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A novel non-invasive colorectal cancer diagnostic method: Volatile organic compounds as biomarkers

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ABSTRACT

Introduction: Population-based fecal tests for colorectal cancer (CRC) screening have shown to reduce mortality thanks to the early detection of the disease. However, currently available fecal tests are limited in their sensitivity and specificity. Our aim is to look for volatile organic compounds in fecal samples as biomarkers for CRC detection.

Material and Methods: Eighty participants were included; 24 had adenocarcinoma, 24 had adenomatous polyps and 32 presented no neoplasms. Fecal samples were collected 48 h preceding the colonoscopy from all participants, except CRC patient samples that were collected after 3–4 weeks from the colonoscopy. Magnetic headspace adsorptive extraction (Mag-HSAE) followed by thermal desorption-gas chromatography-mass spectrometry (TD-GC–MS) was performed on stool samples to identify volatile organic compounds as biomarkers.

Results: p-Cresol was significantly more abundant in the cancer samples ($P < 0.001$) with an area under the curve (AUC) of 0.85 (CI 95%; 0.737–0.953), having a sensitivity and specificity of 83% and 82%, respectively. In addition, 3(4H)-dibenzofuranone,4a,9b-dihydro-8,9b-dimethyl- (3(4H)-DBZ) was also more abundant in the cancer samples ($P < 0.001$) with an AUC of 0.77 (CI 95%; 0.635–0.905), sensitivity of 78% and specificity of 75%. When combined (p-cresol and 3(4H)-DBZ), the AUC was 0.86, sensitivity 87% and specificity 79%. p-Cresol also appeared to be promising as a biomarker for pre-malignant lesions with an AUC of 0.69 (CI 95%; 0.534–0.862), sensitivity 83% and specificity 63%, $P = 0.045$.

Conclusions: Volatile organic compounds emitted from feces and determined by a sensitive analytical methodology (Mag-HSAE-TD-GC–MS), employing a magnetic graphene oxide as extractant phase, could be used as a potential screening technology for CRC and pre-malignant lesions.

1. Introduction

Colorectal cancer (CRC) is a leading cause of mortality and morbidity worldwide, expected to cause 2.2 million new cases and 1.1 million deaths by 2030 [1]. Early identification of CRC and detection and removal of advanced pre-malignant adenomas have been found to decrease CRC incidence and mortality [2,3]. Colonoscopy is the gold standard for the diagnosis of CRC, and fecal immunochemical test (FIT) is the most widely used non-invasive screening tool. Although FIT-based screening has led to a decrease in mortality, its performance is suboptimal, with a fairly good specificity but a high variation in sensitivity leading to misdiagnosis of CRC and unnecessary colonoscopy performance, using an important amount of resources [4]. Due to these limitations, there is a major need to develop new non-invasive and sensitive

Abbreviations: CRC, colorectal cancer; Mag-HSAE, Magnetic headspace adsorptive extraction; TD, thermal desorption; GC–MS, gas chromatography-mass spectrometry; AUC, area under the curve; 3(4H)-DBZ, 3(4H)-dibenzofuranone,4a,9b-dihydro-8,9b-dimethyl-; FIT, fecal immunochemical test; VOCs, volatile organic compounds; SPME, solid-phase microextraction; MGO, magnetic graphene oxide; HGUA, Hospital General Universitario Doctor Balmis de Alicante; IBD, inflammatory bowel disease; ISABIAL, Instituto de Investigación Sanitaria y Biomédica de Alicante; SIM, selected ion monitoring; ROC, receiver operator characteristic; BMI, body mass index; SD, standard deviation; AA, advanced adenomas; SIFT-MS, selective-ion flow-tube mass spectrometry; FOBT, fecal occult blood test.

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tools for early CRC diagnosis.

Odor-related compounds are volatile organic compounds (VOCs), associated with metabolic changes produced during processes such as cancer, necrosis or inflammation [5–7]. Therefore, VOCs are currently determined in different biological samples (i.e., urine, breath, feces) to study their correlation with certain diseases [7]. CRC is a disease in which the metabolites produced in the gut differ from those of a healthy patient probably due to bacterial dysbiosis [8–10]. Accordingly, determination of the VOC profile in fecal samples could be used as a diagnostic and preventive tool for CRC [11–14]. Solid-phase microextraction (SPME) is a rapid, solventless and environmentally-friendly extraction technique for the isolation of compounds from liquid, solid or gaseous matrices. In this study the role of a modality of SPME called Magnetic Headspace Adsorptive Extraction (Mag-HSAE) coupled to thermal desorption-gas chromatography-mass spectrometry (TD-GC–MS) [15] was investigated for fecal analysis for the first time. One of the advantages of this technique is that magnetic graphene oxide (MGO) is employed for solid-phase extraction because of its relatively low production cost, easy manipulation, simple synthesis procedure, wide accessibility, and high extraction capacity of aromatic compounds [16].

Until now, few studies have been carried out searching for the potential of fecal VOC analysis for CRC detection with promising results [11–14]. There is thus a necessity to develop sensitive methodologies for VOC determination in fecal samples in order to make them useful for CRC diagnosis and prevention.

The aim of this pilot study is to determine specific fecal VOCs in CRC and adenoma patients as biomarkers employing the Mag-HSAE-TD-GC–MS methodology [17].

2. Material and methods

2.1. Participants

This cross-sectional study recruited 24 newly diagnosed CRC patients (with different staging I-IV); 24 patients with adenomatous polyps; and, 32 individuals with normal colonoscopy, between July 2017 and July 2020 at the Hospital General Universitario Doctor Balmis de Alicante (HGUA). All of them participated in the CRC screening program of the Valencian Community and colonoscopies were carried out following a positive FIT test. Exclusion criteria were a previous history of CRC, inherited CRC syndromes, prior intestinal resection, and inflammatory bowel disease (IBD).

Research Ethics Committee approval for the study was obtained from the "Grupo de Evaluación de la Comisión de Investigación del Instituto de Investigación Sanitaria y Biomédica de Alicante" (ISABIAL) (Ref. CEIC PI2017/25). All patients were supplied with an information sheet and provided written consent. Specific permission was also granted by the Conselleria de Sanitat Research Committee.

2.2. Sample collection and storage

For individuals with normal colonoscopy and patients with colonic adenomas, samples were collected, at home, before commencing the required bowel preparation and during the 48 h preceding their colonoscopy. For patients with CRC, samples were requested at least 3–4 weeks after diagnosis and before surgical treatment. The stool was initially collected in a recipient, and then participants had to transfer three to four spoonfuls of feces to a plastic container before it was sealed and stored in the fridge. The initial volume of stool supplied by the patient was not specified but could not exceed the volume of the provided 100 mL plastic container. The sample was delivered at an office next to the Endoscopy Department at the HGUA. During the transportation from the patient's home to the hospital, the sample was kept at room temperature. Only patients with CRC, adenomatous polyps and healthy individuals were included in the study.

2.3. Reagents

Iron oxide II, III (Fe_3O_4) (50–100 nm) was obtained from Sigma-Aldrich (Madrid, Spain) and GO from Applynano Solutions S.L. (Alicante, Spain). HNO_3 65% from Merck (Darmstadt, Germany) and NaOH pellets from Scharlau (Barcelona, Spain) were employed for the nanocomposite synthesis.

Deionized water (resistivity of 18.2 MΩ cm at 25 °C) was prepared on a water purification system (GradientA10) supplied by Millipore (Billerica, MA, USA).

2.4. Instruments and apparatus

The Gerstel TDS-2 Thermal Desorption System fitted with a Gerstel CIS-4 Cooled Injection System with a programmable temperature vaporization (PTV) inlet, from Gerstel (Mülheim an der Ruhr, Germany) was used to carry out the thermal desorption process. It was installed in an Agilent 6890 N gas chromatograph coupled to an Agilent 5973 mass spectrometer, both from Agilent Technologies (Palo Alto, CA, USA), which were employed for the analytical measurements. A Gerstel thermal desorption glass tube (187 mm length, 4 mm I.D., 6 mm O.D.) and glass wool from Panreac (Barcelona, Spain) were used to construct the thermal desorption device (Fig. 1), which enabled the desorption of the compounds from the magnetic nanomaterial, while preventing them from entering the GC system.

For the headspace extraction unit, NdFeB rod magnets (Nd) (N48, Ø 3 mm, height 8 mm) with a nickel-plated (Ni-Cu-Ni) coating from Supermagnete Co. (Gottmadingen, Germany), 5 mL plastic vials from Supelco (Bellefonte, PA, USA), and a magnetic stirrer plate with professional heating from VWR (Barcelona, Spain) were used (Fig. 1).

An Elma Schmidbauer GmbH (Singen, Germany) ultrasound bath, a laboratory vacuum oven-450 Watt de Goldbrunn Therm (Poland, UE) and a pH meter from Tecnylab (Valencia, Spain) were used for the nanocomposite synthesis.

2.5. Synthesis of magnetic graphene oxide

The synthesis of the GO/Fe_3O_4 nanocomposite is based on work by Costa dos Reis et al. [16]. 250 mg of Fe_3O_4 nanoparticles were dissolved in 1.25 mL of an aqueous-acidic solution (HNO_3 1 M) in an ultrasonic bath for 30 min obtaining a Fe_3O_4 dispersion with a positively charged surface. 50 mg of GO was dispersed in deionized water using an ultrasonic bath for 60 min, obtaining a GO dispersion (1 mg mL^{-1}) with a negatively charged surface. The Fe_3O_4 and GO dispersions were mixed, and the pH was adjusted to 2. The mixture (GO/Fe_3O_4 ratio 1:5) was continuously magnetically stirred for 5 h. Accordingly, the GO/Fe_3O_4 nanocomposite was separated from the liquid employing an external magnetic field (neodymium magnet). Ultimately, the nanocomposite was vacuum-dried in an oven at 60 °C for 24 h [16].

2.6. TD-GC–MS conditions

The TD system was programmed in splitless mode at an initial desorption temperature from 25 °C (0.5 min) to 240 °C at 60 °C min^{-1}; desorption time, 5 min; helium flow rate, 100 mL min^{-1}; transfer line temperature, 300 °C. The desorbed compounds were cryo-focused in the cooled injection system at 0 °C. Then, the temperature was then rapidly increased at 12 °C s^{-1} up to 250 °C, transferring the compounds to the GC column by operating in solvent vent mode for 1 min. A DB-624 (6% cyanopropylphenyl-94% dimethylpolysiloxane, 30 m, 0.25 mm I.D., 1.4 mm film thickness) analytical column from Agilent Technologies was used with the following oven temperature program: from 50 °C (1 min) at 20 °C min^{-1} to 250 °C (30 min).

The MS detector voltage was set at 1700 V. Electron impact ionization was used at 70 eV ionization energy. The mass source and quadrupole were set at 230 and 150 °C, respectively. Measurements were

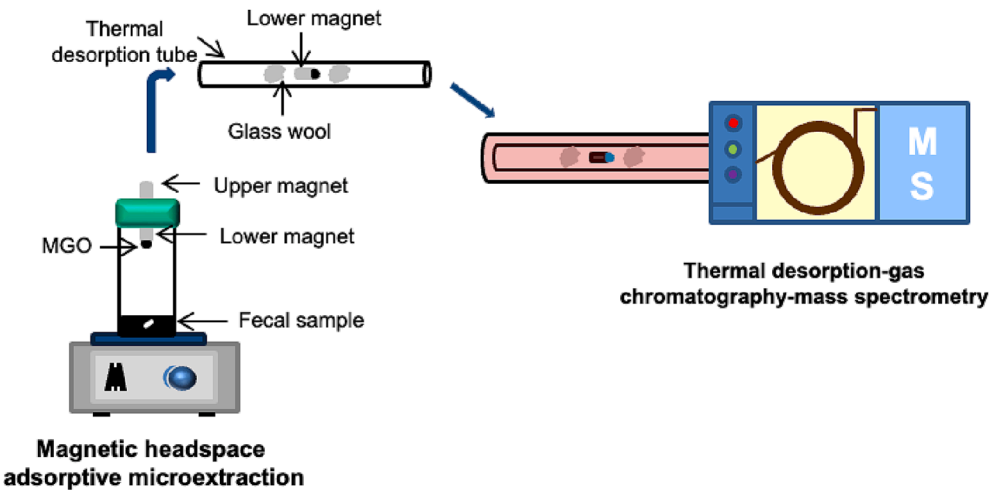

Fig. 1. Developed system for extraction and determination of VOCs extracted from fecal samples [17].

carried out first in SCAN mode, followed by selected ion monitoring (SIM) mode at the following mass/charge ratios (*m/z*): 77 and 107 from minute 6 to 8 for the determination of p-cresol; 89, 90 and 117 from minute 9 to 10 for the determination 1H-indole; and 171, 199 and 214 from minute 12 to 14 for the determination of 3(4H)-DBZ.

2.7. Magnetic headspace adsorptive extraction

450–500 mg of feces were placed in a 5 mL vial. 4.3 mg of MGO was magnetically attached inside the lid of the vial to a neodymium magnet held with the help of another external neodymium magnet (Fig. 1). The vial was then immersed in a water bath at 60 °C, and the MGO was exposed in the vial headspace above the feces for 30 min under static conditions. Subsequently, the sample was stirred for 20 min at the same temperature at 1500 rpm to collect the VOCs released from feces. Finally, VOC-enriched MGO was introduced in the glass tube for TD-GC–MS analysis (Fig. 1).

2.8. Data processing

The GC–MS data were processed using the MSD ChemStation software from Agilent technologies. The WILEY.275L mass spectral library was used to deconvolute chromatograms and identify metabolites. The peak area of each VOC was the response used.

2.9. Statistical analysis

Data analysis was performed by using Statistical Package for the Social Sciences for Windows, SPSS version 19.0 (SPSS® Statistical software, an IBM Company, Chicago, IL, USA) to evaluate biomarker signatures discriminating clinical cases (CRC and adenomas) from their healthy respective controls. Student's *t* test, Mann-Whitey tests, Fisher's exact test, ANOVA with Bonferroni correction and Receiver Operator Characteristic (ROC) analysis together with logistic regression modeling were used to test potential biomarkers. The VOC peak areas were logarithmically transformed and compared by ANOVA.

3. Results

3.1. Descriptive characteristics of patients with colorectal cancer, adenomas and controls

The descriptive data of case-control individuals are summarized in Table 1. A total of 15 (62.5%) men and 9 (37.5%) women in the CRC group, 15 (62.5%) men and 9 (37.5%) women in the adenomatous polyp group and 9 (28%) men and 23 (72%) women in the control group were included. The mean age was lower in those without neoplasia and higher in those with cancer, 60 years and 68 years respectively (P = 0.024). Significant body mass index (BMI) differences were found among the adenomas and control group (adenomas 29 Kg/m^2 vs. controls 26 Kg/m^2, P = 0.019).

Table 1
Descriptive characteristics of patients with colorectal cancer, adenomas and controls.

	CRC (n = 24)	Adenomas (n = 24)	Controls (n = 32)	P-value
Age, mean ± SD (yr)	68 ± 11	61 ± 6	60 ± 12	a) 0.017[a] b) 0.024[a] c) 0.722
Sex, % (n)				
Men	62.5% (15)	62.5% (15)		
Women	37.5% (9)	37.5% (9)	28% (9) 72% (23)	a) 1.0 b) 0.015[a] c) 0.015[a]
BMI[b], mean ± SD (kg/m^2)	28 ± 6	29 ± 4	26 ± 4	a) 0.501 b) 0.282 c) 0.019[a]

a) Association between CRC and adenomas; b) association between CRC and control; c) association between adenomas and control group.
[a] Results are statistically significant different.
[b] BMI: body mass index.

3.2. Characteristics of the lesions found in patients with CRC and adenomas

The characteristics of the patients in the CRC group were compared against those of patients with adenomas (Table 2). Significant differences were found between the size of the lesions in the CRC group compared to the group of patients with adenomas, being 42 mm and 9 mm, respectively (P < 0.001). On the other hand, no differences were found in the location of the lesions; most of them being found in the distal colon, namely 52% of the CRC, and 61% of the adenomas (P =

Table 2
Comparison of the lesions found in both groups of patients.

	CRC (n = 24)	Adenomas (n = 24)	P-value
Mean size ± SD, mm	42 ± 16	9 ± 3	< 0.001
Location, % (n)			0.592
Proximal	47.8% (11)		
Distal	52.2% (12)	38.9% (14)	
		61.1% (22)	

0.592).

Table 3 shows a summary of the descriptive characteristics of both groups. 8.3% of the cancers belong to stage I, 20.8% belong to stage II, while stage III cancers represent the most numerous with almost 42%, and 25% are stage IV cancers. High-grade cancers represented 17%, mucinous cancers were present in 14.3% of cases, and a serrated subtype in 4.8%. We found a median of 2 (1.00–3.00) adenomas per patient. All the adenomas in the study had low-grade dysplasia, 14.3% were tubule villous and the remaining 85.7% were tubular adenomas.

3.3. Volatile organic compounds detected in fecal samples

From 60 VOCs identified in each analysis, only 3 VOCs showed different peak areas between the CRC and control groups: p-cresol, 1H-indole and 3(4H)-DBZ. The identified retention time values (±standard deviation, SD) were: 7.92 min (±0.08) for p-cresol, 9.84 min (±0.06) for 1H-indole, and 13.77 min (±0.13) for 3(4H)-DBZ (Table 4). Peak differences between CRC and controls can be seen in the chromatogram shown in Fig. 2.

3.4. Volatile organic compounds as biomarkers for colonic adenocarcinoma

Initially, the peak area of the three VOC compounds in the three study groups were analyzed. The median peak area values of p-cresol in the CRC group were 4×10^9, 1×10^9 in adenoma and 7×10^8 in controls (Fig. 3). The data were logarithmically transformed and compared using ANOVA: the differences were statistically significant ($P < 0.001$); a post hoc analysis using the Bonferroni test showed that the greatest difference is observed between samples from patients with CRC and healthy controls ($P < 0.001$).

Table 3
Descriptive characteristics of the study participants: patients with CRC and patients with adenomas.

Characteristics of CRC	
Location of CRC, % (n)	
Proximal	47.8% (11)
Distal	52.2% (12)
Stage, % (n)	
I	8.3% (2)
II	20.8% (5)
III	41.7% (10)
IV	25% (6)
Differentiation degree, % (n)	
High grade	17% (4)
Low grade	83% (19)
Special subtypes, % (n)	
Mucinous component	14.3% (3)
Serrated	4.8% (1)
Characteristics of the adenomas	
Size, % (number of adenomas)	
≥ 1 cm	50% (12)
< 1 cm	50% (12)
Median of adenomas/patient[a]	2 (1.00–3.00)
Location of adenomas, % (n)	
Proximal colon	38.9% (14)
Distal colon	61.1% (22)
Degree of displasia, % (n)	
High grade	0% (0)
Low grade	100% (24)
Villious component, % (n)	
Yes	14.3% (3)
No	85.7% (18)

[a] The medians of adenomas/patient are presented as median (P25-P75).

Table 4
Description of the retention time values and *m/z* ratios of the studied VOCs.

Average GC–MS retention time value + SD (min)	m/z	Compound
7.92 min ± 0.08	*77, 107*	*p-cresol*
9.84 min ± 0.06	*89, 90, 117*	*1H-indole*
13.77 min ± 0.13	*171, 199, 214*	*3(4H)-DBZ*

The peak area of 3(4H)-DBZ, was subjected to the same analysis. The median peak area in the CRC group was 4×10^7, 1×10^7 in adenoma and 1×10^7 in controls (Fig. 3). The data were logarithmically transformed and compared using ANOVA: the differences were significant ($P = 0.001$); a post hoc analysis using the Bonferroni test showed that the greatest difference was also observed between samples from patients with CRC and healthy controls ($P = 0.003$).

However, the median peak area of 1H-indole did not present significant differences between the study groups; CRC, adenoma, and controls (1×10^8, 4×10^7, 4×10^7, respectively) with a P value of 0.436.

In the multivariate analyses, after adjusting for sex, age and BMI, only CRC was independently associated with an increase of p-cresol and 3(4H)-DBZ (Table 5).

3.5. Potential single and combined biomarkers for colonic adenocarcinoma prediction

Exploration of potential candidates for biomarker analysis did not include samples from patients with adenomatous polyps: only those with confirmed adenocarcinoma and without neoplasia were included for analyses as similar VOC peak areas were found among the adenoma and control groups (Fig. 3).

p-Cresol showed to be the most promising as a single biomarker for CRC. It achieved an AUC to predict CRC of 0.85 (CI 95%; 0.737–0.953) (Fig. 4) with a sensitivity and specificity of 83% and 82%, respectively ($P < 0.001$). 3(4H)-DBZ showed an AUC to predict CRC of 0.77 (CI 95%; 0.635–0.905) with a sensitivity of 78% and specificity of 75%, respectively ($P < 0.001$) (Fig. 5). These results are summarized in Table 6.

The combination of p-cresol and 3(4H)-DBZ (i.e., sum of peak areas of both compounds) is also optimistic as a combined biomarker (Fig. 6) to predict CRC with an AUC of 0.86 (CI 95%; 0.757–0.964), a sensitivity of 87% and specificity of 79%, respectively ($P < 0.001$) (Table 6).

3.6. Volatile organic compounds as biomarkers for pre-malignant lesions

A Mann Whitney-*U* test showed that only p-cresol was significantly associated with patients with advanced adenomas (AA) (≥1cm adenomas) when compared to healthy controls (1×10^9 vs. 7×10^8, $P = 0.045$). The association remained after adjustments for age, sex and BMI ($P = 0.048$).

As a biomarker for ≥ 1 cm adenomas, p-cresol showed an AUC of 0.70 (CI 95%; 0.534–0.862) with a sensitivity of 83% and specificity of 63%, respectively ($P = 0.045$) (Fig. 7).

4. Discussion

A suitable population-based screening program is the key to reducing the incidence and mortality of CRC [18]. Early identification of CRC and pre-malignant lesions are linked to improved outcomes. In this pilot study, the potential of fecal VOCs as biomarkers for colonic neoplasm is confirmed. Three VOCs whose peak area differ between samples from patients with CRC and controls have been identified; specifically, p-

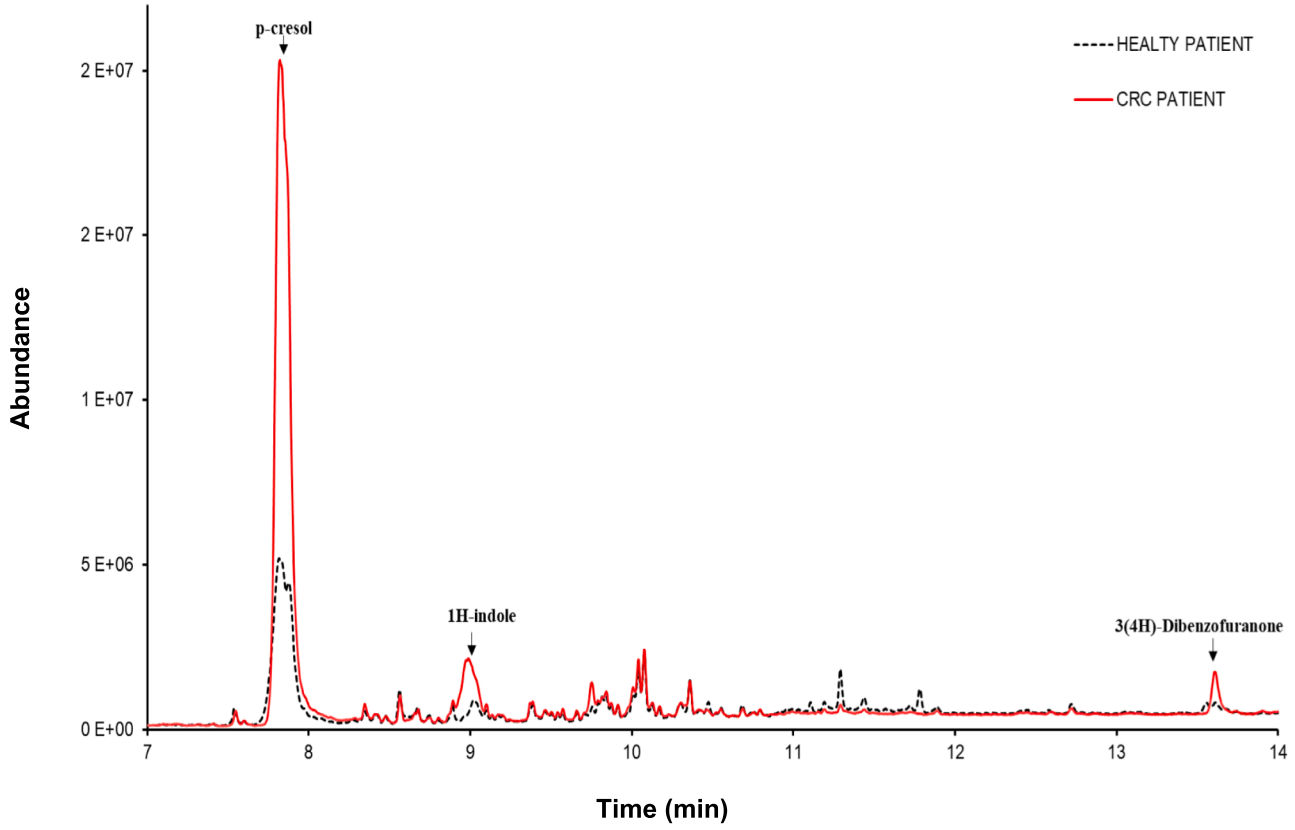

Fig. 2. Chromatograms of a fecal sample from a CRC patient and from a healthy control patient after the extraction.

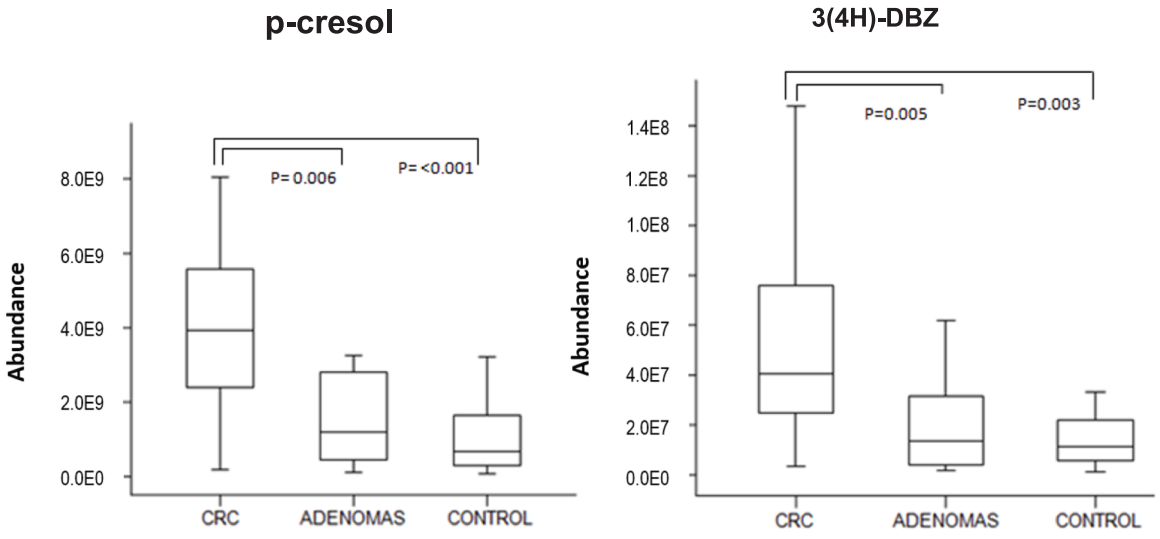

Fig. 3. Box plots show the peak areas of p-cresol and 3(4H)-DBZ in feces from all participants. All patients in each cohort were included (cancer n = 24, adenomas n = 24, control n = 32).

cresol, 3(4H)-DBZ and 1H-indole.

p-Cresol is a carcinogenic compound that is generated from the metabolism of aromatic amino acids such as phenylalanine and tyrosine. On the other hand, 3(4H)-DBZ is generated from the oxidation of p-cresol [19] and is considered a carcinogenic compound that acts as a tumor promoter [20]; and, 1H-indole is generated from the anaerobic metabolism of tryptophan through the intestinal microflora [21], with some studies classifying indole as a carcinogen and associated with CRC [22,23]. Among the different VOCs determined, p-cresol is the best individual biomarker to predict CRC with a good AUC, sensitivity and specificity, better than that found in other compounds according to previous studies [11,24]. Additionally, good results for the combined VOC biomarkers have been found. p-Cresol also shows promise as a biomarker for AA. The results obtained in this pilot study, even if preliminary, point to p-cresol as a potential biomarker for CRC.

The first study to analyze fecal volatolome for CRC detection was a small case-control study (10 cancer patients and 11 controls) conducted by Weir et al. [8]. Stool metabolome analysis revealed that the

Table 5
Linear regression analyses of volatile organic compounds (p-cresol and 3(4H)-DBZ) and their association with the case-control group adjusted by sex, age and BMI.

Dependent variable	Independent variable	P-value	Coefficient (Beta)	Standardized coefficient for B 95% (range)
p-cresol	Case/Control	<0.001*	−0.457	−0.363–0.102
	Sex		−0.032	−0.040–0.164
	Age		−0.134	−0.010–0.010
	BMI		0.032	0.004–0.017
3(4H)-DBZ	Case/Control	0.002*	−0.450	
	Sex		0.031	−0.345–0.103
	Age		−0.089	0.038–0.164
	BMI		0.046	−0.006–0.009
				0.006–0.017

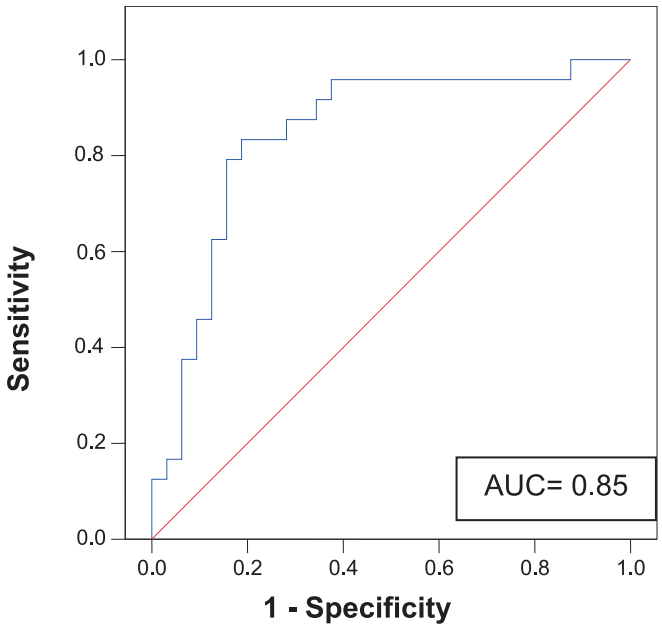

Fig. 4. ROC curve for p-cresol for CRC and no colonic neoplasia patients. Cut point: 1.9×10^9. Higher peak area of CRC and lower of healthy control.

metabolic profiles of the CRC group were distinct from that of the healthy control group. Wang et al. in 2017 also found differences between the metabolic profiles of CRC and control groups [10]. In 2015, Batty et al. used selective-ion flow-tube mass spectrometry (SIFT-MS) to analyze VOCs emitted from feces in fecal occult blood test (FOBT) positive patients [24]. They found that hydrogen sulfide, dimethyl sulfide and dimethyl disulfide were significantly higher in samples from high-risk (CRC or AA at colonoscopy) compared to low-risk subjects (no abnormalities at colonoscopy). They reported an overall classification accuracy of 75% with a specificity of 78% and a sensitivity of 72% for "high-risk group" vs. "low-risk group". SIFT-MS technique is less accurate and provides less information on VOCs compared to GC–MS [25]. More recently, Bond et al. analyzed fecal VOCs in 60 patients without neoplasia, 56 patients with adenoma and 21 CRC patients with SPME technique followed by GC–MS [11]. They found that propan-2-ol was significantly more abundant in the cancer samples ($P < 0.0001$) with an AUC of 0.76, and when combined with 3-methylbutanoic acid, the AUC was 0.82, sensitivity 87.9% (CI 95%; 0.87–0.99) and specificity 84.6% (CI 95%; 0.65–1.0). The authors also reported that 5-methyl-2-propan-2-yl-cyclohexan-1-ol was the only VOC to be negatively associated with CRC and they identified a three-VOCs panel (propan-2-ol, hexan-2-one and ethyl 3-methyl-butanoate) for an AUC of 0.73 ($P < 0.0001$). In the present study, the VOCs found as biomarkers (i.e., p-cresol, 3(4H)-DBZ and 1H-indole) are different from those previously identified [12–14]. The more plausible reason is the methodology employed, with the use of a different sorbent as graphene oxide for the extraction of

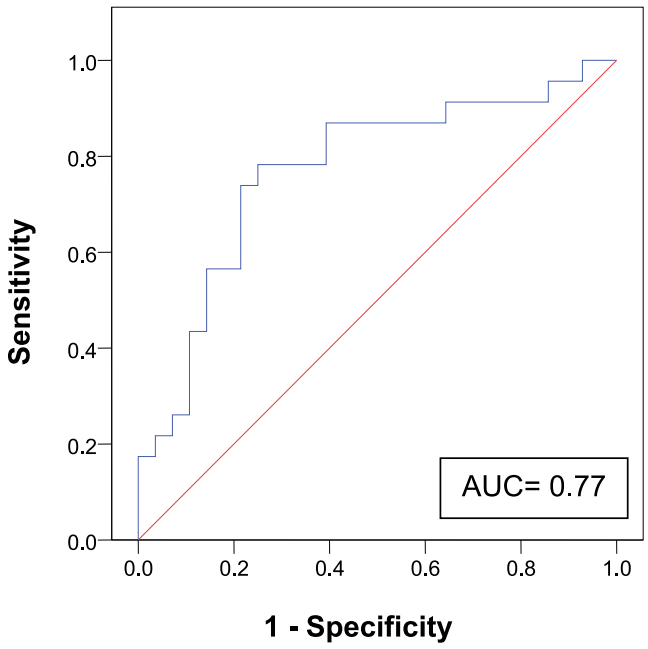

Fig. 5. ROC curve for 3(4H)-DBZ for CRC and no colonic neoplasia patients. Cut point: 2×10^7. Higher peak area of CRC and lower of healthy control.

Table 6
The area under the curve of the VOCs when comparing colorectal cancer and no colonic neoplasia patients.

Compound	AUC	95% CI	Optimal sensitivity (%)	Optimal specificity (%)
p-cresol	0.85	0.74–0.95	83	82
3(4H)-DBZ	0.77	0.64–0.91	78	75
p-cresol/3(4H)-DBZ	0.86	0.75–0.96	87	79

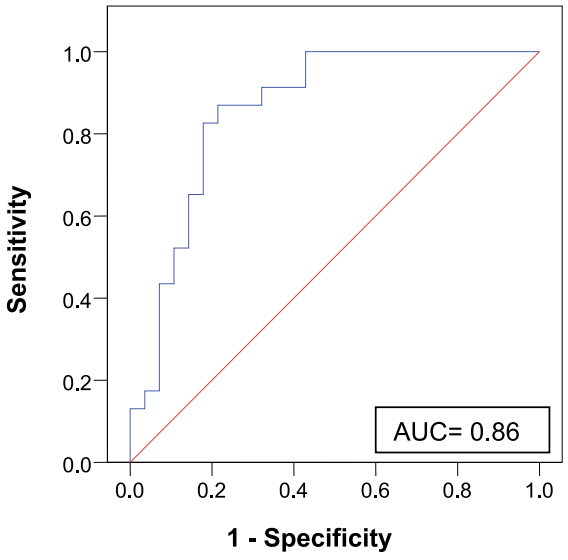

Fig. 6. ROC curve for p-cresol combined with 3(4H)-DBZ for CRC and no colonic neoplasia patients. Cut point: 1.9×10^9. Higher peak area of CRC and lower of healthy control.

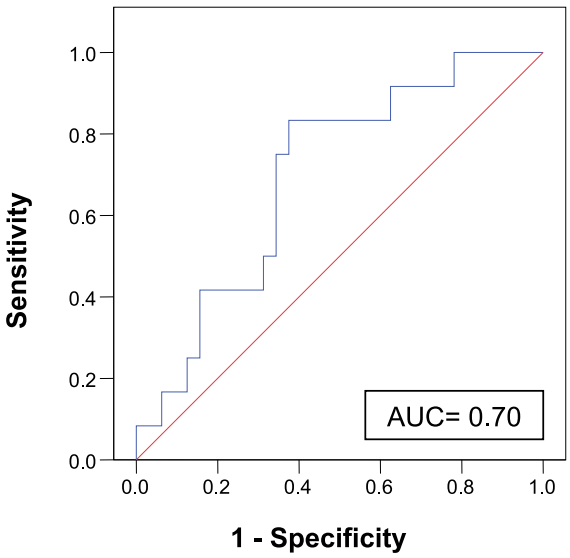

Fig. 7. ROC curve for p-cresol for patients with ≥ 1 cm adenomas and no colonic neoplasia patients. Cut point: 8.4×10^8. Higher peak area of ≥ 1 cm adenomas and lower of healthy control.

human fecal samples as well as the extraction conditions.

In this study, the VOCs were extracted following a magnetic headspace adsorptive extraction method developed by our research group [15,17]. Other studies, using a variety of methods and substrates, have suggested the utility of fecal VOCs for the diagnosis of CRC [12–14]. Most of the studies perform the volatile headspace extraction using commercial polydimethylsiloxane fibers or by fecal metabolome analysis. Despite the ability of the polydimethylsiloxane fibers to detect VOCs, the fibers currently used and marketed have certain disadvantages; they are fragile, break easily, exhibit preferential sorption of heavier material "displacement", lack of good sorbents to detect light materials and have a low effectiveness due to the small amount of polydimethylsiloxane coated on fibers [26]. The system proposed in this study does not present these disadvantages mainly due to the use of a magnetic (nano)sorbent (i.e., MGO) held over a small neodymium magnet as a VOCs sorption technique. This system does not require the use of any commercial fiber; therefore, it does not break and has a long functional life, is capable of adsorbing small compounds and has a lower cost [15,17]. Moreover, the developed methodology is fast, sensitive, green, economical, and easy-to-handle.

Further work is necessary to ascertain the origin of the VOCs that were found in association with CRC in this pilot study. They may be bacterial metabolites. p-Cresol is the metabolite of aerobic bacteria such as Enterobacteriaceae and anaerobic bacteria such as Clostridium and Coriobacteriia. Studies show that Enterobacteriaceae are more abundant in patients with CRC [27–29], also Clostridium subgroups [30], and Coriobacteriia have been found to be increased in the lumen of patients with CRC [31]. Saito et al. analyzed 153 bacterial strains in order to identify which specific bacteria produce p-cresol in the intestine, and they discovered that p-cresol-producing strains were dispersed across seven families, that included Clostridium, Enterobacteriaceae and Coriobacteriacae [32]. Furthermore, p-cresol exhibits cytotoxicity and genotoxicity and reduces endothelial barrier function in vitro [33,34]. Even if the role of p-cresol in CRC as "cause or effect" is unknown, the differences found between healthy and CRC patients make this compound a promising biomarker. In addition, 3(4H)-DBZ is generated from the oxidation of p-cresol, suggesting that high concentrations of p-cresol can lead to increased concentrations of 3(4H)-DBZ. Indole is produced by a wide variety of both Gram positive and Gram negative bacteria [35]. The increased peak area of 1H-indole found in the CRC group may be due to an increase of 1H-indole-producing intestinal bacteria due to the dysbiotic state of these patients. Following the driver-passenger model [36], colonization by specific (harmful) bacteria may be responsible for the increased number of indole-producing bacteria in patients with CRC. All this information is summarized in Fig. 8.

p-Cresol is the metabolite of several aerobic and anaerobic bacteria as Enterobacteria, Coriobacteriia, Clostridium [31] and different putrefaction bacteria [22]. These bacteria have been found to be increased in CRC patients [25,28–30]. Some of the putrefaction products are cresol and indole [22]. Furthermore, p-cresol has been found to exhibit citotoxicity, genotoxicity and reduction of endothelial barrier function "in vitro" [32,33]. 3(4H)-DBZ is generated from the oxidation of p-cresol, suggesting that high concentrations of p-cresol can lead to increased concentrations of 3(4H)-DBZ.

Nevertheless, our study has some limitations. This is an exploratory study including a small number of patients and samples and, therefore, the results must be validated in a larger sample. Moreover, as with current fecal-based screening techniques (FIT or FOBT) proper fecal specimen collection by the patient cannot be guaranteed, errors should be accounted for. Patients collected stool samples at their own homes, kept them in the refrigerator, and brought them to the hospital on the same day as their colonoscopy. Any possible error occurred in this phase of the study may have affected the composition of VOCs detected in the different study groups. Finally, the adenomas group was especially heterogeneous including polyps of different sizes and characteristics [37,38].

In summary, this pilot study has identified for the first time in human fecal samples three VOCs that are differentially detected in patients with CRC, colonic adenomas and controls. These results should be validated in a new cohort of patients in order to demonstrate the utility of these VOCs as future biomarkers for diagnosis and prevention of CRC and colonic adenomas. Moreover, technology must be developed in order to facilitate the detection of these VOCs as a potential CRC screening method, either alone or in conjunction with fecal occult blood tests.

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CRediT authorship contribution statement

Miren Alustiza: Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Laura Ripoll:** Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Antonio Canals:** Conceptualization, Methodology, Resources, Data curation, Writing – review & editing, Project administration, Funding acquisition. **Oscar Murcia:** Resources, Data curation, Writing – review & editing. **Alejandro Martínez-Roca:** Resources, Data curation, Writing – review & editing. **Anabel García-Heredia:** Resources, Data curation, Writing – review & editing. **Mar Giner-Calabuig:** Resources, Data curation, Writing – review & editing. **Rodrigo Jover:** Conceptualization, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Lorena Vidal:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

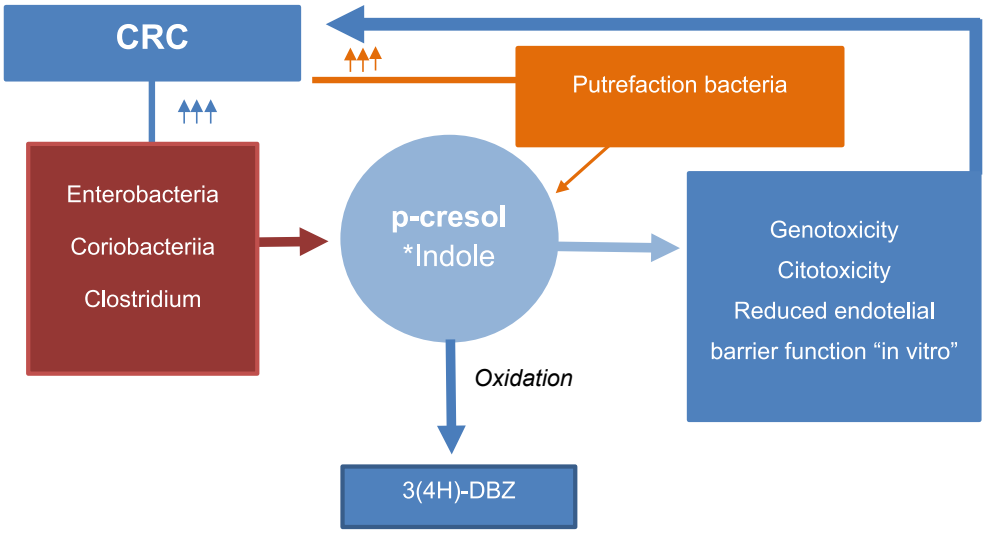

Fig. 8. p-Cresol association with CRC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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