tool which could be suitable for clinical implementation. Analysis by tools which can detect VOCs on individual level are expensive and time-consuming, and can therefore not be sued in daily practice [12].

Our findings implicate that faecal VOC analysis may have potential to serve as non-invasive biomarker to discriminate IBS/FAP-NOS from IBD, with a higher specificity (87%) compared to the currently used FCP (specificity 68%), but not IBS/FAP-NOS from healthy state. Combination of biomarkers like FCP and faecal VOCs could possibly lower the rate of unnecessary colonoscopies in the diagnostic process of IBS/FAP-NOS patients. This was, however, a proof of principle study to explore the diagnostic value of faecal VOCs in IBS/FAP-NOS patients. Whether this technique sufficiently contributes to this diagnostic process needs to be elucidated in a larger 'intention-to-diagnose' cohort, in which patients with suspected IBD should be divided in a case and control group based on FCP, combined with upper- and lower endoscopy findings and radiography (like MR enteroclysis). In addition, discrimination between IBS/FAP-NOS-like symptoms and active disease in the course of IBD patients who present with nonspecific abdominal pain may be challenging in clinical practice, by limited specificity of FCP. Whether VOC analysis could serve as an additional biomarker in this specific population needs to be evaluated in future studies including IBD and non-IBD patients with high FCP levels.

In conclusion, we have shown that patients with IBS/FAP-NOS could be distinguished from IBD with a high diagnostic accuracy, but not from HC, by faecal VOC analysis using FAIMS technology. This signifies its potential role as additional non-invasive biomarker in the diagnostic work-up to discriminate (paediatric) functional gastrointestinal disorders from IBD.

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SUPPLEMENTARY MATERIAL

Supplemental Table 1. Performance characteristics with corresponding area under the curve, sensitivity, specificity, positive and negative predictive value of faecal volatile organic compound analysis for the discrimination of irritable bowel syndrome, functional abdominal pain-not otherwise specified, inflammatory bowel disease and healthy controls using the supervised classification method Sparse Logistic Regression

Analysis	p-value	AUC (± 95% CI)	Sensitivity (± 95% CI)	Specificity (± 95% CI)	PPV	NPV
IBS/FAP-NOS vs IBD	<0.0001	0.92 (0.84 – 1)	0.93 (0.68 – 1)	0.8 (0.61 – 0.92)	0.7	0.96
IBS/FAP-NOS vs CD	0.002	0.8 (0.63 - 0.98)	0.8 (0.52 - 0.96)	0.8 (0.52 - 0.96)	0.8	0.8
IBS/FAP-NOS vs UC	<0.0001	0.94 (0.84 - 1)	1 (0.78 - 1)	0.87 (0.6 - 0.98)	0.88	1
IBS/FAP-NOS vs HC	0.219	0.57 (0.4 - 0.75)	0.8 (0.52 - 0.96)	0.4 (0.23 - 0.59)	0.4	0.8
IBS vs FAP-NOS	0.950	0.76 (0.44 - 1)	1 (0.69 - 1)	0.6 (0.15 - 0.95)	0.83	1
IBD vs HC	<0.0001	0.96 (0.92 - 1)	0.93 (0.78 - 0.99)	0.93 (0.78 - 0.99)	0.93	0.93
UC vs HC	<0.0001	0.98 (0.94 – 1)	0.93 (0.68 - 1)	0.97 (0.83 - 1)	0.93	0.97
CD vs HC	<0.0001	0.95 (0.9 - 1)	1 (0.78 - 1)	0.73 (0.54 - 0.88)	0.65	1
CD vs UC	0.058	0.67 (0.47 – 0.88)	0.6 (0.32 – 0.84)	0.8 (0.52 – 0.96)	0.75	0.67

Supplemental Table 2. Performance characteristics with corresponding area under the curve, sensitivity, specificity, positive and negative predictive value of faecal volatile organic compound analysis for the discrimination of irritable bowel syndrome, functional abdominal pain-not otherwise specified, inflammatory bowel disease and healthy controls using the supervised classification method Random Forrest

IBS/FAP-NOS vs IBD	<0.0001	0.93 (0.85 - 1)	0.93 (0.68 - 1)	0.83 (0.65 - 0.94)	0.74	0.96
IBS/FAP-NOS vs CD	0.0004	0.86 (0.72 - 0.99)	1 (0.78 - 1)	0.6 (0.32 - 0.84)	0.71	1
IBS/FAP-NOS vs UC	<0.0001	0.96 (0.91 - 1)	1 (0.78 - 1)	0.8 (0.52 - 0.96)	0.83	1
IBS/FAP-NOS vs HC	0.282	0.55 (0.37 - 0.74)	0.8 (0.52 - 0.96)	0.4 (0.23 - 0.59)	0.4	0.8
IBS vs FAP-NOS	0.703	0.42 (0.039 - 0.8)	0.7 (0.35 - 0.93)	0.6 (0.15 - 0.95)	0.78	0.5
IBD vs HC	<0.0001	0.96 (0.9 - 1)	0.93 (0.78 - 0.99)	0.97 (0.83 - 1)	0.97	0.94
UC vs HC	<0.0001	0.96 (0.91 - 1)	0.93 (0.68 - 1)	0.97 (0.83 - 1)	0.93	0.97
CD vs HC	<0.0001	0.92 (0.82 - 1)	0.93 (0.68 - 1)	0.87 (0.69 - 0.96)	0.78	0.96
CD vs UC	0.398	0.52 (0.3 – 0.73)	1 (0.79 – 1)	0.13 (0.017 – 0.4)	0.54	1

Supplemental Table 3. Performance characteristics with corresponding area under the curve, sensitivity, specificity, positive and negative predictive value of faecal volatile organic compound analysis for the discrimination of irritable bowel syndrome, functional abdominal pain-not otherwise specified, inflammatory bowel disease and healthy controls using the supervised classification method Gaussian Process

Analysis	p-value	AUC (± 95% CI)	Sensitivity (± 95% CI)	Specificity (± 95% CI)	PPV	NPV
IBS/FAP-NOS vs IBD	<0.0001	0.94 (0.88 - 1)	1 (0.78 - 1)	0.87 (0.69 - 0.96)	0.79	1
IBS/FAP-NOS vs CD	0.0002	0.87 (0.73 - 1)	0.93 (0.68 - 1)	0.8 (0.52 - 0.96)	0.82	0.92
IBS/FAP-NOS vs UC	<0.0001	0.94 (0.85 - 1)	1 (0.78 - 1)	0.87 (0.6 - 0.98)	0.88	1
IBS/FAP-NOS vs HC	0.167	0.59 (0.41 - 0.77)	0.6 (0.32 - 0.84)	0.63 (0.44 - 0.8)	0.45	0.76
IBS vs FAP-NOS	0.570	0.48 (0.14 - 0.82)	0.5 (0.19 - 0.81)	0.8 (0.28 - 0.99)	0.83	0.44
IBD vs HC	<0.0001	0.95 (0.88 - 1)	0.93 (0.78 - 0.99))	0.93 (0.78 - 0.99)	0.93	0.93
UC vs HC	<0.0001	0.98 (0.94 - 1)	0.93 (0.68 - 1)	0.93 (0.78 - 0.99)	0.88	0.97
CD vs HC	<0.0001	0.93 (0.86 - 1)	0.87 (0.6 - 0.98)	0.9 (0.73 - 0.98)	0.81	0.93
CD vs UC	0.659	0.46 (0.24 – 0.67)	1 (0.78 – 1)	0.067 (0.0017 – 0.32)	0.52	1

Supplemental Table 4. Performance characteristics with corresponding area under the curve, sensitivity, specificity, positive and negative predictive value of faecal volatile organic compound analysis for the discrimination of irritable bowel syndrome, functional abdominal pain-not otherwise specified, inflammatory bowel disease and healthy controls using the supervised classification method Support Vector Machine

IBS/FAP-NOS vs IBD	0.000002783	0.89 (0.78 - 1)	1 (0.78 - 1)	0.8 (0.61 - 0.92)	0.71	1
IBS/FAP-NOS vs CD	0.01175	0.74 (0.54 - 0.94)	0.93 (0.68 - 1)	0.67 (0.38 - 0.88)	0.74	0.91
IBS/FAP-NOS vs UC	0.00001021	0.92 (0.79 - 1)	0.93 (0.68 - 1)	0.93 (0.68 - 1)	0.93	0.93
IBS/FAP-NOS vs HC	0.2878	0.45 (0.26 - 0.63)	0.53 (0.27 - 0.79)	0.57 (0.37 - 0.75)	0.38	0.71
IBS vs FAP-NOS	0.9354	0.74 (0.42 - 1)	0.9 (0.55 - 1)	0.6 (0.15 - 0.95)	0.82	0.75
IBD vs HC	0.0000000001787	0.92 (0.84 - 1)	0.93 (0.78 - 0.99)	0.9 (0.73 - 0.98)	0.9	0.93
UC vs HC	0.00000009573	0.93 (0.85 - 1)	0.93 (0.68 - 1)	0.93 (0.78 - 0.99)	0.88	0.97
CD vs HC	0.00000001636	0.95 (0.88 - 1)	0.93 (0.68 - 1)	0.93 (0.78 - 0.99)	0.88	0.97
CD vs UC	0.2062	0.59 (0.38 – 0.81)	0.6 (0.32 – 0.84)	0.73 (0.45 – 0.92)	0.69	0.65

CHAPTER 6

Simultaneous assessment of urinary and faecal volatile organic compound analysis in de novo paediatric inflammatory bowel disease

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ABSTRACT

Endoscopic evaluation is mandatory in establishing the diagnosis of paediatric inflammatory bowel disease (IBD), but unfortunately carries a high burden on patients. Volatile organic compounds (VOC) have been proposed as alternative, noninvasive diagnostic biomarkers for IBD. The current study aimed to assess and compare the potential of faecal and urinary VOC as diagnostic biomarkers for paediatric IBD in an intention-to-diagnose cohort. In this cohort study, patients aged 4–17 years, referred to the outpatient clinic of a tertiary referral centre under suspicion of IBD, were eligible to participate. The diagnosis was established by endoscopic and histopathologic assessment, participants who did not meet the criteria of IBD were allocated to the control group. Participants were instructed to concurrently collect a faecal and urinary sample prior to bowel lavage. Samples were analysed by means of gas chromatography–ion mobility spectrometry. In total, five ulcerative colitis patients, five Crohn's disease patients, and ten age and gender matched controls were included. A significant difference was demonstrated for both faecal (p-value, area under the curve; 0.038, 0.73) and urinary (0.028, 0.78) VOC profiles between IBD and controls. Analysis of both faecal and urinary VOC behold equal potential as noninvasive biomarkers for paediatric IBD diagnosis.

INTRODUCTION

Inflammatory bowel disease (IBD), classically divided into the two phenotypes—Crohn's disease (CD) and ulcerative colitis (UC)—presents in childhood in approximately 25% of de novo cases and its incidence has increased over the past decades to ten per 100,000 children [1,2]. Establishing IBD diagnosis in this population can be challenging, since presenting clinical symptoms are commonly heterogeneous and nonspecific. To date, the gold standard for the diagnosis of IBD remains endoscopic evaluation and histologic examination of mucosal biopsies, however, this is an invasive procedure which carries a high burden on patients [3]. Faecal calprotectin (FCP) has been demonstrated as a useful noninvasive biomarker in the selection of patients eligible for further endoscopic evaluation, however, its specificity (68%) is limited, resulting in false-positive results and, subsequently, the performance of unnecessary colonoscopies [4]. Therefore, the search for noninvasive diagnostic IBD tests remains warranted [5].

Volatile organic compounds (VOC) have emerged as a promising alternative. These gaseous molecules are products of physiologic and pathophysiologic metabolism and are emitted from various bodily excrements. Alterations in cellular metabolic processes (e.g., in diseased state, microbiome metabolism, and microbiota-host interaction) are reflected by changes in the emitted VOC composition [6]. For VOC analyses, gas-chromatography-mass spectrometry (GC–MS) is considered the gold standard, because of its ability to identify specific VOC based on their physiochemical properties [7]. A relatively new method has been introduced for VOC analyses, being gas chromatography–ion mobility spectrometry (GC–IMS), in which traditional GC is combined with an orthogonal separation of molecules based on ion mobility [8]. By combining these two methods, the sensitivity for VOC analyses can be increased.

Faecal VOC are most extensively studied in microbiome-associated gastro-intestinal diseases, since faecal VOC are largely produced during bacterial fermentation in the gut and reflect microbial composition, function, and microbiota–host interaction [9]. Faecal VOC analyses have demonstrated to allow for differentiation of a variety of diseases, including colorectal carcinoma, celiac disease, and IBD [5,10,11]. Similarly, testing of urinary VOC has potential to identify various diseases, such as cancer, diabetes and adult IBD [12–15]. IBD-related VOC can be detected in both exhaled breath and urine, however, exhaled breath analysis has shown to be challenging because of sample instability and the need for comprehensive analytical methods [7,16–19]. Collection of faecal has been described to evoke feelings of embarrassment and concerns about hygiene by patients [20]. In most countries, however, collection of urine samples is considered as more user friendly and, therefore, less of a burden for patients [21]. Furthermore, urine can be more easily provided on demand in situations where fast analysis is required to accelerate the diagnostic process. Therefore, it is aimed in the current study to assess and compare the diagnostic accuracy of faecal and urinary VOC in de novo paediatric IBD by means of GC–IMS.

METHODS

Subjects

The current case-control pilot study was part of an ongoing cohort study, in which patients (aged 4–17 years) are included at the paediatric gastroenterology department outpatient clinic of a tertiary referral centre (Amsterdam University Medical Centre; locations: VU medical centre (VUmc) and Amsterdam medical centre (AMC)) [22–25]. For the current study, patients suspected of IBD in the period May 2017 to February 2019 were eligible to participate. Exclusion criteria were a proven bacterial or viral gastroenteritis during the month prior to inclusion, use of immunosuppressive therapy, antibiotic or probiotic treatment in the last three months prior to inclusion, diagnosis with an immunocompromising disease, and insufficient ability to understand the Dutch language. Furthermore, participants who were not able to deliver both samples (i.e., faecal and urine) were excluded.

Participants, and in case of participants aged under 12 years, parents, were asked for informed consent after their first visit to the outpatient ward. The Medical Ethical Review Committee of the VUmc approved the study protocol (file number 2015.393, amendment number A2017.188).

All patients underwent diagnostic endoscopic evaluation because of suspected IBD based on clinical symptoms and/or biochemical abnormalities (e.g., elevated FCP). Participants were allocated to either the IBD or control group, based on the combination of endoscopy, histology, biochemical, and radiological findings, according to the currently applied international diagnostic criteria [1]. Disease localisation and behaviour in IBD cases were assessed based on the Paris classification [26]. IBD cases were matched to controls based on age and gender.

Sample and Data Collection

Participants were asked to concurrently collect a faecal and urine sample in containers (Stuhlgefäβ 10 mL, Frickenhausen, Germany), prior to bowel lavage. Participants were asked to store the samples in their freezer at home within one hour after collection and bring the samples to the hospital in cooled condition on their next regular appointment at the outpatient clinic. Samples were then directly stored at −20 °C until further handling. In addition to the sample collection, participants were asked to complete an online accessible and secured questionnaire (Castor EDC®) on dietary preferences, clinical symptoms, Bristol stool scale, bowel habits, extra-intestinal symptoms, residency, medical history, and medication use. Additional clinical information was collected from medical files, including IBD disease activity based on the physician global assessment (PGA) and laboratory values (CRP and FCP) [27,28].

Sample Preparation

Sample preparation was performed according to our standard operating procedure, previously described by Rouvroye et al. [29]. In short, a calibrated scale (Mettler Toledo, AT 261 Delta Range, Columbus, OH, USA) was used to weigh approximately 500 mg of faecal and frozen urine sample. Subsequently, the sample was transferred to a glass vial (20 mL, Frickenhausen, Germany), which was restored in a −20 °C freezer [22]. The faecal and

urine samples were sent to the BioMedical Sensors Lab, School of Engineering, University of Warwick (Coventry, UK) on dry ice (−80 °C) for VOC analyses.

Sample Analyses

Samples were analysed following the methods by Rouvroye et al. [29]. Samples were randomly analysed by means of GC-IMS (FlavourSpec®, G.A.S., Dortmund, Germany). This device consists of a GC column front-end, coupled with a drift tube IMS. The complex chemical mixture deriving from the sample headspace were pre-separated by the GC, before detection by IMS. Within the IMS, VOC ions were created by soft chemical-ionization (low-radiation tritium (H3) source). These ions were fed into a drift tube, which were propelled along it by an electric field. Against the flow of ions a buffer gas was added (in our case pure nitrogen). In general, larger molecules were struck more times than smaller molecules, losing momentum and, thus, taking longer to travel along the tube. As a result each substance's drift time is dependent on the interaction of the ion with the electric field and the buffer gas. A Faraday plate was used to measure the resulting ion current, as a function of time [30]. The GC–IMS was connected to an automatic sampling system with a chiller allowing processing of a batch of 32 samples kept in cooled condition (4 °C) until the start of the analyses to minimize sample degradation (PAL RSi, CTC Analytics AG, Zwingen, Switzerland). In the eight minutes before analysis, the samples' temperature was raised to 80 °C. Then, a syringe transported the headspace from the vial into the injector port of the instrument and into the GC column. Nitrogen 99.9% (3.5 bar), served as a carrier gas at 40 °C for GC separation, and as drift gas for IMS at 45 °C. GC flow rate was set at 20 mL/minute (34.175 kPa) for six minutes, while a 150 mL/min flow rate (0.364 kPa) was used for IMS.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS, IBM version 22.0) was used to perform the statistical analyses on demographics. Patient demographics and clinical characteristics were compared using a Fisher's exact test for dichotomous and ordinal data, and an independent t-test for parametric continuous data. In case of non-parametric continuous data, a Mann–Whitney U test was performed. A p-value < 0.05 was considered as a statistical significant difference.

Prior to the statistical analyses of VOC profiles, the GC–IMS data was pre-processed to only crop areas containing relevant chemical data, which is located centrally within the data. Group noise was filtered after setting a threshold limit and, finally, a correction was performed for instrumental disturbances by baseline correction. This reduced the data points per sample from around 11 million to a more manageable 100,000. Classification was performed applying a 10-fold cross-validated approach, in which the data was split into a 90% training set and a 10% test set. The 100 most discriminatory features were identified by Wilcoxon rank-sum test (undertaken within the fold) and then used to train five different classifiers: random forest, Gaussian process, sparse logistic regression, support vector machine, and neural network algorithms. Subsequently, these models were applied to the test set. This process was repeated until every sample was classified as a test sample and from the resultant classification probabilities, statistical results were calculated. This method is part of our standard pipeline used in similar studies [18,29,31]. Statistical analyses

were performed using R packages, including randomForest for random forest, glmnet for sparse logistic regression, kernlab for support vector machines, neuralnet for neural network analysis, and kernlab for Gaussian processes.

RESULTS

Baseline Characteristics

In total, ten IBD (five UC and five CD) and ten matched controls were included from the intention to diagnose cohort (Figure 1). The baseline characteristics are listed in Table 1. Faecal calprotectin was significantly higher (median 1208 µg/g, p-value 0.009) in the IBD group compared to the control group (median 50 µg/g). No differences were found for the remaining variables, as listed in Table 1.

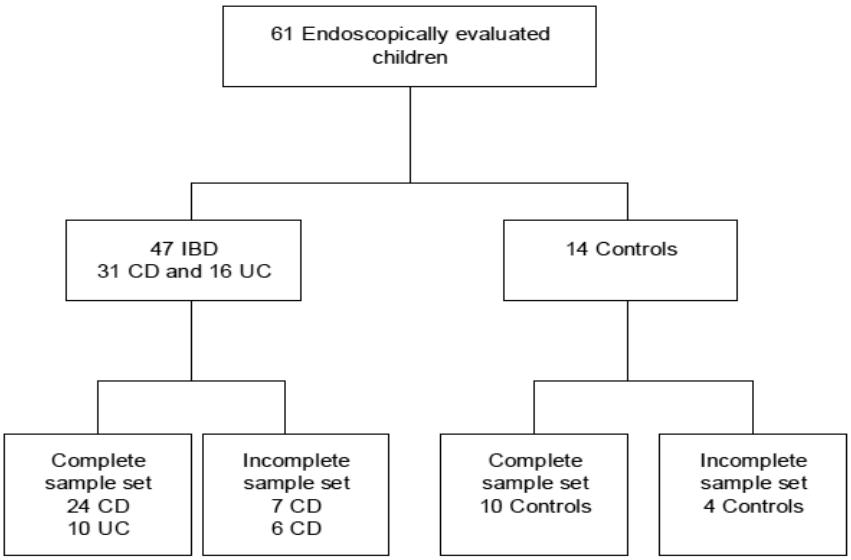

Figure 1. Study design. Inclusion flowchart Abbreviations: n, number; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

In Table 2, the diagnoses established after diagnostic endoscopy in the control group are listed. Three children were diagnosed with irritable bowel syndrome or functional abdominal pain, three had an alternative diagnosis, and in four children no alternative diagnosis was established. Three children in the IBD group used prescribed medication, opposed to six children in the control group (Table 3).

VOC Analysis

The discrimination of IBD from controls based on faecal VOC profiles, was statistically significant when training the algorithm using the neural network analysis (area under the curve (AUC) (95% confidence interval (CI)), p value, sensitivity, specificity; 0.73 (0.47–0.99),

0.038, 0.70, 0.90), Figure 2A. For urinary VOC profiles, a similar accuracy was reached for the discrimination between IBD and controls using the sparse logistic regression classifier (0.78 (0.57–1), 0.028, 0.80, 0.70), Figure 2B. The results of the remaining statistical analyses are listed in Supplemental Tables 1 and 2. An example of GC–IMS output of faecal and urine, collected by the same participant, is displayed in Figure 3.

Table 1. Baseline characteristics.

	IBD (n = 10)	Controls (n = 10)	p-value
Sex male (n, %)	4 (40)	4 (40)	1.00
Age years (median, IQR)	15.0 (10.4–17.1)	14.2 (9.6–16.6)	0.71
BMI kg/m2 (mean, SD)	18.9 (4.35)	21.3 (3.66)	0.20
Bristol stool scale (median, IQR)	6.0 (4.0–6.5)	6.0 (3.0–6.0)	0.97
FCP µg/g (median, IQR)	1208.0 (1023.5–3086.5)	50.0 (14.5–900)	0.01
CRP mg/L (median, IQR)	<2.5 (<2.5–39.5)	<2.5 (<2.5–4.35)	0.15
Sample weight faecal mg (median, IQR)	500.5 (485.3–512.5)	502.5 (486.3–508.8)	0.88
Sample weight urine mg (median, IQR)	644.5 (532.8–726.0)	628.5 (592.0–663.3)	0.79
Storage time months (median, IQR)	6.5 (5.8–6.5)	10 (6.75–15.5)	0.14
Physician's Global Assessment			
Quiescent	0		
Mild	6		
Moderate	4		
Severe	0		
Crohn's Disease (n = 5) localisation			
Ileal (L1)	2		
Colonic (L2)	1		
Ileocolonic (L3)	2		
Proximal disease (L4)	1		
Crohn's Disease behaviour			
B1 (NSNP)	4		
B1p (NSNP +p)	1		
B2 (S)	0		
B2p (S+p)	0		
B3 (P)	0		
B3p (P+p)	0		
Ulcerative Colitis (n = 5) localisation			
Proctitis (E1)	0		
Left sided (E2)	2		
Extensive (E3)	3		

Values were obtained at inclusion. CD and UC localisation and behaviour were determined using the Paris classification, based on findings during ileocolonoscopy and esophagogastroduodenoscopy and magnetic resonance enteroclysis before start of treatment [26]. Abbreviations: CRP, C-reactive protein; FCP, faecal calprotectin; IQR, interquartile range; SD, standard deviation; NSNP, non stricturing non-penetrating; S, stricturing; P, penetrating; p, perianal disease.

Table 2. Diagnoses controls.

Diagnosis	Number
Irritable bowel syndrome	2
Functional abdominal pain	1
Helicobacter pylori infection	1
Juvenile polyp	1
Multiple angiodysplasia	1
IBD excluded without alternative diagnosis	4

All diagnoses in the controls were established after diagnostic endoscopy. In four children, no diagnosis was established. Abbreviations: IBD, inflammatory bowel disease.

Table 3. Medication usage.

IBD	n	Controls	n
Number of participants receiving medication	3		6
Type of prescribed medication			
Ferrous fumarate	1	Macrogol	2
Ethinylestradiol/Desogestrel	1	Ibuprofen/Naproxen	2
Acrivastine	1	Formoterol/Beclamethason*	1
		Mebeverine	1
		Omeprazole	1
		Montelukast	1
		Ferrous fumarate	1
		Ondansetron	1
		Methylphenidate	1

All values were obtained at inclusion. In the control group, more medication usage was reported. Several participants were prescribed more than one type of medication. Abbreviations: n, number; IBD, inflammatory bowel disease; *, inhaler.

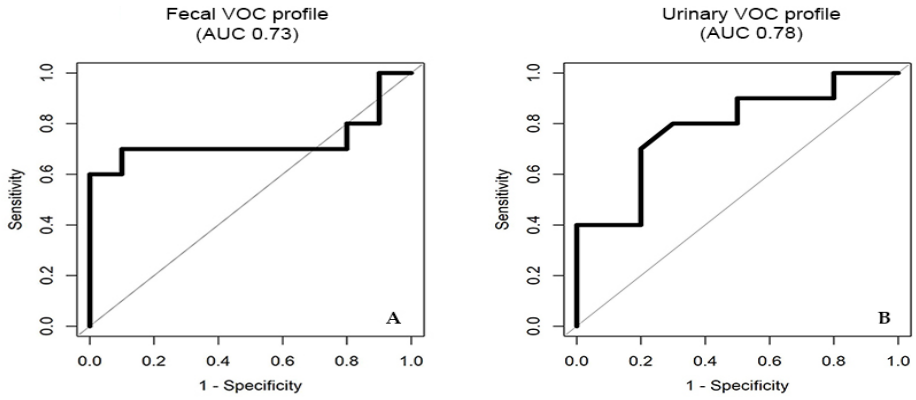

Figure 2. Faecal and urinary VOC profiles for the differentiation between inflammatory bowel disease and controls. (A)Receiver operating characteristic (ROC) curves display the ROC curve for the faecal VOC profile, and (B) displays the ROC curve for urinary VOC profile for the discrimination of IBD from controls. Abbreviations: AUC, area under the curve.

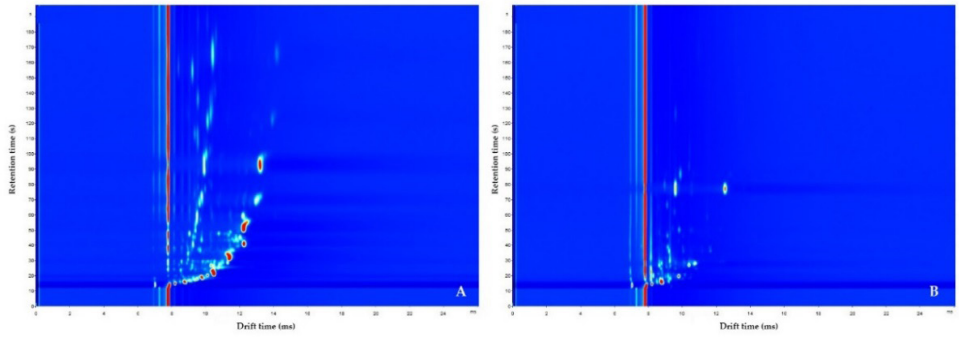

Figure 3. Example output of GC-IMS analysis. Here, the GC-IMS of faecal (A) and urinary (B) VOC profiles of one IBD patient are displayed. On the y-axis, the retention time indicates the separation time of the analyte by means of gas chromatography. The color represents the VOC concentration using color spectrum from blue to red, indicating low concentration to high concentration, respectively.

DISCUSSION

In the current study, the diagnostic accuracies of faecal and urinary VOC profiles to detect IBD were assessed and compared, using an endoscopy-controlled intention to diagnose cohort. Diagnostic accuracies were similar for both faecal and urine VOC profiles.

The potential of faecal VOC analysis in paediatric IBD has been previously studied. Van Gaal et al. used field asymmetric ion mobility spectrometry (FAIMS) to analyse faecal VOC composition, which allowed for discrimination between 36 paediatric IBD patients and 24 healthy controls (HC) (AUC 0.76, sensitivity 0.79, specificity 0.79, p-value < 0.001) [23]. In another study on paediatric IBD, a robust difference in faecal VOC profiles between 26 UC patients and 28 HC (AUC, sensitivity, specificity, p-value; 1.00, 1.00, 1.00, <0.001) and between 29 CD and 28 controls (0.85, 0.86, 0.67, <0.001) were observed [25]. In the current study, similar diagnostic accuracies were identified for the discriminationof IBD from controls. However, due to smaller sample size, no sub-analyses could be performed to assess the diagnostic accuracy of faecal VOC analyses in the discrimination of both entities from controls.

To the best of our knowledge, the current study is the first in which the potential of urinary VOC profiles in paediatric IBD is assessed. In adult IBD, urinary VOC analysis allowed for discrimination between IBD and HC by means of an eNose device (Fox 4000; AlphaMOS, Toulouse, France) and FAIMS [15]. The FAIMS analysis demonstrated similar diagnostic accuracies (AUC 0.75, p-value < 0.001), while VOC profiles analysed by the eNose device (AUC 0.88, p-value < 0.001) exceeded the accuracy as observed in the current study. The variation in diagnostic accuracies can be explained by several aspects. In the current study, a paediatric intention to diagnose cohort was established, whereas in the study by Arasaradnam et al., adult patients with an established IBD diagnosis which received treatment were included. Additionally, IBD cases were compared to healthy controls, rather than with symptomatic patients, in which it is expected that there is a larger difference between healthy controls and cases, than is seen between symptomatic non IBD patients compared to IBD cases. Furthermore, different analytical techniques were applied, Arasaradnam et al. used an eNose device and FAIMS, whereas in the current study a novel technique, GC–IMS, was applied. Lastly, in the current study, a more diverse control group was included, which could possibly have led to a lower diagnostic accuracy.

Since the algorithms applied in the current study have different internal learning structures and certain algorithms tend to perform better in different data collections, multiple algorithms were assessed in order to identify the best performing algorithm per bodily excrement [32]. Additionally, it is hypothesised that different VOCs can be detected in urine and faecal. Analyses of faecal VOCs is believed to reflect the gut microbiota composition and local inflammatory processes. On the contrary, urinary VOCs associated with IBD are more likely to be a combination of compounds that diffused from the intestine into the bloodstream and VOCs that are associated with general inflammation, which therefore can be detected in urine [15,33]. However, to confirm this hypothesis, in-depth studies are needed to identify specific VOCs in both urine and faecal.

The main strength of this study is the inclusion of cases and controls from an endoscopy-controlled intention-to-diagnose cohort. Especially when aiming to find a novel non-invasive biomarker to prevent the performance of (unnecessary) endoscopic assessment of paediatric patients, it is important to include a realistic selection of patients with gastro-intestinal symptoms representing clinical practice, since this is the population that would potentially gain the most from an alternative diagnostic biomarker. In addition we included de novo IBD patients, circumventing bias by VOC altering effects of bowel lavage and immunosuppressive medication [34]. All controls were successfully matched to a case based on age and gender. Additionally, there were no differences in BMI between groups, which is a known factor affecting the VOC profile outcome [35]. By comparison of two different bodily excrements, concurrently collected by participants, we were able to identify the most suitable bodily excrement to analyse. Urine offers practical advantages compared to faecal while maintaining a similar diagnostic accuracy and should, therefore, be considered as a clinically implementable bodily excrement for VOC analysis. To assess the diagnostic accuracy of faecal and urinary VOCs, we chose to perform the analysis by means of GC–IMS. These analyses are quick (approximately ten minutes per sample), sample pre-treatment is not required and the system setup is relatively easy [8,18,36]. Furthermore, machine learning algorithms can be trained to quickly recognize a disease specific profile without the need of the identification of individual VOC, making the GC–IMS output suitable for future clinical application. Lastly, there was no statistical difference between sample storage time between groups, which is demonstrated to affect VOC profile outcome [21,22].

This pilot study is limited by a relatively small sample size; consequently, no sub-analyses could be performed, assessing the diagnostic accuracy of faecal and urinary VOCs in the discrimination of both phenotypes from controls. Additionally, cases and controls could not be matched based on the possible confounding effect of medication use because of the variety of types of medication that were reported. One control patient was using esomeprazole, a proton pump inhibitor, that has been shown to influence the faecal VOC pattern [35,37]. Although the other reported medicines have not been described to affect the VOC profile, this difference might have influenced the VOC outcome.

Both faecal and urinary VOC analyses allowed for the discrimination of de novo IBD from controls by means of GC–IMS, with similar diagnostic accuracies, making them potential adjuvant biomarkers in the diagnostic work up of IBD.

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SUPPLEMENTARY MATERIAL

Table S1. **Statistical analyses of Faecal VOC profiles for the discrimination of IBD from controls**

	AUC [95% CI]	Sensitivity [95% CI]	Specificity [95% CI]	PPV	NPV	p-value	Threshold
Sparse logistic regression	0.53 [0.25-0.81]	0.4 [0.12-0.74]	0.8 [0.44-0.97]	0.67	0.57	0.602	0.140
Random forest	0.53 [0.24-0.82]	0.3 [0.067-0.65]	1.0 [0.69-1.00]	1.00	0.59	0.425	0.936
Gaussian Process	0.69 [0.44-0.94]	0.9 [0.55–1.00]	0.5 [0.19-0.81]	0.64	0.83	0.083	0.435
Support Vector Machine	0.60 [0.34-0.86]	0.8 [0.44-0.97]	0.5 [0.19-0.81]	0.62	0.71	0.218	0.618
Neural Network	0.73 [0.47-0.99]	0.7 [0.35-0.93]	0.9 [0.55-1.00]	0.88	0.75	*0.038*	0.499

Here, all the statistical analyses performed on the faecal VOC profiles are listed. For these analyses, 100 features were used in the 10 fold cross validation. The neural network analysis is identified as the best performing algorithm for the discrimination of IBD from controls based on faecal VOC profiles.
Abbreviations: AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; IBD, inflammatory bowel disease; VOC, volatile organic compounds.

Table S2. **Statistical analyses of urinary VOC profiles for the discrimination of IBD from controls**

	AUC [95% CI]	Sensitivity [95% CI]	Specificity [95% CI]	PPV	NPV	p-value	Threshold
Sparse logistic regression	0.78 [0.57-1.00]	0.8 [0.44-0.97]	0.7 [0.35-0.93]	0.73	0.78	*0.028*	0.502
Random forest	0.69 [0.44-0.94]	1.0 [0.69–1]	0.5 [0.19-0.81]	0.67	1.00	0.070	0.876
Gaussian Process	0.35 [0.09-0.61]	0.6 [0.26-0.88]	0.5 [0.19-0.81]	0.55	0.56	0.879	0.542
Support Vector Machine	0.47 [0.19-0.75]	0.4 [0.12-0.74]	0.8 [0.44-0.97]	0.67	0.57	0.604	0.608
Neural Network	0.41 [0.14-0.68]	0.6 [0.26-0.88]	0.5 [0.19-0.81]	0.55	0.56	0.764	0.293

Here, all the statistical analyses performed on the urinary VOC profiles are listed. For these analyses, 100 features were used in the 10 fold cross validation. The Sparse logistic regression analysis is identified as the best performing algorithm for the discrimination of IBD from controls based on faecal VOC profiles.
Abbreviations: AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; IBD, inflammatory bowel disease; VOC, volatile organic compounds.

CHAPTER 7

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

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ABSTRACT

Background

Inflammatory bowel disease (IBD) is diagnosed and monitored using endoscopic assessment, which is invasive and costly. In this study, potential of faecal volatile organic compounds (VOC) analysis for IBD detection and identification of disease activity was evaluated.

Methods

IBD patients visiting outpatient clinics of participating tertiary hospitals were included. Active disease was defined as FCP ≥250 mg/g, remission as FCP <100 mg/g with Harvey Bradshaw Index <4 for Crohn's disease (CD) or Simple Clinical Colitis Activity Index <3 for ulcerative colitis (UC). Healthy controls (HC) were patients without mucosal abnormalities during colonoscopy. Faecal samples were measured using gas chromatography-ion mobility spectrometry.

Results

A total of 280 IBD patients collected 107 CDa, 84 CDr, 80 UCa and 63 UCr samples. Additionally, 227 HC provided one faecal sample. UC and CD were discriminated from HC which was highly accurate (AUC (95%CI): UCa vs HC 0.96(0.94-0.99); UCr vs HC 0.95(0.93-0.98); CDa vs HC 0.96(0.94-0.99); CDr vs HC 0.95(0.93-0.98)). There were small differences between UC and CD (0.55(0.50-0.6)) and no differences between active disease and remission (UCa vs UCr 0.63(0.44-0.82); CDa vs CDr 0.52(0.39-0.65)).

Conclusion

Our study outcomes imply that faecal VOC analysis holds potential for IBD detection but not for monitoring disease activity.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic gastrointestinal diseases characterised by periods of relapse and remission, and are together referred to as inflammatory bowel disease (IBD). A worldwide increase in incidence as well as prevalence of IBD has been observed. In Europe, prevalence of 505 UC patients per 100.000 persons (Norway) and 322 CD patients per 100.000 persons (Germany) have been reported[1]. 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 a risk of complications. Therefore the identification of a specific, non-invasive IBD biomarker or biomarkers remains warranted.

Volatile organic compounds (VOCs) are gaseous carbon-bound chemicals and are thought to represent both metabolic processes in the human body and the interaction between gut microbiota and host[2]. These molecular end-products can be found in all bodily excretions dependent on their volatility and sample temperature. The great potential of faecal VOC profiles as non-invasive biomarkers have been described for various gastrointestinal diseases[3-5]. The detection of paediatric IBD using faecal VOC patterns has also been subject of various studies, with promising results[6-8]. The literature on its potential in adults, who are generally characterised by more comorbidity and medication use, is limited. The aim of the current study is to validate the potential of faecal 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 Centre (MUMC+) in Maastricht), and two district hospitals (OLVG West in Amsterdam and Spaarne Gasthuis (SG), location Hoofddorp and Haarlem) all located in The Netherlands.

Ethical statement

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval 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 file number 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 completed, 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 were asked to participate in this study[9]. Patients were asked to collect a faecal sample and to complete a questionnaire, which included information on age, gender, BMI, smoking status, abdominal symptoms, medication use, comorbidity and clinical disease activity based on the Harvey Bradshaw Index (HBI) for CD patients and the Simple Clinical Colitis Activity Index (SCCAI) for UC patients [10, 11]. Active disease was defined as FCP level of ≥250 mg/g. Remission was defined as FCP <100 mg/g combined with HBI <4 points or SCCAI <3 points. All IBD patients were included in the primary statistical analysis assessing the diagnostic potential of faecal 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 differences in faecal 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[12].

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 asked to complete a questionnaire on demographics, 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, subjects were only included as HC if no histologic abnormalities were detected. Exclusion criteria were a history of bowel disease (e.g. celiac disease, IBD, CRC), failure to perform complete colonoscopy because of various reasons (e.g. inadequate bowel cleansing, pain) and/or collection of insufficient faecal sample mass to perform VOC analysis.

Sample collection

Inflammatory bowel disease Amsterdam University Medical Centres

Between February 2015 and November 2017, IBD patients were asked to collect two faecal samples (Stuhlgefäß 10ml, Frickenhausen, Germany) from the same bowel movement prior to the consult: one for FCP levels and one for VOC analysis. The FCP sample was sent to the hospital by mail. The sample for VOC analysis was the participant's 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.

Inflammatory bowel disease Maastricht University Medical Centre

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 delivery. One for FCP measurements (using ELISA in Amsterdam UMC and FEIA in MUMC+) and one for research purposes. The second sample was stored in the freezer at -80°C.

Healthy controls

Between February 2015 and November 2017, patients from the Amsterdam UMC, OLVG West and SG collected a faecal 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

For the faecal VOC analysis, subsamples of 500mg per participant (with a maximum deviation 5%) were weighted on a calibrated scale (Mettler Toledo, AT 261 Delta Range, Ohio, United States). This was done whilst keeping the samples on dry-ice to avoid thawing of the samples during preparation. Samples were then labelled and re-stored in glass vials (20ml headspace vial, Thames Restek, Saunderton, UK), in a -24°C freezer until further handling. As confirmed by our research team in a previous sampling method study on faecal VOC analysis, the 500mg weight was carefully chosen to provide an optimum amount of VOCs in the headspace of the sample [13]. The subsamples were shipped to the University of Warwick on dry ice for faecal VOC analysis.

Faecal volatile organic compound analysis

Faecal samples were analysed using gas chromatography ion mobility spectrometry (GC-IMS, FlavourSpec®, G.A.S., Dortmund, Germany) conform our previously published studies[14, 15]. In short, the instrument consists of a gas chromatography column (GC), coupled to an ion mobility spectrometry (IMS) drift tube. First, the GC column provides separation of the mixture of chemicals found in the headspace of the faecal sample. This separation is based on the levels of attraction between the molecules in the faecal volatile mixture (mobile gas phase) and molecules in the stationary phase. These chemicals are then driven to the IMS column. Within the IMS, a low-radiation tritium (H3) source causes to chemicals to create reactant ions with the gas atmosphere in the column. These ionised VOCs then travel at atmospheric pressure against the flow of an inert drift gas, in this case, nitrogen. In general, larger molecules collide more times than smaller molecules, losing momentum and thus, taking longer to travel along the tube. The drift time of each is substance therefore determined by the ion's mass and geometrical structure. The resulting ion current is measured by an electrometer as a function of time[16]. Figure 1 gives an example output from the instrument. Here the y-axes is the retention time and the x-axes is the IMS drift time. As can be seen, the majority of the image is blue, which is the signal when there is no chemicals present. The white and red 'blobs' are chemicals being detected. The red line through the whole image is the output when there no chemicals present and is the response to the carrier gas.

Statistical analysis

Prior to the statistical analyses, a number of pre-processing steps are used to reduce the dimensionality of the data to speed up the analysis steps. As can be seen from Figure 1, the majority of the data (non-background) is located at the central section of the plot, thus there is high data dimensionality, but low information content. Therefore, the data is cropped to only the 'areas of interest'. This area is decided upon by visual inspection of all of the

samples, to ensure that all of the chemical information is included. A threshold was applied to remove background noise and finally corrected for instrumental disturbances by baseline correction. This reduces data points per sample from approximately 11 million to a more manageable 100,000. The data was split into three sets, 70% for training and validation and 30% as test set. A 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 training and validation sets.

RESULTS

Baseline characteristics

A total of 280 IBD patients (164 CD patients, 112 UC patients, 4 IBD-undetermined) were included. Sample collection is depicted in *Figure 2.* In total, 495 faecal 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 (CDa), 84 were CD in remission (CDr), 80 were active UC (UCa) and 63 were UC in remission (UCr) 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 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 statistically significant difference in gender and smoking status between IBD patients and HC. The mean age of the IBD group was 46.1 (±29.8) compared to 60.6 (±11.8) for HC. The 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)*.

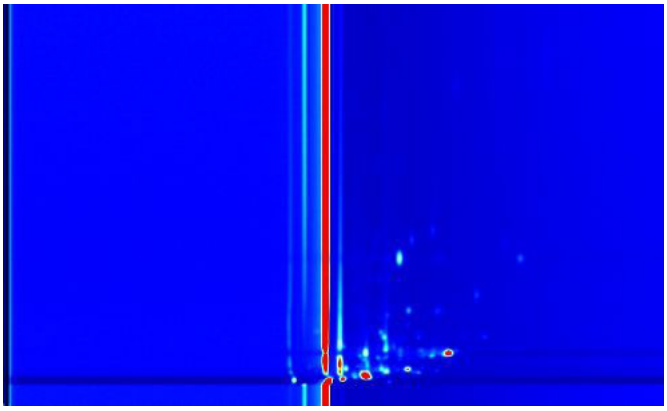

Figure 1. Example output of gas chromatography – ion mobility spectrometer. The y-axes is the retention time and the x-axes is the IMS drift time. White and red blobs are chemicals being detected. The red line through the whole image is the output when there no chemicals present and is the response to the carrier gas.

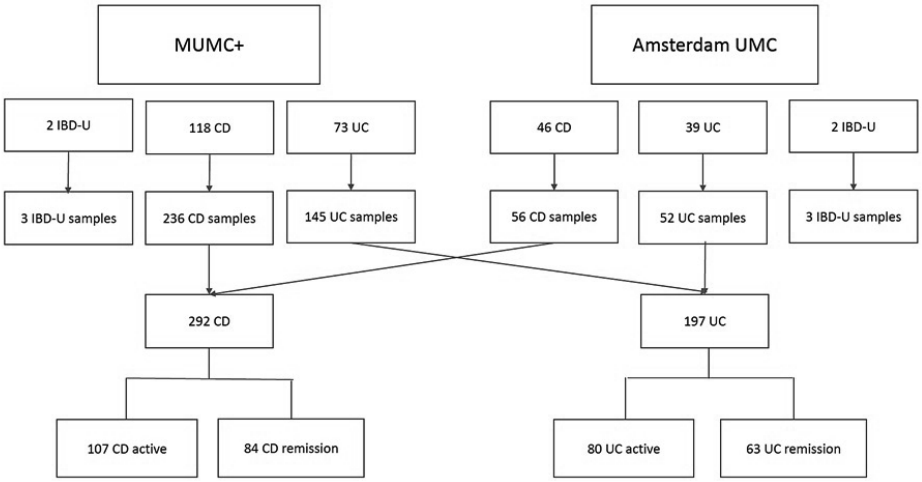

Figure 2. Sample collection scheme. In total, 495 faecal IBD samples (292 CD, 197 UC, 6 IBD-U) were collected during the follow-up period of this study. Of these, 107 were active CD, 84 were CD in remission, 80 were active UC and 63 were UC in remission according to the previously mentioned criteria. Abbreviations: MUMC+, Maastricht University Medical Centre; Amsterdam UMC, Amsterdam University Medical Centres; IBD-U, Inflammatory bowel disease undetermined; CD, Crohn's Disease; UC, ulcerative colitis.

Table 1. Baseline characteristics

	CD (n = 164)	UC (n=112)	IBD-U (n=4)	HC (n=227)
Gender, f (n, [%])	107 [65.2]	54 [48.2]	1 [25]	129 [56.8]
Age, y mean ± s.d.	45.3 [19-82]	51.1 [18-80]	49 [36 – 64]	60.6 ±11.8
Smoking				
Current (n, [%])	37 [22.6]	6 [5.4]	1 [25]	37 [16.3]
Past (n, [%])	56 [34.1]	59 [52.7]	3 [75]	92 [40.5]
Never (n, [%])	67 [40.9]	43 [38.4]	0 [0]	98 [43.2]
Medication use at inclusion				
Aminosalicylates (n, [%])	22 [13.4]	54 [50.0]	1 [25]	NA
Corticosteroids (n, [%])	26 [15.9]	14 [12.5]	0 [0]	NA
Immunosuppresives				
Thiopurines (n, [%])	58 [35.4]	28 [25.0]	2 [5]	NA
Methotrexate (n, [%])	12 [7.3]	3 [2.7]	0 [0]	NA
Biologicals				
Anti-TNF (n, [%])	61 [37.2]	26 [23.2]	1 [25]	NA
Selective (n, [%])	5 [3.0]	0 [0]	0 [0]	NA

Table continues

		CD (n = 164)	UC (n=112)	IBD-U (n=4)	HC (n=227)
Antibiotics 3 months prior to inclusion (n, [%])		7 [4.3]	6 [5.4]	0 [0]	32 [14.1]
Surgery prior to inclusion (n, [%])		72 [43.9]	15 [14.3]	0 [0]	NA
Ileocecal resection (n, [%])		57 [37.8]	2 [1.2]*	NA	NA
(Partial) Colectomy (n, [%])		31 [18.9]	15 [9.1]	NA	NA
Small bowel resection (n, [%])		5 [3.0]	0 [0]	NA	NA
Montreal Classification at inclusion					
Age at diagnosis, y					
A1 ≤ 16 (n, [%])		20 [13.4]	4 [3.6]	1 [25]	NA
A2 = 17 – 40 (n, [%])		93 [56.7]	61 [54.5]	1 [25]	NA
A3 ≥ 41 (n, [%])		51 [31.1]	47 [42.0]	2 [50]	NA
Localisation Crohn's disease					
L1 Ileal (n, [%])	L1 L1 + L4	47 [28.7] 5 [3.0]	NA	NA	NA
L2 colonic (n, [%])	L2 L2 + L4	38 [23.2] 2 [1.2]	NA	NA	NA
L3 ileocolic (n, [%])	L3 L3 + L4	62 [31.7] 6 [3.7]	NA	NA	NA
L4 proximal (n, [%])		4 [2.4]	NA	NA	NA
Behaviour Crohn's disease**					
B1 nonstricturing/ nonpenetrating (n, [%])	B1 B1p	79 [48.1] 20 [12.2]	NA	NA	NA
B2 stricturing (n, [%])	B2 B2p	30 [18.3] 9 [5.5]	NA	NA	NA
B3 penetrating (n, [%])	B3 B3p	17 [10.4] 8 [4.9]	NA	NA	NA
Extent ulcerative colitis**					
E1 Proctitis (n, [%])		NA	9 [8.0]	NA	NA
E2 Left-sided (n, [%])		NA	53 [47.3]	NA	NA
E3 Pancolitis (n, [%])		NA	49 [43.8]	NA	NA

Abbreviations: CD, Crohn's Disease; UC, ulcerative colitis; IBD-U, inflammatory bowel disease undetermined; HC , healthy control; f, female; y, year; n, number of participants; NA, not applicable. *Ileocecal resection in UC group was solely performed in combination with (partial) colectomy **Montreal classification of one participant missing.

Faecal 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 data generated using all five classifiers based on the 20, 50 and 100 most discriminative features are given in Supplemental Table 1-3. In addition, in Supplemental Table 4, the results of our post-hoc

analysis comparing only samples collected in the same period of time are shown.

Inflammatory bowel disease versus healthy controls

IBD patients were 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 1-4)*. Likewise, high diagnostic accuracy was found for 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 1-4)*. 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 1-4)*. Corresponding Receiver Operating Characteristic (ROC) curves are visualised in *Figure 3a-d.*

Table 2. Inflammatory bowel disease activity scores

	CDa (n=107)	CDr (n=84)	UCa (n=80)	UCr (n=63)
FCP (mean ± s.d.)	664.6 ± 448.9	29.9 ± 24.4	1108.5 ± 952	39.5 ± 27.3
SCCAI (mean ± s.d.)	NA	NA	2.9 ± 0.38	0.8 ± 0.20
HBI (mean ± s.d.)	4.1 ± 0.42	1.1 ± 0.16	NA	NA
Medication use at time of sample collection				
Aminosalicylates (n, [%])	11 [10.3]	19 [22.6]	35 [43.8]	26 [41.3]
Corticosteroids (n, [%])	22 [20.6]	8 [8.3]	15 [18.8]	4 [6.3]
Thiopurines (n, [%])	34 [31.8]	32 [39.1]	21 [26.3]	33 [52.4]
Methotrexate (n, [%])	7 [6.5]	3 [3.6]	2 [2.5]	0 [0]
Anti-TNF (n, [%])	46 [43.0]	52 [61.9]	38 [47.5]	26 [41.3]
Selective biologicals (n, [%])†	2 [1.9]	0 [0]	1 [1.3]	0 [0]
Antibiotics (n, [%])	1 [0.9]	1 [1.2]	4 [5.0]	1 [1.6]

IBD Activity scores. Abbreviations: CDa, active Crohn's disease; CDr, Crohn's disease in remission; UCa, active ulcerative colitis; UCr, ulcerative colitis in remission; FCP, faecal calprotectin; SCCAI, simple clinical colitis activity index; HBI, Harvey Bradshaw Index. Active disease was defined as an FCP level of ≥250 mg/g, remission was defined as an FCP level of <100 combined with a HBI <4 points or SCCAI <3 points. †All users of Vedolizumab.

Crohn's disease versus ulcerative colitis

Faecal VOC patterns of CD and UC differed significantly, 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, Supplemental Tables 1-4)*. 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 faecal 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 3i)*. However, when 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)*.

Table 3. Differences in faecal volatile organic compound patterns

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
Inflammatory bowel disease versus healthy controls						
IBD versus HC	0.96 (0.92 - 0.99)	0.97	0.92	0.98	0.87	<0.0001
CD versus HC	0.97 (0.95 - 1)	0.94	0.96	0.97	0.91	<0.0001
CDa versus HC	0.96 (0.94 - 0.99)	1	0.92	0.74	1	<0.0001
CDr versus HC	0.95 (0.93 - 0.98)	1	0.90	0.67	1	<0.0001
UC versus HC	0.95 (0.91 - 0.99)	1	0.91	0.88	1	<0.0001
UCa versus HC	0.96 (0.94 - 0.99)	1	0.92	0.74	1	<0.0001
UCr versus HC	0.95 (0.93 - 0.98)	1	0.88	0.52	1	<0.0001
Active disease versus remission						
IBDa versus IBDr	0.59 (0.51 - 0.67)	0.21	0.96	0.90	0.39	0.019
CDa versus CDr	0.52 (0.39 - 0.65)	0.72	0.43	0.71	0.45	0.645
UCa versus UCr	0.63 (0.44 - 0.82)	0.67	0.57	0.79	0.42	0.082
Crohn's disease versus ulcerative colitis						
CD versus UC	0.55 (0.50 - 0.60)	0.17	0.96	0.90	0.36	0.031
CDr versus UCr	0.52 (0.39 - 0.65)	0.95	0.18	0.67	0.67	0.607
CDa versus UCa	0.56 (0.37 - 0.75)	0.74	0.43	0.76	0.40	0.744

All the results of the VOC analysis are obtained using Sparse Logistic Regression classification based on the 100 most discriminative features. Sensitivities, specificities, p-values and AUC are reported for the respective optimum cut-off points. Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; a, active disease state; r, remission; HC, healthy controls; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

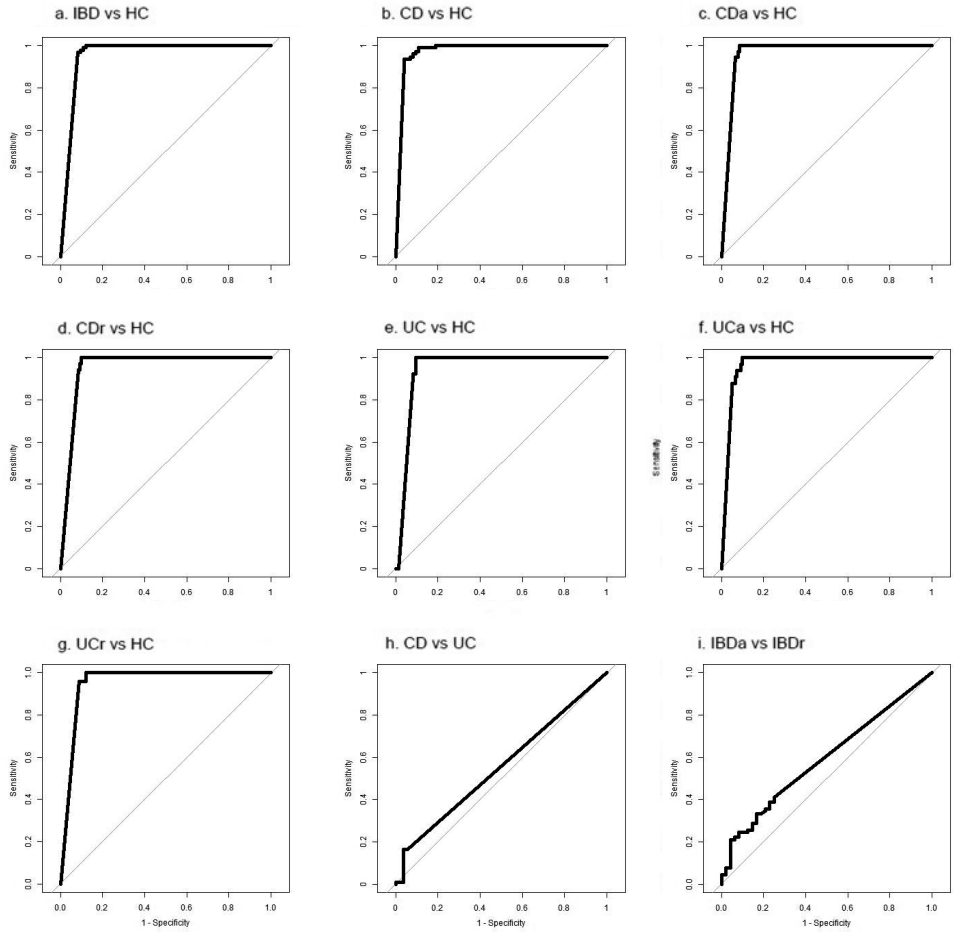

Figure 3. Receiver operator characteristic curves for all comparisons. Receiver operator characteristic curves for the differentiation between study groups based on Sparse Logistic Regression classification using the 100 most discriminative features. A. Inflammatory bowel disease versus healthy controls; B. Crohn's disease versus healthy controls; C Active Crohn's disease versus heathy controls; D. Crohn's disease in remissions versus healthy controls; E. Ulcerative colitis versus healthy controls; F. Active ulcerative colitis versus healthy controls; G. Ulcerative colitis in remission versus healthy controls; H. Crohn's diseases versus ulcerative colitis; I. Active inflammatory bowel diseases versus in remission.

DISCUSSION

The GC-IMS faecal VOC profiles distinguish adults with IBD, and subgroups of UC and CD patients from HC with high diagnostic accuracies, both during active disease state and remission. VOC profiles of IBD patients in active disease state and remission differed statistically, but these small differences were not clinically relevant.

Sensitivity of faecal VOCs to discriminate between IBD and HC in this study are similar to that of the currently used non-invasive biomarker FCP (0.98, 95%CI 0.95-0.99) [17]. However, the specificity of faecal VOC patterns both during active disease and remission is higher compared to the reported values of FCP (0.81-0.91) in adults. This underlines its value for differential diagnosis in clinical practice.

To the best of our knowledge, the potential of faecal VOC profiles to discriminate IBD from HC was previously assessed in only one study in an adult population, including faecal samples of 117 CD, 100 UC and 109 HC analysed by gas chromatography – mass spectrometry (GC-MS)[18]. Although AUC values were not provided, active CD and HC could be separated excellently based on three unique metabolites. In contrast to our findings, study results of Ahmed et. al did not allow discrimination between UC patients compared to HC. Other studies have assessed the diagnostic potential of faecal VOCs for IBD using pattern-based techniques in paediatric cohorts, such as electronic nose (eNose) instruments (CD 29, UC 26, HC 28) and field asymmetric ion mobility spectrometry (FAIMS) (23 CD, 13 UC, 24 HC)([6, 7]. In the study using eNose technology, similar accuracies to the current study were demonstrated for the detection of IBD. In addition, using an eNose there was a clear separation of UC and CD phenotypes. Using FAIMS, CD patients were separated from HC with high accuracy and moderate separation was found for the discrimination of UC compared to both HC and CD samples (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 in adults is both scarce and contradictory. In only one study the assessment of IBD activity based on faecal VOC profiles has been described. Inactive CD was separated significantly from active CD based on faecal VOC profiles, whereas VOC profiles of active and inactive UC were similar[18]. 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 [19]. Though, this accuracy was based on different metabolite alterations. 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)[20]. 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)[21].

Alterations in the faecal 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 these. In a recent study on canine olfaction, in

vitro breast cancer and colon cancer cells 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 print[22]. The same might apply for inflammatory diseases like Crohn's disease and ulcerative colitis of which it may be hypothesised 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.

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 faecal VOCs: the gut microbiota. The faecal microbiota of a healthy individual consists of over 400 different species which play an important role in the defence against invading organisms. There is a large inter-individual diversity in the faecal microbiota composition of healthy individuals [23]. Nonetheless, the microbiome of an individual is remarkably stable, suggesting the presence of a core microbial community, which is dependent of host factors [24, 25]. 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 (*Faecalibacterium Prausnitzii, Roseburia Hominis)* [26-31]. This may well explain the high diagnostic accuracy to discriminate between IBD patients and HC based on faecal VOC profiles in the current study. Remarkably, this variability of microbial composition in IBD patients does not correlate with disease activity. Fluctuations in microbiota composition have been observed both during exacerbation and clinical remission, which has hampered the identification of microbial changes related to the presence of flare-ups [26, 27, 32].

The dissimilarities to other studies considering differentiation between active and remission disease state may also be due to the use of different techniques to analyse VOC profiles. In the current study, we made use of pattern-recognition, since this is a fast and relatively cheap manner to analyse faecal 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 faecal VOCs have previously been demonstrated due to an altered composition of esters, short chain fatty acids (SCFAs) and cyclo-hexanecarboxylic acid, of which the first group is believed to be associated with bacterial dysbiosis[33]. The differences between active disease state and remission in faecal as well as breath VOC profiles originate from a different group of metabolites, mainly aldehydes and ketones [19, 20, 34]. 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 and internal validation set for the development of algorithms using the different machine learning classifiers and an external validation set to test the best available algorithm. In addition, this was a prospective multicentre 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 participants to undergo this investigation without immediate clinical indication. Furthermore, sample age might have been of influence on study outcomes, especially on the IBD samples collected in the period of 2009-2010[13]. However, the results from our post-hoc analysis where we solely compared samples collected in the region of North-Holland within one period of time did not give different outcomes, and we therefore believe that this has not influenced our results significantly (Supplemental Table 4). In addition, the use of probiotics may be of influence on our study outcomes as we did not include a specific question on this and thus we are not able to correct for this in our study results. 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 assessing faecal VOC patterns for IBD detection should include subgroups of patients with non-IBD induced mucosal inflammation for a reliable assessment of its specificity. Additionally, to assess the potential of faecal 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 standardised follow-up moments, ensuring the sole inclusion of patients with active disease and remission based on mucosal appearance and histology findings. These disease (activity) specific profiles would allow for fast and accurate IBD detection and follow-up. Third, it would be interesting to compare the faecal metabolite composition and microbiota in IBD simultaneously in a multi-omics approach, exploring the origin of the faecal VOC pattern alterations.

In conclusion, our results suggest that faecal 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 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 faecal 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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SUPPLEMENTARY MATERIAL

Supplemental Table 1. Overview of the data generated using all five classifiers based on the 20 most discriminative features using all IBD patients

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
IBD versus HC						
Sparse Logistic Regression	0.94 (0.90 – 0.98)	0.99	0.88	0.97	0.96	<0.0001
Random Forest	0.95 (0.92 - 0.99)	0.97	0.92	0.98	0.89	<0.0001
Gaussian Process	0.96 (0.92 – 0.99)	0.99	0.97	0.92	0.98	<0.0001
Support Vector Machine	0.96 (0.93 -0.99)	0.97	0.92	0.98	0.89	<0.0001
Neural Net	0.96 (0.93 – 0.99)	0.97	0.92	0.98	0.89	<0.0001
CD versus HC						
Sparse Logistic Regression	0.95 (0.91-0.98)	0.97	0.91	0.94	0.96	<0.0001
Random Forest	0.95 (0.92 – 0.99)	0.98	0.89	0.93	0.97	<0.0001
Gaussian Process	0.95 (0.92 – 0.99)	0.97	0.92	0.95	0.96	<0.0001
Support Vector Machine	0.95 (0.92 – 0.99)	0.97	0.91	0.94	0.96	<0.0001
Neural Net	0.95 (0.90 – 0.99)	0.97	0.91	0.94	0.96	<0.0001
CDa versus HC						
Sparse Logistic Regression	0.95 (0.93 - 0.97)	1	0.90	0.71	1	<0.0001
Random Forest	0.97 (0.95 - 0.99)	1	0.95	0.82	1	<0.0001
Gaussian Process	0.97 (0.96 - 0.99)	1	0.95	0.82	1	<0.0001
Support Vector Machine	0.97 (0.96 - 0.99)	1	0.95	0.82	1	<0.0001
Neural Net	0.94 (0.91 - 0.97)	1	0.92	0.74	1	<0.0001
CDr versus HC						
Sparse Logistic Regression	0.95 (0.92 – 0.97)	1	0.90	0.67	1	<0.0001
Random Forest	0.97 (0.95 – 0.99)	1	0.92	0.71	1	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	1	0.94	0.76	1	<0.0001
Support Vector Machine	0.97 (0.95 - 0.99)	1	0.92	0.72	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.94	0.76	1	<0.0001
UC versus HC						
Sparse Logistic Regression	0.95 (0.92 – 0.99)	1	0.91	0.88	1	<0.0001
Random Forest	0.96 (0.94 – 0.99)	1	0.93	0.91	1	<0.0001
Gaussian Process	0.96 (0.93 – 0.99)	1	0.92	0.90	1	<0.0001
Support Vector Machine	0.96 (0.94 – 0.99)	1	0.93	0.91	1	<0.0001
Neural Net	0.96 (0.94 – 0.99)	1	0.93	0.91	1	<0.0001

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
UCa versus HC						
Sparse Logistic Regression	0.95 (0.93 - 0.98)	0.97	0.90	0.67	0.99	<0.0001
Random Forest	0.97 (0.95 - 0.99)	1	0.91	0.69	1	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	1	0.90	0.67	1	<0.0001
Support Vector Machine	0.96 (0.93 – 0.93)	0.99	0.97	0.68	0.99	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.90	0.67	1	<0.0001
UCr versus HC						
Sparse Logistic Regression	0.94 (0.91 - 0.96)	1	0.87	0.51	1	<0.0001
Random Forest	0.96 (0.94 - 0.98)	1	0.90	0.56	1	<0.0001
Gaussian Process	0.96 (0.94 - 0.98)	0.96	0.93	0.66	0.99	<0.0001
Support Vector Machine	0.96 (0.94 - 0.98)	0.96	0.93	0.66	0.99	<0.0001
Neural Net	0.96 (0.94 - 0.98)	0.96	0.93	0.66	0.99	<0.0001
IBDa versus IBDr						
Sparse Logistic Regression	0.57 (0.50 - 0.64)	0.27	0.8	0.8	0.39	0.034
Random Forest	0.57 (0.50 - 0.64)	0.27	0.8	0.8	0.39	0.035
Gaussian Process	0.57 (0.51 - 0.64)	0.27	0.8	0.8	0.39	0.025
Support Vector Machine	0.56 (0.49 - 0.63)	0.26	0.9	0.8	0.39	0.054
Neural Net	0.55 (0.48 - 0.62)	0.23	0.92	0.84	0.39	0.095
CDa versus CDr						
Sparse Logistic Regression	0.50 (0.93 - 0.60)	0.19	0.9	0.79	0.37	0.515
Random Forest	0.51 (0.41 - 0.62)	0.28	0.83	0.76	0.38	0.425
Gaussian Process	0.51 (0.40 - 0.61)	0.11	1	1	0.37	0.442
Support Vector Machine	0.51 (0.41 - 0.62)	0.28	0.83	0.76	0.38	0.425
Neural Net	0.51 (0.41 - 0.62)	0.26	0.87	0.79	0.38	0.406
UCa versus UCr						
Sparse Logistic Regression	0.58 (0.41 - 0.75)	0.58	0.64	0.79	0.39	0.194
Random Forest	0.64 (0.46 - 0.82)	0.73	0.57	0.8	0.47	0.062
Gaussian Process	0.61 (0.42 - 0.79)	0.64	0.64	0.81	0.43	0.126
Support Vector Machine	0.63 (0.44 - 0.82)	0.67	0.64	0.81	0.45	0.076
Neural Net	0.61 (0.44 - 0.78)	0.61	0.643	0.8	0.41	0.117
CD versus UC						
Sparse Logistic Regression	0.55 (0.51 - 0.60)	0.17	0.96	0.9	0.34	0.030
Random Forest	0.54 (0.49 - 0.60)	0.14	0.98	0.9375	0.36	0.069
Gaussian Process	0.54 (0.49 - 0.60)	0.09	1	1	0.35	0.077
Support Vector Machine	0.54 (0.49 - 0.60)	0.11	0.98	0.92	0.35	0.073
Neural Net	0.54 (0.49 - 0.60)	0.16	0.94	0.85	0.35	0.080

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
CDa versus UCa						
Sparse Logistic Regression	0.50 (0.37 - 0.63)	0.67	0.45	0.68	0.44	0.496
Random Forest	0.52 (0.39 - 0.65)	0.79	0.36	0.68	0.5	0.610
Gaussian Process	0.48 (0.35 - 0.61)	0.952	0.12	0.65	0.57	0.605
Support Vector Machine	0.51 (0.38 - 0.64)	0.67	0.45	0.68	0.44	0.463
Neural Net	0.48 (0.35 - 0.61)	0.60	0.51	0.68	0.43	0.605
CDr versus UCr						
Sparse Logistic Regression	0.50 (0.50 - 0.50)	1	0	0.71	N/A	1
Random Forest	0.58 (0.40 - 0.76)	0.29	0.93	0.91	0.35	0.811
Gaussian Process	0.53 (0.35 - 0.70)	0.5	0.71	0.81	0.37	0.624
Support Vector Machine	0.57 (0.39 - 0.75)	0.44	0.79	0.83	0.37	0.780
Neural Net	0.64 (0.46 - 0.82)	0.71	0.57	0.8	0.44	0.936

Overview of the data generated using all five classifiers based on the 20 most discriminative features. Abbreviations: IBD inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; a, active disease state; r, remission; HC, healthy controls; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

Supplemental Table 2. Overview of the data generated using all four classifiers based on the 50 most discriminative features using all IBD patients

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
IBD versus HC						
Sparse Logistic Regression	0.95 (0.92 – 0.99)	0.99	0.91	0.98	0.96	<0.0001
Random Forest	0.97 (0.94 – 1)	0.97	0.93	0.98	0.88	<0.0001
Gaussian Process	0.97 (0.94 – 1)	0.97	0.92	0.98	0.88	<0.0001
Support Vector Machine	0.97 (0.95 – 1)	0.95	0.95	0.99	0.82	<0.0001
Neural Net	0.96 (0.92 – 0.99)	0.96	0.93	0.98	0.85	<0.0001
CD versus HC						
Sparse Logistic Regression	0.96 (0.93 – 0.99)	0.96	0.93	0.95	0.95	<0.0001
Random Forest	0.97 (0.95 – 1)	0.97	0.93	0.95	0.96	<0.0001
Gaussian Process	0.97 (0.94 – 1)	0.97	0.93	0.95	0.96	<0.0001
Support Vector Machine	0.97 (0.95 – 1)	0.98	0.92	0.95	0.97	<0.0001
Neural Net	0.96 (0.92 – 1)	0.97	0.93	0.95	0.96	<0.0001
CDa versus HC						
Sparse Logistic Regression	0.96 (0.94 - 0.98)	1	0.92	0.76	1	<0.0001
Random Forest	0.97 (0.96 - 0.99)	1	0.95	0.82	1	<0.0001
Gaussian Process	0.97 (0.96 - 0.99)	1	0.95	0.82	1	<0.0001
Support Vector Machine	0.97 (0.95 - 0.99)	1	0.94	0.80	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.94	0.80	1	<0.0001
CDr versus HC						
Sparse Logistic Regression	0.96 (0.94 - 0.98)	1	0.91	0.68	1	<0.0001
Random Forest	0.97 (0.94 – 0.99)	1	0.92	0.71	1	<0.0001
Gaussian Process	0.97 (0.94 - 0.99)	1	0.92	0.72	1	<0.0001
Support Vector Machine	0.97 (0.94 - 0.99)	1	0.93	0.74	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.93	0.74	1	<0.0001
UC versus HC						
Sparse Logistic Regression	0.95 (0.92 - 0.99)	1	0.91	0.88	1	<0.0001
Random Forest	0.97 (0.94 – 1)	1	0.93	0.91	1	<0.0001
Gaussian Process	0.97 (0.94 – 1)	1	0.92	0.90	1	<0.0001
Support Vector Machine	0.97 (0.94 – 1)	1	0.91	0.88	1	<0.0001
Neural Net	0.97 (0.94 – 1)	0.98	0.93	0.91	0.99	<0.0001
UCa versus HC						
Sparse Logistic Regression	0.97 (0.95 - 0.99)	1	0.90	0.66	1	<0.0001
Random Forest	0.97 (0.95 - 0.99)	1	0.92	0.70	1	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	1	0.91	0.69	1	<0.0001

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
Support Vector Machine	0.97 (0.95 - 0.99)	1	0.90	0.67	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.91	0.69	1	<0.0001
UCr versus HC						
Sparse Logistic Regression	0.95 (0.92 - 0.97)	1	0.88	0.52	1	<0.0001
Random Forest	0.97 (0.95 - 0.99)	0.96	0.95	0.70	0.99	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	0.96	0.95	0.70	0.99	<0.0001
Support Vector Machine	0.97 (0.95 - 0.99)	0.96	0.95	0.70	0.99	<0.0001
Neural Net	0.97 (0.95 - 0.99)	0.96	0.95	0.70	0.99	<0.0001
IBDa versus IBDr						
Sparse Logistic Regression	0.56 (0.50 - 0.63)	0.26	0.88	0.79	0.39	0.042
Random Forest	0.57 (0.50 - 0.65)	0.3	0.85	0.79	0.39	0.028
Gaussian Process	0.56 (0.49 - 0.63)	0.24	0.90	0.81	0.39	0.080
Support Vector Machine	0.57 (0.50 - 0.64)	0.26	0.90	0.82	0.39	0.040
Neural Net	0.53 (0.46 - 0.60)	0.23	0.88	0.78	0.38	0.215
CDa versus CDr						
Sparse Logistic Regression	0.53 (0.41 - 0.65)	0.18	0.93	0.83	0.37	0.292
Random Forest	0.58 (0.44 - 0.70)	0.52	0.63	0.73	0.41	0.116
Gaussian Process	0.56 (0.43 - 0.69)	0.51	0.67	0.74	0.42	0.171
Support Vector Machine	0.56 (0.44 - 0.69)	0.58	0.57	0.72	0.41	0.152
Neural Net	0.62 (0.50 - 0.74)	0.49	0.7	0.76	0.42	0.032
UCa versus UCr						
Sparse Logistic Regression	0.57 (0.39 – 0.75)	0.33	0.86	0.85	0.35	0.230
Random Forest	0.63 (0.45 – 0.81)	0.82	0.43	0.77	0.50	0.090
Gaussian Process	0.57 (0.36 – 0.78)	0.85	0.43	0.78	0.55	0.231
Support Vector Machine	0.56 (0.36 – 0.76)	0.85	0.43	0.78	0.55	0.261
Neural Net	0.54 (0.34 – 0.74)	0.82	0.43	0.77	0.50	0.350
CD versus UC						
Sparse Logistic Regression	0.55 (0.51 - 0.60)	0.17	0.96	0.9	0.36	0.030
Random Forest	0.54 (0.49 - 0.60)	0.16	0.96	0.89	0.36	0.072
Gaussian Process	0.54 (0.49 - 0.60)	0.17	0.92	0.83	0.35	0.078
Support Vector Machine	0.54 (0.49 - 0.59)	0.17	0.92	0.83	0.35	0.093
Neural Net	0.54 (0.49 - 0.59)	0.16	0.92	0.81	0.35	0.088

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
CDa versus UCa						
Sparse Logistic Regression	0.50 (0.37 - 0.63)	0.65	0.48	0.69	0.44	0.500
Random Forest	0.53 (0.40 - 0.67)	0.79	0.36	0.68	0.50	0.305
Gaussian Process	0.49 (0.36 - 0.63)	0.95	0.15	0.66	0.63	0.540
Support Vector Machine	0.51 (0.38 - 0.64)	0.42	0.70	0.71	0.41	0.427
Neural Net	0.52 (0.39 - 0.65)	0.60	0.58	0.71	0.45	0.374
CDr versus UCr						
Sparse Logistic Regression	0.43 (0.25 - 0.61)	0.5	0.57	0.74	0.32	0.773
Random Forest	0.45 (0.27 - 0.64)	0.09	1	1	0.31	0.691
Gaussian Process	0.55 (0.38 - 0.72)	0.5	0.79	0.85	0.39	0.715
Support Vector Machine	0.55 (0.36 - 0.73)	0.53	0.64	0.78	0.36	0.691
Neural Net	0.50 (0.31 - 0.69)	0.82	0.36	0.76	0.45	0.500

Overview of the data generated using all five classifiers based on the 50 most discriminative features. Abbreviations: IBD inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; a, active disease state; r, remission; HC, healthy controls; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

Supplemental Table 3. Overview of the data generated using all four classifiers based on the 100 most discriminative features using all IBD patients

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
IBD versus HC						
Sparse Logistic Regression	0.96 (0.92 - 0.99)	0.97	0.92	0.98	0.87	<0.0001
Random Forest	0.97 (0.95 - 1)	0.97	0.95	0.99	0.88	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	0.98	0.93	0.98	0.93	<0.0001
Support Vector Machine	0.98 (0.95 - 0.99)	0.94	0.95	0.99	0.80	<0.0001
Neural Net	0.97 (0.93 - 0.99)	0.99	0.92	0.98	0.97	<0.0001
CD versus HC						
Sparse Logistic Regression	0.97 (0.95 - 1)	0.94	0.96	0.97	0.91	<0.0001
Random Forest	0.98 (0.95 - 1)	0.96	0.93	0.95	0.95	<0.0001
Gaussian Process	0.98 (0.95 - 1)	0.96	0.93	0.95	0.95	<0.0001
Support Vector Machine	0.97 (0.93 - 1)	0.97	0.92	0.95	0.96	<0.0001
Neural Net	0.96 (0.93 - 1)	0.98	0.92	0.95	0.97	<0.0001
CDa versus HC						
Sparse Logistic Regression	0.96 (0.94 - 0.99)	1	0.92	0.74	1	<0.0001
Random Forest	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Gaussian Process	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Support Vector Machine	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.94	0.80	1	<0.0001

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
CDr versus HC						
Sparse Logistic Regression	0.95 (0.93 - 0.98)	1	0.90	0.67	1	<0.0001
Random Forest	0.97 (0.95 - 0.99)	1	0.93	0.74	1	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	1	0.93	0.74	1	<0.0001
Support Vector Machine	0.97 (0.95 - 0.99)	1	0.94	0.76	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.94	0.76	1	<0.0001
UC versus HC						
Sparse Logistic Regression	0.95 (0.91 - 0.99)	1	0.91	0.88	1	<0.0001
Random Forest	0.97 (0.93 - 1)	1	0.91	0.88	1	<0.0001
Gaussian Process	0.97 (0.93 - 1)	0.96	0.95	0.93	0.97	<0.0001
Support Vector Machine	0.97 (0.94 - 1)	1	0.91	0.88	1	<0.0001
Neural Net	0.97 (0.94 - 1)	0.98	0.92	0.90	0.99	<0.0001
UCa versus HC						
Sparse Logistic Regression	0.96 (0.94 - 0.99)	1	0.92	0.74	1	<0.0001
Random Forest	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Gaussian Process	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Support Vector Machine	0.98 (0.96 - 1)	1	0.95	0.84	1	<0.0001
Neural Net	0.97 (0.95 - 0.99)	1	0.94	0.80	1	<0.0001
UCr versus HC						
Sparse Logistic Regression	0.95 (0.93 - 0.98)	1	0.88	0.52	1	<0.0001
Random Forest	0.97 (0.95 - 0.99)	0.96	0.95	0.72	0.99	<0.0001
Gaussian Process	0.97 (0.95 - 0.99)	0.96	0.95	0.72	0.99	<0.0001
Support Vector Machine	0.97 (0.95 - 0.99)	0.96	0.95	0.72	0.99	<0.0001
Neural Net	0.97 (0.95 - 0.99)	0.96	0.95	0.70	0.99	<0.0001
IBDa versus IBDr						
Sparse Logistic Regression	0.59 (0.51 - 0.67)	0.21	0.96	0.90	0.39	0.019
Random Forest	0.59 (0.51 - 0.68)	0.31	0.88	0.82	0.40	0.017
Gaussian Process	0.58 (0.49 - 0.66)	0.43	0.75	0.76	0.41	0.039
Support Vector Machine	0.58 (0.49 - 0.67)	0.43	0.75	0.76	0.41	0.038
Neural Net	0.57 (0.49 - 0.65)	0.29	0.90	0.84	0.40	0.066
CDa versus CDr						
Sparse Logistic Regression	0.52 (0.39 - 0.65)	0.72	0.43	0.71	0.45	0.645
Random Forest	0.49 (0.36 - 0.62)	0.33	0.77	0.731	0.38	0.562
Gaussian Process	0.54 (0.41 - 0.67)	0.30	0.83	0.771	0.38	0.291
Support Vector Machine	0.53 (0.39 - 0.66)	0.88	0.27	0.691	0.53	0.674
Neural Net	0.52 (0.39 - 0.64)	0.60	0.57	0.721	0.43	0.606

Table continues

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
UCa versus UCr						
Sparse Logistic Regression	0.63 (0.44 - 0.82)	0.67	0.57	0.79	0.42	0.082
Random Forest	0.62 (0.44 - 0.81)	0.85	0.43	0.78	0.55	0.094
Gaussian Process	0.55 (0.34 - 0.77)	0.88	0.43	0.78	0.60	0.288
Support Vector Machine	0.50 (0.29 - 0.71)	0.85	0.36	0.76S	0.50	0.514
Neural Net	0.58 (0.40 - 0.76)	0.82	0.43	0.77	0.50	0.191
CD versus UC						
Sparse Logistic Regression	0.55 (0.50 - 0.60)	0.17	0.96	0.90	0.36	0.031
Random Forest	0.54 (0.49 - 0.60)	0.12	0.98	0.93	0.35	0.074
Gaussian Process	0.54 (0.49 - 0.60)	0.17	0.92	0.83	0.35	0.078
Support Vector Machine	0.54 (0.49 - 0.60)	0.17	0.92	0.83	0.35	0.089
Neural Net	0.54 (0.49 - 0.59)	0.16	0.92	0.81	0.35	0.090
CDa versus UCa						
Sparse Logistic Regression	0.52 (0.39 - 0.65)	0.95	0.18	0.67	0.67	0.607
Random Forest	0.55 (0.41 - 0.68)	0.54	0.67	0.74	0.46	0.228
Gaussian Process	0.53 (0.40 - 0.66)	0.89	0.27	0.68	0.60	0.311
Support Vector Machine	0.53 (0.41 - 0.66)	0.67	0.48	0.69	0.46	0.308
Neural Net	0.56 (0.43 - 0.69)	0.72	0.48	0.71	0.50	0.832
CDr versus UCr						
Sparse Logistic Regression	0.56 (0.37 - 0.75)	0.74	0.43	0.76	0.40	0.744
Random Forest	0.53 (0.33 - 0.72)	0.53	0.64	0.78	0.36	0.393
Gaussian Process	0.54 (0.36 - 0.71)	0.41	0.86	0.88	0.38	0.653
Support Vector Machine	0.58 (0.41 - 0.76)	0.26	1	1	0.36	0.199
Neural Net	0.59 (0.41 - 0.76)	0.59	0.71	0.83	0.42	0.844

verview of the data generated using all five classifiers based on the 100 most discriminative features. Abbreviations: IBD inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; a, active disease state; r, remission; HC, healthy controls; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

Supplemental Table 4. Overview of the data generated using all four classifiers based on the 100 most discriminative features using only the Amsterdam UMC samples

	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
IBD versus HC						
Sparse Logistic Regression	0.95 (0.91 – 0.98)	0.98	0.92	0.92	0.98	<0.001
Random Forest	0.97 (0.95 – 0.99)	1	0.92	0.92	1	<0.001
Gaussian Process	0.96 (0.92 – 0.99)	0.98	0.94	0.93	0.98	<0.001
Support Vector Machine	0.96 (0.93 – 0.99)	1	0.91	0.91	1	<0.001
Neural Net	0.94 (0.90 – 0.98)	0.98	0.92	0.92	0.98	<0.001
CD versus HC						
Sparse Logistic Regression	0.96 (0.93 – 0.99)	1	0.92	0.85	1	<0.001
Random Forest	0.97 (0.94 – 0.99)	1	0.92	0.85	1	<0.001
Gaussian Process	0.95 (0.92 – 0.99)	1	0.92	0.85	1	<0.001
Support Vector Machine	0.95 (0.91 – 0.99)	1	0.92	0.85	1	<0.001
Neural Net	0.93 (0.88 – 0.97)	0.93	0.94	0.87	0.97	<0.001
UC versus HC						
Sparse Logistic Regression	0.96 (0.92 – 0.99)	1	0.90	0.81	1	<0.001
Random Forest	0.96 (0.93 – 0.99)	1	0.91	0.83	1	<0.001
Gaussian Process	0.96 (0.93 – 0.99)	0.96	0.93	0.85	0.98	<0.001
Support Vector Machine	0.96 (0.93 – 0.99)	1	0.91	0.83	1	<0.001
Neural Net	0.90 (0.85 – 0.96)	0.93	0.94	0.86	0.97	<0.001
CD versus UC						
Sparse Logistic Regression	0.63 (0.53 – 0.74)	0.64	0.62	0.63	0.63	0.008
Random Forest	0.58 (0.47 – 0.69)	0.63	0.55	0.58	0.59	0.075
Gaussian Process	0.57 (0.46 – 0.68)	0.59	0.65	0.63	0.61	0.106
Support Vector Machine	0.49 (0.38 – 0.60)	0.43	0.64	0.54	0.52	0.436
Neural Net	0.53 (0.42 – 0.63)	0.54	0.62	0.59	0.57	0.320

Overview of the data generated using all five classifiers based on the 100 most discriminative features using only the Amsterdam UMC samples. Abbreviations: IBD inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; a, active disease state; r, remission; HC, healthy controls; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

CHAPTER 8

Prediction of inflammatory bowel disease course based on faecal volatile organic compounds: a pilot study

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ABSTRACT

Background

In patients with inflammatory bowel disease (IBD), early prediction of changes in disease state provides for timely treatment which improves disease outcome. Aim of this pilot study is to explore the potential of fecal volatile organic compound (VOC) profiles to predict IBD course.

Methods

In this prospective multi-center cohort, IBD patients were asked to collect two fecal samples and a questionnaire at set intervals. Biochemically, active disease was FCP≥250 mg/g, remission FCP<100 mg/g. Clinically, active disease was a Harvey Bradshaw Index (HBI) ≥5 for Crohn's disease or Simple Clinical Colitis Activity Index (SCCAI) ≥3 for ulcerative colitis. Clinical remission was HBI<4 or SCCAI≤2. Fecal VOC profiles were measured using Gas Chromatography – Ion Mobility Spectrometry (GC-IMS). The fecal samples collected at first time-point were included for fecal VOC analysis to predict disease state at the second time-point.

Results

Total of 182 subsequently collected samples met the disease state criteria. Fecal VOC profiles of samples displaying low FCP levels at first measurements differed when preceding exacerbation versus remaining in remission (AUC 0.75; p<0.01). For high FCP levels at the first time-point displayed different VOC profiles when preceding remission compared to when remaining active (B3 vs B4 AUC 0.86; P<0.01). Based upon disease activity scores, there were no significant differences in any of the comparisons.

Conclusion

The alterations in fecal VOC profile preceding changes in FCP level may be useful to detect disease course alterations at an early stage, which would subsequently lead to earlier treatment, decreased numbers of complications, surgery and hospital admission.

PART III

The liquid faecal metabolome as biomarker for inflammatory bowel disease

CHAPTER 9

Faecal amino acid analysis can discriminate de novo treatment-naïve paediatric inflammatory bowel disease from controls

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ABSTRACT

Background

Endoscopy remains mandatory in the diagnostic work-up of inflammatory bowel disease (IBD), but is a costly and invasive procedure. Identification of novel, non-invasive, diagnostic biomarkers remains a priority. The aim of this study was to explore the potential of faecal amino acid composition as diagnostic biomarker for paediatric IBD.

Methods

In this case-control study, treatment–naïve, *de novo* paediatric IBD patients from two tertiary centres were included. Endoscopic severity of ulcerative colitis (UC) and Crohn's disease (CD) was based on global physician assessment scores, substantiated by levels of faecal calprotectin and C-reactive protein at study inclusion. Patients were instructed to collect a faecal sample prior to bowel cleansing. Healthy controls were recruited from primary schools in the same region. Dedicated amino acid analysis was performed on all samples.

Results

Significant differences between 30 IBD patients (15 UC, 15 CD) and 15 age and sex matched healthy controls (HC) were found in six amino acids (histidine, tryptophan, phenylalanine, leucine, tyrosine and valine; all AUC > 0.75 and $p < 0.005$), displaying higher levels in IBD. When distributing the patients according to type of IBD, a similar spectrum of amino acids differed between UC and HC (histidine, tryptophan, phenylalanine, leucine, valine and serine), whereas three amino acids were different between CD and HC (histidine, tryptophan and phenylalanine).

Conclusion

Significantly increased levels of six different faecal amino acids were found in IBD patients compared to controls. Whether these differences reflect decreased absorption or increased loss by inflamed intestines needs to be elucidated.

INTRODUCTION

Inflammatory bowel disease (IBD) comprises the two main phenotypes ulcerative colitis (UC) and Crohn's disease (CD). These chronic relapsing conditions of the gastrointestinal tract usually develop during young adulthood[1-5]. Diagnosis and follow-up of IBD are essentially assessed by endoscopic investigation which is a costly, invasive procedure with a risk of complications[6]. Especially in children the burden is high, since they need to be hospitalised for colonic lavage with administration of laxatives by nasogastric tube and ileocolonoscopy has to be performed under general anesthesia. Therefore, the search for novel non-invasive diagnostic biomarkers in the diagnostic work-up remains warranted.

Various biomarkers have been evaluated as tool in the diagnostic work-up for IBD, with faecal calprotectin (FCP) showing the highest sensitivity for detection of mucosal inflammation (0.98, 95%CI 0.95-0.99)[7]. However, FCP is characterised by a relative low specificity (0.68, 95% CI 0.50-0.86), limiting its use to differentiate between IBD and other gastro-intestinal diseases, such as polyps and infectious gastroenteritis[8-10]. Other widely studied biomarkers include metabolic products, which may potentially have a higher specificity for IBD detection, since they reflect (patho)physiological processes involved in IBD pathogenesis[11]. For example, volatile organic compounds (VOCs), which are carbon based chemicals and considered to reflect microbiota composition, seem to have potential as non-invasive diagnostic biomarkers for diagnosing and monitoring IBD. Promising results have been described in analysis of VOCs deriving from different bodily excretions including exhaled breath, faecal and urine[12-21].

By VOCs analysis, however, exclusively gaseous metabolites are captured. The spectrum of non-volatile organic compounds in IBD remains yet largely unexplored. In a previous study on faecal and serum metabolomic patterns comparing *de novo* paediatric IBD patients and controls, there seemed to be a promising role for especially the faecal metabolome in diagnosing (different subtypes of) IBD[22]. In our earlier (unpublished) study on the faecal metabolome using ultra performance liquid chromatography (UPLC) combined with high resolution mass spectrometry (HR/MS), particularly levels of amino acids differed between IBD patients and controls. Therefore, the aim of this study was to explore these differences in the composition of faecal amino acids between paediatric *de novo* IBD patients and healthy controls further by means of a dedicated amino acid analysis.

METHODS

Study design

This case-control study was performed at the outpatient clinic of the paediatric gastroenterology departments of two tertiary hospitals, VU University medical centre (VUmc) and the Emma Children's Hospital, (Academic Medical Centre), both located in Amsterdam, The Netherlands. All subjects had to be familiar with Dutch language, since questionnaires were provided in Dutch.

Study participants

Inflammatory bowel disease patients

Participants were extracted from a cohort consisting of *de novo* treatment-naïve paediatric IBD patients (94 CD, 56 UC) aged 4 to 17 years, included between December 1st 2011 and March 10st 2016. Patients were diagnosed with IBD based on endoscopic, histologic and radiologic findings using the revised Porto-criteria for paediatric IBD[23]. Localisation and behaviour of disease were classified according to the Paris Classification[24]. Included patients were instructed to collect a faecal sample for microbiota analysis, prior to bowel cleansing[25]. Patients with IBD were randomly selected and matched with controls based on age and gender. Exclusion criteria were the use of anti- or probiotics in the three months prior to inclusion, prior immunosuppressive therapy or a concomitant diagnosis of immunocompromised disease (i.e. HIV, leukemia), underlying gastro-intestinal disease (i.e. celiac disease, Hirschsprung's disease) and history of gastro-intestinal surgery (except appendectomy). In addition, patients with proven infectious colitis during the month before presentation were excluded. Infectious colitis was determined by parasites in stools or positive stool culture for *Salmonella spp., Shigella spp., Yersinia spp., Campylobacter spp.* or *Clostridium spp.* toxins. Disease activity was measured at study inclusion using the global physician assessment (GPA) score, substantiated by faecal calprotectin (FCP), and C-reactive protein (CRP).

Healthy controls

As control group, healthy children aged 4-17 years were recruited between June 2016 and December 2016 from elementary and high schools located in the Dutch provinces North-Holland, South-Holland, and Flevoland. Notably, all included IBD children lived in the same topographic regions. Exclusion criteria were similar to the study group, extended with gastrointestinal symptoms fulfilling the Rome IV criteria of functional gastrointestinal disorders[26, 27]. All children and their parents were asked to complete a questionnaire on subject characteristics, medical history, living environment, use of antibiotics in the last three months, defecation pattern based on Bristol stool chart scores and gastro-intestinal symptoms[28].

Sample collection

Participants were asked to collect a faecal sample in a stool container (Stuhlgefäß 10ml, Greiner Bio-One, Frickenhausen, Germany) and instructed to store the sample within one hour following bowel movement at the freezer at home at -20°C. The samples were transported to the hospital in a cooled condition and stored in a -20°C refrigerator immediately after arrival.

Targeted amino acid analysis

To investigate differences in the amino acid composition, faecal samples of IBD patients and healthy controls were analysed by means of a targeted High Performance Liquid Chromatography (HPLC) technique, specifically amino acid analysis (AAA). For this analysis, approximately 300mg faecal and 1000 µL distilled water were mixed by vortex for one minute to homogenize the samples. Subsequently, these samples were recoded and investigated by an independent laboratory researcher (ES), blinded for the diagnosis. The samples were frozen at minus 30 degrees and subsequently freeze-dried for 24 hours (Christ

Alpha 2-4) to prevent potential bias by differences in faecal water content. The residual, approximately 30-50mg depending on the faecal consistency, was mixed with a quantity of distilled water maintaining a faecal-water ratio of 100mg:5mL. This mixture was again vigorously homogenised using vortex. For the amino acid profile (aminogram) analysis, 400 µL of the mixture was pipetted into a filter and centrifuged for 20 minutes at 14.000g (Hettig Zentrifugen Mikro 2R). The supernatant was subsequently mixed with an internal standard solution with a one-to-one ratio. Finally, this mixture was centrifuged for 10 minutes and filtered (Whatman) into compatible containers for the final amino acid analyses (Biochrome 30). Amino acids were separated by ion-exchange chromatography and detected by UV-absorbance after post-column derivatization with ninhydrin.

Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 22, IBM, NY, USA). The demographic data of each group (UC, CD patients and healthy controls) were compared using the non-parametric Kruskal-Wallis-H tests for continuous data and the Fisher's exact test for dichotomous data. On account of the small sample size, the Mann-Whitney *U* test / Wilcoxon rank-sum test was used to calculate p-values to identify potential differences in aminograms between the IBD phenotypes and the healthy control group. To compensate for multiple testing, a p-value of <0.005 was considered significant. In addition, to circumvent possible over- or underestimation of the discriminative accuracy, amino acids were excluded from the analysis if levels were unquantifiable or undetectable in at least one of the study subjects. Amino acid levels of 5 µmol/l or lower were considered unquantifiable, levels of 0 µmol/l were considered undetectable. A receiver operator characteristic (ROC-)curve was created to predict sensitivity and specificity values. Correlations between levels of amino acids and CRP, FCP and GPA were analysed using the Spearman rank-order correlation coefficient. A formal sample size calculation could not be performed due to a lack of previous data on this topic.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre under file number 2015.393. Written informed consent was obtained from all study participants and their parents.

RESULTS

Baseline characteristics

From the initial 96 CD and 56 UC subjects, a total of 99 IBD patients (59 CD, 40 UC) were not eligible for this study due to insufficient quantities of collected faecal. From the remainder of the cohort, IBD patients were randomly selected and matched with controls based on age and gender. Thirty *de novo*, treatment-naïve IBD patients were included (15 UC, 15 CD) in this study. The control group consisted of 15 healthy children. Baseline characteristics and disease specifics of the study subjects are described in table 1. No statistical significant differences between UC and CD patients were found for the variables FPC, leukocytes and GPA. Higher levels of CRP were observed in the CD group compared to the UC group (p=0.013). In addition, IBD samples were stored for a significantly longer period compared to samples of healthy controls.

Inflammatory bowel disease versus controls

A total of 42 amino acids were detected during AAA of which 23 amino acids were not suitable for statistical analysis due to unquantifiable or undetectable levels in at least one of the study subjects. The results of the amino acid analysis comparing paediatric IBD patients and healthy controls are displayed in table 2, and visualised in upplemental figure 1 in Box Whisker plots. In six of the 19 amino acids, statistically significant differences between IBD patients and healthy controls were observed. In particular, increased levels of tryptophan, histidine, phenylalanine, leucine, tyrosine and valine were strongly predictive for the discrimination of IBD patients versus healthy controls. For all these amino acids, predictive values for the diagnosis of IBD are shown in table 3. Their associated ROC-curves are displayed in supplemental figure 2A. No correlations were found between amino acid levels and disease activity parameters (GPA, CRP and FCP). An overview of all the measured amino acid levels per subject is given in supplemental table 1-3.

IBD subtypes versus controls

Ulcerative colitis versus healthy controls

The same spectrum of amino acids differed significantly between UC and HC, compared to the set of amino acids differentiating IBD (UC and CD together) from controls, except for tyrosine and serine (Table 2, Supplementary figure 1). In UC, increased levels of serine differed significantly from controls (p=.001), whereas tyrosine did not (p=.006). Overall, the accuracy of the aminogram to differentiate between UC and HC improved compared to the discrimination between IBD and HC. Table 3 depicts the AUC for this UC sub analysis. Corresponding ROC-curves are displayed in supplemental figure 2B. In addition, no correlations were found between amino acid levels and disease activity parameters in this sub analysis.

Crohn's disease versus healthy controls

By focusing on CD subjects versus HC, levels of the amino acids histidine, phenylalanine and tryptophan increased significantly in the CD subgroup (Table 2, supplementary figure 1). The predictive value of the Crohn's disease sub analysis is shown in Table 3. Corresponding ROC-curves are visualised in supplemental figure 2C. There was no correlation between amino acid levels and disease activity parameters.

Ulcerative colitis versus Crohn's disease

There were no significant differences in amino acids profiles between UC and CD (Table 2).

Table 1. Baseline characteristics

	Healthy controls (n=15)	Ulcerative colitis (n=15)	Crohn's disease (n=15)
Sample age, yr (median [IQR])	15.5 [7.8 – 16.6]	12.0 [7.9 – 15.7]	14.0 [10.5 – 14.6]
Sex, male [%]	60	53	40
Storage time, yr (median [IQR])	0.6 [0.5 – 0.6]	4.2 [3.4 – 4.9]	3.4 [2.7 – 4.6]
Global Physician Assessment			
Quiescent	NA	0	0
Mild	NA	3	1
Moderate	NA	5	5
Severe	NA	7	9
Faecal calprotectin (µg/g) (median[IQR])	NA	1260 [393-1950]	1111 [627-1366]
CRP (mg/l) (median[IQR])	NA	2.5 [2.5-7.0]	19.4 [2.5-45.0]
Crohn's disease localisation[1]			
Ileal (L1)	NA	NA	0
Colonic (L2)	NA	NA	7
Ileocolonic (L3)	NA	NA	8
Proximal disease (L4)	NA	NA	2
Crohn's disease behaviour[1]			
B1 (NSNP)	NA	NA	6
B1p (NSNP+p)	NA	NA	1
B2 (S)	NA	NA	0
B2p (S + p)	NA	NA	1
B3 (P)	NA	NA	5
B3p (P + p)	NA	NA	1
Ulcerative Colitis[1]			
Proctitis (E1)	NA	3	NA
Left-sided (E2)	NA	2	NA
Extensive (E3)	NA	10	NA

All values were obtained at study inclusion. Localisation was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and magnetic resonance enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. [1]Based on Paris classification for inflammatory bowel disease (24)

Table 2. Differences in amino acid levels between IBD patients and healthy controls

Amino acid	Healthy controls (median [LQR-UQR]) n = 15	IBD patients (median [LQR-UQR]) n = 30	IBD vs HC p-value	UC (median [LQR-UQR]) n = 15	UC vs HC p-value	CD (median [LQR-UQR]) n = 15	CD vs HC p-value	UC vs CD p-value
Alanine	31.5 [28.3-45.0]	60.1 [34.8-81.7]	0.007	71.4 [37.1-94.1]	0.007	48.3 [34.7-66.4]	0.045	0.106
Citrulline	11.1 [8.6-17.5]	17.5 [10.3-24.7]	0.043	12.7 [10.3-27.9]	0.081	18.1 [11.0-23.4]	0.089	0.624
Ethanolamine	2.9 [1.5-4.9]	1.9 [1.1-2.8]	0.177	2.3 [1.6-3.8]	0.902	1.9 [0.9-2.1]	0.026	0.045
Glutamine	7.1 [4.9-10.4]	9.2 [6.6-17.7]	0.056	9.1 [5.5-21.3]	0.137	9.8 [7.1-13.4]	0.074	0.902
Glutamine acid	37.3 [33.1-56.3]	46.5 [24.9-61.7]	0.718	46.5 [25.4-61.5]	0.595	41.0 [22.0-62.3]	0.967	0.935
Glycine	19.5 [15.6-26.9]	21.8 [19.6-50.4]	0.013	33.4 [21.2-53.1]	0.007	31.5 [18.7-46.1]	0.116	0.367
Histidine	1.7 [0.7-3.9]	4.8 [3.8-13.0][1]	<0.001	8.65 [3.85-16.0][2]	<0.001	4.4 [2.8-5.2][2]	0.002	0.089
Isoleucine	17.0 [12.3-24.4]	24.7 [17.8-29.2]	0.025	24.7 [19.3-28.7]	0.037	24.7 [17.5-34.5]	0.081	0.902
Leucine	21.7 [15.6-30.1]	44.6 [26.9-66.8][1]	<0.001	62.5 [30.1-72.3][2]	<0.001	37.9 [24.4-53.7]	0.005	0148
Lysine	21.0 [16.2-33.2]	31.1 [23.5-49.4]	0.034	43.9 [23.5-53.2]	0.021	26.3 [23.5-41.9]	0.187	0.202
Methionine	7.1 [5.2-10.7]	8.1 [5.5-11.4]	0.360	8.2 [5.4-12.7]	0.389	7.9 [5.5-11.3]	0.512	0.838
Ornitine	2.3 [1.3-3.7]	3.0 [1.4-6.5]	0.253	4.0 [2.5-7.4]	0.074	2.1 [1.2-3.6]	0.870	0.174
Phenylalanine	9.7 [7.1-15.3]	22.4 [12.3-31.4][1]	<0.001	25.7 [13.1-34.4][2]	<0.001	19.1 [12.0-26.2][2]	0.002	0.250
Proline	8.7 [6.7-10.8]	16.1 [7.8-22.3]	0.009	16.5 [7.8-25.6]	0.009	15.5 [7.3-20.0]	0.056	0.367
Serine	14.0 [10.3-21.2]	22.2 [15.0-36.2]	0.006	29.4 [16.7-41.7][2]	0.001	20.0 [13.5-24.4]	0.116	0.116
Threonine	14.2 [12.2-22.2]	22.6 [16.2-33.5]	0.018	27.8 [17.7-36.5]	0.011	20.9 [13.9-25.7]	0.126	0.217
Tryptophan	2.1 [1.4-3.4]	4.4 [3.1-5.7][1]	<0.001	4.8 [3.5-6.1][2]	<0.001	3.9 [2.8-5.6][2]	0.003	0.367
Tyrosine	9.7 [7.45-14.6]	17.7 [11.3-22.5][1]	0.003	19.5 [11.4-25.7]	0.006	15.3 [11.0-21.5]	0.013	0.744
Valine	21.0 [16.1-31.0]	41.0 [23.9-55.3][1]	0.003	49.2 [29.2-58.7][2]	0.003	35.4 [20.3-46.5]	0.019	0.187

All levels of amino acids are reported in nmol/mg. [1]IBD significantly different from control subjects (Mann-Whitney-U test) [2] Subgroup analysis significantly different from control subjects (Mann-Whitney-U test). Abbreviations: LQR, lower quartile range; UQR, upper quartile range; IBD: inflammatory bowel disease; HC: healthy controls; UC: ulcerative colitis; CD: Crohn's disease.

Table 3. Predictive value for the diagnosis of IBD and the UC and CD phenotypes

Amino acid	AUC IBD	AUC UC	AUC CD
Histidine	0.868	0.909	0.827
Leucine	0.822	0.849	NA
Phenylalanine	0.836	0.853	0.818
Tryptophan	0.853	0.898	0.809
Tyrosine	0.778	NA	NA
Valine	0.778	0.807	NA
Serine	NA	0.836	NA

Predictive values were measured using a receiver operator curve. Abbreviations: AUC, area under the curve; IBD: inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn's disease; NA: not applicable. Levels of significance are similar to p-values in Table 2.

DISCUSSION

In this study, we compared faecal amino acid profiles of *de novo*, treatment-naïve paediatric IBD patients with matched healthy controls, by means of targeted amino acid analysis. We observed that six faecal amino acid profiles of IBD patients could be discriminated from healthy controls with high accuracy. This high discriminative accuracy remained intact while focusing on the phenotypes UC and CD. However, UC could be discriminated from HC more accurately and based on a broader range of different amino acids compared to the CD subgroup. Lastly, the phenotypes UC and CD could not be discriminated based on their amino acid profiles.

Novel techniques to diagnose *de novo* IBD in paediatric patients have recently been studied in an intention-to-diagnose cohort by analysis of faecal and serum metabolomic patterns of IBD patients (n=69) and endoscopically proven healthy controls (n=29) using liquid chromatography (LC)[22]. In line with our study, levels of the faecal amino acids tryptophan, glycine, citrulline and serine were increased in both UC and CD compared with HC, with the highest concentrations measured in UC patients. Histidine and phenylalanine were also elevated in IBD patients compared to healthy controls, though with a lower discriminative accuracy compared to the other amino acids. Tryptophan levels measured in serum were decreased in the IBD group, whereas levels of serine were increased, compared to healthy controls. Interestingly, concentrations of these two amino acids were elevated in faecal samples of IBD patients, suggesting both a role for a *leaky gut* and for metabolic alterations as potential causes for these differences.

Other studies assessing the faecal human metabolome as biomarker for IBD have solely focused on adult IBD patients. Faecal amino acid profiles of 20 adult IBD patients (10 UC, 10 CD) treated with prednisolone and 5-aminosalicylic acid as part of disease management, were compared to faecal samples of 13 healthy controls by means of nuclear magnetic resonance (NMR) spectroscopy[29]. In line with our study, levels of multiple amino acids were elevated in IBD patients compared to healthy controls. In contrast to our study outcome,

levels of amino acids were significantly increased in CD compared to UC. More specific, faecal concentrations of isoleucine, leucine, lysine and valine were significantly elevated, whereas levels of histidine, tryptophan and phenylalanine were undetectable. Interestingly, increased levels of especially the latter three amino acids (histidine, tryptophan and phenylalanine) were accurate for the differentiation of IBD versus HC in our study. One of the possible explanations for the undetectability of these amino acids in the previous study is the fact that NMR Spectroscopy is a less sensitive technique compared to AAA. Bjerrum et al. assessed the global faecal metabolome of 92 adult IBD patients (48UC, 44CD) and 21 controls by means of NMR spectroscopy[30]. Similar to our study, they found increased amino acid levels in IBD patients compared to healthy controls and related these metabolic changes to malabsorption and dysbiosis.

Studies focusing on the serum metabolome as biomarker for IBD are scarce. In one study analyzing amino acid profiles of IBD patients in blood plasma using targeted AAA on 22 amino acids similar to the ones assessed in our study, decreased plasma amino acid levels of IBD patients (n=387) compared to healthy controls (n=210) were observed[20]. Consistent with our study outcome, these differences were most pronounced for the amino acids histidine and tryptophan. In another study plasma amino acid levels of 43 IBD patients (24 UC, 19 CD) were compared to 17 healthy controls by means of NMR spectroscopy and showed increased levels of phenylalanine, leucine, isoleucine and glycine in IBD patients, whereas levels of histidine, creatine and choline decreased[19]. These studies are in line with the study outcome on paediatric serum amino acids levels in *de novo* IBD patients mentioned earlier.

Based on previous studies it could be hypothesised that the observed increases and decreases in concentration of specific serum amino acids, and increases in all faecal amino acids are due to decreased absorption in the intestines, a loss of amino acids caused by colonic leakage or a metabolic alteration (either by increased catabolism of proteins or by dysbiosis of intestinal microbiota)[25, 31, 32]. However, since levels of faecal amino acids in UC were higher compared to CD in this study, it is more likely that differences between a healthy gut and IBD result from a systemic loss of specific amino acids into the intestines, rather than from a decreased uptake in the jejunum.

Interestingly, all of the significantly increased amino acids in this study are categorised lipophilic to very lipophilic, except for histidine. In addition, phenylalanine, tryptophan, tyrosine and histidine are all (unsaturated) aromatic amino acids. The chemical structure of histidine is also characterised by an electrically charged side chain (basic)[33]. All these chemical characteristics influence cell membrane diffusion and transportation. This might play a role in metabolic alterations due to dysbiosis of intestinal microbiota[34].

Recent evidence demonstrates that the human gut microbiota in the small and large intestine plays a role in dietary protein metabolism[35]. In a study performed by Davenport et al, paired colonic pinch biopsy samples of known inflammation sites were taken from 21 CD and 23 UC patients and from 24 HC, on which PCR analysis of the variable region 4 of bacterial 16S ribosomal RNA was performed. They found that bacteria inhabiting inflamed tissue were related to the metabolism of amino acids[36]. For example, in a recent mice

model study on the CARD9 (caspase recruitment domain family member 9) gene, it was shown that tryptophan metabolism is altered in IBD[37]. Here, it was shown the microbiota of CARD negative mice failed to metabolize tryptophan into aryl hydrocarbon receptor (AHR) ligands. Inflammation decreased once the mice were treated with tryptophan metabolizing bacterial strains or AHR ligands. Based on these studies, it is not possible to address the differences in amino acid levels of faecal IBD samples directly to metabolic alterations. Whether these amino acids play an etiological role rather than reflecting inflamed intestines need to be elucidated in future (longitudinal) studies comparing levels of amino acids in blood plasma and faecal.

It has been shown that medication alters the composition of the faecal metabolome[30]. The strength of the current study is that potential bias by colonoscopy preparation and medication was circumvented by including only patients with *de novo*, treatment-naïve IBD, with samples collected prior to bowel preparation. Potential bias by differences in water content was circumvented by freeze-drying our samples according to a strict protocol. By strictly matching IBD subjects and controls, potential bias by age and sex was prevented. In addition, we were able to detect low concentrations of amino acids due to application of a highly sensitive and advanced technique to detect these metabolites.

A limitation of this study is that clinical activity was assessed using the GPA, an activity scoring system based on a subjective physician's observation with the possibility of inter- and intra-observer variance. However, each IBD subject underwent endoscopy upon inclusion, so we believe this may have only limited influence on the outcome. Second, we did not collect detailed information on dietary intake upon inclusion. The intake of dietary essential and non-essential amino acids possibly influences amino acids levels in the colon. It could be hypothesised that children with *de novo* IBD have a lower dietary intake compared to healthy controls due to dietary alterations linked to their illness, possibly influencing outcome. Therefore, differences in secretion or metabolism of certain amino acids between IBD patients and healthy controls could be masked due to differences in dietary intake. On the other hand, considering the increased amino acid levels of IBD patients compared to HC it is not likely this had a major influence on our study. A third limitation of this study is the difference in duration of sample storage. The samples of IBD patients were stored for an average time of four years, whereas those of the control group were stored for just six months. Since storage time is of potential influence on metabolite degradation, it seems likely that more amino acids may have been degraded in the IBD group compared to the healthy group. However, since levels of amino acids were significantly higher in the IBD groups compared to the controls, metabolite degradation was thought not to be of substantial influence on the outcome of this study.

In conclusion, in this explorative case-control study, faecal amino acid analysis allowed for discrimination between paediatric *de novo*, treatment-naïve IBD and controls. In particular, histidine, tryptophan and phenylalanine were strongly predictive for the discrimination of IBD patients and healthy controls. Since faecal amino acid analysis is a relatively fast, non-invasive and inexpensive technique, it seems warranted to evaluate the potential of the aminogram as novel biomarker for IBD.

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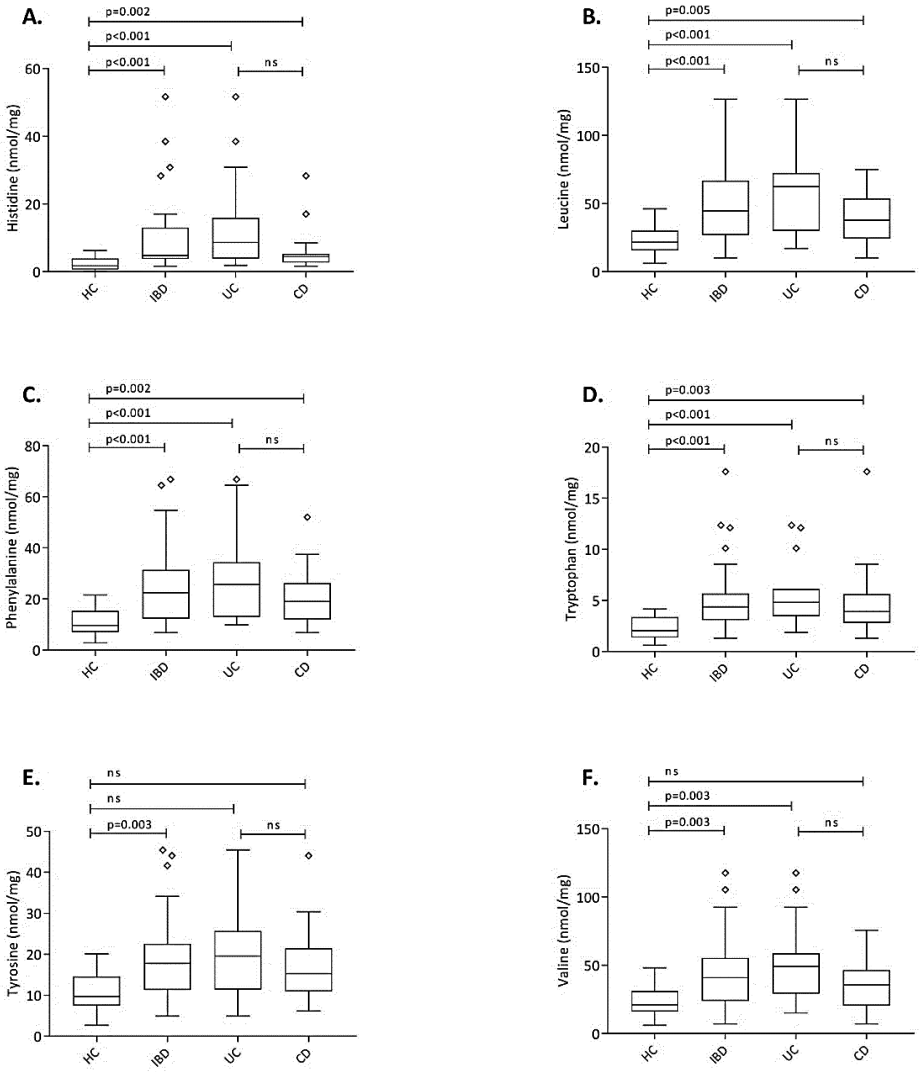
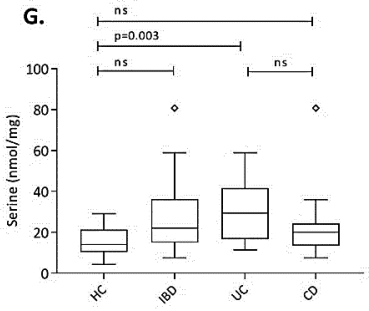
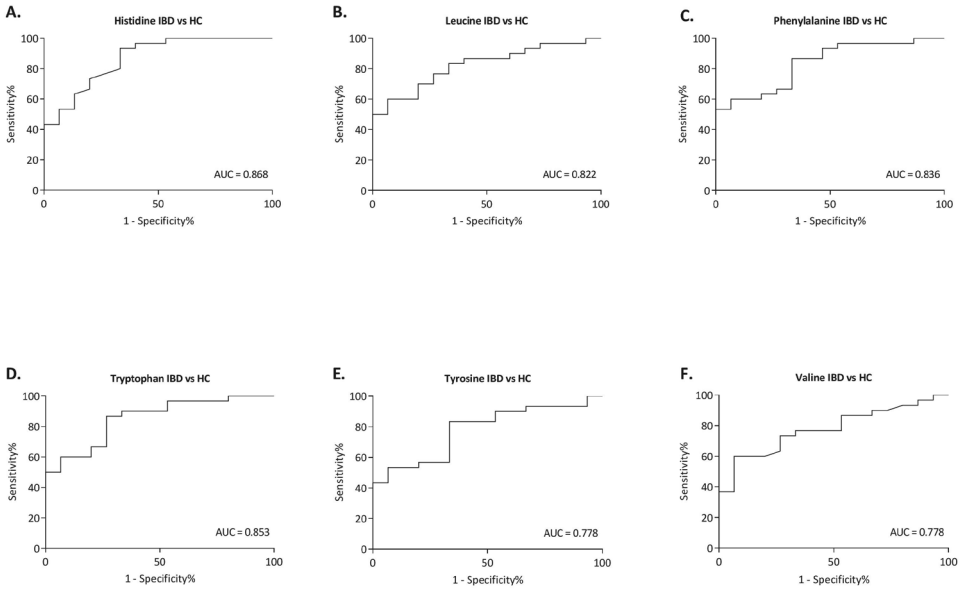
SUPPLEMENTARY MATERIAL

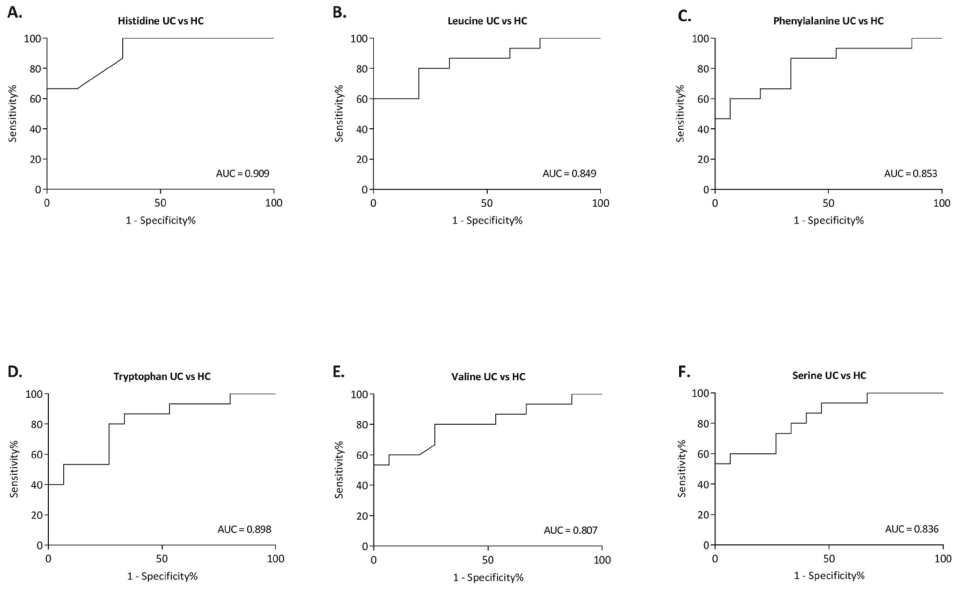




Supplementary figure 1. Box Whisker plots for all significantly different amino acids.
Box Whiskers plots with associated levels of significance. A: Histidine; B: Leucine; C: Phenylalanine; D: Tryptophan; E. Tyrosine; F. Valine; G. Serine. Abbreviations: HC, healthy controls; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease.

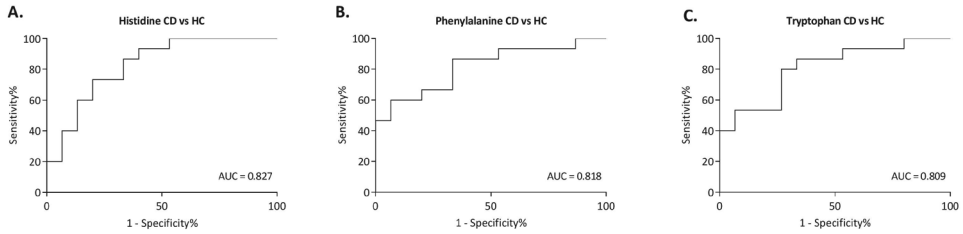


Supplementary figure 2A. Receiver operating characteristics curve for all significantly different amino acids between IBD and HC
A. Histidine IBD versus HC; B. Leucine IBD versus HC; C. Phenylalanine IBD versus HC; D. Tryptophan IBD versus HC; E. Tyrosine IBD versus HC; F. Valine IBD versus HC. Diagonal segments are produced by ties. Abbreviations: IBD, inflammatory bowel disease; HC, healthy controls.



Supplementary figure 2B. Receiver operating characteristics curve for all significantly different amino acids between UC and HC

A. Histidine UC versus HC; B. Leucine UC versus HC; C. Phenylalanine UC versus HC; D. Tryptophan UC versus HC; E. Valine UC versus HC; F. Serine UC versus HC. Diagonal segments are produced by ties. Abbreviations: UC, ulcerative colitis; HC, healthy controls.



Supplementary figure 2C. Receiver operating characteristics curve for all significantly different amino acids between CD and HC

A. Histidine CD versus HC; B. Phenylalanine CD versus HC; C. Tryptophan CD versus HC. Diagonal segments are produced by ties. Abbreviations: CD, Crohn's disease; HC, healthy controls.

CHAPTER 10

Altered tryptophan levels in patients with inflammatory bowel disease owing to colonic leakage, metabolism, or malabsorption?

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To the editor,

With great interest we read the study by Nikolaus *et. al.* concluding that increased tryptophan (TRP) metabolism is associated with disease activity of inflammatory bowel disease (IBD)[1]. We agree with the authors that there still is an unmet need for non-invasive, cost-effective, reproducible and easy-to-perform tests for the diagnosis and risk assessment of IBD. Aromatic amino acids like TRP seem to be a promising candidate in this respect. We would, however, like to add some considerations regarding the underlying pathophysiologic mechanisms causing these differences in serum tryptophan levels.

The authors hypothesised that the observed decreased serum levels of TRP in IBD might be explained by several mechanisms. First, serum TRP might be decreased due to down-regulation of the expression of the neutral amino acid transporter solute carrier family 6 member 19/system B(0) neutral amino acid transporter 1 (SLC6A19/B0AT1) found in their study, which is the main intestinal transporter involved in TRP absorption[2]. Unfortunately, faecal TRP levels were not assessed, hampering to exclude malabsorption as cause for decreased serum levels. In our recent publication on faecal amino acid levels in *de novo*, treatment-naïve paediatric IBD patients we have described that levels of TRP, amongst other amino acids, were elevated in stool samples of IBD patients[3]. Since we observed higher levels of faecal amino acids in UC subjects, in whom small intestines were by definition not affected, compared to CD, we hypothesised that jejunal malabsorption would be an unlikely explanation. However, Nikolaus et. al. reported stronger down-regulation of SLC6A19/B0AT1 in UC compared to CD. This outcome, in combination with the elevated faecal TRP levels observed in our study, reinforces the possibility of malabsorption as a cause for the TRP level changes in IBD.

Another explanation given by the authors is the poorer nutritional state of CD patients compared to UC patients. While dietary intake of TRP was assessed in a subgroup of patients in their study, differences in nutritional status between the groups were not examined. We would like to add the consideration that, based on disease localisation, malabsorption would be expected to be more distinct in CD than in UC, while UC patients are expected to suffer more profoundly from protein loss trough colonic leakage. Our observations of higher faecal TRP levels in UC compared to CD, therefore underlines the hypothesis that colonic leakage might play a major role in causing TRP level differences in active IBD. In addition, 90% of TRP is used in the kynurine (KYN) pathway, which includes the production of quinolinic acid (QUI), picolinic acid (PIC) and kynurenic acid (KYA)[4]. The authors have shown an increased KYN/TRP ratio, increased levels of QUI and lower levels of PIC and KYA in IBD versus controls, illustrating the altered TRP metabolism in IBD. This alteration might have contributed to the decreased levels of serum TRP. However, one would expect decreased faecal TRP levels in upregulated TRP metabolism, and not to increased levels as observed in the IBD subjects in our study. We therefore think that both malabsorption and colonic leakage are more likely to be of influence on TRP levels than alterations in its metabolism.

Furthermore, we would like to emphasize that despite the observed correlation between IBD and TRP, it remains yet unraveled whether this phenomenon is a consequence rather than cause of disease activity. Possibly, TRP plays a role in maintaining IBD activity. Previous

mouse studies have shown that failure of TRP metabolism into aryl hydrocarbon receptor (AHR) ligands after stopping the expression of the caspase recruitment domain-containing protein 9 (CARD9) gene, induced colitis-like symptoms[5]. The gut microbiota metabolizes TRP into indole derivates, used for production of ARH ligands[6]. In addition, it is now known that ARH induces IL22 production, which plays a role in regeneration and protection of the intestinal mucosa[5,6]. We therefore hypothesize that lower levels of TRP, provoked by malabsorption or colonic leakage, could possibly influence IL22 secretion and maintain inflammatory symptoms. In line with this hypothesis, Nikolaus et. al. found increased IL22 levels in IBD patients.

Last, the authors emphasize the potential of tryptophan supplementation as a treatment option in IBD, based on mouse studies[5]. In line with our results, previous studies have shown alterations in serum and/or stool levels of aromatic amino acids in IBD patients [7]. We would therefore like to add the consideration that not only tryptophan, but also other (aromatic) amino acids, like phenylalanine and tyrosine, may hold potential for both diagnostic and treatment purposes in IBD.

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CHAPTER 11

Faecal amino acid profiles exceed accuracy of serum amino acids in diagnosing paediatric inflammatory bowel disease

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ABSTRACT

In this prospective intention-to-diagnose pilot study, we aimed to assess accuracy of serum and faecal amino-acids to discriminate *de novo* paediatric inflammatory bowel disease (IBD) and non-IBD children. Patients with suspected IBD were allocated the IBD (n=11) or non-IBD group (n=8) following laboratory testing or endoscopy according to the revised Porto-criteria. Faecal calprotectin levels were obtained, an addition blood and faecal sample were collected. Faecal and serum amino-acid profiles were analysed using High Performance-Liquid Chromatography. Nine faecal amino-acids (alanine (area under the curve 0.94), citrulline (0.94), glutamine (0.89), leucine (0.98), lysine (0.89), phenylalanine (0.99), serine (0.91), tyrosine (0.96) and valine (0.95) differed significantly between IBD and non-IBD. In serum, no significant differences were observed. This study underlines the potential of faecal amino-acids as novel, adjuvant non-invasive and low-cost biomarkers in the diagnostic work-up of paediatric IBD detection.

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic gastrointestinal tract inflammation and comprises ulcerative colitis (UC) and Crohn's disease (CD). Diagnosis and classification of paediatric IBD is primarily established by means of endoscopy and histopathology. This invasive procedure carries high burden on children, since endoscopy is performed under general anesthesia [1].

Faecal calprotectin (FCP) is the commonly used non-invasive biomarker in the diagnostic work-up of IBD because of its high sensitivity for mucosal inflammation (0.98, 95% CI 0.95-0.99). This biomarker is, however, hampered by low specificity values, especially in paediatric patients (0.68, 95% CI 0.50-0.86)[2]. This leads to a large number of false positive tests and subsequently to unnecessary invasive procedures.

Recent studies have demonstrated high sensitivity of amino acid (AA) composition in serum and faecal to discriminate (paediatric) IBD patients and healthy controls[3, 4]. Specificity values have not yet been addressed as no intention-to-diagnose population has been used as control group so far. We aimed to assess which biological sample holds the highest accuracy in the diagnostic work-up of paediatric IBD in an intention-to-diagnose population.

METHODS

Study design

This prospective intention-to-diagnose pilot study was performed between February 2018 and February 2019 at the outpatient clinic of the paediatric gastroenterology department of the tertiary hospital Amsterdam UMC, The Netherlands.

Participants

Children aged 4-17 years, referred to the outpatient clinic because of suspected IBD, who were sent for routine laboratory blood tests after their first appointment, were asked to participate in this study. Exclusion criteria were proven infectious colitis one month prior to inclusion (defined by a positive stool culture for Salmonella spp., Shigella spp., Yersinia spp., Campylobacter spp. or Clostridium spp. Toxins), use of antibiotics, probiotics or immunosuppressive therapy three months prior to inclusion, co-morbidity with autoimmune diseases or other gastro-intestinal diseases (i.e. celiac disease, Hirschsprung's disease), gastro-intestinal surgery except for appendectomy. According to the revised Porto-criteria, patients were allocated to the IBD group when IBD was proven based on endoscopic, radiologic and/or histologic findings[5]. Non-IBD patients were defined as children referred to our centre because of suspicion of IBD either based on medical history, laboratory testing, but in whom the diagnosis of IBD was not established after additional laboratory testing, radiological and/or endoscopic assessment.

Sample and data collection

All patients were asked to complete an online questionnaire including items on medical history and somatic symptoms. Clinical disease activity of the IBD-cases was determined

by the Paediatric Crohn's Disease Activity Index (PCDAI) [6] or the Paediatric Ulcerative Colitis Activity Index (PUCAI) [7]. Localisation and behaviour of paediatric IBD were classified according to the Paris classification [8]. Blood samples were collected during routine laboratory tests, prior to initiation of therapy. Stool samples were collected and subsequently frozen at -20 degrees within 24 hours following blood sampling and before bowel cleansing was performed. From the obtained faecal samples, 500mg was freeze-dried for further analysis.

Amino acid analysis

Faecal samples were measured using targeted High-Performance Liquid Chromatography (HPLC) conform our previously established standardised protocol[9]. Serum AA composition was determined by the Standard Operating Procedure of the Amsterdam UMC for routine serum AA analysis. Separation of AAs was done by ion-exchange chromatography followed by post-column derivatization with ninhydrin and detection by UV-absorbance.

Statistical analysis

Statistical analysis was conducted conform our previous study[9]. On account of the small sample size, the Mann Whitney U test / Wilcoxon rank-sum test was used to calculate p-values and area under the curve (AUC) values to identify differences in aminograms between the IBD and non-IBD. To correct for multiple testing, a p-value of <0.005 was considered statistically significant. Kruskall wallis tests, followed by Mann-Whitney U tests in case of significance, were used to explore differences in profiles between UC, CD and non-IBD groups. Sensitivity and specificity of faecal AAs were compared to those of calprotectin. Spearman's Rho test was used to calculate correlation coefficients between calprotectin and significantly different AAs.

Ethical considerations

This study was approved by the Medical Ethical Review Committee (METc) of the VU University Medical Centre with file number 2015.393. Written informed consent was obtained from children and/or parents (only parents in case children <12 years).

RESULTS

Baseline characteristics

Written informed consent was given by 20 participants. One patient was excluded as the faecal sample was not sufficiently collected. A total of 19 patients were consecutively included in this study of whom 11 were diagnosed with IBD (6 UC, 5 CD) and 8 were non-IBD controls. Demographics are listed in Table 1. There were no significant differences in age, body-mass index (BMI), gender, symptoms duration, average stool consistency, sample storage time and levels of C-reactive protein between IBD and non-IBD. Calprotectin levels differed significantly between groups (median IBD 2050 µg/g vs. median 75.5 µg/g non-IBD, p=0.001). Three out of eight non-IBD patients had elevated faecal calprotectin levels (202, 642 and 1300 µg/g). Non-IBD patients with high calprotectin levels were monitored at the outpatient clinic for a median period of 3 months (min-max 2-10 months).

Table 1. Demographics

	Ulcerative colitis (n=6)	Crohn's disease (n=5)	Inflammatory Bowel Disease Total (n=11)	Controls (n=8)
Sex, male (n [%])	5 [83.3]	2 [40]	7 [63.6]	4 [50]
Age (median [IQR]), years)	10.4 [8.1-14.3]	15.9 [14.6 – 17.2]	14.4 [9.2 – 16.6]	12.8 [9.4 – 16.7]
Storage time (median [IQR], months)	3.5 [2.3 – 10.3]	5 [1.5 – 7]	4 [3 – 9]	8 [8 – 9]
BMI (median [IQR])	15.6 [14.5 – 19.2]	17.2 [15.0 – 20.1]	16.1 [14.9 – 19.1]	19.1 [15.6 – 24.2]
Bristol stool chart (n [%])				
Type 1	0 [0]	0 [0]	0 [0]	0 [0]
Type 2	0 [0]	0 [0]	0 [0]	0 [0]
Type 3	0 [0]	0 [0]	0 [0]	2 [25]
Type 4	0 [0]	1 [20]	1 [9.1]	3 [37.5]
Type 5	0 [0]	2 [40]	2 [18.2]	0 [0]
Type 6	3 [50]	1 [20]	4 [36.4]	3 [37.5]
Type 7	3 [50]	1 [20]	4 [36.4]	0 [0]
Duration of symptoms (median [IQR], months)	3.5 [1.5 – 4.5]	4 [0.5 – 14.5]	4 [1 – 5]	6 [3 – 16.5]
Faecal calprotectin¥ (µg/g) (median[IQR])	3210 [850.5 – 5739.8]	1312 [870.5 – 2113.5]	2050 [1010 – 3680]¥	75.5 [17.3 – 532]¥
CRP (mg/l) (median[IQR])	2.5 [2.5 – 3.4]	36 [22 – 43]	6 [2.5 – 36]	2.5 [2.5 -20.5]
Disease Activity Index[1] (median[IQR])	60 [46.3 – 61.3]	22.5 [21.3 – 32.5]	NA	NA
Disease Activity Index[1] (n [%])			NA	NA
Remission	0 [0]	0 [0]		
Mild	1 [16.7]	4 [80]		
Moderate	4 [66.7]	1 [20]		
Severe	1 [16.7]	0 [0]		
Crohn's disease localisation[2]				
Ileal (L1)	NA	2 [40]	NA	NA
Colonic (L2)	NA	0 [0]	NA	NA
Ileocolonic (L3)	NA	3 [60]	NA	NA
Proximal disease (L4)	NA	1 [20]	NA	NA
Crohn's disease behaviour[2]				
B1 (NSNP)	NA	3 [60]	NA	NA
B1p (NSNP+p)	NA	2 [40]	NA	NA
B2 (S)	NA	0 [0]	NA	NA
B2p (S + p)	NA	0 [0]	NA	NA
B3 (P)		0 [0]	NA	NA
B3p (P + p)		0 [0]	NA	NA

Table continues

	Ulcerative colitis (n=6)	Crohn's disease (n=5)	Inflammatory Bowel Disease Total (n=11)	Controls (n=8)
Ulcerative Colitis[2]				
Proctitis (E1)		NA	NA	NA
Left-sided (E2)		NA	NA	NA
Extensive (E3)		NA	NA	NA

All values were obtained at study inclusion. Localisation of IBD was obtained by ileocolonoscopy and esophagogastroduodenoscopy before treatment initiation, and MR enteroclysis. Abbreviations: IQR, interquartile range; NA, not applicable; NSNP, non-stricturing non-penetrating; S, stricturing; P, penetrating; p, peri-anal disease. [1] Based on Paediatric Crohn's Disease Activity Index (PCDAI) and Ulcerative Colitis Activity Index (PUCAI). PCDAI defined as remission <10, mild 10-34, moderate 35-64, severe 65-85 points; PUCAI defined as remission <10, mild 10-27.5, moderate 30-37.5, severe ≥40. [2]Based on Paris classification for inflammatory bowel disease [¥]Significant differences between IBD and controls p<0.001, analysed using the Mann-Whitney U test.

Amino acid composition

Inflammatory bowel disease versus controls

A total of 42 unique AAs were detected in all samples together of whom 20 could not be used of statistical analysis due to undetectable levels in more than 50% of the cases. From the 22 AAs included in the statistical analysis of faecal and serum, nine faecal AAs (alanine (AUC 0.94), citrulline (AUC 0.94), glutamine (AUC 0.89), leucine (AUC 0.98), lysine (AUC 0.89), phenylalanine (AUC 0.99), serine (AUC 0.91), tyrosine (AUC 0.96) and valine (AUC 0.95) differed significantly between IBD and non-IBD (Figure 1). Six faecal AAs were significantly different when comparing UC patients to the non-IBD group (alanine (AUC 0.96), leucine (AUC 1.00), phenylalanine (AUC 1.00), serine (AUC 0.90), tyrosine (AUC 0.98), valine (AUC 0.98). In CD patients versus non-IBD, these same faecal AAs had lowest p-values, though, none of them reached a level <0.005. No differences between subgroups of UC and CD were observed. In serum, no significant differences between IBD, subgroups of IBD and non-IBD were observed. We calculated cut-off values of faecal AAs to reach optimum specificity and sensitivity values. When aiming to obtain specificity levels of 100%, we observed high sensitivity of faecal alanine (80%; cut-off value 42.1 nmol/mg), leucine (90%; 24.1 nmol/mg), phenylalanine (90%; 12.9 nmol/mg), tyrosine (80%; 12.3 nmol/mg) and valine (80%; 25.9 nmol/mg).

Comparison of faecal amino acids and calprotectin

Based on the widely used cut-off value of 150 µg/g for calprotectin, all IBD children were classified correctly (sensitivity 100%) and three out of eight non-IBD children incorrectly (specificity 66.7%). Of these incorrectly classified non-IBD patients displaying elevated levels of calprotectin, only one displayed high levels of faecal AAs as well. This patient underwent a second endoscopic assessment a year after this study was closed, and is now treated for IBD-unclassified.

Correlation between faecal amino acids and calprotectin

The faecal AAs citrulline (r=0.486, 95%CI 0.125 – 0.724), leucine (r=0.583, 95%CI 0.249 – 0.788), phenylalanine (r=0.575, 95%CI 0.165 - 0.802) and valine (r=0.529, 95%CI 0.149 –

0.782) revealed a significant correlation to faecal calprotectin.

DISCUSSION

We compared simultaneously collected faecal and serum AA profiles of *de novo* treatment-naïve paediatric IBD patients and controls by means of targeted AA analysis and found statistically significant differences in nine faecal AAs. There were no differences in serum AA levels.

In our previous study, faecal amino acid composition of 30 *de novo* treatment-naive paediatric IBD patients (15 UC, 15 CD) and 30 healthy controls (HC) were assessed using the same methodology. A significant increase in six faecal AAs was observed in IBD subjects when compared to HC(8). These AAs were amongst the most increased AAs in the current study, but were not all significantly altered in our current study. Contrary, our outcomes on serum AA composition were different to the currently available literature. In a previous study on assessment of serum AA levels, 387 adult IBD patients were compared to 210 asymptomatic healthy controls[4]. A decrease in serum tryptophan and histidine were observed. In the current study, we did find negative z-scores for these AAs (-0.713 and -2.03, respectively), however, these differences were not significant. This may be due to our smaller sample size. We did find high AUC-values for faecal AAs, suggesting that these differences may be more explicit and therefore more useful as noninvasive biomarkers.

The etiology and pathogenesis of IBD is still largely unknown. Studies focusing on metabolomics have contributed to a better understanding of IBD pathogenesis. Proteins, consisting of AAs, play an important regulatory role in metabolic pathways and maintaining intestinal health[10]. Current hypotheses on the underlying mechanisms of alterations in faecal AA levels in IBD patients include malabsorption, alterations in metabolism, gut dysbiosis and colonic leakage [11, 12]. As some AAs are essential, meaning they cannot be synthesized and must be obtained from the diet, IBD patients suffering from malabsorption would be expected to have decreased serum levels of essential amino acids. Since no differences in serum AA levels between IBD and controls were observed and nearly all serum values lay within the referency ranges of healthy children, malabsorption can be considered an unlikely explanation for the observed differences. The increase in faecal AA levels whilst sustaining normal levels in serum is possibly caused by a loss and degradation of proteins in infected and damaged enterocytes and colonocytes[13]. Another explanation may be a change in intestinal metabolism. Accumulating evidence shows a correlation between faecal AA levels and alterations in gut microbiota in IBD patients [14, 15].

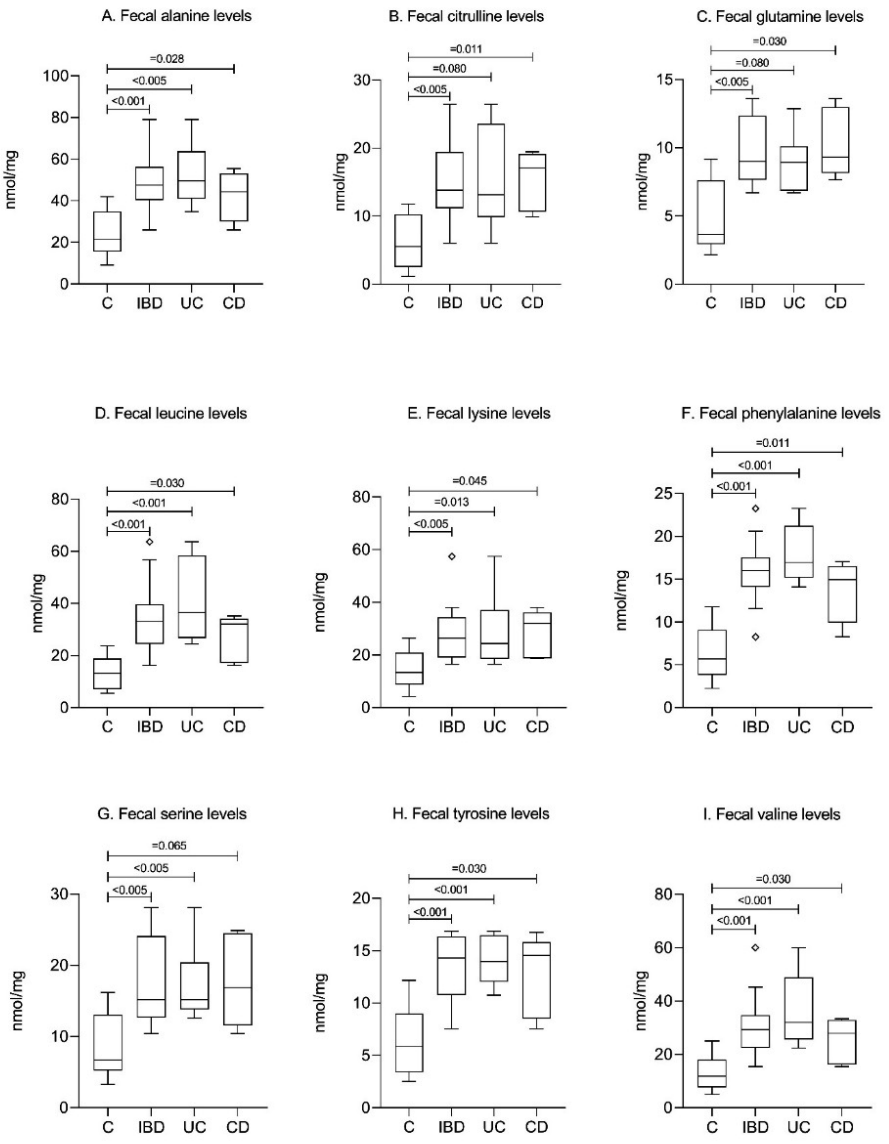

Figure 1. Box Whisker plots for faecal amino acids differentiating between inflammatory bowel disease patients and controls

Faecal amino acid levels are given in μmol/L. Levels were considered significant at a p-value of <0.005. Abbreviations: IBD inflammatory bowel disease, UC ulcerative colitis, CD crohn's disease. C controls

This study has several strengths. This was the first study in which simultaneous analysis of faecal and serum AA profiles in IBD patients was performed. We included participants in an intention-to-diagnose setting, which warranted inclusion of patients generalizable to the population presented at outpatient clinics. *De novo* treatment naïve paediatric IBD patients were included, avoiding potential bias by medication, colonoscopy preparation and long term disease activity. Furthermore, as we freeze-dried our faecal samples, potential bias by differences in stool consistency was circumvented. In addition, targeted AA analysis was done by a high sensitivity platform and based on standard operating protocols. As an explorative pilot study, we also encountered some limitations. Our sample size was small and therefore our study had low power. This may have resulted in false-negative results. In addition, we did not assess dietary intake so we were not able to correct for specific food products despite the knowledge of that lower protein intake negatively effects serum levels of specific AAs (e.g. tryptophan, phenylalanine, tyrosine)[16, 17]. As correction for dietary intake would lead to hundreds of variables, correction for this would not have been feasible based on the current sample size. However, as all children are residential in the same area in The Netherlands, we do not expect large differences in dietary intake. Last, the three non-IBD patients displaying high levels of faecal calprotectin were monitored for a median period of time of 3 months to exclude development of IBD later on. Only one non-IBD displayed high levels of faecal AAs upon inclusion. Remarkably, after closing the study, this patient underwent a sesecond endoscopic assessment because of persisting symptoms and is now treated for IBD-undetermined. This illustrated the high sensitivity of faecal AAs and their potential additional value as non-invasive biomarkers.

We have planned a large intention-to-diagnose cohort study to validate faecal AAs as biomarkers, since this test is cost-effective, reproducible and easy to perform[9]. Targeted AA analysis and calprotectin have comparable costs (approximately 28 [95% CI 15-50] American dollar), whereas endoscopy with biopsies cost approximately 1171 [700-2000] dollars, excluding anesthetics[18]. As demonstrated, high specificity values of faecal AAs may be reached whilst maintaining acceptable sensitivity values. Upon validation of faecal AAs, a diagnostic algorithm may be established combining calprotectin and multiple faecal AAs to improve test accuracy. The best performing AAs may be combined with calprotectin in a 2-step approach to lower the number of false-positive and false-negative tests and subsequently lower the number of unnecessary endoscopic assessments. Another subject of interest may be the correlation between faecal AAs and IBD localisation and to predict disease exacerbation or response to therapy using AAs allowing for development of personalised follow-up strategies.

In conclusion, we observed that levels of nine faecal AAs were significantly increased in IBD versus intention-to-diagnose controls, while serum levels were comparable. This finding underlines the potential of faecal AA levels as additional non-invasive, low-cost biomarker in the diagnostic work-up of paediatric IBD.

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PART IV

The role of the faecal metabolome and microbiota in colorectal neoplasia

CHAPTER 12

Faecal volatile organic compounds for early detection of colorectal cancer: where are we now?

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ABSTRACT

Background

The faecal volatolome, which is composed of faecal volatile organic compounds (VOCs), seems to hold potential as non-invasive biomarker for the detection of colorectal cancer (CRC) and its precursor lesions advanced adenomas (AA). The potential of the faecal volatolome has been subject of various studies using either chemical analytical or pattern-recognition techniques. The available literature on the potential of the faecal volatolome as CRC and AA biomarker was reviewed.

Methods

A systematic literature search was conducted in PubMed, Embase, the Cochrane Library, Google Scholar and ResearchGate using the following keywords: Colorectal Cancer, Advanced Adenoma, Volatile Organic Compound, Metabolome, Gas Chromatrography-Mass Spectrometry, Selected-Ion Flow-Tube Mass-Spectrometry, eNose, and Faecal Biomarkers.

Results

Eighty-eight titles or abstracts were identified from the search, of which eleven papers describing the potential of the faecal volatolome for CRC detection were selected. In these studies, different techniques were used for the headspace analyses of faecal VOCs, limiting the possibility to compare outcomes. Increased levels of amino acids and short chain fatty acids, and decreased levels of bile acids and polyol alcohols in the gas phase of faeces were observed repeatedly. All selected papers reported high diagnostic value for the detection of both CRC and AA based on faecal VOCs.

Conclusion

Based on the included studies, faecal VOC analysis seems promising for future screening of CRC and AA, with potentially improved test performances allowing for earlier detection of AA and CRC and consequently earlier initiation of treatment, possibly reducing morbidity and mortality rates next to lower rates of (unnecessary) colonoscopies.

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy with an incidence rate of 40.7 per 100.000 in the US, and has the highest cancer-related mortality rate in the industrialised world [1-3]. The 5-year survival rate for colon cancer and rectum cancer are 64.4% and 66.6%, respectively[3]. Early detection and treatment are critical factors in the course and prognosis of CRC, as the survival rate decreases with disease progression[4]. Most CRC develops from advanced adenomas (AA), and early detection and removal of these adenomas has been found to decrease CRC incidence and mortality [5, 6]. The most widely used screening modalities for CRC and its neoplastic precursors are faecal immunochemical testing (FIT) and endoscopic evaluation of the colon. Although this screening program has led to a decrease in mortality, the performance of this test is suboptimal, with a sensitivity and specificity for CRC of 56-89% and 94-97%, respectively, depending on used cut-off values [7]. This results in a substantial number of false negative tests, and as a consequence missed diagnosis of colorectal cancer in 11-44% of the cases. In addition, 3-6% of the healthy participants undergoing population based screening still receive false positive test results, which leads to the performance of unneeded colonoscopies. These colonoscopies carry a high burden on patients and create a small risk of complications (e.g. bleeding or perforation)[7]. Because of these limitations, a clear unmet need exists for a more accurate and non-invasive test to select high-risk individuals who need to undergo colonoscopy.

The use of gas molecules as noninvasive disease biomarkers stems from a long history of medicine in which Hippocrates characterised the distinct smell of melena as early as 400 years BC, and patients with diabetes were described as have urine with a smell of rotten apples in ancient Chinese medicine [8]. Nowadays, gaseous molecules are analysed using highly sensitive techniques, resulting in smellprints comprised of over a thousand different gaseous molecules, referred to as volatile organic compounds (VOCs), or the 'volatolome'. These VOCs are produced during metabolic processes such as inflammation, cancer degeneration and necrosis and can be measured in all conceivable bodily excrements including breath, urine and faecal dependent on their volatility and temperature of the sample. The faecal volatolome is also believed to reflect alterations in gut microbiota by a change in VOCs created during gut-microbiota interactions[9]. Multiple studies have focused on the use of the faecal volatolome as biomarker for CRC and AA with promising results[10, 11]. In this systematic review we have aimed to summarize the available literature on faecal volatolome analysis for the differentiation between CRC, AA and controls, and philosophize about the clinical implications for improved screening on colorectal cancer.

METHODS

An electronic literature search was performed systematically, by using the electronic database of the National Centre for Biotechnology Information (PubMed), Embase, Cochrane Library, Google Scholar and ResearchGate to collect publications before June 2018. The following search terms for colorectal cancer and advanced adenoma, including synonyms and closely related words, were used as index terms and/or free words: 'colorectal carcinoma', 'colon cancer', 'rectal cancer', 'colorectal neoplasia', 'colorectal tumor', 'advanced adenoma',

'high-risk adenoma'. These terms were combined with index terms and/or free words for VOC analyses ('gas chromatography-mass spectrometry', 'ion mobility spectrometry', 'selected-ion flow-tube mass-spectrometry', 'electronic nose', 'volatile organic compounds', 'volatolome', 'gas molecules', 'metabolome') and faecal biomarkers ('faecal', 'faecal marker', 'faecal biomarker'). After the search, the collected literature was screened on title and abstract by two authors independently, and included in this study after selection by both of the authors (SB and NKHDB). The reference lists of identified papers were checked on additional studies missed during the original search. Full-text articles, abstracts and posters were only included if they focused on the faecal VOC composition, excluding other bodily fluids and metabolites. Non-original articles, reviews, duplicates and articles in other languages than English or Dutch were excluded from this review.

RESULTS

Eighty-six titles or abstracts were identified from the primary electronic search and reference lists after the literature search was performed in PubMed, Embase and the Cochrane Library and two articles were found using Google Scholar and ResearchGate (*Figure 1*). Of the identified records, 77 publications did not meet the criteria for inclusion for various reasons; a total of 11 publications could be included in this systematic review. Reasons for exclusion were the use of mucosal biopsies and other material rather than faecal, newly described methods without statistics provided, replies on papers or editorial comments and papers not relevant to the topic. Included literature consisted of nine full-texts original articles, one abstract and one poster presentation (*Table 1)*. In six studies, gas chromatography (GC) was used for VOC identification, of which three used GC combined with mass spectrometry (GC-MS). Two studies made use of a self-made VOC sensor: SCENTA1. Other studies made use of selected-ion flow-tube mass spectrometry (SIFT-MS), electronic Nose (eNose) and dogs for scent detection.

Volatolome analytical techniques

Analytical methods can be separated into chemical analytical techniques and pattern-recognition technology. In chemical analytical techniques, alterations in the presence and concentrations of specific molecules can be detected, whereas pattern-recognition technologies focus on the discrimination of VOC profiles by differences in sensor resistance to specific VOCs. In this section of this review an overview of the used analytical methods for faecal VOC analyses for the detection of CRC is given. In table 2, technical characteristics per analytical method are summarised.

Chemical analytical techniques

Gas-chromatography Mass-Spectrometry

This method has been proved to successfully analyse the VOC composition of various bodily fluids and is considered the gold standard for volatolome analyses[12]. Coupling chromatography to spectrometry allows for separation and quantification of individual VOCs. These analytes are transported through the chromatography column in a carrier gas and interact with the column surface. VOCs with different chemical characteristics have specific interactions with the column, resulting in a distinct transportation time for

sets of VOCs with comparable characteristics. VOCs are then ionised and pushed into the second (spectrometry) column, consisting of an electric field with varying voltage. The transportation time of the analytes is influenced by their electric charge, resulting in a distinct transportation time for sets of VOCs with comparable masses[13]. By combining separation on chemical characteristics and mass, the VOC composition can be measured very accurately which allows for biomarker recognition.

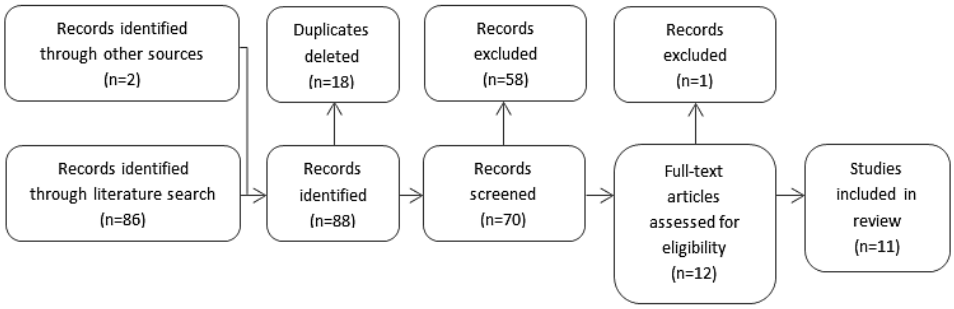

Figure 1. PRISMA flow diagram

Selected Ion Flow Tube-Mass Spectrometry

Another technique used for faecal volatolome analyses for the detection of CRC is selected ion flow tube-mass spectrometry (SIFT-MS). This technique allows for real-time measurements and is therefore faster compared to the gold standard. Mass spectrometry is coupled to a selected ion flow tube, in which VOCs are ionised by precursor ions (H_3O^+, NO^+ and O_2^+) in a defined order[14]. The precursor ions are generated using a microwave discharge and their order for the analysis is chosen using quadrupole mass spectrometry. Before the sample is injected, the selected precursor ions are introduced in the flow tube using helium as carrier gas. The precursor ions and VOCs interact in the flow tube and enter a second quadruple mass spectrometer where the product ions and precursors are separated. Real-time data analyses is done by scanning a specific spectrum of mass-to-charge-ratios defined by the user. The absolute concentration can be calculated from the ratios using the precursor and product ion signals. This technique provides less detailed information on VOC composition compared to GC-MS, since VOCs remain less separated using this analysis, but allows for real-time measurements, has lower maintenance costs and does not require specialised personnel.

Pattern-recognition techniques

eNose technology

There are many sorts of different electronic nose (eNose) devices, which all have in common that VOC analysis is based on pattern-recognition. In eNose technology, VOCs are presented to an array of sensors made from specific material. Analytes interact with the individual sensors based on their chemical characteristics. This sensor-interaction is analysed with different techniques dependent on which eNose is used (e.g. conducting-polymer sensors, electrochemical sensors, metal oxide sensors). The differences in sensor-

interaction create a pattern which is called the VOC 'smellprint', and can be analysed with pattern recognition algorithms to identify specific diseases[15]. The main benefits of eNose technology are measurement speed and low costs and although disease-specific patterns can be recognised, individual VOC biomarkers cannot be identified on a molecular level. Furthermore, sensor drift over time may hamper reproducibility.

SCENT A1

A new eNose device for faecal VOC analyses which has solely been described by one research group so far, is the patented SCENT A1[16]. This is a device made of an array of five chemo resistive sensors which are capable of changing their resistance once in contact with specific VOCs, and can detect tumor biomarkers in low concentrations. The sensor material used in this device are $SmFeO_3$ (Iron and samarium oxides), ST25 + Au (Tin and Titanium), ST20 (Tin and Titanium oxide 20%), TiTav (Titanium, Tantalum and Vanadium oxides) and In_2O_3 (Indium oxide). These sensors have been selected after thorough testing of 20 sensors composed of different material. The array of 5 sensors with the best diagnostic accuracy to differentiate between CRC and HC was selected for further development and validation[17]. Information on practicality, sensor stability and costs are lacking.

Study characteristics and outcomes

Chemical analytical techniques

Gas chromatography-mass spectrometry

The first study to explore the use of the faecal volatolome for the detection of CRC using GC-MS was conducted by Weir in 2013[10]. Stool samples were collected from 10 CRC patients prior to colon resection surgery and 11 healthy controls (HC). Global volatile metabolite profiling was performed on faecal samples of 9 CRC and 10 HC, and targeted short chain fatty acid (SCFA) profiling on 9 CRC and 11 HC,. The global volatolome of CRC patients showed increased levels of 11 amino acids (alanine, glutamate, glycine, aspartic acid, leucine, lysine, proline, serine, threonine, valine and phenylalanine), one carboxlylic acid (benzeneacetic acid), one SCFA (propionic acid) and one saturated fatty acid (myristic acid), one vitamin B5 derivate (pantothenic acid) and one steroid (cholesterol derivate). In addition, three unsaturated fatty acids (oleic, linoleic and elaidic acid) as well as polyol and its derivates (glycerol and monooleoylglycerol), and one bile acid (ursodeoxycholic acid) were decreased. The SCFA profiles of CRC patients showed increased levels of acetic acid, valeric acid, isobutyric acid, isovaleric acid and decreased levels of butyric acid compared to profiles of HC. This study did not report any outcomes on accuracy, specificity and sensitivity for one (or a combination) of these metabolites as CRC biomarker. Another study applying GC-MS was reported by Bond, who analysed faecal samples from symptomatic patients and patients referred after they were tested positive during the UK Bowel Cancer Screening Program[18]. A total of 20 CRC patients and 60 HC were included. Four discriminating compounds were reported, of which compound A showed an area under the curve (AUC) of 0.76 (P-value < 0.0001), and the combination of A and B increased the AUC to 0.82. After a 10-fold cross validation, an AUC of 0.82 with a sensitivity of 87.9% and a specificity of 84.6% was found. During further logistic regression analyses a combination of three compounds (A, X and Y) was found, of which the AUC was 0.86 (P-value <0.0001). Unfortunately, no specific VOCs were named in this abstract, due to potential future intellectual property.

Table 1. An overview of study design, number of patients included, analytical techniques, identified compounds and test specifics of the 11 selected papers

Author (year)	Study design	Number of inclusions	Analytical technique	Identified compounds*	Chemical Classes	AUC	Sensitivity	Specificity	p-Value	Article type
Chemical analytical techniques										
Weir (2013)	Case-control study	Global metabolome 9 CRC 10 HC	GC-MS	↑Alanine, Glutamate, Glycine, Aspartic acid, Leucine, Lysine, Proline, Serine, Threonine, Valine, Phenylalanine, Benzeneacetic acid, propionic acid, myristic acid, pantothenic acid, cholesterol derivate	Amino acids, Carbocylic acid, SCFA, saturated fatty acid, Vitamine B5, Steroid	-	-	-	All p-values <0.05 - <0.0001	Full-text original article
				↓Oleic acid, linoleic acid, elaidic acid, glycerol, monooleoylglycerol, ursodeoxycholic acid	Unsaturated fatty acids, polyol and polyol derivate, bile acid					
		Targeted SCFAs 9 CRC 11 HC	GC-MS	↑Acetic acid, valeric acid, isobutyric acid, isovaleric acid	SCFAs					
				↓Butyric acid						
Batty (2015)	Case-control study	62 FOBT+: 31 AA 31 HC	SIFT-MS	↑Hydrogensulphide Dimethylsulphide Dimethyldisulphide m/z 90 (unknown)	Sulphides	-	72% AA	78% AA	-	Full-text original article
Bond (2016)	Prospective case-control cohort	21 CRC 56 Adenoma 60 HC	GC-MS	No specific VOCs described due to potential future IP	-	0.82 CRC	87.9%	84.6%	<0.0001 CRC	Abstract

Table continues

Author (year)	Study design	Number of inclusions	Analytical technique	Identified compounds*	Chemical Classes	AUC	Sensitivity	Specificity	p-Value	Article type
Wang (2017)	Case-control study	15 CRC 12 HC	GC-MS	↑Acetic acid, valeric acid, butyric acid and isovaleric acid, Glutamic acid, glycine, aspartic acid, leucine, glycerin, proline, serine, valine, phenylalanine, phenylacetic acid, cholesterol derivates ↓isobutyric, oleic acid, elaidic acid, linoleic acid Glycerin, monoacyl glycerol, myristic acid, ursodesoxycholic acid, pantothenic acid	SCFAs, amino acids, steroids SCFA, unsaturated fatty acids, polyhydric alcohols, Saturated fatty acid, bile acid, vitamin B	-	-	-	All p-values <0.05 - <0.0001	Full-text original article
Ishibe (2017)	Prospective case-control study	30 CRC 26 HC	GC-SCD GC-TCD	↑Methyl mercaptan (MM) ↓Hydrogen (H_2)	Sulphide Hydrogen	0.78	-	-	P=0.036 MM P=0.008 H_2	Full-text original article
Song (2018)	Prospective case-control study	26 CRC 28 HC	GC-MS (for long chain fatty acids) GC-MSD (for SCFAs)	↑Oleic acid, Linoleic acid only in male subjects -	Mono and polyunsaturated fatty acids -	-	-	-	Only significant increases in male subjects (p<0.04 – P<0.01)	Full-text original article
Pattern-recognition techniques										
Sonoda (2011)	Prospective case-control study	37 CRC 148 HC	Canine scent judgement	-	-	-	97%	99%	-	Full-text original article

Table continues

Author (year)	Study design	Number of inclusions	Analytical technique	Identified compounds*	Chemical Classes	AUC	Sensitivity	Specificity	p-Value	Article type
De Meij (2014)	Case-control study	40 CRC 60 AA 57 HC	eNose (Cyranose® 320)	Pattern-recognition	-	0.92 CRC 0.79 AA 0.82 AA vs CRC	85% CRC 62% AA 75% AA vs CRC	87% CRC 86% AA 73% AA vs CRC	<0.001 CRC <0.001 AA <0.001 AA vs CRC	Full-text original article
Bond (2016)	Prospective case-control study	18 CRC 18 AA 67 Adenomas 78 HC	GC + metal oxide gas sensor	Pattern recognition based on sensor resistance	-		75% CRC 100% AA 100% CRC vs AA	66.7% CRC 100% AA 100% CRC vs AA	-	Poster
Zonta (2016)	Case-control cohort	6 CRC 10 HC	SCENT A1	Pattern-recognition	-	-	-	-	-	Full-text original article
Zonta (2017)	Not clear from text	Feasibility: 86 CRC 71 CRC	SCENT A1	Pattern-recognition	-	-	95% overall	95% overall	-	Full-text original article
	Prospective case-control cohort	Validation: 6 CRC + AA 20 low risk adenomas 60 HC	SCENT A1	Pattern-recognition	-	-	-	-	-	

*Changes in metabolite levels are noted as measured in CRC/AA samples. Abbreviations: CRC, colorectal carcinoma; AA, advanced adenoma; HC, health control; GC-MS, gas chromatography-mass spectrometry; GC, gas chromatography; SIFT-MS, selected ion flow tube-mass spectrometry; eNose, electronic Nose; SCD, Sulfur Chemiluminescence Detector; TCD, thermal conductivity detector; MSD, Mass selective detector; SCFA, short chain fatty acids.

Table 2. Overview of technical characteristics per volatolome analyses method

	GC-MS	SIFT-MS	Cyranose 320 ®	SCENT A1
Accuracy	Very good separation of analytes using two techniques to separate based on different characteristics	Good separation but some discrimination of VOCs is lost since there is no chromatography column	High accuracy, however no conclusions about specific volatiles can be drawn	High accuracy, however no conclusions about specific volatiles can be drawn
Stability	Needs calibration daily	Very stable, no calibration required	Easily influenced by environment	No information
Speed	10 - 45 minutes per sample	A few seconds – minutes per sample	6 minutes per sample including air blank to clean machine	Average measurement time of 1 hour per sample
Costs	Expensive: Mainly because of costs for technical operators, maintenance and sample preparation	Expensive: Machine is double the price of GC-MS, however less extra expenses because less maintenance and sample preparation	Inexpensive	No information
Usability/ Practicality	Technical operators only	Technical and non-technical operators	Very easy to use for non-operators	No information
Maintenance	High maintenance	Low maintenance	Low maintenance	Sensor renewal needed every 2 years
Sample preparation	Requires preparation and preconcentration	No preparation required	No preparation required	No preparation required

Overview of technical characteristics per volatolome analyses method. Abbreviations: GC-MS, gas chromatography-mass spectrometry; SIFT-MS, Selected-ion Flow-tube Mass-Spectrometry; eNose, electronic Nose.

In 2017, Wang performed a study on the volatolome for CRC detection using GC-MS analyses on faecal samples of 15 CRC patients and 12 HC[19]. The levels of SCFAs acetic acid, valeric acid, butyric acid and isovaleric acid were increased in CRC patients, whereas isobutyric acid was decreased in this group. There was no difference in the level of propionic acid between CRC and HC. Furthermore, levels of 9 amino acids (glutamic acid, glycine, aspartic acid, leucine, glycerin, proline, serine, valine, phenylalanine), phenylacetic acid and

cholesterol derivates were significantly increased in the CRC group compared to HC, and the unsaturated fatty acids (oleic acid, elaidic acid and linoleic acid), two types of glycerin (glycerin and monoacyl glycerol), one saturated fatty acid (myristic acid), one vitamin B5 derivate (pantothenic acid) and one bile acid (ursodeoxycholic acid) were decreased.

In the most recent study on the use of GC-MS for faecal volatolome analyses, samples from 26 newly diagnosed CRC patients were compared to 28 healthy individuals[20]. Analyses of long-chain fatty acids using GC-MS was combined with the analyses of SCFAs using GC coupled to a Mass Selective Detector (GC-MSD). In contrast to the previous studies, no significant differences were found in the levels of both these short and long-chain fatty acids when comparing CRC to HC. Interestingly, sub-analyses by gender did reveal an increase in faecal concentrations of both oleic acid and linoleic acids in male CRC patients whereas in previous studies decreased concentrations of these fatty acids were observed.

Other chemical analytical techniques

In 2015, selected ion flow tube linked to mass spectrometry (SIFT-MS) was used for the identification of individual faecal VOCs by Batty, who included 62 patients with a positive faecal occult blood test (FOBT) resulting in a subsequent performed colonoscopy[21]. During endoscopy, at least one advanced adenoma (referred to as high grade adenoma) or adenocarcinoma was found in 31 of these participants (high risk group), while no abnormalities were observed in the residual of the participants (low risk group). In this article it is not noted how many patients included in the 'high risk' group had CRC or AA. Four analytes (m/z 35 and m/z 90 using H_3O^+ as precursor ion, m/z 62 and m/z 94 using NO^+ as precursor ion) were found to be significantly increased in the high risk group. Possible identities of these analytes were hydrogen sulphide, dimethyl sulphide and dimethyl disulphide. The compound with mass-to-charge ratio 90 remained unknown. Based on the whole dataset created by analyses of all three precursors, the researchers found an overall classification accuracy of 75%, with a specificity of 78% and a sensitivity of 72% for 'high risk' patients versus healthy controls.

Another study was conducted by Ishibe who included 30 CRC patients and 26 healthy volunteers. A gas sampling apparatus was built into a toilet which collected gas components during defecation. Faecal gas was analysed on methyl marcaptan (MM) and hydrogen sulphide (H_2S) using Gas Chromatography coupled to a Sulfur Chemiluminescence Detector (GC-SCD). Next Hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) were analysed by Gas Chromatography coupled to a Termal Conductivity Detector (GC-TCD). Levels of MM were found significantly increased in CRC patients, whereas levels of H_2 were significantly decreased compared to HC. Using a logistic regression analyses, a discriminant formula was calculated with values of AUC, sensitivity, specificity and accuracy of 0.78, 90%, 57.7% and 75%, respectively.

Pattern recognition techniques

Canine scent

Dogs are renowned for their excellent sense of smell. Their olfactory capacities have been mainly used for hunting and protection of property, but trained dogs are also able to accurately detect drugs and explosives at borders, airplane fields or in prisons. For

disease detection, multiple studies have focused on the use of dogs for various carcinomas (e.g. prostate cancer, ovarian cancer, bladder cancer), all showing high accuracy to detect diseases[22-24]. A canine trial with a Labrador retriever aimed at identifying CRC was performed in 2013 and demonstrated a remarkable diagnostic accuracy. The sensitivity and specificity of faecal samples was high (97% and 99%, respectively)[25]. The correctness proved to be better than FOBT.

Electronic Nose technology

Cyranose

In 2014, the VOC profiles, of faecal samples of 40 CRC patients, 60 patients with advanced adenomas (described as polyps sized >1.0cm or exposing villous features or high grade dysplasia) and 57 healthy controls were analysed and compared using an eNose (Cyranose 320®)[26]. The Cyranose 320® is a portable machine consisting of an array of 32 nanocomposite carbon sensors. The interaction of specific VOCs with the sensor material causes swelling of the sensors, which induces a change in the electrical resistance. This change is measured and creates a specific 'smellprint'. Faecal VOC profiles of CRC patients could be discriminated from controls based on their smellprint with a sensitivity of 85% and a specificity of 87% (AUC 0.92). Patients with advanced adenomas could be discriminated from controls with a sensitivity of 62% and specificity of 86% (AUC 0.79). Differentiation between CRC and AA was possible with a sensitivity and specificity of 75% and 73%, respectively (AUC 0.82).

SCENT A1

This fairly new eNose apparatus to discriminate CRC from HC has been described in two trials by the same research group. First, a total of 20 sensors composed of different material were tested in different arrays of 5 units, to find the best performing array for the detection of CRC[17]. Faecal samples of 6 CRC patients and 10 HC were used, of which all CRC patients and 9 out of 10 HCs were classified correctly using the best performing array. No statistics were described. In their following publication, a feasibility and validation study for this sensor array was performed[16]. In the feasibility study, the best performing array for the detection of CRC was again chosen after testing different combinations of sensors on a total of 157 samples in the laboratory (86 CRC, 71HC). An overall accuracy, sensitivity and specificity of 95% was computed when considering all the samples tested with all the sensor combinations tried. Then, the best performing array was validated in a new prospective case-control cohort including faecal occult blood test (FOBT) positive patients. A total of 6 CRC/AA patients, 20 low-risk adenoma patients and 60 healthy controls were included. Using this sensor-based eNose, all CRC and AA patients were classified correctly. Healthy controls were classified correctly in 58 out of 60 cases, 1 was classified in the low-risk adenoma group and 1 was classified in the CRC and AA group. No values on sensitivity or specificity were given in this section of the paper.

Gas Chromatography coupled to Metal Oxide Gas Sensor

GC coupled to a Metal Oxide Gas sensor has been described in an abstract by Bond and is similar to eNose technology, in which resistance patterns are identified by computer algorithms. In their study performed in 2016, the VOC patterns of 18 CRC and 18 AA patients were compared to 78 healthy controls with a positive bowel screening[27].

Advanced adenoma was described as any polyp sized >1.0 cm. High accuracy values of 83.3% and 87.5%, were observed for the discrimination of CRC and AA patients compared to HC, respectively. Differentiation between CRC and AA patients was feasible with accuracy, sensitivity and specificity levels of 100%.

DISCUSSION

Analysis of the 11 selected papers demonstrated that the composition of the faecal volatolome is different in the presence of CRC and AA compared to a healthy state. In addition, the clinical value of the faecal volatolome as colonic neoplasia biomarker has been demonstrated in all published studies, with sensitivity values ranging between 75-97% for CRC and 62-100% for AA, and specificity values between 66.7-99% for CRC and 78-100% for AA. The metabolic 'smellprint' was analysed using multiple techniques, which limits the possibility of a detailed comparison of the papers.

Three of the studies selected for this review used GC-MS, of which two investigated the global metabolic profile and one investigated only short-chain and long-chain fatty acids. Although research groups were small, ranging from 9 to 26 CRC cases, there are some interesting similarities in potential biomarkers found. For example, increased levels of similar amino acids (glycine, leucine, proline, serine and phenylalanine) were found in both studies evaluating the global metabolite profile, which could be caused by various reasons. First, an increase of the mucin-degrading bacteria *Bacteriodes* and *Akkermansia muciniphila* in CRC patients has been observed in various studies[10, 28]. These bacterial strains are known to break down mucin, which consists of glycoproteins built from amino acids. Enrichment of these mucin-degrading strains can cause an accumulation of free amino acids. In addition, both mucin degradation and colon cancer itself have been linked to intestinal inflammation[29-31]. Inflammation of the colon can cause a reduction in the absorption of amino acids, amongst other nutrients. Last, autophagy is known to be activated in colorectal cancer cells, leading to the release of free amino acids[32].

Other similarities were the increased levels of the SCFAs valeric acid, isovaleric acid and acetic acid. Dissimilation of leucine by anaerobe bacteria results in the production of isovaleric acid, whereas valine dissimilation results in the production of isobutyic acid[33, 34]. Both these amino acids have been found increased in stool of CRC patients. Therefore, the increase in isobutyric and isovaleric acid may be explained by the fact that they are final products of bacterial amino acid metabolism. Acetic acid may be used by colonic bacteria to produce butyric acid. Low levels of butyric acid producing bacteria have been found in CRC patients in multiple studies[10, 19]. High levels of acetic acid may be caused by a decrease in bacterial consumption. Another explanation given by Weir is the degradation of butyric acid to acetic acid in the colon[10]. Although these SCFAs may seem promising biomarker for CRC, it has to be noted that differences in levels of SCFAs were not shown by Song et al., who specifically focused on that section of the volatolome using the same analytical platform[20].

In addition, the role of butyric acid is of importance. Butyric acid is a widely studied microbial metabolite, which is notorious for its prevention against colorectal cancer[11, 35, 36]. Previous studies have shown decreased levels of butyric acid producing bacterial strains in faecal samples of CRC patients[10, 19]. It could be hypothesised that levels of butyric acid may also decrease in faecal of CRC patients, and that this compound may hold potential as CRC biomarker. Results of the publications included in this review are, however, inconclusive on this particular metabolite. One study has indeed shown decreased levels of butyric acid in faecal samples of CRC patients, whereas the other two studies focusing on SCFAs have either shown increased levels or no differences. These differences could at least partly be explained by differences in sampling methods, storage conditions and machine settings, underlining the need for the implementation of standardised protocols in faecal VOC analyses. Interestingly, the contradicting studies both found lower levels of butyric acid-producing bacterial strains in the faecal samples of their participants.

Last, decreased levels of polyhydric alcohols (e.g. glycerol) and one bile acid (ursodeoxycholic acid) were found. Ursodeoxycholic acid is a secondary bile acid produced by colonic bacteria, and is considered to have a chemopreventive effect on colorectal cancer cells by inhibiting tumor development[37]. Polyhydric alcohols are used by human cancer cells, which possess a well-known transport system for increased glycerol absorption[38]. Uptake of these alcohols could explain the decreased levels in stool of CRC patients.

In colon cancer screening programs, the costs/quality ratio of biomarker detection is of great importance. Although there have been several studies published on the use of dogs for disease detection, all showing very high sensitivity, no studies have ever compared the accuracy of dogs and analytical techniques in a systematic manner. However, implementation of canine olfaction in daily practice is obviously hampered by obstacles as training is costly and laborious. It seems even more challenging to train sufficiently enough dogs for (nationwide) CRC screening programs[39]. The quality of the GC-MS technique is high, though it is expensive, time-consuming and needs specialised laboratory personnel for its operation and maintenance which limits its use for population based screening or large scale research[12, 15, 40]. Less expensive compared to GC-MS is SIFT-MS since it requires less maintenance and sample preparation, but this technique provides less detailed information on VOC composition. It is dependent of the chemical and mass characteristics of potential faecal CRC biomarkers, whether this technique could accurately differentiate between CRC and HC, and thus holds potential for population based screening. The publications on pattern-based techniques all report a high diagnostic value for the detection of both CRC and AA. The compact size, low costs, user-friendliness and speed of eNose analyses underline their potential for the high-throughput analyses required in population based screening. However, their main limitation is their inability to identify specific VOCs.

To date, VOC tests for the detection of AA and CRC are still far from the use in a public health setting. For this, the number of subjects studied in the current available literature is rather small, and the use of various techniques for VOC analyses in combination with the use of different criteria used for advanced adenomas, hampers reliable comparison between studies. In addition, data on the VOC profiles of non-advanced polyps is lacking. It is well-known that the tendency of polyps to develop into malign neoplasia is dependent

of multiple characteristics as epithelial differentiation, size, basis and the presence of villous features. It would be of interest to assess whether these specific characteristics influence the VOC profile and whether there is similarity with the VOC profile of CRC patients. Another challenge to encounter before developing a disease specific eNose, is the correction for other factors influencing VOC profile. Though for breath analyses it is known that there is only small similarity in VOC profile when measured in a single individual at consecutive moments, information is lacking on the intraindividual stability of faecal VOC profiles[41]. In addition, it is known that lifestyle factors as smoking, dietary intake and use of medication have an effect on the faecal VOC composition [42-44]. Future studies should therefore take these variables into account by either matching of study participants or calculating correction factors per variable. The next step for developing CRC specific VOC tests, is to combine measurements with a chemical analytical technique such as GC-MS and a pattern-recognition based technique in a cohort including CRC, AA, non-advanced adenomas and healthy controls, to identify which disease specific molecules interact with which sensors. This allows for the development of a highly accurate, inexpensive, easy-to-use, CRC-specific eNose. The CRC-specific eNose should then be tested on suitability for clinical implementation in mass screening programs, preferably by comparing the eNose to the presently used screening program test on performance characteristics, user friendliness and costs in a large population study.

In conclusion, an increasing number of studies demonstrate the potential of the faecal volatolome as non-invasive biomarkers for the detection of CRC and advanced adenomas. The use of different techniques limits comparability, however, all studies demonstrated a high diagnostic value of the faecal volatolome for CRC detection. This holds promise for future screening on CRC and advanced adenoma, with potentially better test performances allowing for earlier detection of AA and CRC and consequently earlier initiation of treatment, possibly reducing morbidity and mortality rates next to lower rates of (unnecessary) colonoscopies. Future studies should focus on validation of previously found faecal VOC biomarkers in a large prospective cohort linked to the population based screening, preferably by combining a chemical analytical technique with pattern-recognition, so CRC- and AA-specific biomarkers can be identified which can be used to develop tailor-made eNose sensors to be used in clinical practice.

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CHAPTER 13

Early detection and follow-up of colorectal neoplasia based on faecal volatile organic compounds

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ABSTRACT

Background
Early detection and removal of colorectal cancer (CRC) and advanced adenomas (AA) decreases incidence and mortality. We aimed to evaluate potential of faecal volatile organic compounds (VOC) for colorectal adenomas detection and follow-up using advanced electronic nose technology.

Methods
This was a prospective multi-centre case-control cohort including two district hospitals and one tertiary referral hospital. Patients undergoing colonoscopy were instructed to collect a faecal sample prior to bowel cleansing and were included when CRC, AA, large adenomas (LA; 0.5-1.0cm), small adenomas (SA; 0.1-0.5cm) or no endoscopic abnormalities (healthy controls; HC) were observed. Patients undergoing polypectomy and HC were asked for a second sample after three months. Faecal VOCs were measured with gas chromatography-ion mobility spectrometry. Random Forest, Support Vector Machine, Gaussian Process and Neural Net classification were used to evaluate accuracy.

Results
In total, 14 CRC, 64 AA, 69 LA, 127 SA and 227 HC were included. Second sample was collected by 32 polypectomy patients and 32 HC. Faecal VOCs discriminated CRC and adenomas from HC (AUC(95%): CRC vs HC 0.96(0.89-1); AA vs HC 0.96(0.93-1); LA vs HC 0.96(0.92-0.99); SA vs HC 0.96(0.94-0.99)). There were no significant differences between CRC and adenoma groups. Patients with adenomas and HC were discriminated prior to polypectomy, whereas three months after polypectomy VOC profiles were similar (T0 adenoma vs HC 0.98(0.95-1); T1 adenoma vs HC 0.55(0.40-0.69)).

Conclusions
Faecal VOC profiles may be useful for early CRC and adenoma detection, and timing of polyp surveillance as polypectomy led to a normalization of VOC profile.

INTRODUCTION

Colorectal cancer (CRC) is one of the three malignancies with the highest incidence in the industrialised world, with a 5-year survival rate of 64.4% and 66.6% for colon and rectum cancer, respectively[1]. Majority of CRC originates from dysplastic adenomatous polyps, so-called advanced adenomas[2]. Early detection and removal of these precancerous polyps is essential for improvement of CRC course and prognosis, illustrated by a gradually decrease of survival rates with increasing cancer stage[3]. Faecal immunochemical test (FIT) is widely used for population-based screening but lacks sensitivity, indicated by the missed diagnosis of CRC in 1-47% and advanced adenomas in 43-61% of the tests[4]. In addition, specificity is suboptimal, as approximately 7% of the performed tests provide false-positive results leading to the performance of unneeded colonoscopies. This emphasizes the need for improvement of CRC/adenoma bowel screening tools.

Endoscopic assessment is advised after FIT positivity, but also remains key for surveillance after removal of polyps (polypectomy) and following CRC treatment[3], as these patients remain at increased risk for development or recurrence of dysplastic lesions. Thus far, no non-invasive biomarkers have been validated for this purpose.

Analysis of the faecal volatolome is a novel approach within the field of biomarker exploration. The faecal volatolome consists of volatile organic compounds (VOCs), which are carbon-bound molecules. Composition of the faecal volatolome is considered to reflect metabolic processes in the human body, like inflammation, necrosis, change in dietary intake, gut microbial dysbiosis and cancer growth[5, 6]. Several studies have focused on the application of the faecal volatolome as a biomarker for detection of CRC and advanced adenoma, with promising results. However, none of the available studies have included patients with (low-risk) adenomas to explore the specificity of VOC analysis. Aim of this study was to assess the potential of faecal VOC as non-invasive biomarker to detect colonic neoplasia and precursor lesions. In addition, we aimed to explore its potential for secondary non-invasive surveillance following polypectomy.

METHODS

Study design

This multi-centre prospective case-control study was performed between February 2015 and November 2017 at outpatient clinics of Gastroenterology and Hepatology departments in one tertiary referral hospital (Amsterdam UMC, location VUmc) and two district hospitals (OLVG West, Amsterdam and Spaarne Gasthuis, Hoofddorp and Haarlem), all located in The Netherlands. This study was approved on 04-09-2014 by the Medical Ethical Review Committee (METc) of Amsterdam UMC (2014.404), and by local METcs of OLVG West and Spaarne Gasthuis. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Written informed consent was obtained from all participants.

Study participants and sample collection

Detection of colorectal adenomas and cancer

All patients aged ≥18 years with a scheduled colonoscopy were asked to participate in this study, irrespective of endoscopy indication. Patients were divided into five subgroups based on observations during endoscopy, combined with histology reports in case biopsies or polypectomies were performed: (a) CRC, histologically confirmed carcinoma of the colon or rectum; (b) Advanced adenoma (AA), according to the European Society of Gastrointestinal Endoscopy (ESGE), that is, characterised by polyps ≥1cm in diameter, or with villous histology, or high grade dysplasia; (c) Large adenoma (LA), adenomas sized 0.5-1.0cm in diameter without villous histology or high grade dysplasia; (d) Small adenoma (SA), adenomas sized <0.5cm in diameter without villous histology or high grade dysplasia; (e) Healthy controls (HC), characterised by no abnormalities observed during endoscopy (excluding small anal fibroma, haemorrhoids and/or diverticula), and in case of mucosal biopsies, no histopathological abnormalities[7]. Participants were asked to collect a faecal sample (Stuhlgefäß 10ml, Frickenhausen, Germany) prior to bowel preparation, store the sample in their own freezer at home within one hour following bowel movement and bring it to the hospital on the day of their endoscopic assessment. Samples were stored at -24°C directly upon arrival at the hospital. Participants were asked to complete a questionnaire on the day of sample collection, which included items on age, gender, body mass index (BMI), smoking habits, comorbidity and medication use. Exclusion criteria were presence of a known underlying gastrointestinal disease, incomplete endoscopic assessment due to various reasons (e.g. inadequate bowel cleansing, pain) and/or inability to collect or store sufficient faecal sample mass to perform VOC analysis.

Monitoring of patients post-polypectomy

Between May and November 2017, patients who underwent a successful polypectomy during endoscopy were asked to participate in the follow-up part of this study. Participants were excluded from this group in case of incomplete removal of polyps. Remaining polypectomy patients were randomly matched to HC in a 1:1 ratio. All patients included in the second part of this study were asked to collect a follow-up faecal sample and complete a second questionnaire (same procedure as the first sample and questionnaire). These samples and questionnaires were collected by one of the researchers and transported to the hospital on dry ice where they were stored at -24°C upon arrival.

Endoscopic and histologic evaluation

Endoscopy reports and histologic outcome of mucosal biopsies and/or polypectomy were checked using electronic patient files. These outcomes were used as standard reference for localisation and total number of removed adenomas in this study. Endoscopies were either performed or supervised by trained gastroenterologists. Histopathological reports were used as the standard reference for size, differentiation grade (e.g. hyperplasia, dysplasia), villous histology and type of CRC in this study. Mucosal biopsies were noted as sized 0.2 cm. The presence of sessile and/or serrated characteristics was noted for all non-advanced adenomas. When multiple polyps were present, classification was based on the most advanced or largest lesion.

Sample preparation and faecal volatile organic compound analysis

One frozen subsample of 500 mg per participant was weighted and transferred into glass vials (20ml headspace vial, Thames Restek, Saunderton, UK). Samples were transported to the University of Warwick (Coventry, UK) for faecal VOC analysis. Gas chromatography-ion mobility spectrometer (GC-IMS, FlavourSpec®, G.A.S., Dortmund, Germany) was used to measure the faecal VOC patterns. In GC-IMS, analytes are pre-separated by retention time in a GC column (SE54 column) before entering the ion mobility spectrometer (IMS). Within the IMS system, soft chemical-ionization is performed using a low-radiation tritium (H3) source, subsequently creating reactant ions with injected gas. Ionised VOCs travel against flow of an inert drift gas. Drift time of each substance is determined by ions mass and geometrical structure due to interactions with the drift gas. In general, the larger the molecule, the more it loses its momentum and thus, the longer it takes to travel along the tube. Resulting ion current is measured as a function of time[8]. Prior to analysis, the samples were heated to 80°C for 8 minutes. After this, GC was performed at 40°C using nitrogen 99·9% (3·5 bar) as carrier gas and IMS was performed at 45°C using nitrogen 99·9% as drift gas. Flow rates were set at 150ml/min (0·364 kPa) (IMS), and 20 ml/minute (34,175 kPa) for 6 minutes (GC). A schematic overview of the setup is depicted in *Figure 1.*

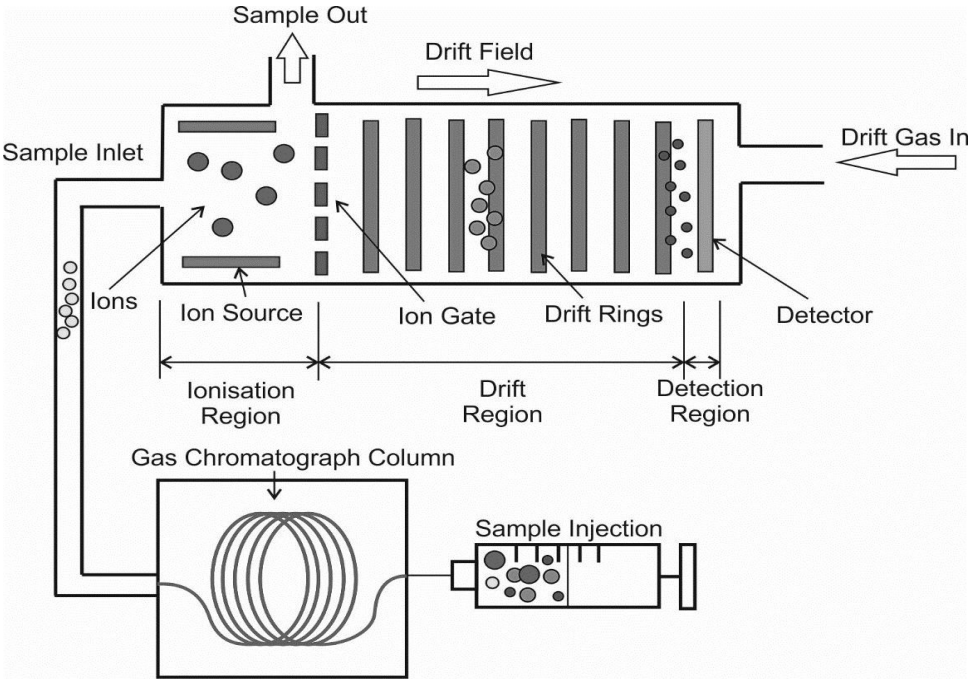

Figure 1. Gas Chromatography Ion Mobility Spectrometry. Samples are heated to 80°C for 8 minutes prior to analyses. Headspace of sample is injected into GC-IMS. VOCs enter the GC column where they are separated based on their interaction with carrier gas. VOCs with similar retention times enter the IMS column simultaneously and are ionised using a soft-chemical ionization. Ionised VOCs travel against flow of inert gas. Drift time of VOCs is determined by ion's mass and geometrical structure. The resulting ion current is measured by electrometer as function of time.

Statistical analysis

Using IBM SPSS Statistics (version 22), demographic data of each group were calculated and compared. One way ANOVA or Kruskall Wallis tests were used to compute differences in baseline demographics between groups. For the secondary part of this study, t-tests, Fisher's exact test, chi-square and Mann-Whitney U tests were used to compute differences between groups. Raw GC-IMS data were pre-processed to crop areas that contained chemical information. A threshold was applied to remove background noise. Then, a correction for instrumental disturbances was performed by baseline correction, where reactive ion peaks were aligned on all samples. Data were split into three sets, 70% for training and validation and 30% as a test set. Wilcoxon rank-sum test was used to find the 20, 50 and 100 most discriminatory features and subsequently Sparse Logistic Regression, Random Forest, Gaussian Process, Support Vector Machine and Neural Net classification were used to provide statistical results. In the case of small subgroups of interest, subsets of HC were chosen randomly to avoid skewed analyses.

RESULTS

Baseline characteristics

In total, 1039 patients collected a faecal sample prior to colonoscopy of which samples from 14 CRC patients, 64 AA, 69 LA and 127 SA were included in this study. 227 HC were included as they did not have any mucosal abnormalities during endoscopy. Baseline demographics of all study participants are depicted in *Table 1.* Age differed significantly between groups ($p<0.001$), with HC displaying the lowest mean age (60 ± 11.8) and advanced adenoma the highest (68.8 ± 6.7). Gender differed significantly between HC, SA, LA and advanced adenoma ($p<0.0001$) but not between HC and CRC or any of the adenoma groups. There were no significant differences in BMI, smoking status and use of antibiotics between groups. For the follow-up part of this study, all 32 patients undergoing a complete polypectomy and 32 HC subjects were included and collected a second faecal sample three months after endoscopy. Baseline demographics of the follow-up study are given in *Table 2.* There were no significant differences in BMI, smoking status and use of AB three months prior to sample collected. Gender and age did differ significantly between groups ($p<0.014$, $p<0.001$, respectively).

Faecal volatile organic compound analysis

Results of VOC analyses by means of GC-IMS are shown in *Table 3.* Results from the Random Forest classifier based on the 20 most discriminative features are presented. Data generated based on all five classifiers using the 20, 50 and 100 most discriminative features are given in Supplemental Table 1-3.

Table 1. Demographics of all study participants

	Colorectal Cancer (n=14)	Advanced adenomas (n=64)	Large adenomas (n=69)	Small adenomas (n=127)	Healthy controls (n=227)
Age (mean, ± s·d·)	66·6 ± 8·7	68·8 ± 6·7	68·7 ± 7·2	63·7 ± 10·0	60 ± 11·8
Gender, f (n, [%])	6 [42·9]	17 [26·3]	21 [30·4]	44 [34·6]	129 [56·8]
BMI* (mean, ± s·d·)	25·9 ± 5·3	27·0 ± 4·1	26·6 ± 3·9	27·3 ± 6·5	26·6 ± 7·0
ABx 3 months prior to inclusion (n, [%])	0 [0]	6 [9·4]	3 [4·3]	21 [16·5]	31 [13·7]
Smoking Status (n, [%])					
Active	2 [14·3]	9 [14·1]	15 [21·7]	23 [18·1]	37 [16·3]
Quit	10 [71·4]	37 [57·8]	36 [52·2]	64 [50·4]	92 [40·5]
Never	2 [14·3]	18 [28·1]	17 [24·6]	40 [31·5]	98 [43·2]
Size largest adenoma (mean, ± s·d·)	0·7 ± 0·63	1·4 ± 0·64	0·7 ± 0·26	0·4 ± 0·32	NA
Localisation of Largest adenoma† (n, [%])					
Caecum	0 [0]	4 [6·3]	7 [10·1]	12 [9·4]	NA
Colon Ascendens	1 [7·1]	8 [12·5]	12 [17·4]	32 [25·2]	NA
Flexura Hepatica	1 [7·1]	2 [3·1]	0 [0]	1 [0·8]	NA
Colon Transversum	0 [0]	3 [4·7]	15 [21·7]	18 [14·2]	NA
Flexura Lienalis	0 [0]	1 [1·6]	0 [0]	1 [0·8]	NA
Colon Descendens	1 [7·1]	4 [6·3]	6 [8·7]	9 [7·1]	NA
Sigmoid	5 [35·7]	30 [46·9]	19 [27·5]	24 [18·9]	NA
Rectosigmoid	2 [14·3]	3 [4·7]	1 [1·4]	4 [3·1]	NA
Rectum	2 [14·3]	6 [9·4]	6 [8·7]	14 [11·0]	NA
Terminal ileum	1 [7·1]	0 [0]	0 [0]	1 [0·8]	NA
CRC type (n, [%])					
Adenocarcinoma	12	NA	NA	NA	NA
Neuroendocrine	2	NA	NA	NA	NA
Advanced adenoma characteristics (largest adenoma) (n, [%])					
≥ 10 mm	NA	54 [84·4]	NA	NA	NA
Villous histology	NA	31 [48·4]	NA	NA	NA
HGD	NA	6 [9·4]	NA	NA	NA
Polyp characteristics (largest adenoma) (n, [%])					
No dysplasia	NA	1 [1·6]	9 [13·0]	9 [7·1]	NA
Hyperplasia	NA	2 [3·1]	4 [5·8]	14 [11·0]	NA
LGD	NA	55 [85·9]	55 [79·7]	100 [78·7]	NA
Sessile/serrated	NA	NA	8 [11·6]	10 [7·9]	NA

Table continues

	Colorectal Cancer (n=14)	Advanced adenomas (n=64)	Large adenomas (n=69)	Small adenomas (n=127)	Healthy controls (n=227)
Total number adenomas removed (n, [%])					
1	6 [42·8]	11 [17·2]	18 [26·1]	59 [46·5]	NA
2	1 [7·1]	14 [21·9]	20 [29·0]	34 [26·8]	NA
3	3 [21·4]	11 [17·2]	14 [20·3]	14 [11·0]	NA
4-5	2 [14·3]	14 [21·9]	8 [11·6]	15 [11·8]	NA
6-10	1 [7·1]	13 [20·3]	9 [13·0]	4 [3·2]	NA
>10	0 [0]	1 [1·6]	0 [0]	0 [0]	NA

Baseline characteristics. Abbreviations: CRC, colorectal cancer; AA, advanced adenoma, LA, large adenomas; SA, small adenomas; HC, healthy controls; BMI, body mass index; NA, not applicable; HGD: high grade dysplasia; LGD: low-grade dysplasia; AB: antibiotics. *Insufficient documentation of 2CRC, 6AA, 6LA, 10SA; 11 HC.

Table 2. Demographics of participants included in follow-up study

	Polypectomy group (n=32)	Healthy controls (n=32)
Age (mean, ±SD)	71·0 ± 5·9	60·5 ± 11·3
Gender (n females, %)	5 [15·6]	15 [46·9]
BMI (mean, ±SD)	26·8 ± 4·2	26·3 ± 3·4
Smoking status (n, %)		
Active	6 [18·8]	4 [12·5]
Quit	18 [56·3]	19 [59·4]
Never	8 [25·0]	9 [28·1]
Indication for endoscopic assessment (n, %)		
Positive FIT	10 [31·3]	4 [12·5]
Rectal blood loss	4 [12·5]	1 [3·1]
Change in bowel habits	2 [6·3]	3 [9·4]
Surveillance	5 [15·6]	3 [9·4]
Abdominal Pain	2 [6·3]	11 [34·4]
Diarrhea	3 [9·4]	1 [3·1]
Weight Loss	1 [3·1]	0 [0]
Anaemia	0 [0]	2 [6·3]
Constipation	0 [0]	2 [6·3]
Family history CRC+	1 [3·1]	8 [25]
Monitoring previous diverticulitis/abscess	0 [0]	2 [6·3]
Other	4 [12·5]	5 [15·7]
ABx 3 months prior to inclusion (n, %)	2 [6·3]	6 [18·8]
ABx 3 months prior to second sample (n, %)	1 [3·1]	3 [9·4]

Table continues

	Polypectomy group (n=32)	Healthy controls (n=32)
Size adenoma (mean, ±SD)	1·1 ± 0·5	NA
Localisation of adenoma (n, %)		
Caecum	3 [9·4]	NA
Colon Ascendens	6 [18·8]	NA
Flexura Hepatica	0 [0]	NA
Colon Transversum	2 [6·3]	NA
Flexura Lienalis	1 [3·1]	NA
Colon Descendens	2 [6·3]	NA
Sigmoid	11 [34·4]	NA
Rectosigmoid	1 [3·1]	NA
Rectum	4 [12·5]	NA
Ileocecal valve	1 [3·1]	NA
Adenoma characteristics (largest adenoma) (n, [%])		
≥ 10 mm	13 [40·6]	NA
Villous histology	9 [28·1]	NA
HGD	0 [0]	NA
No dysplasia	3 [9·4]	NA
Hyperplasia	0 [0]	NA
LGD	29 [90·6]	NA
Sessile/serrated	3 [9·4]	NA
Total number adenomas removed (n, [%])		
1	7 [21·9]	NA
2	11 [34·4]	NA
3	7 [21·9]	NA
4-5	3 [9·4]	NA
6-10	4 [12·6]	NA

Baseline characteristics of participants in polypectomy follow-up study. Abbreviations: HC, healthy controls; BMI, body mass index; ABx: antibiotics.

Detection of colorectal cancer, advanced adenomas and non-advanced adenomas

Based on faecal VOC profiles, CRC was discriminated from HC with high diagnostic accuracy (AUC ± 95%CI: 0·96(0·89 – 1)(*Table 3, Supplementary table 1-3)*. Likewise, high diagnostic accuracy was observed for discrimination of AA, LA and SA when compared to HC (Advanced adenoma 0·96(0·93 –1); LA 0·96(0·92 – 0·99); SA 0·96(0·94-0·99))(*Table 3, Supplementary tables 1-3)*. There were no significant differences between any of the CRC, AA, LA and SA groups based on faecal VOCs (*Table 3, Supplementary table 1-3)*. Receiver operator characteristic (ROC) curves are constructed in *Figure 2.*

Faecal volatile organic compounds for surveillance after polypectomy

Faecal VOC profiles of patients with adenomas differed significantly from HC before polyp removal (AUC ± 95%CI: 0·98(0·95-1)). Remarkably, there was no difference between the faecal VOC profiles of patients who underwent a polypectomy and HC three months after endoscopic intervention (AUC ± 95%CI: 0·55(0·40-0·66)). There was a highly significant difference in the profiles of patients with adenomas before and after polypectomy, whereas no significant differences were present in faecal VOC profiles of HC before and three months after endoscopy (T0 vs T1 polyps 0·94(0·88-1); T0 vs T1 HC 0·58(0·44-0·73)). ROC-curves for the follow-up study are depicted in *Figure 3.*

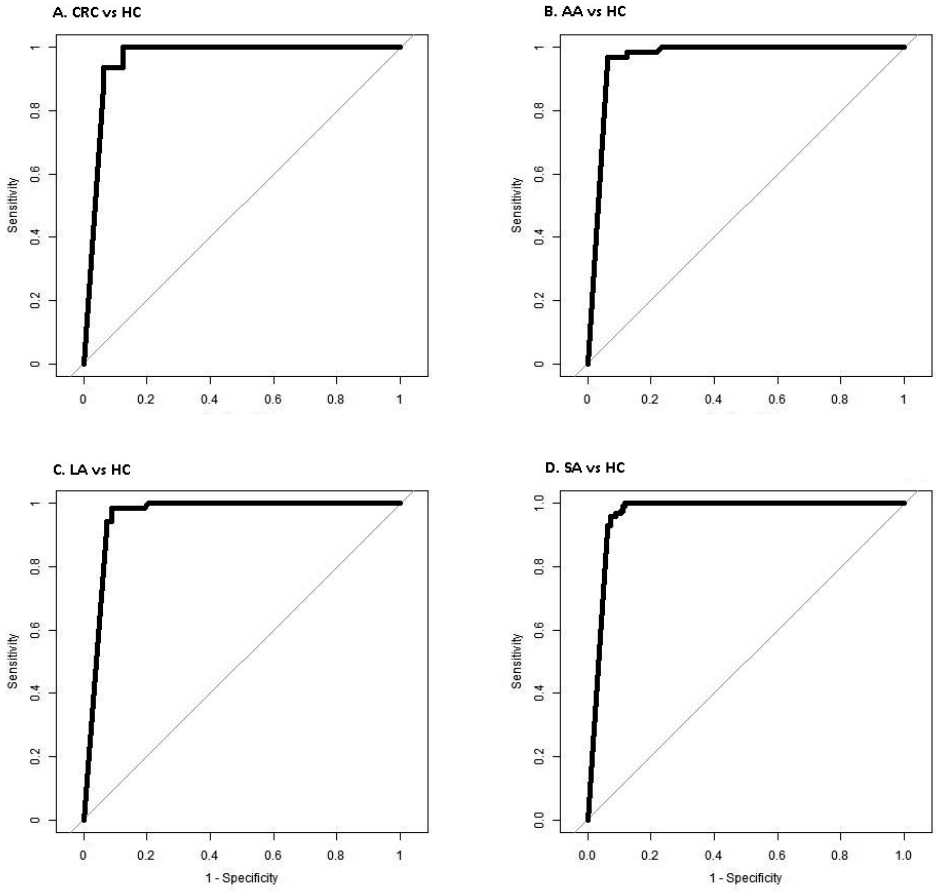

Figure 2. Receiver operator characteristic curves for the comparison between colorectal cancer, advanced adenoma, large adenoma and small adenoma and healthy controls. Abbreviations: CRC, colorectal cancer; AA, advanced adenomas; LA, large adenoma; SA, small adenoma; HC, healthy controls; AUC, area under the curve.

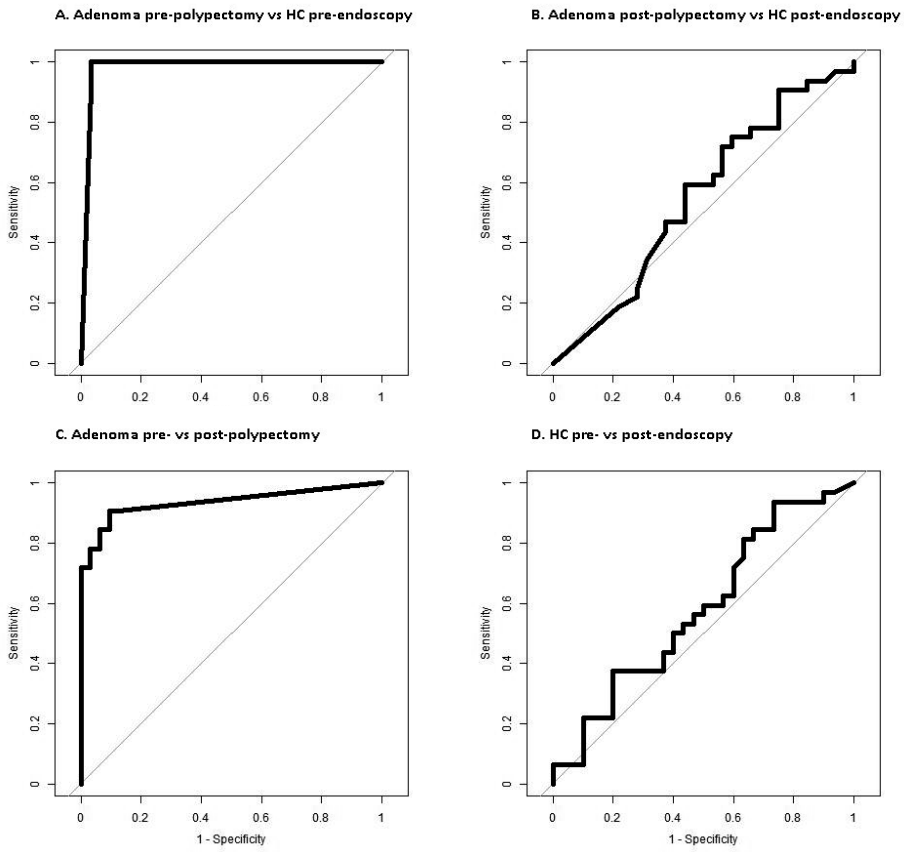

Figure 3. Receiver operator characteristic curves for the polypectomy follow-up. Abbreviations: AUC, area under the curve; HC, healthy controls.

DISCUSSION

We observed high diagnostic accuracies for faecal VOC-based differentiation between CRC and HC and between adenomas and HC. Faecal VOC profiles of patients with adenomas converted towards those of HC after polypectomy. VOC profiles of CRC could not be discriminated from different adenoma types. These results demonstrate the potential of faecal VOC analysis for both colonic neoplasia screening and surveillance.

This is the first study to assess faecal VOC profiles for CRC and advanced adenoma detection, which included patients with non-advanced adenomas as unique control groups. No previous studies have been performed on the potential of faecal VOC profiles for surveillance after polypectomy. Surveillance of patients after polypectomy using FIT has been subject of a previous study comparing stool haemoglobin levels with colonoscopy outcomes[9]. A total of 5225 participants completed a first FIT one year after polypectomy, demonstrating sensitivity and specificity values of 27·6% and 94·1% at 40 µg/g, and 51·7% and 86·2% at 10

µg/g for CRC, respectively. For AA, sensitivity and specificity values were 17·0% and 95·1% for 40 µg/g and 33·0% and 88·0% for 10 µg/g, respectively. Replacing colonoscopy with FIT would reduce colonoscopies by 71%, but would lead 30-40% missed CRC cases and 40-70% missed cases of advanced adenomas. Observed discriminative accuracies for CRC and advanced adenoma detection based on faecal VOC analysis exceed these reported accuracies[4, 10]. In addition, accuracy to distinguish patients with adenomas from HC was high prior to polypectomy, whereas intra-individual profiles changed to physiological state three months following polypectomy, indicating the potential of faecal VOCs as biomarker for timing of polyp surveillance, and tight control in high risk populations (e.g. Lynch syndrome).

Table 3. Differences between all subgroups of colorectal neoplasia, polyps and healthy controls based on faecal volatile organic compounds

Comparison	AUC (95% CI)	Sensitivity	Specificity	PPV	NPV	P-value
Colorectal cancer vs healthy controls	0·961 (0·891-1)	1	1	0·889	1	<0·001
Advanced adenoma vs healthy controls	0·964 (0·932-0·996)	0·969	0·938	0·939	0·968	<0·001
Large adenomas vs healthy controls	0·957 (0·923-0·992)	0·985	0·912	0·918	0·984	<0·001
Small adenomas vs healthy controls	0·964 (0·940-0·987)	0·960	0·929	0·931	0·959	<0·001
Colorectal cancer vs advanced adenomas	0·541 (0·382-0·700)	0·984	0·188	0·829	0·75	0·294
Colorectal cancer vs large adenomas	0·41 (0·31 - 0·51)	0·06	0·96	0·25	0·81	0·920
Colorectal cancer vs small adenomas	0·413 (0·379-0·446)	1	0	0·113	NA	0·965
Advanced adenomas vs large adenomas	0·53 (0·43 - 0·63)	0·75	0·36	0·55	0·58	0·278
Advanced adenomas vs small adenomas	0·578 (0·495-0·662)	0·719	0·444	0·397	0·757	0·039
T0 Healthy controls vs T0 pre-polypectomy	0·982 (0·946-1)	1	0·967	0·970	1	<0·001
T1 Healthy controls vs T1 post-polypectomy	0·548 (0·404-0·691)	0·906	0·25	0·547	0·828	0·256
T0 vs T1 pre- and post-polypectomy	0·936 (0·875-0·996)	0·906	0·906	0·906	0·906	<0·001
T0 vs T1 Healthy controls	0·581 (0·436-0·726)	0·938	0·267	0·577	0·8	0·139

Outcomes obtained using Random Forest analyses based on 20 most discriminating features. Abbreviations: AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; CRC, colorectal cancer; AA advanced adenoma; LA, large adenomas; SA, small adenomas; LGD, low-grade dysplasia; HC healthy controls.

Few studies have demonstrated the potential to discriminate CRC and advanced adenomas from HC based on faecal VOC analysis. Current study outcomes are in line with this literature. However, reliable comparison with other studies is restricted due to the small number of included subjects, lack of knowledge on VOC profiles of advanced adenomas and non-advanced adenomas, and the use of various different techniques and sampling protocols. Four previous studies have focused on detection of CRC using the analytical platform gas chromatography—mass spectrometry (GC-MS), which is considered the gold standard for the detection of specific metabolites [11-14]. In the most recent publication, a group of adenomatous polyps patients (n=56) was included in addition to CRC (n=21) and no neoplasia as HC (n=60). Multiple differences in metabolite levels were found. Highest diagnostic accuracy was found for the combination of Propan-2-ol and 3-methylbutanioc acid discriminating CRC samples from polyps and HC (AUC 0.82). These differences specifically provided discrimination between CRC patients and other groups, whereas in the current study, VOC profiles differed between CRC and adenoma groups compared to HC. Possible explanations for this are the differences in faecal VOC analysis technique, and in inclusion criteria per subgroup. In the publication by Bond et. al., polyp characteristics are not reported, which hampers reliable comparison to our study groups. All other studies using GC-MS reported relatively small groups of subjects, ranging from n=9-26 CRC and n=10-60 HC. There were interesting similarities in study outcomes (e.g. increased levels of amino acids and short-chain fatty acids and decreased levels of polyhydric alcohols and bile acid), though, none of these metabolite levels were consequently altered. In one previous publication, VOC profiles of 40 CRC, 60 advanced adenoma and 57 endoscopy controlled HC were compared using pattern-recognition (eNose, Cyranose 320 ®)[15]. Based on faecal VOC patterns, CRC and advanced adenomas were discriminated from HC with sensitivity of 85% and 62%, and specificity of 87% and 86%, respectively. These test characteristics were below characteristics found in the current study, however, using the Cyranose 320®, subgroups of CRC and advanced adenomas were discriminated with moderate accuracy (sensitivity 75% and specificity 73%). A possibility for this apparent discrepancy is the number of CRC patients included in the study by de Meij *et al.*, increasing power to find differences between groups. Another possibility is the instrumental difference between the Cyranose 320® and the GC-IMS system. The Cyranose 320® uses an array of nanocomposite sensors, whereas the GC-IMS system is using drift time. Although GC-IMS is more sensitive and repeatable, it cannot measure molecules with low proton affinities. Should differentiation between CRC and advanced adenomas be based on these type of molecules they could not have been detected in the current study.

Alterations in faecal VOC patterns represent metabolic shifts that may be explained by various mechanisms (e.g. alterations in dietary intake, microbial dysbiosis, inflammatory processes, cancer degeneration). In a recent study, metabolic waste was retrieved from benign cells, colon cancer cells and breast cancer cells that were grown *in vitro*. It was observed that dogs were able to differentiate cancer cells from benign cells, but not the cell waste of breast from colon cancer, implying that both cancers phenotypes seem to share a common smell print[16]. This may also apply for different phenotypes of adenomas; adenoma and eventually CRC degeneration are possibly based on a shared (metabolic) pathway, explaining the similarities in VOC patterns observed in the current study. Apart from excretion of metabolic end-products, our findings may be explained by the presence of

intestinal dysbiosis. Faecal microbiota has important function in protection against invading pathogens and strong evidence exists for the association between microbial dysbiosis and polyp-associated tissue and colonic neoplasia[17]. Causality remains unclear; it is unknown whether this phenomenon is triggered or maintained by carcinogenesis. Intriguingly, in the present study it was found that faecal VOC profiles of patients three months after polypectomy were altered to normalcy following this intervention, suggesting that the alleged faecal microbial dysbiosis returns to physiological state after polyp removal.

Design of this large, multi-centre prospective cohort contributed to generalizability of the outcomes. Bias by colonic abnormalities was avoided by including an endoscopy controlled HC subgroup. This study also had some limitations. Most importantly, CRC subgroup was relatively small which may have contributed to the inability to discriminate CRC and adenoma profiles. In addition, even though most variables expected to influence faecal VOC profiles were evenly present in the subgroups (i.e. smoking status, BMI and use of antibiotics), gender and age did differ between groups. Data on the influence of age and gender on faecal VOC profiles are lacking and we were therefore not able to exclude this possible bias.

Future research aimed at creating a disease-specific faecal VOC algorithms for CRC and adenoma detection may focus either on pattern-recognition or on metabolite specific analytical platforms. The use of pattern-recognition may be favourable for this purpose since this allows for fast, easy-to-perform, high-throughput and low-cost analyses, underlining its suitability for application in clinical practice. Using machine learning, algorithms may be built for the detection of CRC and its precursor lesions, which improve continuously with increasing numbers of samples measured. Once the algorithm reaches satisfactory accuracy, the device may be implemented not only for population based screening but also for intra-individual surveillance after polyp or CRC removal. For surveillance, the device should be validated coupled to endoscopy outcomes in the surveillance program. Last, our data have raised the hypothesis that faecal VOCs associated with CRC and advanced adenoma may be a consequence rather than the cause of adenoma presence. It would be interesting from a pathophysiological and therapeutic point of view to unravel the underlying entities causing the faecal VOC differences pre- and post-polypectomy.

In conclusion, because of its high sensitivity, this study highlights the potential faecal VOC analysis for population based screening. Additionally, intra-individual faecal VOC profiles of patients with adenomas altered towards a physiological state following polypectomy, emphasizing its potential for intra-individual surveillance and timing of endoscopy.

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CHAPTER 14

Data integration of stool microbiota, proteome and amino acid profiles to discriminate patients with adenomas and colorectal cancer

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ABSTRACT

Background

Screening for colorectal cancer (CRC) reduces its mortality but has limited sensitivity. We aimed to explore potential biomarker panels for CRC and adenoma detection and to gain insight into the interaction between gut microbiota and human metabolism in the presence of these lesions by integrating faecal microbiota, proteome and amino acid profiles.

Methods

Between February 2016 and November 2019, patients ≥18 years with a scheduled colonoscopy in two district hospitals and one tertiary referral hospital were eligible for this multi-centre cohort. Participants were divided into subgroups of CRC (n=12), adenomas (n=21) and controls without endoscopic abnormalities (n=20) and collected faecal samples for proteome (LC-MS/MS), microbiota (16S rRNA profiling) and amino acid (HPLC) assessment. Feature selection was performed to obtain best predictive biomarker panels. Pearson correlation was used to create networks of all omics platforms.

Results

Maximum area under the curve (AUC) for individual markers were 0.95 for CRC versus controls (HBA1), 0.89 for adenoma versus controls (ethanolamine) and 0.86 for CRC versus adenoma (sulfo-l-cystine). Integration of data sets revealed markers associated with increased blood excretion, stress- and inflammatory responses and pointed towards downregulation of epithelial integrity. Combining omics platforms led to selection of new panels which outperformed AUC of hemoglobin in this cohort, which is currently used in FIT (AUC 0.98, 0.95 and 0.87 for CRC vs controls, adenoma vs controls and CRC vs adenoma, respectively).

Conclusions

Integrating faecal microbiota, proteome and amino acids platforms provides for new biomarker panels that may improve non-invasive screening for adenomas and CRC, and may subsequently lead to lower incidence and mortality of colon cancer.

CHAPTER 15

Detection and surveillance of adenoma patients undergoing polypectomy using faecal microbiota and amino acid composition

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ABSTRACT

Background

The risk of recurrent dysplastic colonic lesions is increased following polypectomy. Yield of endoscopic surveillance after adenoma removal is low whilst interval colorectal cancers occur. Aim of this study was to longitudinally assess dynamics of faecal microbiota and amino acids in the presence of adenomatous lesions and after their endoscopic removal.

Methods

In this prospective multicentre case–control cohort study, patients undergoing colonoscopy, irrespective of indication, were instructed to collect a faecal sample prior to bowel preparation. Patients with advanced adenomas and non-advanced adenomas (0.5-1.0cm) who underwent polypectomy during endoscopy (n=19) were strictly matched on age, body-mass index and smoking habits to controls without endoscopic abnormalities (n=19). All subjects collected a follow-up faecal sample 3 months after adenoma removal. On all paired samples, microbial taxa were measured by 16S RNA sequencing and using linear discriminant analysis effect size (LEfSe), amino acids (AA) were measured by High Performance Liquid Chromatography (HPLC).

Results

Adenoma patients could be discriminated from controls based on AA and microbial composition. Levels of proline (p=0.001), ornithine (p=0.02) and serine (p=0.02) were upregulated in adenoma patients compared to controls, but expressed levels similar to those of controls after adenoma removal. These AAs were combined as potential adenoma specific panel and area under the curve (AUC) value of 0.84 (95%CI 0.81 - 0.89) and 0.79 (0.64 – 0.94) were found for the training and test set, respectively. For bacterial taxa, differences between patients with adenomas and controls were found, (*Bifidobacterium*↓, *Sutterella* ↑ *and Catenibacterium* ↑), independently on performance of polypectomy.

Conclusion

We were able to describe an amino acids panel specifically increased only in the presence of adenomas and a microbial signature present in adenoma patients, irrespective of polypectomy. Upon validation, the amino acid panel may serve as non-invasive marker to determine timing of surveillance endoscopy. The microbial signature may be of help for the detection of high-risk individuals. These panels may both improve effectiveness of the surveillance program, leading to less unnecessary endoscopies and less interval-cancer.

CHAPTER 16

Summary and general discussion

This thesis focused on the exploration and development of non-invasive biomarkers for the detection and follow-up of inflammatory bowel disease and colorectal neoplasia. To this end, we made use of faecal bacterial composition (microbiota), protein spectra (proteomics) as well as metabolite spectra (metabolomics), in specific amino acid profiles and faecal volatile organic compounds (VOC), which are referred to as 'omics' platforms. Throughout this thesis, deep phenotyping of study participants based on these omics strategies has led to the exploration of potential tools for novel, accurate and non-invasive disease detection, surveillance and prediction of disease course at an early stage, which, if clinically implemented, may lead to earlier initiation of treatment and consequently decreased numbers of complications, surgery and hospital admission. In addition, detailed characteristics of metabolic end-products are essential to enlarge knowledge on pathological pathways and to develop scientifically based therapeutic interventions linked to the gut microbiota and human metabolism. Within this thesis we used various analytical techniques for the omics measurements. For faecal VOC measurements, pattern-recognition was assessed based on different electronic nose (eNose) techniques: sensor resistance (Cyranose 320©), field asymmetric ion mobility spectrometry (FAIMS) and gas chromatography – ion mobility spectrometry (GC-IMS). In addition, the faecal microbiota was assessed using 16S ribosomal DNA Sequencing, the human faecal proteome was investigated using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) and the amino acid profiles were assessed using High Performance Liquid Chromatography (HPLC).

PART I

Optimizing volatile organic compound analysis

In the first part of this thesis we assessed the influence of methodological variables and environmental factors on composition and dynamics of faecal volatile organic compound analysis for different gastro-intestinal diseases. In **Chapter two**, the influence of sampling conditions on diagnostic accuracy of VOC measurements was assessed in a cohort of *de novo* treatment-naïve paediatric IBD patients and asymptomatic healthy controls and measured using FAIMS. Here, we defined sampling conditions to reach the optimum accuracy of faecal VOC as diagnostic biomarker. We observed that faecal sample mass, as well as number of thaw-freeze cycles and time-interval between sample preparation and VOC analysis all significantly influenced outcome. Comparable variations in VOC composition after different sampling procedures have previously been described[1]. We also observed that prolonged sample storage in frozen condition, up to 20 months did not affect accuracy of faecal VOCs to discriminate IBD from controls. A larger faecal sample mass resulted in an increased diagnostic accuracy, with 500mg providing optimum test characteristics. In addition, we observed that an increase in thaw-freeze cycles negatively influenced diagnostic accuracy, with a loss of 20% after one cycle and even a complete loss of discriminative value after two thaw-freeze cycles. Another severe loss in diagnostic accuracy was presented when a delay in analysis of 180 minutes had taken place after sample thawing. These findings underlined the importance of a standardised and strict operating procedure for faecal VOC measurements and formed the basis of our sampling protocol of the VOC studies throughout this manuscript.

The aim of **chapter three** was to assess the stability of faecal VOC composition under

different inter-individual variations in a cohort of healthy individuals, defined as individuals with no gastrointestinal diseases and mucosal abnormalities as by the Cyranose 320©. Here, we described variations in VOC profiles based on age, gender, body-mass index, smoking status, dietary preferences, medication use and co-morbidity. Similar to these outcomes, the influence of smoking on faecal VOC profiles has previously been described. In the current study, we also presented that the factors 'low BMI' (<18.5), 'younger age' (18-35) and a vegetarian or other specific diets strongly influenced VOC profiles, whereas the least influence was found for the variables gender, age > 51 years, previous smoking and use of antibiotics within three months before measurements. The importance of correction for these 'lifestyle' variations when performing faecal VOC analysis was underlined by this study. We proposed methodological correction models for future research as for example matching on the most important variables or the calculation of correction factors within a clinical study. In addition, we presented that most sensors of the Cyranose 320© demonstrated similar changes in electrical resistance when measuring one singular faecal sample. As the sensor outcomes were illustrated by a common tendency per sample to either have outcomes above or below their mean sensor outcome across samples, the question was raised whether the sensors react differently to specific VOCs or whether short-term sensor drift may affect sample outcomes. This finding was the basis for the next chapter.

Although short-term sensor drift over time is a limitation of eNose technology, for which correction may be needed, its high diagnostic accuracy presented in previous studies, combined with its low costs and fast measurements are appealing for routine testing in clinics[2]. In **chapter four**, we therefore investigated the effects of sensor drift on faecal VOC profiles as measured using the Cyranose 320© in a cohort of adult patients with IBD. Mucosal IBD disease activity was assessed based on endoscopic evaluation and the control group consisted of age, gender, smoking habits and hospital matched patients who underwent colonoscopy for various reasons and had no endoscopic abnormalities. In this study, we presented that short-term sensor drift more profoundly influenced VOC patterns than any other clinical variable, such as dietary intake, and disease state of interest, in this case IBD, irrespective of disease activity and localisation. Most correction methods for sensor drift are solely reported once and are mostly based on available, public and mostly artificial online environments, whereas in this study, the effects of eNose sensor drift on diagnostic accuracy were illustrated in a real-life cohort. In addition, we proposed a correction method for this sensor drift which led to improved diagnostic accuracy of faecal VOC profiles to detect IBD. Furthermore, various methodological methods to avoid bias by sensor drift were proposed. These outcomes emphasize the importance of sensor drift correction to improve reliable eNose measurements. Raising awareness amongst clinicians on this phenomenon and how to deal with it in a standardised manner is important as correction for drift improves reliability and repeatability of eNose study outcomes, both within and across studies.

In part one of this thesis, the possible confounding effects of environmental factors, inter- and intraindividual variations and sensor drift have been assessed. Based on these studies, we have developed a standard operating procedure and have proposed correction methods for confounding lifestyle and sensor drift factors. Awareness of these effects and how to adjust study protocols accordingly is important amongst clinicians performing eNose

measurements, as this improves consistency, as well as repeatability and reliable comparison between studies. Standardization of protocols may facilitate future implementation of eNose technology in clinical practice.

PART II

Volatile organic compound analysis as biomarker for inflammatory bowel disease

Both paediatric and adult Inflammatory bowel disease (IBD) are diagnosed and monitored using endoscopic assessment, which are invasive and costly. The differentiation between paediatric IBS and IBD is often challenging in daily practice and faecal calprotectin testing is characterised by a low specificity of IBD, leading to a significant number of (unnecessary) invasive endoscopic procedures performed. Previous studies have highlighted the potential of faecal VOCs to differentiate between paediatric IBD patients and controls[3, 4]. Studies on its discriminative value when including symptomatic paediatric IBS patients were lacking in the available literature, limiting to reliably explore the specificity of VOC analysis to discriminate IBD from an intention to diagnose population. In **chapter five**, a proof of principle study is described in which we were able to differentiate paediatric patients with inflammatory bowel disease from symptomatic irritable bowel syndrome (IBS) patients and asymptomatic controls based on faecal VOC profiles, as measured with FAIMS. In addition, it was concluded that the faecal VOC profiles of IBS patients were similar to those of asymptomatic controls. This latter finding suggested that these faecal profiles may possibly serve as additional non-invasive biomarker to differentiate between paediatric IBS and IBD patients, which would subsequently lead to less unnecessary endoscopic procedures. As this was a proof of principle study with limited number of inclusions, a larger endoscopy-controlled intention-to-diagnose cohort needs to be performed in the future, including study groups with low and high faecal calprotectin values to reliably compare accuracy of the currently used markers to faecal VOC profiles and to assess the value of VOC profiling as (additional) diagnostic biomarker for IBD in clinical practice.

In the literature, urinary VOC profiles have been described to evaporate within few hours and after nine months of storage in the freezer[5]. Therefore, collection of urinary samples for VOC study purposes may, although possibly more patient-friendy to collect and store, be less reliable compared to faecal samples. In a previous (unpublished) study, we provided questionnaires to participants including items on their experience with faecal sample collection. We observed that for most children, the collection of a faecal sample caused a level of embarrassment, whereas they indicated that sampling of urine would be less of an issue. As on-demand collection of urinary samples during clinical appointments has practical advantages over faecal samples, we conducted a study to assess the differences in diagnostic accuracy between faecal and urinary volatile organic compound profiles for IBD. In **Chapter six**, the potential of these urinary and faecal VOC profiles were compared in an intention-to-diagnose cohort of patients aged 4–17 years, referred to the outpatient clinic of a tertiary centre under suspicion of IBD. Here, we assessed urinary and faecal VOC profiles as measured with gas chromatography–ion mobility spectrometry and found that both bodily excrements performed similarly for the detection paediatric IBD diagnosis.

Although faecal VOC samples are considered to be more robust and stable over time and would therefore be more suitable for large-batch measurements and less urgent matters, the development of disease-specific VOC algorithm based on urine samples may be a valuable addition for bed-side, on demand analysis.

In **chapter seven**, we assessed the potential of faecal VOC profiles to detect adult IBD patients in a large multi-centre cohort, using an advanced eNose technology system for pattern-recognition (GC-IMS). We were able to compose and internally validate a diagnostic algorithm based on faecal VOC data that discriminated IBD patients from controls, irrespective of their disease activity, with similar sensitivity and higher specificity to that of faecal calprotectin levels. An algorithm for the discrimination between active disease and remission could, however, not be established based on faecal VOC profiles. Faecal VOC profiles are thought to partly reflect the gut microbiota[6]. Consistent patterns for specific IBD-related bacterial species have not yet been validated due to differences of methodology used to describe microbiota composition in the available literature[7]. However, in multiple studies, the microbial stability has been found higher in healthy individuals compared to IBD patients, who are characterised by a decreased diversity and higher variability over time[8-13]. Remarkably, this variability of microbial composition in IBD patients does not correlate with disease activity and as fluctuations in microbiota composition have been observed both during exacerbation and clinical remission, no typical microbial signatures related to flare-ups have been identified [8, 9, 14]. This may well explain the high diagnostic accuracy of faecal VOC profiles to discriminate between IBD patients and HC, as well as the inability to discriminate between active and quiescent disease in the current study. In a previous study on breath VOC profiling, differences between active IBD and remission were presented based on metabolites related to inflammatory processes[15]. These findings combined implicate that faecal VOCs may serve as valuable biomarker for IBD detection.
As the course of IBD is characterised by periods of relapse and remission and insufficient control of (sub)clinical mucosal inflammation leads to irreversible bowel damage, early prediction of changes in disease state adds to timely management adjustment which subsequently improves disease outcome and prevents drug-related side effects[16, 17]. Gut microbial markers and faecal calprotectin (FCP) have been presented as predictive markers for IBD course, however, validation of these markers is lacking for this purpose[18]. In **chapter eight**, we conducted a follow-up cohort study of IBD patients in which we aimed to predict biochemical disease course (based upon faecal calprotectin levels) and clinical disease course (based on clinical activity scores) using faecal VOC profiles. We observed differences in faecal VOC profiles of IBD patients preceding a change in disease activity based upon faecal calprotectin levels, both prior to going into exacerbation and going into remission. There were no differences preceding a change in the clinical disease course as based upon disease activity scores. In this study, the groups were too small to draw firm conclusions regarding the potential of VOC profiling to monitor IBD, however, the suggestion is made that faecal VOC profiles may be useful to predict disease course and optimize treatment an early stage. A larger cohort is required to validate our findings and may further improve the predictive algorithm.

In conclusion, part two of this thesis presents the potential of faecal VOC profiles to detect and monitor IBD patients. Some of the newly obtained algorithms have already been internally validated. To implement these algorithms in a clinical setting, external validation needs to be performed. For this, it is important to choose one or more specific eNose technologie(s) for further implementation steps, as pattern-recognition algorithms will vary upon the technology and device used and this will influence outcome of external validation. Alongside this, faecal VOC profiles should be assessed next to faecal calprotectin and endoscopy outcomes in an intention-to-diagnose setting, to investigate their additional value and to conduct a reliable cost/benefit analysis for clinical implementation. Once these aspects have been addressed, the results are externally validated and VOC profiles appear to be cost-effective, measurements may be performed for clinical purposes whilst simultaneously adding to a growing pattern-recognition database further improving diagnostic accuracy.

PART III

The liquid faecal metabolome as biomarker for paediatric inflammatory bowel disease

Metabolomics is a branch of chemistry that studies all molecules with a low molecular weight within cells, fluids, tissues or organisms and their interaction with metabolic processes. Similar to faecal VOC profiles, in which gaseous metabolites are measured, these liquid metabolites are believed to directly reflect the underlying biochemical state of the medium measured. The metabolite composition of any medium changes continuously and rapidly over time as the composition is dependent of numerous processes such as synthesis, degradation, absorption, transportation and excretion. Still, it is believed that in the event of predominant pathophysiologic processes, changes in the overall composition of the metabolome may be useful for disease detection. In the third part of this thesis, we illustrated the potential of metabolomic methods for detection of children with inflammatory bowel disease and their additional value to unravel pathophysiological processes.

In chapter nine, the potential of the faecal amino acid composition for IBD detection was explored in a cohort of *de novo* paediatric IBD patients. IBD patients were distinguished from controls based on six specific amino acids varying between the phenotypes UC and CD. The faecal and serum metabolomic patterns of paediatric IBD patients have recently been subject to another study assessing both the serum and faecal metabolome[19]. In line with our study, levels of the faecal amino acid tryptophan were increased in both UC and CD compared to HC. We additionally found elevated faecal levels of histidine, phenylalanine, leucine, tyrosine and valine in paediatric IBD patients. Based on these outcomes, faecal amino acids do seem to hold potential as diagnostic biomarker, which may be more disease-specific and personalised compared to FCP. Since only small study groups were used for this explorative study, larger intention-to-diagnose studies should be performed in a longitudinal setting before firm conclusions can be drawn on the clinical potential of faecal amino acids for IBD detection and for monitoring of disease activity.

In **chapter ten**, we hypothesised on the underlying pathophysiology causative for the change in faecal amino acid composition in a letter to the editor. Based on the available literature, we opted that colonic leakage may be the most likely cause for the increased faecal amino acid levels, though, other contributing factors as downregulation of absorption transporters, a poor nutritional state and a change in metabolism were described.

In **chapter eleven,** we presented a pilot study including *de novo* paediatric IBD children and symptomatic children without IBD to validate our previous results in an intention-to-diagnose setting, whilst simultaneously comparing amino acid composition of both faeces and serum to levels of faecal calprotectin. We presented significantly increased levels of nine amino acids in paediatric IBD children when compared to the non-IBD children, whereas there were no differences in serum amino acid levels. This latter finding was contrary to the available literature on adult IBD patients, in which a decrease of tryptophan and histidine have been described[20]. We did present negative z-values for these AAs. These were, however, non-significant. We demonstrated that high specificity values of faecal AAs may be reached whilst maintaining acceptable sensitivity values. Based on calprotectin, all IBD children were classified correctly and three out of eight non-IBD children were classified incorrectly. Of these incorrectly classified non-IBD patients displaying elevated levels of calprotectin, only one displayed high levels of faecal AAs. Remarkably, this patient underwent a second endoscopic assessment a year after this study was closed, and is currently being treated for IBD-unclassified. Though this pilot study was small, these outcomes illustrate the high specificity of faecal AAs and their potential additional value as non-invasive biomarkers.

In conclusion, we repeatedly presented elevated levels of faecal amino acids in IBD patients, irrespective of their phenotype. The etiology and pathogenesis of this phenomenon is still largely unknown. Current hypotheses on the underlying mechanisms of alterations in faecal AA levels in IBD patients include malabsorption, alterations in metabolism, gut dysbiosis and colonic leakage [21, 22]. In addition, accumulating evidence shows a correlation between faecal AA levels and alterations in gut microbiota in IBD patients [23, 24]. Since testing for faecal AA composition seems reproducible, is easy-to-perform and has low costs, this technology would be suitable for clinical use. We have planned a large intention-to-diagnose cohort study to validate faecal AAs as biomarkers. Upon validation, a diagnostic algorithm may be established combining calprotectin and multiple faecal AAs to improve test accuracy.

PART IV

Using multi-omics strategies to our advantage in the search for early stage colorectal cancer biomarkers

Early detection and treatment are critical factors in the course and prognosis of CRC, as the survival rate decreases with disease progression[25]. Most of the CRC lesions develop from adenomas in the so-called adenoma-carcinoma sequence, in which it is thought that a sequence of mutations result in carcinogenic development. Identification and removal of high-risk adenomas (advanced adenomas) has been described to decrease CRC incidence and mortality [26, 27]. To date, the most widely used screening test for CRC and its precursor

lesions is the faecal immunochemical test (FIT). Although this screening program has led to a decrease in mortality, the performance of this test has suboptimal sensitivity, indicated by the missed diagnosis of CRC in 1-47% and AA in 43-61% of the tests[28]. In addition, specificity is suboptimal, as approximately 7% of the performed tests provide false-positive results leading to the performance of unneeded colonoscopies. Endoscopic assessment is advised after FIT positivity, but also remains key for surveillance after removal of polyps (polypectomy) and following CRC treatment, as these patients remain at increased risk for development or recurrence of dysplastic lesions[25]. In the fourth part of this thesis we describe the potential of faecal volatolomics as well as metabolomics, proteomics and microbial profiling as diagnostic tool for CRC and adenomas and for polyp surveillance. In **chapter twelve** we have provided a review of the available literature on the evidence of faecal VOCs as biomarker for CRC and its precursor lesions. In all studies, promising results for future screening of adenomas and CRC based on faecal VOC analyses were described, yet, the lack of standardised sampling procedures, the use of various analytical techniques and the lack of correction for participant variables as smoking and diet hampered a reliable comparison of results.

In **chapter thirteen**, we demonstrated differences between well-characterised patients with CRC, advanced adenomas, large adenomas and small adenomas when compared to healthy controls (defined as patients with no colonic abnormalities) based on faecal VOC profiles in a large cohort, measured with a GC-IMS system. Additionally, we described that patients with adenomas and controls can be discriminated prior to polypectomy, however, three months after polypectomy faecal VOC profiles are similar. This suggests that the faecal VOC profiles of patients three months after polypectomy alter to more closely resemble healthy controls following this intervention and is substantiated by the fact that faecal VOC profiles of controls did not differ pre- and post-endoscopy, whereas profiles of polypectomy patients did differ pre- and post-polypectomy. This study implied that faecal VOC profiles may be useful for early CRC and adenoma detection when added to the currently available screening methods, but it should not be used for screening as single marker, as large numbers of false positive results will be obtained, leading to a large number of unnecessary endoscopies. Furthermore, this study highlighted that faecal VOC profiles may be used for timing of polyp surveillance as polypectomy led to a normalization of VOC profile. Strong evidence exists for the association between microbial dysbiosis and polyp-associated tissue and colonic neoplasia[29]. As faecal VOC profiles partly reflect the gut microbiota composition, alterations in the microbial composition may be underlying the differences in VOC profiles. So far, causality of the association between gut microbial composition and polyp-associated tissue remains unclear. It is unknown whether microbial changes trigger carcinogenesis, or, whether specific species are attracted to the gut when malignant processes are evolving. As in **chapter thirteen**, faecal VOC profiles were described to return to normal state after polyp removal, the suggestion is raised that the alleged faecal microbial dysbiosis returns to physiological state after polyp removal. Based on these findings, it could be hypothesised that gut microbial changes may rather result from the underlying adenoma-carcinoma sequence than being the causative factor. This study formed the basis of the next two chapters, in which the metabolomic, proteomic and microbial profiles are more closely assessed to further investigate the change in VOC patterns.

In **chapter fourteen**, we integrated data on faecal microbiota, human faecal proteome and faecal amino acid profiles in a multi-centre cohort of CRC-patients, adenoma-patients and controls who underwent colonoscopy (for various reasons) without endoscopic abnormalities. We presented potential diagnostic biomarkers within the proteomic, microbial and amino acid platforms. In addition, we presented that a combination of markers from the proteomic and amino acid platforms outperformed accuracy of the (proteomic) haemoglobin markers in discriminating both CRC and adenomas from controls, and in discriminating CRC from adenomas. As these haemoglobin markers are currently used for population based screening, we believe that after validation in a new cohort, the above mentioned new panels may improve screening for adenomas and CRC. This would subsequently lead to lower incidence and mortality of bowel cancer. Furthermore, we demonstrated new insights into the complex interplay of human metabolism and gut microbial composition in the presence of adenomas and CRC. In line with the available literature, we revealed markers associated with increased blood excretion in the presence of adenomas and CRC. In addition, we presented an upregulation of end-products associated with inflammatory- and stress responses and a downregulation of epithelial integrity. We described associations between these end-products and specific anti- and pro-carcinogenic microbial taxa and hypothesised on the aetiology of these associations based on the available literature.

In **chapter fifteen**, we assessed the adenoma-associated gut microbiota and amino acid profile and investigated their regulation prior to and after adenoma removal in a prospective cohort, including adenoma patients undergoing polypectomy and controls undergoing colonoscopy. Patients with adenomas and controls were distinguished prior to intervention both based on microbial taxa and amino acid profiles. Overall, amino acids returned to physiological state after adenoma removal, whereas the adenoma-associated alterations in bacterial taxa remained largely similar, irrespective of polypectomy. Adenoma-specific amino acids were combined into one biomarker panel which outperformed diagnostic accuracy of the currently used population based FIT test. Based on these study findings, the suggestion was made that amino acids may serve as biomarkers to determine the right timing of surveillance endoscopy, whereas the microbial signature may be used to select high-risk individuals. Upon validation of these panels, these findings may improve effectiveness of the surveillance program, leading to less unnecessary endoscopies and less interval-cancer.

In conclusion, in part four of this thesis, we presented that the use and integration of various omics platforms as faecal VOC profiles, microbiota, metabolome and proteome hold potential to improve both our currently used CRC screening program and timing of our post polypectomy endoscopic surveillance program. Similar to part two of this thesis, some of the newly obtained algorithms have already been internally validated, however, to implement these algorithms in a clinical setting, external validation needs to take place. In the future, we therefore intend the enrollment of a validation cohort in which we aim to repeat measurements and validate currently established algorithms. After validation, these markers should be tested next to the currently used gold standards to provide for a reliable cost/benefit analysis setting and to choose the best available omics strategy. Improvement of the screening and surveillance program may then lead to lower CRC incidence and mortality and to less unnecessary endoscopic assessments.

Future perspectives

It has now become evident that profiling of faecal metabolic end products holds tremendous potential as biomarker for disease detection, disease course prediction and disease surveillance. Apart from the need for a high diagnostic accuracy, for a screening test to be valuable, a high level of reproducibility is warranted. Therefore, a biomarker should be stable and detectable against changes in environmental circumstances. Moreover, it should be cost-effective and easy to use. Third, in case of population based screening, a test that can be transported via regular mail would be preferred. With regards to the omics platforms used in this thesis, especially protein analysis, has been proven to remain unaffected by circumstances such as storage, transport and dietary intake, hereby meeting up to the criterion of robustness. For microbial composition, it is known that dietary intake, storage time and temperature all influence outcomes, however, various studies on CRC have shown comparable outcomes for some specific bacterial strains, making it unlikely that environmental differences significantly affect these CRC-specific bacteria. The influence of storage temperature on microbiota composition, for which minus 80 degrees seems to be preferred, brings along logistic challenges for transportation via regular mail. Amino acid analysis seems to hold high potential as non-invasive CRC and adenoma biomarker. Costs of these analysis are low compared to both microbial and protein profiling, underlining its potential for large-batch population-based screening. However, this platform is at the early stage of development for faecal analysis. The influence of diet and variations in storage and transportation have not yet been elucidated.

A downside of the above mentioned metabolite specific analytical platforms is, however, that measurements are relatively time-consuming, and usually require expensive equipment and expertise. Electronic nose technology may overcome these limitations, as, once a diagnostic algorithm is developed, results can be obtained within minutes after collection. In addition, the use of pattern-recognition may be favourable for diagnostic purposes since this allows for easy-to-perform, high-throughput and low-cost analysis, underlining its suitability for application in clinical practice. Non-invasive testing for the detection and monitoring of IBD, advanced adenomas or CRC based on faecal VOC profiles is, however, still far from the use in a public health setting. The use of various techniques between different studies for faecal VOC analyses in combination with the use of different criteria used for specific diseases, hampers reliable comparison between studies. In addition, as alterations in VOC profiles have been presented for sampling circumstances, lifestyle variations and sensor drift during measurements, collaboration to establish standardization of methodology and technique should be given priority in future studies. Using machine learning, we have presented algorithms specific for the detection of IBD, CRC and precancerous polypous lesions. The accuracy of these algorithms will improve continuously with increasing numbers of samples measured. Once the disease-specific algorithm reaches satisfactory accuracy, it should be validated in a new cohort of patients and compared to the currently used gold standard, to calculate a cost-benefit ratio. The device and corresponding disease-specific algorithms may then be implemented not only for population based screening but also for intra-individual surveillance or follow-up. For population based screening or large batches of samples, it would be preferable to analyse all samples at one central collection point where the VOC profiles may be measured and based on the existing algorithm, chance of disease may be calculated. For smaller numbers of samples, eNose devices may even

allow for bedside analysis. Though, the limitation of sensor drift first needs to be addressed by standardizing correction methodology before reliable diagnostic algorithms can be developed for these smaller devices.

Development of a reliable cost/benefit analysis is limited in this early phase of biomarker development, as targeted analysis and batch measurements change the costs of each omics strategy dramatically and increasing specificity and sensitivity of the proposed test will subsequently alter the costs of the following health care steps. In the future, presented biomarker panels and algorithms should be validated in new datasets, covering all omics platforms. In addition, the new biomarker panels should be tested separately and in combination with the currently used gold standards for screening and surveillance. This may lead to a selection and development of a set of optimal, diagnostic biomarkers to be used as clinical tools.

CONCLUSION

In conclusion, this thesis highlights the potential of faecal VOC analysis for the detection of IBD and the prediction of its disease course. In addition, its potential for CRC and adenoma detection is presented and proposed to be a reliable tool for intra-individual surveillance and to estimate the timing endoscopy after polyp removal. We have described combined omics panels to increase diagnostic accuracy for detection of both CRC and adenomas compared to the use of solely one omics platform. Future studies should focus on the following aspects: a standardised method for VOC measurements, robustness assessment of amino acid profiles, a prospective validation study comparing all biomarkers and algorithms to the gold standard and a cost/benefit assessment. This way the best biomarker(s) and/or algorithms may be selected for further development as clinical tools, improving detection of disease at an early stage which subsequently leads to earlier treatment, lower numbers of complications, surgery, hospital admission and eventually morbidity.

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APPENDICES

NEDERLANDSE SAMENVATTING

In dit proefschrift worden biomarkers onderzocht voor de niet-invasieve vroegdetectie en het monitoren van inflammatoir darmlijden (IBD), dikke darmkanker (CRC) en poliepen. Hiervoor wordt gekeken naar faecale eindproducten van ons metabolisme. Deze eindproducten, ookwel biomarkers, bestaan onder andere uit vluchtige organische componenten (VOCs), de bacteriële samenstelling (faecale microbioom), de eiwitsamenstelling (het faecale proteoom) en hiervan de bouwstenen, de aminozuur profielen, welke behoren bij het faecale metaboloom. De overkoepelende term voor deze biomarkers wordt '*omics*' genoemd. In dit proefschrift hebben deze *omics* strategieën gezorgd voor nieuwe manieren om darmaandoeningen op een niet-invasieve manier in een vroeg stadium te detecteren, te monitoren en het beloop van ziekte te voorspellen. Dit is van belang omdat dit in de kliniek kan leiden tot vroegtijdige behandeling, vermindering van complicaties, operaties en opnames in het ziekenhuis. Daarnaast is gedetailleerde ziekte-specifieke informatie over de eindproducten van het metabolisme essentieel om ons begrip van de pathologie te vergroten en om wetenschappelijk onderbouwde therapeutische interventies te ontwikkelen, gelinkt aan metabolisme en het faecale microbioom. Hiertoe wordt in dit proefschrift gebruik gemaakt van verscheidene technieken. Allereerst, VOCs zijn gasvormige organische moleculen waarvan wordt gedacht dat zij zowel de samenstelling en functie van het microbioom als de interactie met het humane metabolisme reflecteren. Om de faecale VOC profielen te meten wordt in deze thesis gebruikt gemaakt van patroonherkenning op basis van *machine learning* algoritmes met verscheidene electronische neus (eNose) technieken: sensor resistentie (Cyranose 320©), asymmetrische veld - ionmobiliteitsspectrometrie (FAIMS) en gas chromatrografie - ionmobiliteitsspectrometrie (GC-IMS). De faecale bacteriële samenstelling wordt geanalyseerd met 16S ribosomale DNA sequencing, het humane proteoom middels vloeistof chromatografie – massa spectrometrie / massa spectrometrie (LC-MS/MS) en de aminozuurprofielen met hogedrukvloeistofchromatografie (HPLC).

DEEL I

Het optimaliseren van de vluchtige organische componenten analyse

In het eerste deel van dit proefschrift wordt de methodologie en de stabiliteit van de VOC analyse voor de detectie van gastro-intestinale aandoeningen onderzocht. In **hoofdstuk twee** wordt onderzocht wat het effect is van verschillende onderzoeksprotocollen op de diagnostische accuratesse van VOC profielen voor het detecteren van nieuw-gediagnosticeerde, nog onbehandelde kinderen met IBD. Zij worden vergeleken met een groep klachtenvrije gezonde kinderen. De faecale VOC profielen worden gemeten met gebruik van FAIMS. Het werd duidelijk dat de accuratesse toeneemt bij het verhogen van de massa van het faecale monster. Hierbij gaf een massa van 500mg een zeer hoge diagnostische nauwkeurigheid. Een toename van de hoeveelheid dooi-vries cycli had een drastisch effect op de accuratesse, waarbij een verlies van 20% werd gezien na één dooi-vries cyclus en een totaal verlies van discriminatieve waarde na twee dooi-vries cycli. Een vertraging in de meting van 180 minuten na ontdooien van het faecale monster gaf tevens drastisch verlies van diagnostische mogelijkheden. Het bevroren bewaren van de

samples voor 20 maanden had geen duidelijk effect op de diagnostische waarde van de VOC profielen. Gebaseerd op deze studie resultaten hebben we een strikt studie protocol ontwikkeld dat een optimale diagnostische accuratesse bewerkstelligt bij het gebruik van faecale VOC profielen als diagnosticum. Dit protocol vormde de basis voor de rest van dit manuscript.

Het doel van **hoofdstuk drie** was om de stabiliteit van faecale VOC profielen te onderzoeken bij verschillen in de leefstijl. Dit werd onderzocht in een cohort van gezonde individuen, welke in deze studie zijn gedefinieerd als asymptomatische individuen die om verschillende redenen een endoscopie kregen, waarbij geen afwijkingen aanwezig waren. Faecale geurprofielen zijn in deze studie onderzocht met gebruik van de Cyranose 320© gebaseerd op sensor resistentie. De invloed van variabelen als leeftijd, geslacht, BMI, rookgedrag, dieet, gebruik van medicatie en co-morbiditeit op faecale VOC samenstelling werd onderzocht. We vonden dat zowel roken als een laag BMI (<18), een lage leeftijd (18-35 jaar), een vegetarisch of ander specifiek dieet en het gebruik van antibiotica in de drie maanden voorafgaand aan de meting allen van invloed waren op het faecale geurprofiel. In deze studie werd het belang van de correctie voor deze 'leefstijl' variaties bij het doen van faecale VOC analyses duidelijk. Tevens werden verschillende correctie methodes voor toekomstig onderzoek voorgesteld, zoals het matchen van cases en controles op de meest belangrijke leefstijl variaties of het berekenen van correctie factoren binnen een studie. Daarnaast werd aangetoond dat de sensor uitslagen van de Cyranose 320© allen een soortgelijke verandering in elektrische resistentie hadden wanneer zij aan een fecaal monster werden blootgesteld, terwijl de 32 sensoren op verschillende VOCs een andere reactie behoren te hebben. Hierdoor werd de mogelijke invloed van zogenaamde 'sensor drift', het tijdelijk afdrijven van sensor uitslagen, op de VOC profielen blootgelegd. Deze bevinding vormde de basis van de volgende studie.

Ondanks dat het verschuiven van sensoruitslagen een bekende beperking is van de eNose, is de diagnostische accuratesse van deze techniek in verscheidene studies aangetoond. Gecombineerd met de lage kosten en snelheid van de metingen is dit een aantrekkelijke techniek om te gebruiken in de kliniek. In **hoofdstuk vier** wordt het effect van sensor afdrijving op de diagnostische accuratesse van faecale VOC profielen onderzocht, gemeten met de Cyranose 320©. Dit wordt gedaan op basis van een cohort met volwassen IBD patiënten waarbij de ziekte activiteit gescoord wordt op basis van de endoscopie afbeeldingen. Hierbij hebben wij ons gehouden aan het eerdere protocol en zijn de cases en controles voorafgaand aan de analyse gematcht op leeftijd, geslacht, rookgedrag en ziekenhuis van inclusie. In dit hoofdstuk wordt aangetoond dat, wanneer er niet gecorrigeerd wordt voor sensor drift, deze variabele een grotere invloed heeft op de faecale VOC patronen dan IBD zelf, ongeacht de ziekteactiviteit en lokalisatie van de ziekte. In de literatuur zijn de meeste sensor drift correctie methoden maar eenmalig gerapporteerd, en deze zijn vaak gebaseerd op publiekelijk beschikbare, artificiële online databases. In de huidige studie wordt dit effect onderzocht op een levensecht cohort. Tevens wordt een correctie methode voorgesteld en uitgevoerd, waarbij de accuratesse om IBD te detecteren verbetert. De uitkomsten van de huidige studie benadrukken het belang van sensor drift correctie bij eNose metingen om de betrouwbaarheid van de uitkomsten te verbeteren.

In **deel een** van dit proefschift worden mogelijke confounders van faecal VOC profielen gezocht in onderzoeksprotocollen, inter-individuele variaties en eNose technieken zelf. Gebaseerd op de studies uit deel I hebben we een standaard bedrijfsprotocol ontwikkeld voor faecale VOC profielen en hebben we mogelijke correctie methoden voorgesteld voor sensor drift. Wetenschap van deze mogelijke confounders en hoe met deze om te gaan is belangrijk onder artsen en onderzoekers die gebruik maken van eNose metingen, omdat dit de consistentie, herhaalbaarheid en betrouwbaarheid van de metingen bevorderd.

DEEL II

De vluchtige organische componenten analyse als biomarker voor inflammatoir darmlijden

Inflammatoir darmlijden is een chronische ontstekingsziekte van het maag/darmstelsel. Er zijn twee vormen waarin IBD voorkomt, dit zijn de ziekte van Crohn (CD) en colitis ulcerosa (CU). De ziekte wordt bij zowel kinderen als volwassenen gediagnostiseerd en gemonitord door middel van endoscopische onderzoeken. In de kliniek is het soms een uitdaging om het onderscheid te maken tussen patiënten met een prikkelbaar darmsyndroom (IBS) en met IBD. Fecaal calprotectine wordt in de kliniek gebruikt om een actieve darmontsteking op te sporen, en kan daarmee de aannemelijkheid dat er IBD speelt vergroten. Het calprotectine is echter een marker voor alle vormen van mucosale darmontstekingen en heeft daarom een lage specificiteit voor IBD. Dit leidt tot een aantal onnodig uitgevoerde endoscopische onderzoeken. Deze zijn niet alleen invasief en duur, maar zijn ook zwaar voor kinderen omdat zij moeten worden opgenomen in het ziekenhuis en laxatie middelen krijgen toegediend via een neusmaag sonde. In eerdere studies is de toepasbaarheid van faecale VOC profielen voor de detectie van IBD patiënten aangetoond. Echter, dit is steeds gedaan in vergelijking met asymptomatische kinderen. In **hoofdstuk vijf** wordt aangetoond dat kinderen met IBD kunnen worden onderscheiden van symptomatische kinderen met IBS gebaseerd op faecale VOC profielen gemeten met de FAIMS techniek. Daarnaast wordt aangetoond dat er geen onderscheid is tussen de faecale VOC profielen van kinderen met IBS en asymptomatische (gezonde) kinderen. Deze bevindingen benadrukken dat de geurprofielen van toegevoegde waarde zijn voor het differentiëren tussen symptomatische kinderen met IBD en zonder IBD. Dit kan in de toekomst leiden tot het verrichten van minder (onnodige) invasieve endoscopische onderzoeken bij kinderen met buikklachten.

In de literatuur wordt beschreven dat de VOC profielen van urine binnen enkele uren diffunderen. Het verzamelen van urine monsters lijkt daarom minder geschikt dan faecale samples. Echter, in een eerdere (ongepubliceerde) vragenlijst studie bleek dat er met name voor kinderen schaamte speelt bij het verzamelen van faecale monsters. Zij gaven aan dat dit een minder grote rol speelt bij het verzamelen van urine. Omdat het 'op aanvraag' verzamelen van een urine monster tijdens een afspraak op de polikliniek tevens praktische voordelen heeft, ontwikkelden we een studie om de verschillen in diagnostische accuratesse te onderzoeken tussen urine monsters en faecale monsters voor het detecteren van kinder-IBD. In **hoofdstuk zes** werd deze accuratesse vergeleken in een 'intention-to-diagnose' cohort van patiënten tussen de 4 en 17 jaar oud. Deze patiënten werden allen verwezen naar een tertiair centrum vanwege verdenking op IBD. Kinderen met symptomen,

maar waarbij geen IBD gediagnosticeerd werd, vormden de controle groep. Faecale geurprofielen werden gemeten met een geavanceerde, zeer stabiele eNose techniek: GC-IMS. Voor zowel ontlasting als urine werd hetzelfde protocol gevolgd, waarbij tevens bewaartijd van samples in de vriezer gelijk was. Er werden geen verschillen aangetoond in de diagnostische accuratesse tussen faecale en urinaire VOC profielen voor IBD. Ondanks dat er wordt gedacht dat faecale VOC profielen stabieler en meer robust zijn over de tijd en hierdoor meer geschikt zijn voor het doormeten van grote partijen monsters, zou een ziekte-specifiek VOC algoritme voor urine een goede optie zijn voor snelle 'bed-side' metingen.
In **hoofdstuk zeven** is onderzocht of faecale geurprofielen het onderscheid kunnen maken tussen volwassenen met IBD en gezonde controles in een groot multi-centrum onderzoek waarbij we gebruik maakten van de GC-IMS techniek. In deze studie werd een hoge diagnostische accuratesse aangetoond voor de vergelijking tussen IBD patiënten en controles, ongeacht de ziekteactiviteit van deze patiënten. Onze bevindingen toonden aan dat de geurprofielen mogelijk behulpzaam zijn bij het opsporen van IBD. De detectie van de mate van ziekteactiviteit op basis van faecale geurprofielen was matig. Omdat een opvlamming progressief verloopt, werd de hypothese gesteld dat geurprofielen mogelijk wel een rol kunnen spelen bij de vroeg detectie van een verandering in ziekteactiviteit. Dit is belangrijk omdat periodes van (sub)klinische mucosale ontsteking kunnen leiden tot onomkeerbare darmschade en complicaties. In **hoofdstuk acht** onderzochten we dit vraagstuk in een follow-up studie van IBD patiënten die op gezette momenten faecale samples en vragenlijsten verzamelden voor de klinische activiteit score, het fecaal calprotectine en faecale geurprofielen. In deze studie werd ontdekt dat de faecale geurprofielen van patiënten voorafgaand aan een verandering in ziekteactiviteit, gebaseerd op het fecaal calprotectine, verschillend zijn dan patiënten waarvan de ziekteactiviteit stabiel blijft. Dit gold voor patiënten die van een exacerbatie in remissie gingen als voor patiënten in remissie die een exacerbatie kregen. De onderzoeksgroepen geïncludeerd in deze studie waren te klein om uitspraken te doen over het gebruik van faecale geurprofielen met als doel het voorspellen van het ziektebeloop van IBD in de kliniek. Echter suggereren de bevindingen van deze studie wel dat faecale geurprofielen mogelijk een bijdrage zouden kunnen leveren aan de optimalisatie van IBD behandeling. Een groter cohort is echter nodig om deze bevindingen te valideren.

Concluderend, in deel twee van dit proefschrift wordt beschreven hoe faecale geurprofielen kunnen bijdragen aan de klinische zorg voor kinderen en volwassenen met IBD. Sommige van de voorgestelde algoritmes zijn al intern gevalideerd. Voordat verdere implementatiestappen gezet worden, is het van groot belang om allereerst een keuze te maken welke de eNose techniek het meest geschikt is voor het beoogde doeleinde, omdat patroonherkenning algoritmes specifiek zijn voor het bijbehorende meetinstrument. Wanneer deze keuze is gemaakt, zal het verkregen algoritme gevalideerd moeten worden in een extern prospectief cohort, waar het vergeleken moet worden met het fecaal calprotectine en de gouden standaard, de endoscopie. Op deze manier kan de toegevoegde waarde van faecale VOC profielen als biomarkers berekend worden en een betrouwbare kosten/baten analyse verricht worden.

Deel III Het faecale metaboloom als biomarker voor inflammatoir darmlijden bij kinderen
Het metaboloom omvat alle moleculen met een laag moleculair gewicht binnen cellen, vloeistoffen, weefsels of organismen en diens interactie met metabole processen. Vergelijkbaar met faecale VOC profielen, waarin gasmoleculen gemeten worden, wordt gedacht dat deze vloeibare moleculen een directe reflectie zijn van de onderliggende biochemische status van het medium dat gemeten wordt. Het metaboloom van een medium verandert continue, vaak en snel omdat de samenstelling afhankelijk is van verscheidene processen zoals synthese, degradatie, absorptie, transport en excretie. Toch wordt er gedacht dat, in het geval van predominante (patho)fysiologische processen, globale veranderingen in deze samenstelling bruikbaar kunnen zijn voor ziekte-detectie. In dit derde deel illustreren we de potentiële toegevoegde waarde van het faecale metaboloom voor het vaststellen van inflammatoir darmlijden bij kinderen.

In **hoofdstuk negen** wordt de accuratesse van faecale aminozuren voor de detectie van IBD onderzocht in een cohort van nieuw gediagnosticeerde kinderen. We tonen aan dat IBD van controles kan worden onderscheiden met een hoge accuratesse op basis van zes aminozuren, welke verschillen tussen de fenotypes CD en UC. Gelijk aan de eerdere literatuur was een van deze verhoogde aminozuren het molecuul tryptofaan. Wij vonden daarnaast een verhoging van histidine, phenylalanine, leucine, tyrosine an valine in kinderen met IBD. In een brief worden in **hoofdstuk tien** hypotheses uitgewerkt over de onderliggende pathofysiologie die ten grondslag ligt aan de verschillen in faecale aminozuur profielen tussen kinderen met IBD en hun gezonde leeftijdsgenoten. Gebaseerd op onze eerdere studie en de beschikbare literatuur, stellen we dat lekkage uit het colon de meest waarschijnlijke oorzaak is voor de verhoging van faecale aminozuren bij IBD patiënten. Andere mogelijke bijdragende factoren als de verminderde expressie van absorptie-transporters, een verminderde voedingsstatus en een veranderd metabolisme worden tevens beschreven. In **hoofdstuk elf** wordt een kleine pilot studie gepresenteerd waarbij de faecale en serum aminozuren van een groep nieuw-gediagnosticeerde IBD kinderen worden vergeleken de profielen met symptomatische kinderen zonder IBD in een 'intention-to-diagnose' setting. We presenteren wederom significante verhogingen van, dit keer negen, verschillende faecale aminozuren bij IBD patiënten, terwijl er geen verschillen worden aangetoond in de aminozuurprofielen van het serum. Deze laatste bevinding is anders dan eerder gepubliceerde literatuur, waarbij een vermindering van tryptofaan en histidine werd beschreven in het serum. In onze studie werden negatieve Z-waarden gevonden voor deze specifieke aminozuren in het serum, echter waren onze bevindingen niet significant. Gedemonstreerd wordt dat de hoge specificiteit van faecale aminozuren behaald wordt terwijl een acceptabele sensitiviteit behouden blijft. Gebaseerd op het faecale calprotectine, een veelgebruikte biomarker, werden 3 van 8 niet-IBD kinderen verkeerd gediagnosticeerd. Van deze patiënten had maar één kind verhoogde aminozuren. Deze patiënt is een half jaar na de studie een tweede endoscopie ondergaan vanwege aanhoudende klachten en is alsnog gediagnosticeerd met IBD. Ondanks dat dit een kleine pilot studie was, illustreren deze uitkomsten de hoge specificiteit en hiermee de toegevoegde waarde van faecale aminozuren als niet-invasieve IBD biomarkers.

In deel III zijn herhaaldelijk verhoogde faecale aminozuurprofielen gevonden bij kinderen met IBD, onafhankelijk van het fenotype. De etiologie en pathogenese van dit fenomeen is nog onduidelijk. Huidige hypotheses zijn lekkage uit het colon, malabsorptie, ondervoeding, een veranderd metabolisme of een veranderde microbiële samenstelling van de ontlasting. Omdat het testen van aminozuren reproduceerbaar lijkt, makkelijk te verrichten is en weinig kosten met zich mee brengt, is deze technologie uitermate geschikt voor klinisch gebruik. We zijn voornemens een grote, prospectieve, 'intention-to-diagnose' cohort studie op te zetten om deze faecale aminozuurprofielen te valideren als biomarkers voor kinderen met inflammatoir darmlijden. Een combinatie met het faecale calprotectine zou dan in de toekomst gebruikt kunnen worden om niet-invasieve risico inschatting te verbeteren hiermee en minder (onnodige) endoscopieën uit te voeren.

DEEL IV

Multi-omics strategieën als biomarkers voor de vroege opsporing van colorectaal kanker en risicopoliepen

De vroegdetectie en behandeling van colorectaal kanker (CRC) zijn belangrijke factoren voor het verloop en de prognose, omdat de overlevingskans vermindert bij een toegenomen ziekteprogressie. De meeste CRC lesies ontstaan vanuit adenomateuze poliepen in de zogenoemde adenoom-carcinoom reeks. Hierbij wordt gedacht dat een serie van mutaties resulteert in CRC. Het identificeren en verwijderen van hoog-risico adenomen (advanced adenomen) leidt tot een verlaging van CRC incidentie en mortaliteit. Momenteel wordt er in Nederland gebruikt gemaakt van het bevolkingsonderzoek, waarbij er wordt gescreend op CRC en hoog-risico adenomen op basis van de faecale immunochemische test (FIT). Dit screeningprogramma heeft tot een verlaging van CRC mortaliteit geleid, maar de test heeft een suboptimale sensitiviteit en specificiteit. Op basis van de FIT test wordt CRC in 1-47% van de gevallen gemist, afhankelijk van de afkapwaarde die gebruikt wordt. Daarnaast worden hoog-risico poliepen in 43-61% van de gevallen gemist door deze test. Ook is 7% van de uitslagen fout-positief, wat ertoe leidt dat er veel onnodige coloscopieën worden uitgevoerd. Endoscopie wordt niet alleen uitgevoerd na een positieve FIT test, maar blijft ook de standaard interventie voor surveillance na het verwijderen van adenomen en CRC. In het vierde deel van deze thesis wordt onderzocht of patiënten met CRC en poliepen kunnen worden opgespoord met behulp van verscheidene omics platforms. Daarnaast wordt onderzocht of faecale geurprofielen, het microbiota en faecal aminogrammen van toegevoegde waarde kunnen zijn in het huidige surveillance programma. In **hoofdstuk twaalf** geven we een overzicht van de bestaande literatuur over faecale geurprofielen als biomarker voor CRC en voorstadium adenomen. Ondanks dat het niet mogelijk was een goede vergelijking te maken tussen de studies door een gebrek aan gestandardiseerde methoden en gebruik van verschillende technieken, beschreven al deze studies allen veelbelovende resultaten. In **hoofdstuk dertien** wordt de diagnostische waarde van faecale geurprofielen beschreven voor de detectie en monitoring van CRC en adenomen, waarbij gebruik is gemaakt van onze gestandardiseerde methodologie. In deze studie wordt gebruik gemaakt van patroon-herkenning met een geavanceerde electronische neus, een GC-IMS systeem. Op basis van de geurprofielen worden patiënten met CRC, voorloper adenomen, grote goedaardige adenomen en kleine adenomen met hoge accuratesse gekarakteriseerd

en onderscheiden van gezonde controles. Daarnaast wordt beschreven dat de faecale geurprofielen van patiënten met adenomen die een radicale poliepectomie ondergaan, 3 maanden na het verwijderen van het adenoom een geurprofiel hebben dat gelijk is aan die van controlepatiënten terwijl de geurprofielen van controlepatiënten niet veranderen pre- en post endoscopie. Op basis van deze bevindingen kan gesuggereerd worden dat er een adenoom-specifiek geurprofiel bestaat, welke mogelijk gebruikt kan worden voor screening en surveillance doeleinden. Omdat de faecale bacteriële samenstelling deels gereflecteerd wordt door faecale VOC profielen, kan het zijn dat dit geassocieerde microbioom ten grondslag ligt aan de uitkomsten van **hoofdstuk dertien**. Eerdere studies hebben een associatie aangetoond tussen bacteriële dysbiose en poliep-geassocieerd weefsel / CRC. De causaliteit van deze associatie is tot op heden nog onduidelijk. Enerzijds kunnen de microbiële veranderingen carcinogenese in het colon stimuleren, anderzijds is het mogelijk dat specifieke bacteriën zich nestelen in het colon in de aanwezigheid van neoplastische laesies. Omdat in dit hoofdstuk wordt beschreven dat de faecale VOC profielen normaliseren na een radicale poliepverwijdering, is de suggestie gewekt dat het faecale microbiota tevens normaliseert. Op basis van deze bevinding kan gesuggereerd worden dat de microbiële dysbalans in de aanwezigheid van adenomen meer waarschijnlijk een resultaat is van de onderliggende adenoom-carcinoom reeks dan een oorzaak. Deze studie vormt de basis van de opvolgende twee hoofdstukken, waarin het metaboloom, proteoom en microbiota nader onderzocht worden.

In **hoofdstuk veertien** wordt data van de faecale microbiota, het proteoom en de aminogrammen geintegreerd in een multi-centrum cohort van patienten met CRC, adenomen en controles zonder afwijkingen bij endoscopie. Potentiële biomarkers voor ziekte detectie worden gepresenteerd binnen elk van deze platforms. Daarnaast worden deze geselecteerde biomarkers gecombineerd tot potentiële 'diagnostische panels'. Deze overschreiden de accuratesse van het huidige gebruikte FIT zowel voor de detectie van CRC patienten als van patienten met adenomen. Na validatie van deze zogenoemde panels zouden deze het bevolkingsonderzoek naar CRC kunnen verbeteren. Tevens worden nieuwe inzichten gepresenteerd betreffende de interactie tussen het humane metabolisme en het microbiota in de aanwezigheid van neoplastische lesies. Vergelijkbaar met eerdere literatuur worden markers gepresenteerd welke een associatie hebben met verhoogde bloed excretie en afbraak. Daarnaast presenteren we markers geassocieerd met een verhoogde inflammatoire status, verhoogde stress reactie en een vermindering van epitheliale integriteit. Associaties worden beschreven met zowel pro- als anticarcinogene microbiële taxa.

In **hoofdstuk vijftien** onderzoeken we het adenoom-geassocieerde aminozuurprofiel en microbiota voorafgaand en na poliepectomie in een prospectief cohort van adenoma patienten en controle patiënten zonder afwijkingen bij endoscopie. Patiënten met adenomen werden onderscheiden van controles op zowel op basis van het aminozuurprofiel als op basis van de microbiota. Er werd een adenoom specifiek aminozuur profiel gevonden, dat na verwijderen van de adenomen terugkeerde naar de 'normale staat', terwijl de bacteriële samenstelling van de ontlasting onveranderd bleef na de interventie. De adenoom-specifieke aminozuren werden gecombineerd tot één biomarker panel waarvan de accuratesse die van de huidig gebruikte FIT test overschreedt. Gebaseerd op deze resultaten suggereren

we dat het faecale aminogram kan dienen als biomarker voor adenomen, waarbij de juiste timing van een surveillance endoscopie bepaald kan worden. Klinisch betekent dit een verbetering van de efficiëntie van het surveillance programma met een vermindering van onnodige coloscopiën, een vermindering van interval kanker en een verlaging van de zorgkosten.

Concluderen, in deel IV van deze thesis presenteren we dat het gebruik en de integratie van verscheide omics platforms zoals het faecale VOC profiel, de microbiota, het metaboloom en proteoom van toegevoegde waarde kunnen zijn voor ons huidige bevolkingsonderzoek darmkanker en het surveillance programma post-poliectomie. Sommige van deze algoritmes zijn reeds intern gevalideerd. Voordat implementatie in de kliniek kan plaatsvinden moet externe validatie worden bewerkstelligt. Een doel voor de komst is daarom om een validatiecohort uit te rollen waarin niet alleen de huidige bevindingen gevalideerd kunnen worden, maar ook naast de huidige gouden standaard kan worden gelegd voor een gedegen kosten-baten analyse. Na validatie kan de implementatie van deze nieuwe biomarkers een verbetering van zowel het screening als het surveillance programma opleveren, met een vermindering vsan onnodige coloscopiën, een vermindering van interval kanker en een verlaging van de zorgkosten.

Toekomstperspectief

Faecale metabole eindproducten hebben de potentie om gebruikt te worden als gastro-intestinale biomarkers, zo is duidelijk geworden uit de studies in dit proefschrift. Zowel voor het diagnosticeren als voor vervolgen van darmziekten. Zowel voor inflammatoir darmlijden als voor colorectaal kanker en zijn voorloper poliepen. Echter zijn er een aantal voorwaarden waaraan deze biomarkers moeten voldoen, voordat dit kan resulteren in een gebruiksklare, klinische test. Ten eerste moeten de ontwikkelde algoritmes uit dit proefschrift ofwel nog intern en extern, ofwel alleen extern gevalideerd worden. Ten tweede, om een screeningtest of surveillancetest grootschalig te kunnen implementeren zijn er een aantal karakteristieken vereist. Zo moeten de uitkomsten van een test stabiel zijn, ongeacht de omgevingsomstandigheden. Daarnaast moet een test kosteneffectief zijn en is de mogelijkheid om materiaal per post te versturen een pre- bij het verwerken van grote hoeveelheden monsters.

Met het oog op de omics strategieën in deze huidige thesis, is met name de stabiliteit van de eiwitspectra reeds aangetoond. Transport, opslagtemperatuur en voedselinname lijken geen effect te hebben op de uitkomsten van deze test. Deze omgevingsfactoren zijn wel van invloed op de bacteriële samenstelling van de ontlasting. Belangrijk is daarom om te onderzoeken of deze factoren een invloed hebben op de specifieke bacteriële stammen die als biomarker kunnen dienen voor de beoogde aandoening. Omdat verscheidene studies naar het colorectaal kanker-geassocieerde microbiota dezelfde bacteriële stammen aanwijzen, lijkt een verschil in dieet en transportwijze geen grote invloed te hebben. Wel is aangetoond dat een bewaartemperatuur van -80 graden nodig is, om de bacteriële samenstelling betrouwbaar te kunnen meten. Een grote hoeveelheid materiaal per post versturen is hierdoor niet mogelijk. Aminozuur profielen hebben in tegenstelling tot de hiervoor benoemde methoden relatief zeer lage kosten. Hiermee lijken faecale aminozuurprofielen geschikt om te fungeren als diagnosticum. Echter, deze strategie staat

nog in de kinderschoenen van biomarker exploratie en robuustheid van uitkomsten moet nog onderzocht worden.

Een nadeel van bovengenoemde metaboliet-specifieke analytische platforms is dat de materiaal voorbereiding en metingen relatief veel tijd kosten, de techniek kostbaar is en speciaal getrainde laboranten nodig zijn voor het analyseren van het materiaal. De elektronische neus techniek kan dit soort limitaties ondervangen. Nadat een diagnostisch algoritme is ontwikkeld, kunnen metingen binnen enkele minuten worden gedaan, zonder dat het faecale materiaal voorbereid hoeft te worden voor de analyse. Daarnaast kan patroonherkenning voordeliger zijn voor diagnostische doeleinden omdat de metingen makkelijk uitvoerbaar zijn, er grote hoeveelheden in een keer gemeten kunnen worden en de analyses relatief goedkoop zijn. Toch zijn faecale VOC profielen voor diagnostische doeleinden nog niet klaar voor klinisch gebruik. Door het toepassen van de vele verschillende technieken zijn de voor- en nadelen van eNose technologie onderzocht, maar wordt de mogelijkheid om verschillende studies van verschillende onderzoeksgroepen met elkaar te vergelijken bemoeilijkt. Daarnaast zijn veranderingen in VOC profielen aangetoond bij sensor variaties, leefstijl variaties en het gebruik van verschillende studieprotocollen. Samenwerking om tot standaardisatie van de methodologie en gebruikte eNose techniek te komen, zou daarom prioriteit moeten hebben bij toekomstige studies.

Gebaseerd op 'machine-learning' hebben we in deze thesis een aantal VOC algoritmes getoond, specifiek voor de detectie van IBD, CRC en poliepen. De accuratesse van deze algoritmes verbetert naarmate er een groter aantal ontlastingsmonsters gemeten wordt. Zodra een algoritme voldoende accuratesse bereikt, kan deze gevalideerd worden in een extern cohort van patiënten, naast de huidige gouden standaard, om een kosten-baten analyse op te maken. Het apparaat en bijbehorende ziekte-specifieke algoritme kan daarna worden geïmplementeerd als klinische tool, zowel voor screening als voor surveillance. Voor het uitvoeren van bevolkingsonderzoek zou het voordelig zijn om het ontlastingsmateriaal te verzamelen op centrale punten in Nederland. De kans op specifieke ziekten kan dan berekend worden aan de hand van de ziekte-specifieke algoritmen. Voor kleinere patiënt aantallen kan een kleinere en gebruiksvriendelijkere eNose gekozen worden welke 'aan het bed' ingezet kan worden. Echter, voor deze kleinere eNose apparaten moet de sensor drift in de gaten worden gehouden en moet er een goede, gestandaardiseerde correctiemethode ontwikkeld worden alvorens betrouwbare algoritmes kunnen worden ontwikkeld.

Het berekenen van een goede kosten-baten analyse is moeilijk in deze vroege fase van biomarker exploratie. Gerichte metingen kunnen ontwikkeld worden voor metaboliet-specifieke technieken wanneer bepaald is welke metabolieten interessant zijn. Grote hoeveelheden metingen kunnen de kosten van een techniek drastisch veranderen. Een verbeterde accuratesse van een test kan de kosten voor de gezondheidszorg op de lange termijn verminderen. Deze factoren zullen allemaal van invloed zijn op de uiteindelijke waarde van een nieuwe biomarker. Om een goede vergelijking van de diagnostische waarde te maken is het van belang de geselecteerde biomarkers en algoritmes tezamen te valideren in een nieuwe dataset en te vergelijken met de huidige gouden standaard. Op deze manier kan (een combinatie van) de beste biomarker(s) gekozen worden voor verdere implementatie in de kliniek.

CONCLUSIE

In deze thesis wordt de potentiële waarde getoond van faecale VOC profielen voor de detectie van inflammatoir darmlijden en het voorspellen van veranderingen in het ziektebeloop. Daarnaast wordt getoond dat faecale VOC profielen waardevol kunnen zijn voor de detectie van colorectaal kanker en wordt de patroonherkenning voorgesteld als techniek voor het bepalen van de surveillance timing na het verwijderen van poliepen. We beschrijven dat verscheidene omics platforms gecombineerd kunnen worden om de diagnostische accuratesse voor de detectie van colorectaal kanker en poliepen te verbeteren. In de toekomst zullen studies zich moeten focussen op een aantal aspecten: de standaardisatie van VOC metingen, robuustheid van aminozuur metingen, een prospectieve gezamenlijke validatie van de biomarkers en algoritmes gecombineerd met de huidige gouden standaard en een kosten/baten analyse. Op deze manier kunnen de beste biomarker(s) en/of algoritmes geselecteerd worden voor de verbetering van ziekte detectie en monitoring, wat leidt tot vroegere behandelingen, een verminderd aantal complicaties, operaties, ziekenhuisopnames en uiteindelijk morbiditeit.

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ABOUT THE AUTHOR

Sofie Bosch was born in Eindhoven in 1992. She completed secondary school with focus on technical science and health at the Stedelijk College Eindhoven, and took optional courses in Spanish, and management and organisation. She studied medicine at the VU University in Amsterdam from which she graduated in 2016. During her medical training, she took elective internships in Gastroenterology and Hepatology at the Haga Hospital in The Hague (NL) and the endoscopy unit and outpatient clinic of the Queen Elizabeth Hospital in Birmingham (UK).

After completing her medical degree, Sofie was accepted as a PhD candidate at the Amsterdam UMC, location VUmc in 2017, where she worked on research for 3 years. In July of 2019 she started working as a junior house officer at the gastroenterology wards of the NWZ Hospital in Alkmaar and in July 2020 she started her residency in Gastroenterology and Hepatology at the Spaarne Gasthuis in Haarlem and Hoofddorp and the Amsterdam University Medical Centres in Amsterdam.

Sofie has keen interest in translational research, focussing on omics strategies and machine learning for detection and monitoring of gastrointestinal illnesses. She incorporates this interest in her scientific work.

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LIST OF PUBLICATIONS

Sofie Bosch, Renée Menezes, Suzanne Pees, Dion Wintjens, Margien Seinen, Gerd Bouma, Johan Kuijvenhoven, Pieter CF Stokkers, Tim GJ de Meij, Nanne KH de Boer. The influence of short-term sensor drift in a real-life clinical cohort: Limitations of electronic nose analysis hidden in a black box. Submitted.

Sofie Bosch, Dion SJ Wintjens, Alfian Wicaksono, Marieke Pierik, James A Covington, Tim GJ de Meij, Nanne KH de Boer. Prediction of inflammatory bowel disease course based on faecal volatile organic compounds. Submitted.

Sofie Bosch, Animesh Acharjee, Mohammed Nabil Quraishi, Irene Bijnsdorp, Patricia Rojas, Abdellatif Bakkali, Erwin EW Jansen, Pieter Stokkers, Johan Kuijvenhoven, Thang V Pham, Andrew D Beggs, Connie R Jimenez, Eduard A Struys, Georgios B Gkoutos, Tim GJ de Meij, Nanne KH de Boer. Data integration of microbiota, proteome and amino acids for the detection of colorectal cancer and adenomas. Submitted.

Sofie Bosch, Animesh Acharjee, Mohammed Nabil Quraishi, Patricia Rojas, Abdellatif Bakkali, Erwin EW Jansen, Marina Brizzio Brentar, Johan Kuijvenhoven, Pieter Stokkers, Eduard A Struys, Andrew D Beggs, Georgios V Gkoutos, Tim GJ de Meij, Nanne KH de Boer. Detection and surveillance of adenoma patients undergoing polypectomy using faecal microbiota and amino acid composition. Submitted.

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