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Breath testing as potential colorectal cancer screening tool

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Although colorectal cancer (CRC) screening is included in organized programs of many countries worldwide, there is still a place for better screening tools. In this study, 418 breath samples were collected from 65 patients with CRC, 22 with advanced or nonadvanced adenomas, and 122 control cases. All patients, including the controls, had undergone colonoscopy. The samples were analysed with two different techniques. The first technique relied on gas chromatography coupled with mass spectrometry (GC-MS) for identification and quantification of volatile organic compounds (VOCs). The T-test was used to identify significant VOCs (p values < 0.017). The second technique relied on sensor analysis with a pattern recognition method for building a breath pattern to identify different groups. Blind analysis or leave-one-out cross validation was conducted for validation. The GC-MS analysis revealed four significant VOCs that identified the tested groups; these were acetone and ethyl acetate (higher in CRC), ethanol and 4-methyl octane (lower in CRC). The sensor-analysis distinguished CRC from the control group with 85% sensitivity, 94% specificity and 91% accuracy. The performance of the sensors in identifying the advanced adenoma group from the non-advanced adenomas was 88% sensitivity, 100% specificity, and 94% accuracy. The performance of the sensors in identifying the advanced adenoma group was distinguished from the control group was 100% sensitivity, 88% specificity, and 94% accuracy. For summary, volatile marker testing by using sensor analysis is a promising noninvasive approach for CRC screening.

Globally colorectal cancer (CRC) is the third most prevalent cancer and the fourth leading cause of death from malignant diseases.[1] The 5-year survival rate for colorectal cancer when diagnosed at an early stage before it has spread is about 90%. But only about four out of 10 colorectal cancers are found at that early stage. In advanced stages, the survival rates are substantially lower. Therefore, regular CRC screening or testing are considered as some of the most powerful tools for preventing colorectal cancer caused mortality.[2] Not only does CRC screening save lives, but it also is cost effective. Studies have shown that the cost-effectiveness of CRC screening is consistent with many other kinds of preventive services and is lower than some common interventions.[3]

The laboratory-based faecal immunochemical test (FIT) is the test-of-choice currently in the EU.[4] Alternatively, several recent studies have demonstrated the ability of flexible-sigmoidoscopy to decrease the disease-specific mortality when used as a screening tool.[5–7] However, colonoscopy is considered better than sigmoidoscopy in preventing mortality.[8] Poland, Germany and Czech Republic are using colonoscopy as their primary screening tool. Nevertheless, the major problem with endoscopic methods for CRC screening is low compliance.[9] A systemic review of participation in CRC screening within trial settings after the first-time invitation has reported only 28% participation when colonoscopy was used as the primary screening tool.[10] In real-life, one would expect even lower participation rate, and that has been confirmed by the German CRC screening program.[11]

A potential approach for diagnosing CRC with potentially high compliance rates can be provided by the analysis of volatile organic compounds (VOCs).[12–32] Several studies have studied CRC-related VOCs in different body media such as blood,[33] faeces,[34] urine[17,35,36] and breath[37,38], by using spectrometry techniques, sensor analysis and even canine scene

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What's new?

A breath test could help diagnose colorectal cancer, according to this new report. Because a breath test is minimally invasive, it could inspire better compliance than colonoscopy. These authors tested volatile organic compounds in breath samples from colorectal cancer cases and controls, using two different methods of analysis. They successfully identified four compounds that accurately identified the cancer patients, establishing a distinctive "breath-print" for colorectal cancer, possibly leading the way to a cheap, effective, non-invasive screening tool.

detection.[39] Arguably, the most useful of these tests is exhaled breath for the VOC source in monitoring the CRC and its related chemistry. This is because exhaled breath can be obtained noninvasively; it provides a matrix of relative low complexity, as well as being associated in higher compliance of the target group. Several works have analysed breath samples with spectrometry techniques. Altomare *et al.*[37] have studied exhaled breath in 37 patients with CRC and 41 controls by using gas chromatography/mass spectrometry (GC-MS). The accuracy for CRC detection in the blinded validation stage of this study was 76%, though only samples from 25 subjects got included to the blinded analysis. Wang *et al.*[38] have analysed 20 CRC breath samples with solid-phase microextraction-GC-MS (SPME/GC-MS) by demonstrating significant differences in the VOCs between the CRC and control groups.

Here, we elaborate on previous findings with CRC diagnostics *via* breath analysis in two major complementary approaches. The first approach is based on chemical analysis by gas chromatography linked with mass spectrometry (GC-MS) for the identification and quantification of the variety of breath VOCs existing in each studied group. The second approach is based on cross-reactive nanoarrays in combination with pattern recognition methods. This approach provides collective VOC patterns rather than specific VOC identification and quantification and, as a result, has the realistic potential for future fast, cost-effective, and high-throughput diagnostics. The second avenue focuses not only on the discrimination between CRC and control groups, but also on sub-classification of each of these two categories to aid the decision-making process of the oncologist and/or treating medical staff.

Patients and Methods

The study was approved by the Ethics Committee of the Riga East University Hospital Support Foundation (approval No. 4-A/11); it was registered in the clinicaltrials.gov database registration identifier: NCT02332213.

Patients

Patients referred for CRC surgery or for diagnostic colonoscopy in Riga East University hospital or Digestive Diseases Centre GASTRO (Riga, Latvia) were recruited to the study group. Altogether 209 subjects were recruited, 65 had CRC, and 22 were included in the adenoma group. The remaining 122 patients were in the control group (See detailed information in Table 1 and in the "results" section below). All patients provided signed consent prior to enrolment. Breath samples were collected before surgery or colonoscopy, that is, prior to the potential removal of any lesions revealed during the procedure. In the control group without removable lesions volatile marker sampling was also performed starting from 1 week after colonoscopy.

Patients were included in one of three main groups: (*i*) CRC group, that is, histologically confirmed adenocarcinoma of colon or rectum; (*ii*) adenoma group, that is, histologically confirmed presence of an adenomatous polyp; and (*iii*) control group, that is, patients without adenocarcinoma or adenomatous polyps revealed during colonoscopy. The adenoma group were further separated into two subgroups: nonadvanced adenomas (of low risk potential) and advanced adenomas (considered a high-risk group); the latter was composed of patients carrying any of the following lesions: (*i*) >1 cm in size; (*ii*) with high-grade dysplasia; (*iii*) with villous component. No patients with severe or active respiratory disease or active other malignancy at the time of sampling, were included. However, patients were not excluded due to previous cured other malignancies in their medical history. Individuals having undergone major gastrointestinal surgery were excluded. Patients with inflammatory bowel disease (either known disease or suspected during the study colonoscopy) were excluded as well.

Breath sampling

Detailed information on the breath sampling and procurement procedures is described elsewhere[22,23] and in the Supporting Information, Section 1. Collection of the samples was carried out after withholding from smoking for at least 2 hrs. Sampling following overnight fasting was recommended. However, postprandial sampling was also allowed in 15% of the cases, to examine the effect of this parameter as a confounding factor. To test the reproducibility of the breath collection procedure, several experiments were conducted; both short and long term experiments and detailed information is described in previous work.[18] Two breath samples were obtained from each patient to be analysed with two different methods: (*i*) GC-MS for identification and quantification of particular VOCs (for more details, see Supporting Information, section 2 and refs 12,18,40); and (*ii*) sensor technology by utilizing cross-reactive nanoarrays in combination with

Table 1. Clinical characteristics of all patients tested in the current study

	Classification		Number of patients	Age (years)	Gender (M:F)	Smokers (%)
All samples	Controls		122	60 ± 14	31:91	16
	CRC[1]		65	66 ± 10	41:24	8
	Adenoma	NAA[2]	10	66 ± 6	3:7	10
		AA[3]	12	64 ± 7	4:8	17

[1]CRC: colorectal cancer.
[2]NAA:– nonadvanced adenoma.
[3]AA: advanced adenoma.

pattern recognition methods (for more details, see Supporting Information, section 3 and refs. 13,41). Room-air samples were analyzed by in parallel to assure that the breath samples were not affected by volatile contents already in the room.

General study design

GC-MS analysis was conducted to identify and quantify the chemical differences between the patients with CRC and the control group. In the sensor analysis, five different models were built to construct a breath pattern for the discrimination of the tested groups: (*i*) CRC versus controls; (*ii*) CRC versus patients with adenomas; (*iii*) patients with adenomas versus controls; (*iv*) patients with nonadvanced adenomas versus those with advanced adenomas; (*v*) patients with advanced adenomas versus controls. All the relevant patients for whom the breath sample was available for analysis were included in the CRC versus control comparison analysis. When considering the relatively small group size of the other comparisons (2–5), for the remaining models the number of the sample within the analysis was determined by the group size of the smallest comparator; correspondingly a similar group size was randomly chosen for the analysis from the larger comparator group.

Statistical analysis

In the GC-MS analysis, VOCs showing significant the differences (cut-off p values: 0.017) between the studied subpopulations were determined using Student's t test for the VOCs normally distributed. Bonferroni correction with three multiple corrections was used with level of significance set at 0.017 (0.05/3). For the sensor analysis, 70% of randomly selected samples were chosen to build the discriminant function analysis (DFA) model as a training set, while the remaining 30% of the samples were used for the blinded analysis (for more details on DFA, please see Supporting Information, Section 3). The performance results were based on this blinded analysis (validation set). For the analysis of the studied small groups, leave-one-out cross validation analysis was done for validation (for more details on the statistical analysis, please see Supporting Information, Section 3). Statistical analysis was performed using JMP, version 10.0.0 (SAS Institute Inc., Cary, NC, 1989–2005).

The DFA model was used to discriminate CRC from the control group as well as in addressing the potential influence of the confounding factors, in particular, age (below or above 60 years), gender, smoking, sampling of fasting state or after food intake. Area under the curve (AUC) in the receiver operating characteristics (ROC) analysis was used to characterize the impact of these potential confounding factors.

Results

Patient characteristics

After excluding six patients that had suspected inflammatory bowel disease during colonoscopy, the study group was compiled of in total, 418 breath samples recruited from 209 cases (Table 1). Of these, 65 had CRC, and 22 were included to the adenoma Group (10 patients bearing advanced adenomas, but 12 were nonadvanced). The remaining 122 patients complied the control group.

In the CRC group, one patient was Stage 0 (carcinoma *in situ*), 21 patients were Stage I, 19 patients—Stage IIA, 2—Stage IIB, one—Stage IIC, 5—Stage IIIA, 4—Stage IIIB, 9—Stage IIIC, 2—Stage IVA, and one patient had an undetermined prognostic stage. Most of the cancers (49 cases or 75.4%) were left-side localization. Four patients in the group were on neoadjuvant radiation and/or chemo- therapy, others had not been treated with radiation and/or chemotherapy.

Nine patients in the control group and four in the cancer group were known to have a history of other nongastrointestinal cancer in the past. No patients (in either the adenoma or other groups) had sessile serrated lesions. Samples for the VOC testing in postprandial state were obtained in 33 patients (19 CRC patients, five patients with adenoma, and nine patients in the control groups). The remaining were sampled after an overnight fast. Altogether eight breath samples (two CRC cases, four patients with advanced adenomas, and two—with nonadvanced adenomas) were not available for the sensor analysis due to technical issues (glass-tubes broken during the analytical process).

Chemical analysis

The GC-MS analysis revealed four substances in different concentrations in the exhaled breath from CRC patients and the control group. Acetone and ethyl acetate were found in higher concentrations in CRC patients (999.6 ± 116.8 ppb and 128.4 ± 4.01 ppb, respectively) compared to the controls (731.2 ± 63.8 ppb and 41.80 ± 10.00 ppb, respectively) with a p values (p) of $p = 0.010$ and $p = 0.005$, respectively. Ethanol

Table 2. The volatile organic compounds (VOCs) found at different concentrations in the exhaled air between the CRC patients and the control group.

VOC	Chemical group	Retention time (min.)	m/z[1]	Limit of detection (ppb)[2]	Limit of quantitation (ppb)[2]	Median concentration			*p*-values (student *t*-test)
						CRC[3] (ppb)	Control[4] (ppb)[2]	Room air (ppb)[2]	CRC *vs.* Control
Ethanol	Alcohol	2.4	31	1.8	6.0	95.9 ± 48.1	464.± 61.7	48.3± 20.1	<0.0001 (Control)
Acetone	Ketone	2.6	43	2.3	7.7	999.6 ± 116.8	731.2± 63.8	295.1 ± 38.1	0.010 (CRC)
Ethyl acetate	Ester	4.1	43	1.4	4.7	128.4 ± 37.0	41.8 ± 10.0	7.3 ± 2.1	0.005 (CRC)
4- methyl octane	Branched alkane	17.9	43	0.6	1.9	16.0 ± 0.6	19.1 ± 0.8	12.5 ± 0.6	0.004 (Control)

[1]m/z: mass to charge ratio.
[2]ppb: Part Per Billion.
[3]CRC: Colorectal Cancer.
[4]Control: Group of control, that is, excluding patients with CRC or adenomatous polyps.

and 4-methyl octane were higher in the control group (464.7 ± 61.7 ppb and 19.1 ± 0.8 ppb, respectively) compared with the CRC group (95.9 ± 48.1 ppb and 16.0 ± 0.63 ppb, respectively) with a *p*-value of $p < 0.0001$ and $p = 0.004$, respectively. The concentrations of all the above mentioned four substances was higher ($p < 0.01$) in the exhaled air of the patients (both CRC patients and controls) than in the room-air samples, indicating a lack of significant influence of the room-air upon the results (for more details, see Table 2).

Sensor analysis

The differences between the groups were addressed by using five different DFA models as described in the General study design section. Plotting of the test results between the different comparisons in the training set is demonstrated in Figure 1. According to the results obtained from the blinded validation set (see Table 3), the performance of nanoarray technology in discriminating between CRC and the group of control was characterized by 85% sensitivity and a 94% specificity, but the overall accuracy was 91%. The performance indicators for the minor groups were as follows: the CRC versus adenomas comparison exhibited 94% sensitivity, 88% specificity and 91% accuracy. The control group versus adenomas comparison exhibited 94% sensitivity, 94% specificity and 94% accuracy. The nonadvanced versus advanced adenomas comparisons exhibited 100% sensitivity, 88% specificity and 94% accuracy. The controls versus advanced adenomas comparison exhibited 88% sensitivity, 100% specificity, and 94% accuracy.

The results of the potential confounding factor (age, gender, current smoking, and fasting state of at least 12 hrs prior to sampling) are presented in Figure 2. None of the factors demonstrated significant influence on the results. In the ROC analysis, the AUC was 56% for age (above and below 60 years); 59% for gender; 59% for smoking habits; and 65% for having fasted before the sample collection. The low AUC values emphasize that the diagnostic model is not affected by different confounding factors, showing that the model cannot discriminate between the above confounding factor subgroups.

Discussion

As mentioned in the introduction, the compliance rates of participation in CRC screening programs can be increased by offering a noninvasive test, and referring only those that have a positive with this test for colonoscopy.[10,11,42,43] Although currently the FIT test is the noninvasive test of choice for organized CRC screening programs in Europe, it is not an ideal test. FIT tests may be false-negative in a substantial proportion of early-stage cancer as well as with small and nonpolypoid adenomas.[44] The participation rates of 42% for FIT-based studies following the initial invitation[10] are also far from ideal, and there is a substantial variation between different countries and social groups. Stool sampling procedure is among the barriers for optimal participation in CRC screening.[45] There is still a place in improving the accuracy of the screening tools and new screening modalities are

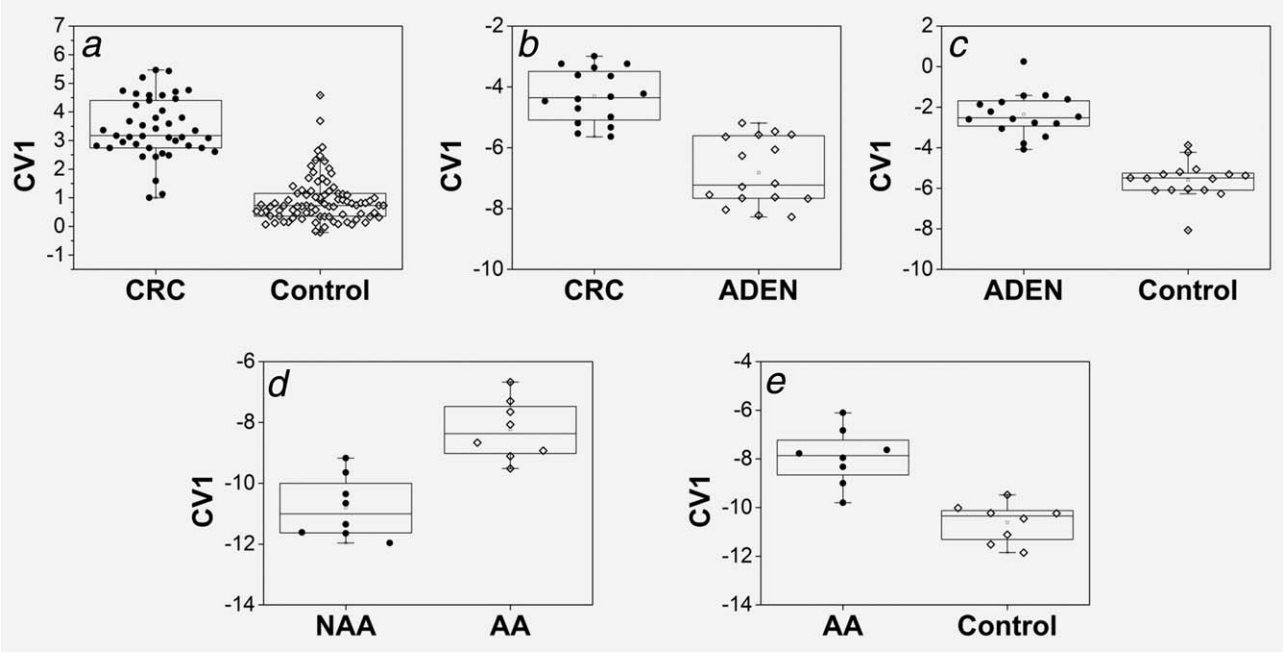

Figure 1. Discriminant factor analysis (DFA) models for different comparisons of the sensor measurements. The output of the training set data for the discrimination of (*a*) CRC patients from the group of control; (*b*) CRC from patients with adenomatous polyps; (*c*) patients with adenomatous polyps from control group patients; (*d*) patients with nonadvanced adenomas from those with advanced adenomas; (*e*) patients with advanced adenomas from the control group patients. CRC: Colorectal Cancer; ADEN: patients with adenomatous polyps; NAA: nonadvanced adenomas; AA: advanced adenomas.

Table 3. The performance results of volatile marker testing in detecting target lesions: the training set and blind evaluation results.

	Analysis parameters	[1]CRC[3] *vs.* Control[4]	[2]CRC[3] *vs.* ADEN[5]	[2]Control[4] *vs.* ADEN[5]	[#]NAA[6] *vs.* AA[7]
Training set results	Sensitivity (%)	93	95	94	100
	Specificity (%)	88	90	94	89
	Accuracy (%)	90	92	94	95
Blind results *or* Leave-one-out results	**Sensitivity (%)**	**85**	**94**	**94**	**100**
	Specificity (%)	**94**	**88**	**94**	**88**
	Accuracy (%)	**91**	**91**	**94**	**94**
	TP[8]	**17**	**15**	**15**	**8**
	FN[9]	**3**	**1**	**1**	**0**
	TN[10]	**34**	**14**	**15**	**7**
	FP[11]	**2**	**2**	**1**	**1**

Bold values in the table are validation test results
[1]Blind analysis for validation.
[2]Leave-one-out- cross validation.
[3]CRC: colorectal cancer.
[4]Control: Group of control, that is, excluding patients with colorectal cancer or adenomatous polyps.
[5]ADEN: Group of patients with adenomatous polyps.
[6]NAA: nonadvanced adenoma.
[7]AA: advanced adenoma.
[8]TP: true positive.
[9]FN: false negative.
[10]TN: true negative.
[11]FP: false positive.

being developed with this objective; these include colon capsule endoscopy[46,47] and molecular/multitarget DNA stool analysis methods.[48] High cost is among the major limitations of these methods, and therefore it cannot be expected that such methods will find their place in large population-based CRC screening programs in the foreseeable future.

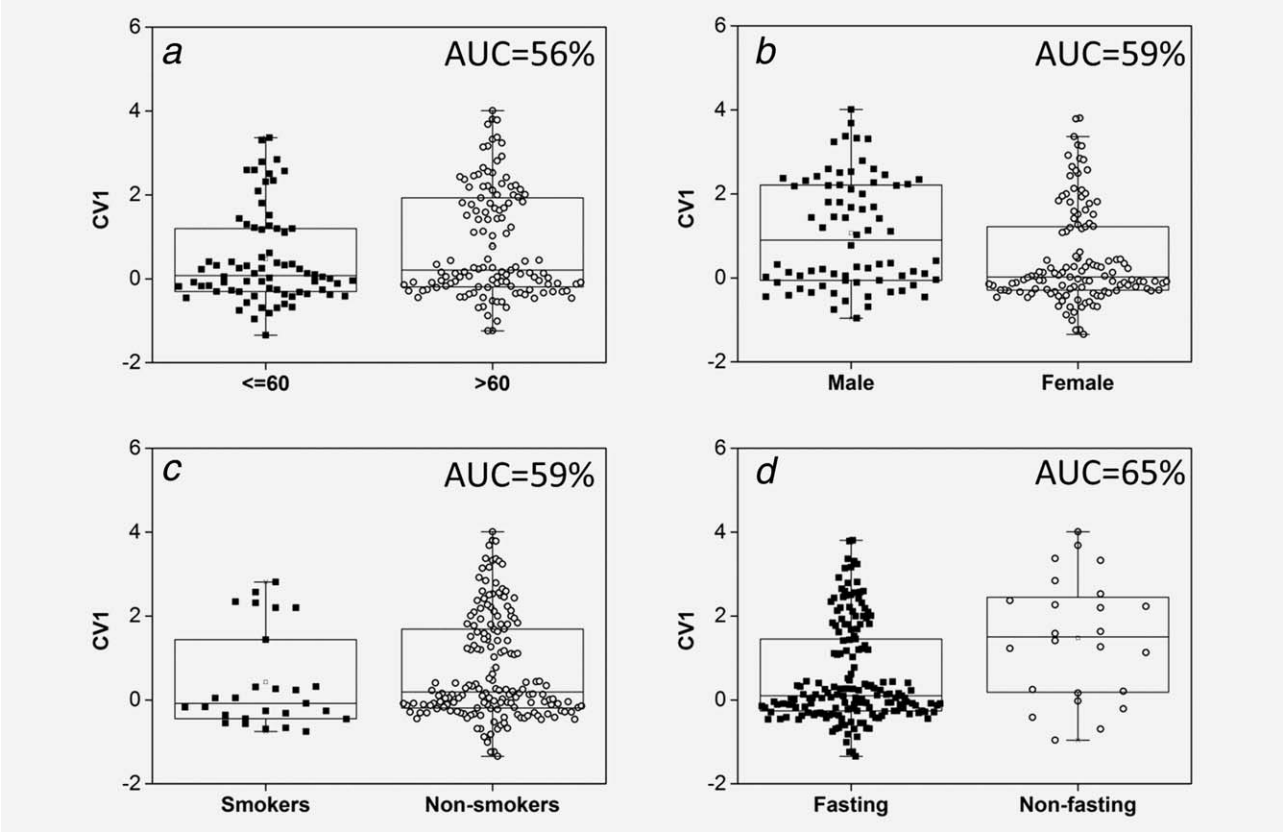

Figure 2. Analysis of the effect of confounding factors upon the first DFA model (colorectal cancer *vs*. control): (*a*) Age (above 60 years or not); (*b*) Gender; (*c*) Current smoking; and (*d*) fasting (fasting [mt]12 hrs or not). CV1 and CV2 stand for canonical variable 1 and 2, respectively.

The breath VOC testing approach may defeat most of these limitations. Although the method itself cannot overcome some of the barriers such as fear of cancer and unwillingness to learn bad news about a health status, it may provide a low-cost, highly accurate method unrelated to any discomfort and not requiring special preparation with a potential to be applied as the primary screening method to select the individuals at high risk for cancer (patients with cancer and those carrying precancerous lesions) for colonoscopy.

The results of our study suggest that VOC detection with sensor technology could have comparable or even better accuracy for CRC and possibly also precancerous lesion detection than the currently recommended FIT test. The recent systemic review and meta-analysis by Lee *et al.*[49] has demonstrated a moderate pooled sensitivity of 79% for CRC detection with good specificity—94%. This study showed that VOC detection has 85% sensitivity and a 94% specificity to detect cancer.

The explanation of the biological relevance of chemical substances identified in the exhaled air with the GC-MS method is somewhat more complicated. Comparing our chemical analysis to prior charts in the literature[33,35,37,38] they showed that the VOCs identified in our study were different from those reported in previous studies. These differences may be explained by the diversity of the mass-spectrometry devices, sample collection process, samples origin (breath, blood, urine or faeces) and geographical differences. Different biochemical processes lead to the release of VOCs in human breath. The potential biological role of alkenes, alcohols, ketones and esters in the exhaled breath have been described elsewhere by Hakim *et al.*[50] The main mechanism which affects the emission of hydrocarbons is oxidative stress which is the overall balance between formation and scavenging of reactive oxygen species (ROS) and free radicals in the body. The hydrocarbons that are not metabolized are excreted in the breath due to their low solubility in the blood. The alcohols mostly originate from food and alcohol beverages; they are also derived from the metabolism of hydrocarbons by cytochrome p450 enzymes. Acetone which is related to the ketones family is a secondary product of lipid peroxidation and in general is a good predictor for ketosis, since these compounds are formed under metabolic conditions associated with a high oxidation rate of fatty acids. Finally, esters can be found in large amounts in natural sources such as fats and fatty oils, waxes, and fruit ethers. However, in order to correlate these biochemical pathways with our results, further chemical analysis should be done for the headspace of CRC tissues, blood of CRC patients and breath samples trying to compare the three origins in order to understand the elevation and decrease of the different VOCs in the CRC patients.

Several limitations of our study should be indicated; first of all - the limited number of patients included to the adenoma group, in particular in the subgroup of advanced adenoma. Although the results obtained in our study suggest that the method may have a capacity for detecting adenomas and also advanced adenomas, this has to be proven in further studies. Larger studies will enable the conduction of blind analysis on all the different comparisons and will provide more robust results.

In summary, the study demonstrated that the breath-print of CRC patients differ from those without the disease. The sensor technology discriminated between the CRC patients and controls with a high accuracy; in addition the technology was similarly promising in identification of high-risk lesions in the colon, that is, high-risk adenomas. By considering the above, the results suggest a promising noninvasive, safe, fast predictive and cheap tool for CRC screening.

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