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Reduction of faecal immunochemical test false-positive results using a signature based on faecal bacterial markers

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Summary

Background: Colorectal cancer is the second commonest cause of cancer mortality. Some countries are implementing colorectal cancer screening to detect lesions at an early stage using non-invasive tools like the faecal immunochemical test. Despite affordability, this test shows a low sensitivity for precancerous lesions and a low positive predictive value for colorectal cancer, resulting in a high false-positive rate.

Aim: To develop a new, non-invasive colorectal cancer screening tool based on bacterial faecal biomarkers, which in combination with the faecal immunochemical test, could allow a reduction in the false-positive rate. This tool is called risk assessment of intestinal disease for colorectal cancer (RAID-CRC).

Methods: We performed both the faecal immunochemical test and the bacterial markers analysis (RAID-CRC test) in stool samples from individuals with normal colonoscopy (167), non-advanced adenomas (88), advanced adenomas (30) and colorectal cancer (48). All the participants showed colorectal cancer-associated symptoms.

Results: Performance of the faecal immunochemical test for advanced neoplasia (ie advanced adenoma and colorectal cancer) was determined by using the cut-off value established in Catalonia (20 μg haemoglobin/g of faeces) for a population-based screening approach. Sensitivity and specificity values of 83% and 80%, respectively, and positive and negative predictive values of 56% and 94%, respectively, were obtained. When both the immunological and the biological analysis were combined, the corresponding values were 80% and 90% for sensitivity and specificity, respectively, and 70% and 94% for positive and negative predictive values, respectively, resulting in a 50% reduction of the false-positive rate.

Conclusions: RAID-CRC test allows a substantial reduction in the faecal immunochemical test false-positive results (50%) in a symptomatic population. Further validation is indicated in a colorectal cancer-screening scenario.

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1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide, and a leading cause of cancer mortality.[1] It affects 6% of individuals by the age of 75, with incidence much greater in developed than in developing countries.[2] Although cancers show a strong hereditary component, most of CRC are sporadic and their development is slow.[3] In most patients, there is absence of symptomatology until advanced disease is present. Regular bowel cancer screening has been shown to reduce the risk of dying from CRC by 26%, when a faecal test is used, and up to 50% when flexible sigmoidoscopy is applied.[4,5] However, CRC screening programs are only implemented in some countries around the world.

Guidelines recommend routine screening for CRC in asymptomatic adults starting at age 50.[6] Current screening programs are based on two strategies: invasive procedures based on endoscopy examinations and non-invasive procedures based on faecal tests.[7] There are different endoscopy procedures for CRC screening such as flexible sigmoidoscopy and colonoscopy. The main advantage of colonoscopy is that it enables direct visualisation of the whole colon making it possible to perform an accurate diagnostic and, therefore, to prevent CRC through the early detection of pre-cancerous lesions.[8] Nevertheless, this procedure requires bowel preparation and sedation, there is a risk of bowel perforation and other adverse effects, and it is time-consuming and expensive.[9,10] Flexible sigmoidoscopy is an alternative approach that enables the direct visualisation of the distal colon, that is, rectum, sigmoid and descendent colon. This method avoids sedation and reduces risk of bowel perforation.[11] Faecal tests are becoming the preferred CRC screening procedure in most countries due to its non-invasiveness and the lower costs compared to endoscopy strategies. Subjects with a positive result of any of the alternative faecal test should undergo a colonoscopy in order to make an accurate diagnosis. Therefore, non-invasive tests are used as a preliminary step in the screening approach. One of the faecal tests used in screening programs is the faecal occult blood test that uses guaiac methods (guaiac Faecal Occult Blood Test, gFOBT), which are based on a chemical oxidation reaction between heme and alpha guaionic acid. The main disadvantage of these kind of tests is the requirement of a prescribed diet in order to avoid false-positive results that can occur because of the consumption of specific foods, alcohol or nonsteroidal anti-inflammatory drugs.[12] Despite its non-invasiveness, it has shown to have a low sensitivity for CRC (25%-38%) and for pre-cancerous lesions (16%-31%).[13] Faecal immunochemical test (FIT) was developed to overcome gFOBT low sensitivity. This test is specific for human blood haemoglobin and does not require dietary restrictions. Several studies have demonstrated a higher sensitivity of FIT screening compared to gFOBT.[14-17] Although overall sensitivity of FIT for CRC is around 61%-91% and for advanced adenomas between 27%-67%,[13] these figures still imply a high false-positive rate.

Recently, it has been proved that bacterial communities in the intestinal mucosa of CRC patients are different from those of healthy individuals.[18,19] Evidences suggest that gut microbiota may play an important role in CRC pathogenesis.[20,21] To date, two possible mechanisms through which gut microbiota could induce tumourigenesis have been described. On the one hand, gut microbiota may promote chronic inflammation which in turn can lead to tumour formation.[22,23] On the other hand, it has been shown that some dietary components metabolised by gut microbiota, such as red meat, result in the production of carcinogenic compounds.[24] Moreover, a number of recent studies have been done to elucidate whether or not there is a CRC specific dysbiosis, or any particular species that can be associated to CRC development.[25-27]

In 2012, a preliminary, prospective study performed by our group with 60 individuals (41 CRC patients and 19 patients with normal colonoscopy) defined a bacterial cluster in mucosal biopsies which prevalence was correlated with CRC risk.[28] From the 55 phylotypes analysed, six showed significantly higher frequencies in CRC patients than in control subjects. Five of them shared similarity with uncultured bacterial sequences retrieved from the human gastrointestinal tract or human faeces, and one shared 97% similarity with *Parabacteroides merdae*. In contrast, there were two phylotypes, B34 (99% similarity to *Clostridium nexile*) and B35 (97% similarity to *Roseburia faecalis*), which were more prevalent in healthy subjects than in CRC patients.

Subsequently, we designed quantitative polymerase chain reaction systems (qPCR) specifically targeted to those bacterial markers.[28] Bacterial signatures were later tested on stool samples (7 from healthy controls and 9 CRC patients) looking for different abundances to check which one was suitable to be used as a non-invasive tool for CRC screening.[29] A retrospective clinical study including 46 patients of the Hospital Universitari de Girona Dr Josep Trueta (Girona, Spain) confirmed the suitability of some bacterial signatures as CRC markers (Data S1).

The aim of this work was to develop a new non-invasive CRC screening tool based on faecal bacterial markers capable of complementing FIT and particularly decreasing its false positive rate.

2 | MATERIALS AND METHODS

2.1 | Study population

A cohort consisting of 333 consecutive patients with CRC-related symptoms referred for a diagnostic colonoscopy from primary and secondary health care to Complexo Hospitalario de Ourense (Ourense, Spain) was recruited (Table 1). Exclusion criteria were: (a) asymptomatic subjects undergoing colonoscopy for CRC screening, (b) patients with a previous history of colonic disease undergoing surveillance colonoscopy, (c) patients requiring hospital admission, (d) patients whose symptoms had ceased within 3 months before evaluation, and (e) patients who had received antibiotic treatment within the last month prior to inclusion. The study protocol was approved by the Biobanco del Complexo Hospitalario Universitario de Vigo (Vigo, Spain). Written informed consent was obtained from all study patients.

TABLE 1 Patients characteristics classified according to colonoscopy diagnostic

Characteristics	CRC	AA	NAA	NC
n (%)	48 (14.4)	30 (9)	88 (26.4)	167 (50.2)
Age (mean, range)	73 (53-91)	65 (44-83)	67 (37-89)	61 (20-87)
Gender, female (%)	17 (10)	15 (8.8)	32 (18.8)	106 (62.4)
FIT100 (%)	47 (97.9)	18 (60)	30 (34.1)	21 (12.6)

Hb, haemoglobin; FIT100 (20 µg Hb/g of faeces); CRC, colorectal cancer; AA, advanced adenoma; NAA, non-advanced adenoma; NC, normal colonoscopy.

All subjects underwent colonoscopy in order to determine their colorectal status. According to the endoscopic examination and the pathology results, diagnosis was classified into four groups: normal colonoscopy (colonoscopy with no findings or with sigma and/or rectum hyperplastic polyps < 10 mm), non-advanced adenomas (tubular adenomas < 10 mm with low grade dysplasia, and serrated polyps < 10 mm without dysplasia), advanced adenomas (adenomas > 10 mm or with villous component or high grade dysplasia, serrated polyps > 10 mm or with dysplasia, and pTis adenocarcinoma) and invasive CRC. Patients diagnosed with CRC were also classified according to the stage of the tumour (Table 2). Individuals were also asked to answer a questionnaire in order to record clinical and epidemiologic data.

TABLE 2 Patients with colorectal cancer according to tumour TNM stage

CRC stage	n (%)
0	3
I	6
II	10
III	21
IV	8

CRC, colorectal cancer.

2.2 | Faecal sample collection

Participants were asked to collect a stool sample from one bowel movement in a sterile faeces container before colonoscopy and prior to bowel cleanse. Samples were immediately frozen after deposition. Then, subjects brought samples to the hospital, where they were kept frozen at −20°C for short-term storage and stored at −80°C upon arrival at the GoodGut SL facilities in Girona (Spain). A total of 11 subjects were excluded from the study due to the wrong stool samples collection.

2.3 | DNA extraction from stool samples

Genomic DNA was extracted from frozen faecal samples after homogenisation using the NucleoSpin Soil Kit (Macherey-Nagel GMbH & Co., Duren, Germany). The instructions of manufacturer were followed, DNA was finally eluted in a 100 µl final volume of SE Elution Buffer and stored at −20°C until use. DNA concentration was determined with Qubit fluorometric quantification (ThermoFisher Scientific, Waltham, USA). All samples were adjusted to a final concentration of 8 ng/µl and quantified again.

2.4 | qPCR assay for CRC biomarkers

The specific bacterial sequences targeted were 10: Eubacteria (EUB) as the total bacterial load; B10 (best BLAST match *Faecalibacterium prausnitzii*), B46 (best BLAST match *Subdoligranulum variabile*), B48 (best BLAST match *Ruminococcus*, *Roseburia*, *Coprococcus*) and *Roseburia intestinalis* (RSBI); *Gemella morbillorum* (GMLL), *Peptostreptococcus stomatis* (PTST) and *Bacteroides fragilis* (BCTF); *Collinsella intestinalis* (CINT); and *Bacteroides thetaiotaomicron* (BCTT).

Quantification of the different biomarkers was performed by preparing single reaction for each biomarker using SYBR Green Master Mix (Promega, Madison, USA). Each reaction consisted of 20 µl containing 1 × GoTaq qPCR Master Mix, between 150 and 300 nmol/L of each primer, and up to 20 ng of genomic DNA template. The species-specific primers used in this study are shown in Table 3 and were purchased at Macrogen (Macrogen, Seoul, South Korea). All quantitative PCR were run on an AriaMx Realtime PCR System (Agilent Technologies, Santa Clara, USA). Thermal profiles were different according to the biomarker analysed (Table 4). A melting curve step was added to the end of each qPCR to verify the presence of the expected amplicon size as well as to control primer dimmer formation. Data were collected and analysed with the Aria Software version 1.3 (Agilent Technologies, Santa Clara, USA). All samples were amplified in duplicates, which were considered valid when the difference between threshold cycles (Ct) was less than 0.6. A dynamic range consisting of 8 logs from 10 to 10^8 genome units/µL was established for each biomarker and was used to calculate the relative abundance. A No-template control reaction was included in each PCR run.

2.5 | FIT analysis

FIT analysis was performed at Complexo Universitario de Ourense employing the same sample used in the CRC-specific biomarkers analysis. Stool samples for faecal haemoglobin determination were analysed using the OC-Sensor tube collector and the assay was performed using the automated OC-Sensor, which detects gastrointestinal bleeding associated with disorders such as CRC, polyps and diverticulitis (Eiken Chemical Co., Tokyo, Japan).[30] Positive tests

were those with a concentration of faecal haemoglobin equal or higher than 100 ng/mL (20 μg Hb/g of faeces; FIT100).

2.6 | Statistical analysis

In terms of qualitative analysis, absence of biomarker was considered if the obtained C_t value was not comprised within its dynamic range. All statistical analyses were performed using SPSS 23.0 statistical package (IBM, NYC, USA). Significance levels were established for $P \leq 0.05$.

Data normality was assessed through the Kolmogorov-Smirnov test. The nonparametric Kruskal-Wallis test was used to test differences in variables with more than two categories. Pairwise comparisons of subcategories of these variables were analysed using a Mann-Whitney test. The Bonferroni correction was used to correct for multiple comparisons. All comparisons using bacterial markers were performed between the relative abundances, which were normalised by the dynamic range of each bacterial marker.

The receiver operating characteristic (ROC) curve analysis was applied to determine the usefulness of each biomarker to distinguish among different colonic neoplasia status. The accuracy of discrimination was measured by the area under the ROC curve (AUC).

Machine learning was used to determine which of the studied variables (gender, age, BMI, smoking, bacterial markers, FIT) in combination were capable of distinguishing subjects with advanced neoplasia lesions from those with normal colonoscopy or non-advanced adenomas. The specific methodology consisted of an initial training iteration on 100 aleatory partitions of the dataset and a further validation of the predictive models generated using four different machine learning algorithms (neural network, logistic regression, gradient boosting tree, random forest). RAID-CRC was eventually designed using the combination of four of the bacterial markers analysed together with FIT results. The final algorithm is based on a Decision Abundance (DA) calculated using following Equation (1):

$$DA = \frac{\frac{Ct_{ind} - b_{ind}}{m_{ind}}}{\frac{Ct_{EUB} - b_{EUB}}{m_{EUB}}}, \quad (1)$$

where Ct is the threshold cycle; b is the intercept point; m is the slope; ind, is the bacterial marker; and EUB are eubacteria (total bacterial load).

3 | RESULTS

3.1 | Faeces biomarkers in neoplasia progression

The relative abundance of each bacterial marker was determined for each diagnostic (normal colonoscopy, non-advanced adenoma, advanced adenoma, CRC) (Figure 1). Regardless of the colonoscopy diagnosis, three different butyrate producing species (B10, B46 and B48) were the most prevalent biomarkers with relative abundance values of 20.4%, 19.0% and 20.0%, respectively. GMLL and PTST were significantly more abundant in CRC population than in normal

TABLE 3 Forward and reverse primers used in this study

Target	Primers	Sequence 5'→3'	Primer concentration (nmol/L)	References
EUB	EUB_F	ACT CCT ACG GGA GGC AGC AGT	200	modified[69]
	EUB_R	GTA TTA CCG CGG CTG CTG GCA C		
B10	B10_F	CAA CAA GGT AAG TGA CGG C	300	[28,29]
	B10_R	CGC CTA CCT GTG CAC TAC TC		
B46	B46_F	TCC ACG TAA GTC ACA AGC G	300	[28,29]
	B46_R	CGC CTA CCT GTG CAC TAC TC		
B48	B48_F	GTA CGG GGA GCA GCA GTG	300	[28,29]
	B48_R	GAC ACT CTA GAT GCA CAG TTT CC		
GMLL	GMLL_F	AAG AGT TCC AAG GCG TTC TC	150	This study
	GMLL_R	CCA TTT CAA GAT CCG CTT TCT ATT T		
PTST	PTST_F	AGG TTG ATG CTC TGA GTA GTA G	150	This study
	PTST_R	ATG AAT ACT AGC CTC TCC TCT TT		
BCTF	BCTF_F	TGA AAG CGT GCT CTT ACT ATT G	150	This study
	BCFT_R	TAT TGG CTG TTG TGC TTT GT		
CINT	CINT_F	GAC CAT CAT GAA CTC TTC CTC	150	This study
	CINT_R	CCG TTG CCT TCC AGT TC		
BCTT	BCTT_F	AGT GAC CTG AAA GAA TCC TAA T	150	This study
	BCTT_R	GAC CGT CAA TAC CGA GAA AC		
RSBI	RSBI_F	GTG CCA GTA ACA GTC CAT ATT	150	This study
	RSBI_R	TAG CAA AGC AGA GTG GAA AG		

EUB, Eubacteria; GMLL, *G morbillorum*; PTST, *P stomatis*; BCTF, *B fragilis*; CINT, *C intestinalis*; BCTT, *B thetaiotaomicron*; RSBI, *R intestinalis*.

TABLE 4 qPCR conditions

		Denaturing		Annealing and extension		Melting curve	
Bacterial markers	**Total cycles**	**Tª (°C)**	**Time (min:sec)**	**Tª (°C)**	**Time (min:sec)**	**Tª (°C)**	**Time (min:sec)**
EUB	40	95	10:00	95 54	00:15 01:00	95 55 95	01:00 00:30 00:30
B10, B46, B48	40	95	10:00	95 62	00:15 00:45	95 55 95	01:00 00:30 00:30
GMLL, PTST, CINT, BCTT, RSBI	40	95	10:00	95 60	00:15 01:00	95 55 95	01:00 00:30 00:30
BCTF	40	95	10:00	95 55 72	00:15 00:30 01:00	95 55 95	01:00 00:30 00:30

colonoscopy individuals ($P = 0.006$ and $P < 0.001$, respectively) or non-advanced adenoma subjects ($P = 0.047$ and $P < 0.001$, respectively). Although with no significant differences, it could be observed a tendency of B46, being more abundant in CRC patients rather than in subjects with advanced adenomas ($P = 0.087$). Interestingly, EUB abundance was maintained constant regardless of neoplasia status. Comparison among the different CRC stages (0, I, II, III and IV) did not show significant differences in the abundance of any bacterial marker.

3.2 | CRC specific biomarkers can detect advanced neoplasia lesions

The relative abundance of bacterial markers was compared after grouping subjects as follows: (a) normal colonoscopy, (b) neoplasia (non-advanced adenoma + advanced adenoma + CRC), (c) advanced neoplasia (advanced adenoma + CRC), and (d) CRC (Figure 2). PTST was found to be highly correlated with neoplasia lesions ($P < 0.001$). Regarding the detection of advanced neoplasia lesions, GMLL, PTST and BCTF were potential biomarkers for their detection ($P = 0.006$, $P < 0.001$, and $P = 0.030$, respectively). In terms of prevalence, these three opportunistic pathogens were found more often in patients with advanced neoplasia (GMLL, 64.9%; PTST, 58.4%; and BCTF, 44.7%) than in healthy subjects (GMLL, 53.5%; PTST, 26.1%; and BCTF, 29.8%).

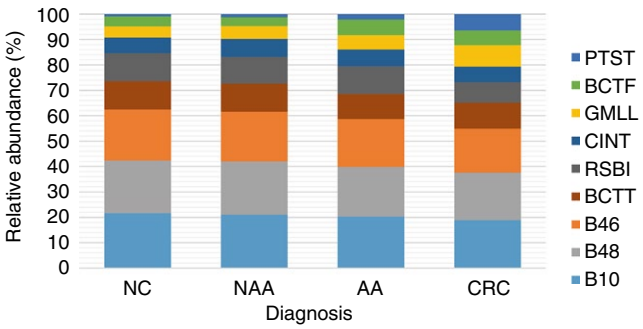

FIGURE 1 Relative abundance in percentage of the analysed biological markers (B10, B46, B48, *G morbillorum* (GMLL), *P stomatis* (PTST), *B fragilis* (BCTF), *C intestinalis* (CINT), *B thetaiotaomicron* (BCTT) and *R intestinalis* (RSBI)); for subjects with normal colonoscopy (NC), non-advanced adenoma (NAA), advanced adenoma (AA) and colorectal cancer (CRC)

3.3 | Combination of CRC bacterial markers and FIT allows a substantial reduction in false-positive results

One the one hand, when FIT100 was used, significant differences were observed between subjects with normal colonoscopies or non-advanced adenomas and advanced neoplasia ($P < 0.001$). A 17.1% (19) of the subjects who showed a normal colonoscopy and a 24.3% (27) of those who had non-advanced adenomas showed FIT positive values. These results led to obtain a sensitivity and specificity of 84% and 81%, respectively, and positive and negative predictive values of 59% and 94%, respectively (AUC = 0.828, 95% CI [0.773-0.883]) for the detection of advanced neoplasia. On the other hand, when FIT50 was used, a 21,1% (27) of the subjects who showed normal colonoscopy and a 24.2% (31) of those who had non-advanced adenomas showed a false-positive result. FIT50 let to obtain a sensitivity and specificity of 91% and 76%, respectively, and positive and negative predictive values of 55% and 96%, respectively (AUC = 0.836, 95% CI [0.787-0.886]) for the detection of advanced neoplasia. Sensitivity values for bacterial markers alone were much lower being 39% for GMLL (AUC = 0.622, 95% CI [0.541-0.694]), 53% for PTST (AUC = 0.710, 95% CI [0.628-0.776] and 33% for BCTF (AUC = 0.571, 95% CI [0.499-0.656]), while specificity values were comparable.

FIT results, both FIT100 and FIT50, were combined with the faecal bacterial markers in order to know which offered higher performance in terms of sensitivity and specificity values for the detection of advanced neoplasia lesions. The combination of the bacterial markers and FIT100 led to obtain a sensitivity of 76% and a specificity of 91% (Table 5). Nevertheless, these results were slightly improved by FIT50 as it showed a 4% higher sensitivity for the detection of advanced neoplasia, which therefore was the cut-off

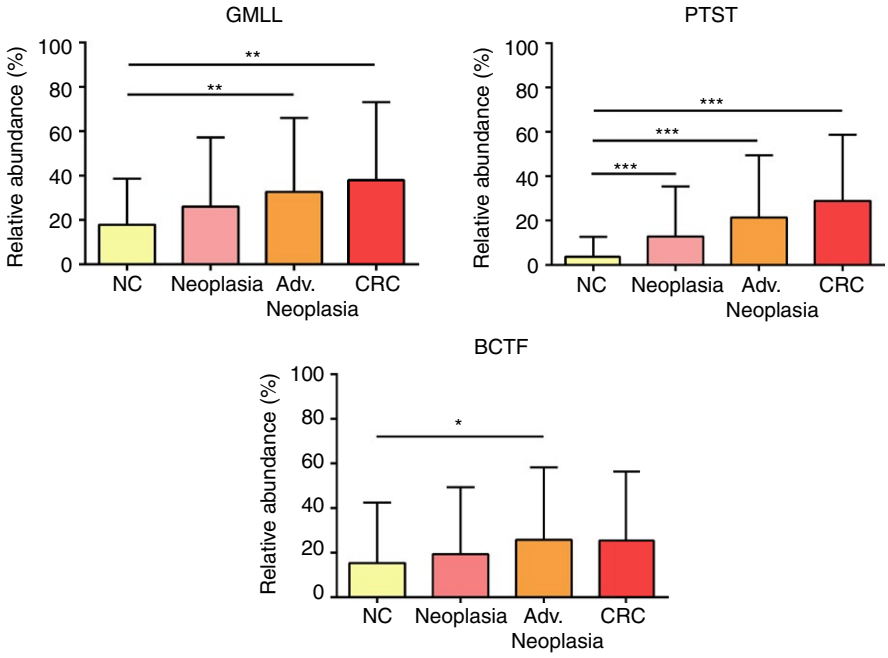

FIGURE 2 Biomarkers abundances comparison among different diagnoses. NC, normal colonoscopy; neoplasia, non-advanced adenoma + advanced adenoma + colorectal cancer; advanced neoplasia, advanced adenoma + colorectal cancer; CRC, colorectal cancer. Level of significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

value of choice. Thus, RAID-CRC test is based on the combination of EUB, PTST, BCTF and BCTT with a faecal haemoglobin concentration equal or higher than 50 ng/μL (10 μg Hb/g of faeces). Although BCTT did not show significant differences between subjects with normal colonoscopies or non-advanced adenomas and advanced neoplasia subjects, once in combination with EUB, PTST, and BCTF, it was able to increase specificity.

The final algorithm consists of the combination of FIT50 and three ratios between bacterial markers (PTST/EUB, BCTF/EUB, BCTT/EUB). The application of the algorithm to the detection of advanced neoplasia resulted in a decrease in the number of false positive results, with a 9.7% of the subjects showing a normal colonoscopy and an 11.7% of subjects having non-advanced adenomas. Altogether, we obtained a sensitivity and a specificity of 80% and 90% (AUC = 0.837, 95% CI [0.730-0.944]), respectively, and positive and negative predictive values of 70% and 94%, respectively. More importantly, the false-positive rate was reduced by 50%, being 46 subjects the false-positive results for FIT100 and 23 subjects for RAID-CRC.

4 | DISCUSSION

Early detection of advanced colorectal neoplasia through population-based screening and surveillance strategies is a critical step to reduce CRC mortality.[26,31-33] The ideal technique should be non-invasive, cost-effective, reproducible and capable to detect premalignant lesions with high risk of tumour development and high sensitivity and specificity values. In this study, we have defined a faecal bacterial signature that complements FIT and is able to reduce FIT-associated false positive results by increasing its specificity, in a symptomatic population.

Analysis of CRC-specific bacterial markers revealed that subjects with different colonoscopy diagnosis (ie normal colonoscopy, non-advanced adenoma, advanced adenoma and CRC) showed different microbiological patterns. The total bacterial load does not seem to be affected when neoplasia appears, according to Sobhani et al.[20] Therefore, tumour lesions affect gut microbiota diversity but not its total amount. Using a qPCR-based approach, our results clearly indicate the existence of a bacterial dysbiosis in patients with CRC. The studied bacterial markers were classified according to gut health related phenotypes: butyrate producers (B10, B46, B48, RSBI), opportunistic pathogens (GMLL, PTST, BCTF), hydrogen and oxygen producers (CINT), and saccharolytic species (BCTT) (Figure 3). Relative abundance of these phenotypes was found to change progressively as progression of the disease status. In particular, between subjects with normal colonoscopies and those with CRC we found a decrease in relative abundance of butyrate producers which were replaced by pathogenic bacteria group, being more abundant in CRC and advanced adenoma individuals than in subjects with normal colonoscopies. It was already reported that patients with CRC show a reduction in butyrate producers and an increase in opportunistic pathogens, which constitutes a major structural imbalance of their gut microbiota.[34] Bacterial dysbiosis can alter the balance of host cell proliferation and death, guide the immune system function and influence the metabolism of host-produced factors, ingested foodstuffs and pharmaceuticals.[35] Changes in bacterial composition, represented by a decrease in the amount of butyrate producing species and an increase in the opportunistic pathogens load, are likely to be a consequence of neoplastic lesion progression.[18,20,22,26,36,37] However, it has been reported that the increase in the abundance of opportunistic pathogens can lead to the release of bacterial toxins that can directly damage host DNA.[35] Other factors like reactive oxygen and nitrogen species, chemokines and cytokines released by these microorganisms can also contribute to tumour growth.[35,39,41] Therefore, we proposed to combine bacterial markers

TABLE 5 Diagnostic performance of RAID-CRC (using FIT100 and FIT50), FIT100 and FIT50 of the studied symptomatic population compared to FIT100 of screening population

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
RAID-CRC (using FIT100)				
Precancerous lesion	50	91	40	94
Colorectal cancer	93	87	55	99
Advanced neoplasia	76	91	72	92
RAID-CRC (using FIT50)				
Precancerous lesion	59	90	43	95
Colorectal cancer	94	85	51	99
Advanced neoplasia	80	90	70	94
FIT100 (this study)				
Precancerous lesion	62	81	28	95
Colorectal cancer	98	75	42	99
Advanced neoplasia	84	81	59	94
FIT50 (this study)				
Precancerous lesion	76	76	28	96
Colorectal cancer	100	71	38	100
Advanced neoplasia	91	76	55	96
FIT100[46,47]				
Precancerous lesion	28	93	13	97
Colorectal cancer	78	92	2	99
Advanced neoplasia	30	93	15	97

FIT100 (20 μg haemoglobin/g of faeces); FIT50 (10 μg haemoglobin/g of faeces); PPV, positive predictive value; NPV, negative predictive value.

with FIT in a new tool, called RAID-CRC, in order to increment the specificity values and consequently reduce the number of false positive results translated to unnecessary colonoscopies.

The RAID-CRC algorithm combines three bacterial markers abundance ratios (PTST/EUB, BCTF/EUB, BCTT/EUB) with FIT50. Our results show that high abundances of PTST and BCTF correlate with advanced neoplasia, whereas BCTT abundance is correlated with healthy individuals. BCTT is a commensal bacterium commonly found in the gut microbiota of healthy individuals. Commensal bacteria have been observed to attenuate gut inflammation and to contribute to colonisation resistance.[43,44] Hence, high abundances of BCTT correlate with a good intestinal health. Using ratios allowed data normalisation, which is critical to control qPCR-associated variables in order to differentiate true biological changes from experimentally induced variation.[45] Reduction in the faecal haemoglobin concentration from 100 ng/μL to 50 ng/μL allows capturing positive subjects that otherwise would be considered false negative with a cut-off value of 100 ng/μL, at expenses of increasing the false-positive rate. However, the RAID-CRC algorithm led to an important reduction in false negative results due to an increase of the sensitivity for detection of precancerous lesions with respect to FIT (Table 5). It has been reported that the sensitivity for precancerous lesions obtained by FIT100 in a screening population is substantially lower than the one which could be obtained by RAID-CRC (increase of 30%),[46,47] as well as in terms of advanced neoplasia (increase of 50% by RAID-CRC). In terms of advanced neoplasia, sensitivity might be also much higher for RAID-CRC (80%) than for FIT100 (30%). It is important to highlight that comparisons have been made using different populations: patients with clinical symptoms and average-risk population. Therefore, RAID-CRC results point out the use of this new tool as a potential alternative to FIT100 in a CRC-screening population, nevertheless it must be validated in a screening scenario.

Although gut microbiota and its effects on the human body are increasingly investigated, bacteria have been little studied as indicators of change in the bowel physiology such as the development of

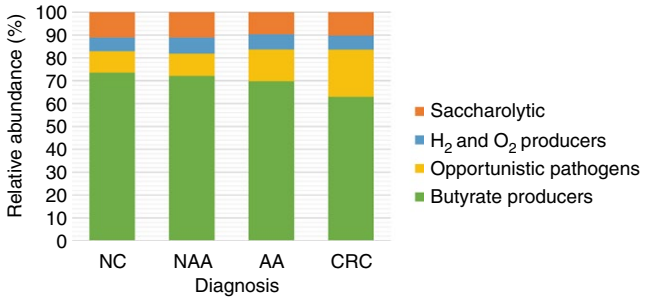

FIGURE 3 Relative abundance in percentage of the analysed biological markers (butyrate producing species: B10, B46, B48, and *R intestinalis* (RSBI); opportunistic pathogens: *G morbillorum* (GMLL), *P stomatis* (PTST), and *B fragilis* (BCTF); H_2 and O_2 producers: *C intestinalis* (CINT); and saccharolytic bacteria: *B thetaiotaomicron* (BCTT)) for subjects with normal colonoscopy (NC), non-advanced adenoma (NAA), advanced adenoma (AA) and colorectal cancer (CRC)

TABLE 6 Comparison of costs associated to follow-up colonoscopies among different CRC-screening programs

Factor	CRC-screening FIT-based[68]	CRC-screening with combined gFOBT and microbiota profiling[26]	CRC-screening with combined FIT and faecal bacterial signature (RAID-CRC)
N° of colonoscopies due to false positive results (per 100 000 screening participants)	47 600	24 750[a]	22 000 (this study)
Costs associated to follow-up colonoscopies after a positive screening result	111 M €[b]	70 M €[b]	51 M €[c]

[a]The savings have been calculated assuming that combining gFOBT with microbiota profiling can increase screening sensitivity more than a 45% relative to gFOBT alone.
[b]Associated costs have been calculated considering that the cost of the test is 25 €.
[c]Associated costs have been calculated considering that the cost of the test is 10 €.

a neoplasm. In the present work we developed a new methodology suitable to be used in national CRC screening programs using stool samples. Bacterial signatures used in this work were originally retrieved from mucosa samples. Therefore, their presence in faeces is not heavily subjected to the variability caused by diet and some external factors[49,50] but is a measure of the real abundance in the colonic mucosa. This helps to overcome the enormous background noise present in stools and provides physiological meaningfulness to the biomarkers.

No metadata on body mass index (BMI), smoking or feeding habits were available on our dataset. Although these parameters have been reported to influence the microbiota composition of faecal samples,[51,52] our biomarkers arise from mucosa samples which are not so dependent on external factors.[56,57] Biedermann et al reported that smoking withdrawal increases microbial diversity.[58] Other studies observed that chronic alcohol consumption leads to an increase in Proteobacteria and a decrease in Bacteroidetes.[59,60] Regarding BMI, its effect on microbiota is controversial.[61,62] Since RAID-CRC is not addressed to a specific population, which includes a variety of conditions and habits, a nonstratification strategy on the basis of these variables is a good way to reproduce with utmost reliability the CRC screening scenario. Another limitation of the study is the method used by patients to collect and conserve stool samples, as they had to collect the sample in a sterile faeces container, froze it using home freezers and transport it to the hospital under cooling conditions. Although acceptability among study participants was high, the procedure followed for sample collection, conservation and transport may be too complex to be implemented in massive CRC screening programs. Moreover, it would take longer to obtain the final results as not only the FIT value would have to be determined but also the relative abundance of the bacterial markers. To overcome this limitation, we have considered to set up the detection of RAID-CRC (FIT and bacterial signature analysis) in the FIT tube collector.

Cost-effectiveness is also a critical issue in population-based screening.[64,65] Wong and co-workers made a comparison of FIT and colonoscopy in this scenario, showing that FIT was cost-effective in average-risk screening, whereas colonoscopy was cost-effective among higher-risk subjects.[67] Therefore, combination of FIT with faecal bacterial markers may be superior in terms of cost-effectiveness, since the use of RAID-CRC would save up to 30% of total colonoscopies. More specifically, the implementation of RAID-CRC in a CRC screening program would result in a reduction of 33 000 colonoscopies due to false positive results when compared to a screening program based on the FOBT (55 000 vs 22 000 false positives, respectively).[68] Considering the cost of RAID-CRC comparable to that of FOBT, the estimated savings in follow-up colonoscopies after positive screening results would be 77 million € per 100 000 participants in the screening program (Table 6). In addition, using the CRC biomarkers presented in this work may achieve both in developed and in resource-deprived regions, where colonoscopy facilities are limited, since RAID-CRC represents a potentially viable, cost-effective tool in a CRC screening scenario.

In conclusion, RAID-CRC is a promising tool for CRC screening because it may achieve a similar sensitivity as the current methodology used in most of the CRC-screening programs (FIT100), with a higher specificity and PPV. We will next seek validation in a screening setting.

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Declaration of personal interests: Prof. Garcia-Gil, Dr. Aldeguer, Dr. Serra-Pagès, Dr. Serrano, Dr. Ramió-Pujol, Mr. Amoedo, Ms. Oliver are employees from GoodGut, company who has received

private and public funding. Prof. Garcia-Gil, Dr. Aldeguer, Dr. Serra-Pagès, Dr. Serrano, Dr. Ramió-Pujol, Mr. Amoedo, Ms. Oliver and Ms. Malagón report grants from MINECO and from CDTI, during the conduct of the study. Prof. Garcia-Gil, Dr. Aldeguer and Dr. Serra-Pagès are also GoodGut shareholders, outside the submitted work; and all of them together with Dr. Mas-de-Xaxars have two licensed patents to GoodGut: EP14382074.4 and PCT/EP2015/054451. The rest of the authors have nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section at the end of the article.

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