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Volatile organic compounds in breath can serve as a non-invasive diagnostic biomarker for the detection of advanced adenomas and colorectal cancer

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Summary

Background: Colorectal cancer (CRC) is the third most common cancer diagnosis in the Western world.

Aim: To evaluate exhaled volatile organic compounds (VOCs) as a non-invasive biomarker for the detection of CRC and precursor lesions using an electronic nose.

Methods: In this multicentre study adult colonoscopy patients, without inflammatory bowel disease or (previous) malignancy, were invited for breath analysis. Two-thirds of the breath tests were randomly assigned to develop training models which were used to predict the diagnosis of the remaining patients (external validation). In the end, all data were used to develop final-disease models to further improve the discriminatory power of the algorithms.

Results: Five hundred and eleven breath samples were collected. Sixty-four patients were excluded due to an inadequate breath test (n = 51), incomplete colonoscopy (n = 8) or colitis (n = 5). Classification was based on the most advanced lesion found; CRC (n = 70), advanced adenomas (AAs) (n = 117), non-advanced adenoma (n = 117), hyperplastic polyp (n = 15), normal colonoscopy (n = 125). Training models for CRC and AAs had an area under the curve (AUC) of 0.76 and 0.71 and blind validation resulted in an AUC of 0.74 and 0.61 respectively. Final models for CRC and AAs yielded an AUC of 0.84 (sensitivity 95% and specificity 64%) and 0.73 (sensitivity and specificity 79% and 59%) respectively.

Conclusions: This study suggests that exhaled VOCs could potentially serve as a non-invasive biomarker for the detection of CRC and AAs. Future studies including more patients could further improve the discriminatory potential of VOC analysis for the detection of (pre-)malignant colorectal lesions. (https://clinicaltrials.gov Identifier NCT03488537)

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1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer diagnosis and a major cause of mortality in the Western World.[1-3] CRC usually develops from focal changes within benign polyps via a multistep process involving a series of genetic, histological and morphological changes that accumulate over time.[4] This dwell time allows for early detection and removal of precursor lesions to prevent development of CRC. Moreover, detection of early-stage CRC improves survival rates which is why screening programs for CRC are increasingly adapted worldwide.[5,6]

Nowadays, several CRC screening modalities are available, and each test has its own performance characteristics and acceptability profile. Preferably, a screening test should be affordable, non-invasive and precise to achieve a high rate of cooperation from asymptomatic individuals. Faecal immunochemical testing (FIT) is currently the most commonly used non-invasive screening test for CRC. This stool-based test is relatively cheap and easy to perform, but population screening with FIT results in missed cancers and unnecessary colonoscopy procedures due to suboptimal sensitivity and specificity of 56%-89% and 92%-97% respectively, for detection of CRC.[7-9] Furthermore, ideally a screening test should detect treatable precursor lesions to prevent development of cancer, but sensitivity of FIT for advanced adenomas (AAs) is low, only 39%-57%.[7,8] These suboptimal test characteristics of FIT and sometimes disappointing adherence rates to FIT screening programs (37%-62%) illustrate the need for a new, more accurate, non-invasive diagnostic test for CRC and its precursor lesions.[10-13]

Analysis of volatile organic compounds (VOCs) might be a promising new technique for early detection and surveillance of various diseases, including CRC. VOCs are gaseous carbon-based end products of physiologic and pathologic metabolic processes which can be detected in all biological specimens (eg breath, saliva, urine, faeces, blood).[14,15] It has been shown that VOC concentration profiles and/or VOC composition differ between patients with and without certain diseases, ranging from infectious diseases[16,17] to malignancies.[18-20] Some potential CRC-associated VOCs have already been identified in small pilot studies,[21-35] but the VOCs identified differed among studies and (external) validation of the results is lacking. Data on VOC's associated with colorectal polyps is even more scarce.[25,29,32,33]

Thus far, most studies that evaluated the potential of VOCs to serve as a non-invasive biomarker for CRC used faecal samples and gas chromatography-mass spectrometry (GC-MS) for VOC analysis. We hypothesised that breath analysis with electronic nose (e-nose) technology may be more suitable for clinical practice since breath sampling may be more acceptable for (asymptomatic) individuals. In addition, contrary to GC-MS, e-nose technology is relatively low-cost, easily operated and it allows for point-of-care diagnosis.

Therefore, the objective of this study was to evaluate if exhaled VOCs can serve as a non-invasive biomarker for CRC and its precursor lesions using e-nose technology. In this study, breath analysis was performed with Aeonose devices (The eNose Company). This specific type of e-nose allows for datasets of multiple devices to be combined which facilitates the generation of large data sets to develop disease models. Furthermore, once-developed models can be transferred to new e-noses which will further aid in clinical implementation of e-nose technology.[36]

2 | MATERIALS AND METHODS

2.1 | Protocol and registration

The study protocol was approved by the ethics committee of Enschede, the Netherlands, and thereafter by the local ethics committees of the participating centres. This study was performed in compliance with the Declaration of Helsinki and registered at https://clinicaltrials.gov (Identifier NCT03488537). All study patients provided written informed consent before breath testing.

2.2 | Study design and population

This cross-sectional, proof-of-principle study was conducted in two secondary care hospitals in the Netherlands (Medisch Spectrum Twente, Enschede and Bernhoven, Uden). All adult patients referred for elective colonoscopy were invited for study participation. Patients with known inflammatory bowel disease (IBD) or (previous) malignancy were excluded (not including basal cell carcinoma or squamous cell carcinomas of the skin) from breath testing.

2.3 | Breath test

All members of the research team attended a training session for study instructions and e-nose training before the start of the study. Patients were invited for breath testing during a pre-colonoscopy consultation and/or on the day of the examination just before the colonoscopy procedure. Some patients performed two breath tests; one before and one after bowel preparation to evaluate the influence of bowel preparation on VOC profiles. Baseline characteristics were collected from all eligible patients by research nurses using standardised forms for patient interviews and by evaluation of their medical records.

Five e-noses were used for breath testing and all tests were performed in the same room at each hospital location (Figure 1). Patients were instructed to breathe into an e-nose for five consecutive minutes using a disposable mouthpiece while wearing a nose clip. Failed breath tests were excluded from analysis and the reason for exclusion was documented by the research nurse.

2.4 | Aeonose

In this study, breath analysis was performed with Aeonose devices (The eNose Company), which are portable, battery-powered devices, that contain three metal-oxide sensors (AMS AG) with

FIGURE 1 Electronic nose used in the study; the Aeonose (The eNose Company). Patients inhale through a carbon filter to prevent the entry of nonfiltered environmental air and breathe into the device through a disposable mouthpiece while wearing a nose clip

different material properties. VOCs present in exhaled breath cause redox-reactions at the sensor surfaces of the e-nose which results in conductivity changes that are measured and analysed. VOCs interact competitively with these sensors, depending on the physical and chemical characteristics of the sensor material. As the reaction kinetics are temperature-dependent, the metal-oxide sensors of the device are guided through a sinusoidal temperature profile to optimise the amount of information gathered from exhaled breath. Basically, in this way a virtual sensor array is built mimicking identical sensors at different temperatures.[37] During each cycle, conductivity is measured 32 times. In the end, all conductivity values are combined to represent the composition of the total VOC mixture, called a "breathprint", and each breathprint consists of approximately 7000 conductivity values.

Breath analysis with this e-nose takes 15 minutes in which the patient breathes into the device for 5 minutes. Inhaled air is filtered by two carbon filters to minimise contamination of the measurements by environmental VOCs and the mouthpiece has a High Efficiency Particulate Air filter (HEPA; 3M) that prevents contamination of the e-nose by bacteria or viruses.

2.5 | Endoscopic evaluation (reference test)

Patients received bowel preparation prior to colonoscopy. The bowel preparation prescribed was left to the discretion of the treating physician. Colonoscopy data and histopathology reports were evaluated for the presence and size of colorectal lesions. Patients were categorised into one of the following subgroups; controls (patients without any colonic neoplasia or other abnormality), hyperplastic polyps (HPs), non-NAs, AAs (polyps ≥10 mm, >25% villous pathology or high-grade dysplasia) or CRC. If multiple neoplastic lesions were present, classification was based on the most advanced lesion found. All diagnoses were double checked by two members of the research team.

Patients with an incomplete colonoscopy procedure, poor bowel preparation (Boston Bowel Preparation Score [BBPS] <6) or new diagnosis of colitis were excluded from analysis.

2.6 | Blinding

Patients, endoscopists and pathologists remained blinded for the outcome of the breath test throughout the study.

2.7 | Study outcomes

The primary outcome was the ability of the e-nose to distinguish patients with CRC from controls based on VOC patterns. Secondary outcomes included the ability of the e-nose to distinguish patients with precursor lesions (AAs, NAs, HPs) from CRC and controls. In addition, the effect of bowel preparation on VOC patterns was explored.

2.8 | Power calculation

The nature of the statistical method used in e-nose technology makes an exact sample size calculation impossible. Previous studies conducted with this specific e-nose technology have suggested that at least 25 cases and 25 control patients are required to build a disease-specific model.[38-47] For this study, we therefore aimed to include at least 60 patients with CRC; 40 CRC patients to develop a disease-specific model and 20 to validate the diagnostic accuracy of the algorithms.

2.9 | Statistical analysis

2.9.1 | Baseline characteristics

Data on demographic and baseline characteristics were summarised for continuous variables, in case of normal distribution by mean and standard deviation, and in case of nonnormal distribution by median and interquartile range. For each subgroup (eg HP, NA, AA, CRC), data were compared with control patients to detect significant differences in baseline characteristics using either the independent sample *t*-test (normal distribution) or Mann-Whitney *U* test

(nonnormal distribution) for continuous variables and a chi-squared test for categorical data. A two-sided $P < 0.05$ was considered statistically significant.

Data analysis of the baseline characteristics was performed by using the Statistical Program for the Social Sciences (SPSS) version 22.0.

2.9.2 | VOC analysis

Machine Learning was applied to distinguish between breath profiles of controls and patients with (pre-)cancerous lesions. During each breath measurement 64 × 36 × 3 data points were recorded. Strict temperature controls of the sensors limits variability between sensors of different devices.[36] Potential small differences are tackled by pre-treatment of the data including standardisation.[48] After pre-processing, data were compressed using a Tucker3-like solution which resulted in a single vector of limited size per participant.[49] Together with the disease diagnosis, the vector was used to train an artificial neural network. Using the data analysis package Aethena (The eNose Company) several permutations of data pre-processing, sensor combinations, vector lengths and network topologies were investigated to optimise results. More details on the data analysis have been published elsewhere.[48]

2.9.3 | Primary outcome

Breath sample analysis was performed in three phases for CRC versus controls. Only breath tests conducted before bowel preparation were used for these analyses.

- Phase 1: Training models: Development of disease-specific training models.

An important aspect of e-nose technology is that the artificial neural network used to analyse breath tests first needs to be trained to recognise disease-specific mixtures of VOCs. Two-thirds of the breath tests were used to develop "training models" which were validated in the remaining data (phase II). Breath samples were randomly assigned to either the "training" or "validation" phase. The artificial neural network was trained using the "leave-10%-out" cross validation technique to verify that training was successfully applied at the disease classification, and not on some artefact.

- Phase 2: External validation: Blind validation of the disease-specific training models.

After training, the artificial neural network-parameters were fixed and used to blindly predict patient diagnosis of the remaining patients to validate the diagnostic accuracy of the disease-specific training models.

- Phase 3: Final models: Development of final disease-specific models.

Another important aspect of e-nose technology is that the diagnostic accuracy of an artificial neural network generally improves when more data become available for training until a certain level of stability is reached. Therefore, after the validation phase, all data was made available to further train the artificial neural network using the "leave-10%-out" cross validation technique.

2.10 | Secondary outcomes

The three phases described above were also followed to develop algorithms to differentiate between AAs and controls. For all other secondary outcomes only final disease models were developed (phase III) because we only aimed to explore if VOC analysis could be used to differentiate between these subgroups. Again, only breath tests conducted before bowel preparation were used for model development except for the analyses in which the influence of bowel preparation was evaluated.

3 | RESULTS

3.1 | Baseline characteristics

Breath samples of 511 patients were collected over a study period of 10 months. Sixty-four patients were excluded due to a failed breath test (n = 51), incomplete colonoscopy (n = 8) or newly diagnosed colitis (n = 5). Of the 447 patients remaining patients 58.8% were male with a mean age of 65 years (standard deviation [SD] 9.0). Most patients included were referred for CRC screening through the Dutch population screening program (63.5%), followed by diagnostic colonoscopy (24.2%) and surveillance colonoscopy (12.3%). Adequate bowel preparation was achieved in all patients after 3 days of dietary restrictions and a split-dose of either 2L Moviprep or 4L Klean-prep. Seventy patients were diagnosed with CRC, 117 with one or more AAs, 117 with NAs, 15 with HPs and 128 had normal colonoscopy (Figure 2).

Baseline characteristics were categorised per outcome and are shown in Table 1. All groups showed a male sex predominance and controls were slightly younger ($P < 0.05$) than patients with neoplastic lesions.

3.2 | CRC versus controls

Breath tests of 42 CRC patients and 68 control patients were used to train an artificial neural network to recognise CRC specific VOC mixtures. This training model for CRC had an Area under the curve (AUC) of 0.76, with a sensitivity of 83% and specificity of 60% and this model was able to predict patient diagnosis of the blinded patients with similar results, AUC of 0.74. The final model for CRC had an AUC of 0.84, with a sensitivity of 95% and specificity of 64%.

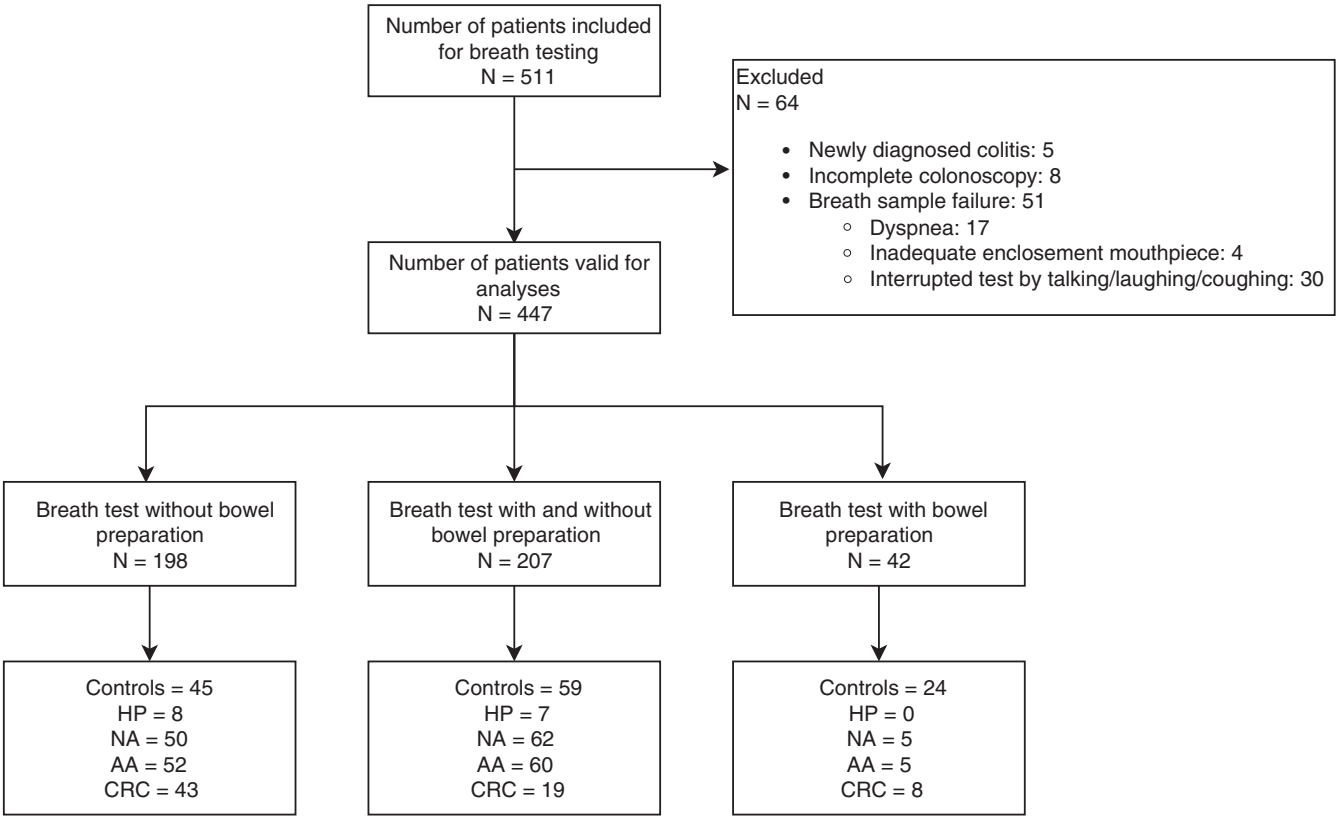


FIGURE 2 Flowchart. AA, advanced adenoma; CRC, colorectal cancer; HP, hyperplastic polyp; N, number; NA, non-advanced adenoma

(Table 2; Figure 3) Baseline characteristics of the development group and validation group did not differ significantly (data not shown).

3.3 | AAs versus controls

The training model for AAs had an AUC of 0.71 with a sensitivity of 82% and a specificity of 59%. Accuracy of this model was slightly lower in the validation group, AUC of 0.61. The final model for AAs versus control had an AUC of 0.73, with a sensitivity of 79% and a specificity of 59% (Table 2). Baseline characteristics of the development group and validation group did not differ significantly (data not shown).

3.3.1 | Correctly versus incorrectly predicted patients

Baseline characteristics such as age, gender, body mass index (BMI), co-morbidities, fasting meal and use of antibiotics or proton pomp inhibitors (PPI) were not significantly different between correctly and incorrectly predicted patients (Table 3). In addition, time of measurement did not significantly influence accuracy of the blind predictions. CRC predictions were slightly better in the first half (P = 0.38) while predictions of AAs were more accurate in the second half (P = 0.10). Control patients with a history of polypectomy (n = 10) were misclassified as AA in 20% (false positives, P = 0.32).

3.4 | Secondary outcomes

All final disease models for secondary outcomes (except for AAs versus controls) are shown in Table 4. The e-nose was able to differentiate between CRC and NAs with an AUC of 0.85, but was unable to differentiate between CRC and AAs. The e-nose was also able to distinguish patients with CRC and AAs from controls (AUC of 0.72), but VOC profiles of AAs and NAs were too similar. In addition, the e-nose was unable to distinguish patients with adenomatous polyps (NA + AA) from controls.

With regards to bowel preparation; 207 patients performed a breath test before and after bowel preparation. Four patients diagnosed with AAs failed their second breath tests. These patients were therefore excluded from this specific analysis. The e-nose was able to differentiate between all patients before and after bowel preparation, AUC of 0.72. Analysis of breath tests before and after bowel preparation within subgroups yielded an AUC of 0.82 for controls and 0.70 for AAs. Only 19 CRC patients performed a breath test before and after bowel preparation which was too little for analysis.

4 | DISCUSSION

This multicentre study suggests that breath analysis with e-nose technology could become a promising, non-invasive, diagnostic tool for the detection of CRC. The e-nose technology used in this study was able to differentiate between VOC profiles of patients with CRC

TABLE 1 Baseline characteristics

	Control	NA	AA	CRC
N (%)[a]	128 (28.6)	117 (26.2)	117 (26.2)	70 (15.7)
Male gender, n (%)	66 (51.6)	77 (65.8)[b]	70 (59.8)	43 (61.4)
Age, y, median (IQR)	62 (56-69)	66 (62-72)[b]	65 (60-70)[b]	68 (62-73)[b]
BMI, median kg/m^2 (IQR)	27.4 (24.4-30.1)	26.5 (24.1- 29.5)	27.1 (24.7- 29.6)	26.5 (23.7- 29.2)
Hypertension, n (%)	47 (36.7)	45 (38.5)	53 (45.2)	36 (51.4)[b]
Diabetes, n (%)	14 (10.9)	12 (10.3)	19 (16.2)	11 (15.7)
COPD/asthma, n (%)	19 (14.9)	23 (19.7)	11 (9.4)[b]	4 (5.7)
Abdominal surgery, n (%)	22 (17.2)	19 (16.2)	20 (17.1)	15 (21.4)
Smoking status[c], n (%)				
Current	20 (15.6)	23 (19.7)	31 (26.5)	8 (8.6)
Ex-smoker	58 (45.3)	55 (47.0)	51 (43.6)	33 (47.1)
Never smoker	50 (39.1)	39 (33.3)	35 (29.9)	31 (44.3)
Alcohol use in IE/week, median (IQR)	1 (0.0-7.0)	3 (0.0-14.0)[b]	4 (1.0-14.0)[b]	1 (0.0-13.0)
Use of antibiotics (≤3 mo before breath testing)	15 (11.7)	19 (16.2)	11 (9.4)	(8.6)
PPI use	32 (25.0)	26 (22.2)	33 (28.2)	19 (27.1)
Location most advanced lesion				
Proximal		40 (34.2)	29 (24.8)	18 (25.7)
Distal		44 (37.6)	75 (64.1)	52 (74.3)
Both		33 (28.2)	13 (11.1)	0 (0)
Cancer type				
Adenocarcinoma				67 (95.7)
Other				3 (4.3)
Stage CRC				
Stage I				22 (31.4)
Stage II				22 (31.4)
Stage III				23 (32.9)
Stage IV				3 (4.3)
Cancers and advanced AA				
CRC without AA				53 (75.7)
CRC with proximal AA				7 (10.0)
CRC with distal AA				10 (14.3)
Type of AA				
≥10 mm			68 (58.1)	8 (47.1)
≥25% villous pathology			11 (9.4)	6 (35.3)
HGD			0 (0)	1 (5.9)
≥10 + HGD			3 (2.6)	0 (0)
≥10 + ≥ 25% villous			31 (26.5)	1 (5.9)
≥10 + ≥ 25% villous + HGD			4 (3.4)	1 (5.9)
Type of NA				
1-5mm		140	126	36
6-9mm		100	87	27
Number of HP		28	39	8

Abbreviations: AA, advanced adenoma; CRC, colorectal cancer; IQR, interquartile range; NA, non-advanced adenoma.

[a]Patients with only hyperplastic polyps are not shown due to small patient numbers (n = 15).

[b]Significance $P < 0.05$, subgroups were compared with controls to determine significance.

[c]Ex-smokers were defined as those who had not smoked for at least 6 months before study commencement.

TABLE 2 Colorectal cancer and Advanced Adenomas versus Controls

Analysis[a]	Cases	Controls	AUC	Sens (%)	Spec (%)	PPV (%)	NPV (%)
CRC vs. controls							
Training model	42	68	0.76	83	60	56	85
Blind predictions (validation)	20	36	0.74	80	64	41	85
Final model with all data	62	104	0.84	95	64	61	96
AA vs. controls							
Training model	74	68	0.71	82	59	69	75
Blind predictions (validation)	38	36	0.61	67	45	69	59
Final model with all data	112	104	0.73	79	59	67	73

Abbreviations: AA, Advanced adenomas; AUC, area under the curve; CRC, colorectal cancer; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; vs, versus.

[a]These analysis only included breath tests performed before the bowel preparation.

and patients with normal colonoscopy with an AUC of 0.84 which indicates that exhaled VOC profiles differ between these patients and that these differences can be captured with an e-nose. Furthermore, the results of this study also suggest that patients with AA have a distinct VOC profile.

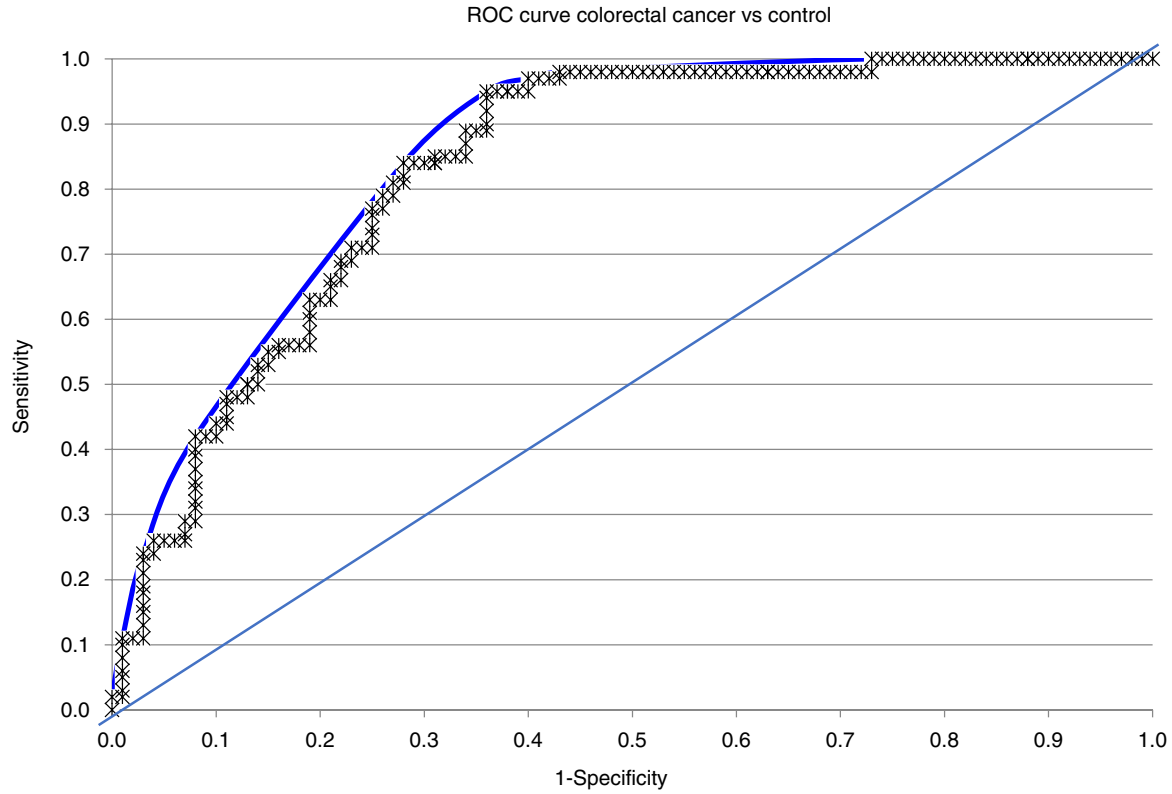
In the last decade, multiple proof-of-principle studies have demonstrated the efficacy of utilising VOC profiles for clinical diagnostics. VOC analysis has not only been used successfully for the detection of several types of cancers such as lung,[18,19] breast,[20,50] prostate,[51,52] gastric and oesophageal cancer,[53,54] but also for the detection of various endoluminal gastrointestinal diseases like IBD,[55,56] *Helicobacter pylori* in the stomach[57] and coeliac disease.[58] Furthermore, some studies have also suggested that VOC analysis can be used to differentiate between various diseases.[18,23,34,39,59] Although the findings of these studies still need to be validated in larger studies, the results seem to support the hypothesis that pathological processes in the body have the potential to influence VOC profiles and that each disease has its own specific VOC signature.[18-20,23,34,39,50-59]

The exact mechanism behind the generation of disease-specific VOCs is complex. With regard to CRC-related VOCs it is thought that uncontrolled cell growth at the tumour site and systemic responses associated with carcinogenesis (ie increased catabolism, increased oxidative stress and immune activation) contribute to the observed changes in VOC profiles.[60,61] In addition, there is also growing evidence linking the gut microbiome to CRC development through interactions with the host's immune system, production of cancer-associated metabolites and release of genotoxic virulence factors.[62] It is therefore plausible that changes in VOC profiles of CRC patients are, at least in part, the result of changes in the gut microbiome. Furthermore, concomitant disease, diet-, lifestyle- and medication-related factors also have the potential to influence VOC profiles, which is why it will take some time before the mechanisms behind the generation of CRC-associated VOCs will be clarified.[63-66]

Meanwhile, the evidence of potential VOCs that might serve as biomarkers for CRC is accumulating. Several proof-of-principle studies have already reported significant differences in VOCs between CRC patients and controls using various biological specimens for VOC analysis ie breath,[21-24] faeces,[25-30] urine[31-35] and blood.[22] These pilot studies reported an AUC for CRC ranging between 0.67 and 0.98, with a sensitivity and specificity ranging between 63%-100% and 58%-94% respectively, similar to our study. Unfortunately, it is not possible to compare the results of these studies because some studies used a chemical analytical technique with the aim to detect the occurrence, identity and changes in quantities of VOCs, while other studies used sensor arrays with pattern recognition technologies with the aim to detect collective differences in the chemical composition of all VOCs. Furthermore, the lack of standardised methods for all phases of VOC analysis further complicates comparison of study data even among studies that used the same technique for VOC analysis. Nevertheless, the results of these pilot studies suggest that CRC has a specific VOC signature which can be detected in various biological specimens.[21-29,31-35,67]

Hitherto, five studies have evaluated the potential of exhaled VOCs to serve as a non-invasive biomarker for CRC.[21-24,68] GC-MS was used in two studies,[21,22] one study used an e-nose[68] and two studies evaluated both techniques[23,24] for VOC analysis. All four GC-MS studies were able to identify a pattern of discriminatory VOCs, consisting of 4-15 VOCs. No single discriminatory VOC could be identified which likely reflects the complexity of the metabolic derangements and systemic responses associated with CRC.[21-24,68] Interestingly, the VOCs identified differed among studies, which may be explained by a combination of differences in patient characteristics, breath sampling methods, storage conditions, GC-MS devices and statistical methods used among these studies. Studies that evaluated the use of a cross-reactive nanosensor reported an AUC of 0.38-0.91 for CRC.[23,24,68] Only Altomore and colleagues[68] were unable to detect a significant differences between the exhaled VOC profiles of patients with CRC and controls, AUC of 0.38. Sensors used in their PEN3 e-nose may have been less sensitive to CRC-associated VOCs than sensors used in the other two studies, and our study.[23,24]

Our study also evaluated the ability of VOCs to serve as biomarkers for precursor lesions of CRC, since cost-efficacy of screening



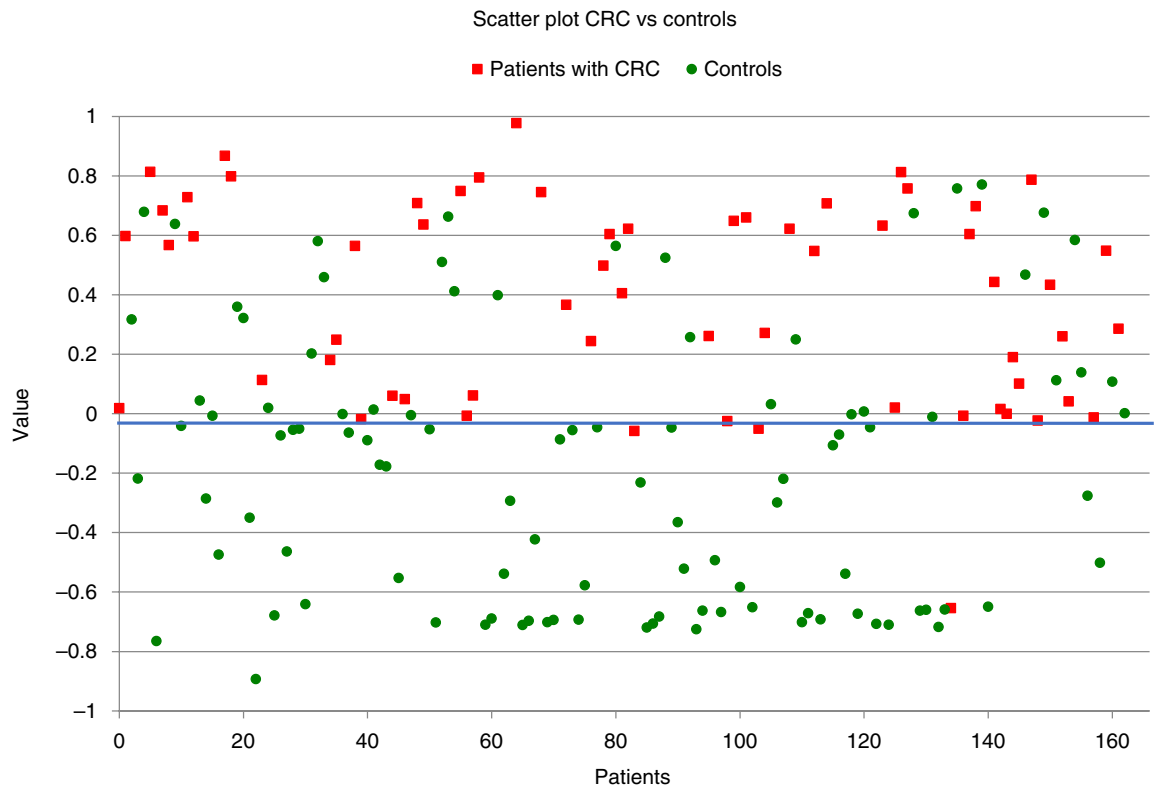


FIGURE 3 ROC curve plus scatter plot of the final disease-specific model for colorectal cancer (CRC). In the scatter plot the individual e-nose value of each patient and control is plotted. Patients with histopathologically confirmed CRC are represented with a red square and all healthy controls are represented with a green dot. The blue line at −0.03 indicates the cut-off point used in this study. All patients with a value greater than −0.03 were indicated as positive for CRC by the e-nose

TABLE 3 Baseline characteristics blind validation group

	CRC versus controls			AA versus controls		
	Correctly predicted (n = 39)	Incorrectly predicted (n = 17)	*P*-value	Correctly predicted (n = 41)	Incorrectly predicted (n = 33)	*P*-value
Male	23 (59.0)	11 (64.7)	0.69	23 (56.1%)	22 (66.7%)	0.36
Age, median, IQR	64 (59-73)	65 (59-71)	0.93	63 (58-71)	65 (59-70)	0.73
BMI, median, IQR	26.7 (23.5-29.1)	27.5 (24.6-31.0)	0.40	27.3 (24.3-28.9)	26.2 (23.5-30.4)	0.72
Hypertension	21 (53.8)	10 (58.8)	0.73	17 (41.5))	17 (51.5)	0.39
Diabetes	4 (10.3)	2 (11.7)	0.87	7 (17.1)	4 (12.1)	0.55
COPD/Asthma	5 (12.8)	0 (0)	0.12	4 (9.8)	2 (6.1)	0.56
Cardiac failure (NYHA≥3)	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A
Abdominal surgery[a]	10 (25.6)	6 (35.3)	0.46	12 (29.3)	8 (24.2)	0.58
Current smoker	5 (12.8)	3 (17.6)	0.64	12 (29.3)	7 (21.2)	0.43
Pack years, median, IQR	3.2 (0-14.0)	7.5 (0.1-14.7)	0.33	10 (0-25.5)	9 (7.8-17.3)	0.25
Alcohol use, median, IQR	1 (0-6)	1 (0-7)	0.83	2 (0-6)	4 (0-14)	0.23
Use of antibiotics[b]	2 (5.1)	2 (11.8)	0.38	3 (7.3)	2 (6.1)	0.83
PPI use	13 (33.3)	5 (39.4)	0.77	16 (39.0)	10 (30.3)	0.44
Previous polypectomy	4 (10.3)	2 (11.8)	0.87	7 (17.1)	3 (9.1)	0.32
Last meal (<3 h)	21 (54.8)	11 (64.7)	0.45	27 (65.9)	21 (63.6)	0.84
Time of measurement	(n = 28)	(n = 28)		(n = 37)	(n = 37)	
First half	21 (75.0)	7 (25.0)	0.38	17 (45.9)	20 (54.1)	0.10
Second half	18 (64.3)	10 (58.8)		24 (64.9)	13 (35.1)	
CRC stage						
Stage I-II	8	4	0.07			
Stage II-III	8	0				

Abbreviations: AA, advanced adenomas; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRC, colorectal cancer; IQR, interquartile range; n, number; NYHA, New York Hart association classification; PPI, Proton pump inhibitor.

[a]Abdominal surgery does not include (partial) colon resection as those patients were excluded in our study.

[b]Use of antibiotics within 3 mo prior to study inclusion.

programs for CRC could significantly improve if a non-invasive test would be able to accurately detect these lesions to prevent the development of CRC. The e-nose used in this study was able to detect AAs with an AUC of 0.73, which is in line with the study of de Meij et al,[29] who reported an AUC of 0.79 for the detection of AAs using faecal samples. Three other studies were unable to differentiate between patients with AAs and controls using faecal[25] and urinary samples[32,33] for VOC analysis. Contrary to our results, de Meij et al[29] were also able to differentiate between CRC and AA with their e-nose (Cyranose 320), which may be explained by differences in biological specimens used, sensor selection, statistical methods and pathological tumour stages. Furthermore, we were able to distinguish CRCs from NAs, but it was not possible to differentiate AAs from NAs. We therefore hypothesise that VOCs associated with AAs with villous features and/or high-grade dysplasia may be more similar to CRC-associated VOCs while AAs without these features may be more similar to NAs. Nevertheless, this hypothesis still needs to be investigated in a larger study.

Co-morbidities, lifestyle factors, such as smoking, BMI and use of antibiotics and/or PPIs were equally matched between patient groups and did not seem to influence accuracy of the blind predictions. In addition, control patients with a history of polypectomy were misclassified as AAs in 20%, but larger studies should further evaluate the influence of polypectomy on VOC mixtures. The influence of bowel preparation was also addressed in this study, because bowel preparation could potentially alter the gut microbiome and thereby influence VOC profiles. Leja et al[69] already evaluated the influence of bowel preparation on VOC profiles and only reported a significant increase in acetone levels which may have been a consequence of fasting. We were able to detect a collective difference in VOC profiles of all patients before and after bowel preparation, but only with a moderate accuracy of 0.72. In the control group, bowel preparation was detected with the highest accuracy, AUC of 0.82. However, e-nose technology does not allow for the identification of specific VOCs which is why we can only speculate that these differences reflect dietary restrictions and/or use of laxatives. Nonetheless, it is reassuring that fasting did not seem to influence the accuracy of the disease models (P = 0.45 for CRC and P = 0.84 for AA-model) since this will make the implementation of breath analysis in clinical practice easier.

This study has several strengths. All consecutive adult colonoscopy patients were invited for study participation in two different

TABLE 4 Secondary outcomes

Analysis[a]	Cases (n)	Control (n)	AUC	Sens (%)	Spec (%)	PPV (%)	NPV (%)
CRC vs NA	62	112	0.85	90	65	59	92
CRC vs NA + HP	62	127	0.91	98	52	50	98
CRC vs AA	62	112	No model	No model	No model	No model	No model
CRC vs AA + NA + HP	62	239	No model	No model	No model	No model	No model
CRC vs AA + NA + HP + Control	62	343	No model	No model	No model	No model	No model
CRC + AA vs Control	174	104	0.72	83	54	75	66
CRC + AA vs NA + HP + Control	174	343	No model	No model	No model	No model	No model
AA vs NA	112	112	No model	No model	No model	No model	No model
NA vs Control	112	104	0.72	67	64	67	64
AA + NA vs Control	224	104	No model	No model	No model	No model	No model
Bowel preparation[b]							
Control before vs after bowel prep	59	59	0.82	77	70	0.72	0.76
AA before vs after bowel prep	56[c]	56[c]	0.70	80	59	68	73
CRC before vs after bowel prep	19	19	No model	No model	No model	No model	No model
All patients with two breath tests before and after bowel prep	203[c]	203[c]	0.72	65	74	71	68
All CRC breath tests versus all Control breath tests[d]	89	187	0.76	88	71	59	92

Abbreviations: AA, advanced adenomas; AUC, area under the curve; C, controls; CRC, colorectal cancer; n, number; NA, non-advanced adenomas; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; vs, versus.

[a]This analysis only included breath tests performed before the bowel preparation.

[b]This analysis included all patients with an adequate breath test performed before and after bowel preparation.

[c]Four patients with advanced adenomas did not have an adequate before and after breath test.

[d]This analysis included all of the breath tests of CRC patients and Controls. This means that if CRC patients or controls performed two breath tests then both tests were included (see flowchart).

hospitals to improve generalisability of the results. All diagnoses were double checked to avoid misclassification and the results were blindly validated in new study patients, which yielded similar results suggesting potential reproducibility of the results. In addition, we were able to exchange disease models between e-noses of the same brand which will render extensive training of individual e-noses unnecessary which can accelerate implementation of e-nose technology in clinical practice.

Some limitations of our study should also be indicated. Most importantly, the number of failed breath tests, almost 10%, raises concern regarding the feasibility of the breath sampling technique used. However, our breathing protocol may have been too strict since other studies using the same e-nose did not report such high failure rates,[38-47,70-72] neither in children with severe asthma or cystic fibrosis (<2%),[72] nor in adult patients with acute exacerbation of chronic obstructive pulmonary disease (0%).[71] In this study, two-thirds of the failed tests were suboptimal tests; ie interrupted tests by talking, laughing, coughing or inappropriate enclosement of the mouthpiece. In clinical practice these suboptimal tests can be repeated immediately (this was not allowed in our study) and additional training of research nurses may further reduce the number of failed tests. Nonetheless, the results of this study do underline the importance of an objective tool to evaluate the quality of breath samples before clinical implementation of breath analysis is considered. Another limitation of our study was the use of an imperfect reference test, up to 25% of all polyps are still missed with conventional colonoscopy, which may have led to misclassification of patients limiting the discriminatory power of the developed algorithms.[73] In addition, this study did not collect information on medication use, but only evaluated the presence of co-morbidities between patient groups. Other study limitations are inherent to e-nose technology such as potential reproducibility issues, sensor drift, instrument variability and loss of sensitivity in the presence of alcohol which need to be addressed in further studies.[74] Nevertheless, in this study five different e-noses were used and accuracy of the training models did not change over time suggesting that inter-device variability and sensor drift were minimal. In addition, sensors used in our e-nose have a proven track record in terms of stability and lifetime in other sectors (ie automotive).[75]

Future studies in the field of VOC analysis should focus on standardising all phases of VOC analysis among different gas analysis techniques to obtain a translatable approach for future VOC research; ie standardisation of gas analysis instruments, sampling protocols, statistical methods, etc. Large scale studies are required to evaluate the influence of covariates on VOC profiles (eg co-morbidities, medication use, lifestyle factors) and to

explore the full diagnostic potential of VOC analysis, preferably with external validation. Eventually, the use of different biological samples and techniques for VOC analysis should be compared to evaluate feasibility, reliability and cost-effectiveness of the different methods of VOC analysis. In this respect, breath analysis with e-nose technology might be preferred over other biological specimens and use of chemical analytical techniques because e-nose devices are relatively low-cost, easily operated and have the potential for a point-of-care diagnosis which makes them more suitable for clinical practice. In addition, breath tests may have high acceptability among patients improving adherence to CRC screening programs.

In conclusion, this multicentre study suggests that breath analysis using e-nose technology has the potential to serve as an easy-to-use, non-invasive diagnostic tool for the detection of CRC and its precursor lesions.

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