Original Article

Analysis of blood markers for early colorectal cancer diagnosis

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Background: Colorectal cancer (CRC) is a very common tumor worldwide. Its mortality can be limited by early diagnosis through screening programs. These programs are based on fecal occult blood testing and colonoscopy. Our objective was to find a model based on the determination of blood biomarkers that was efficacious enough to become part of the early diagnosis of CRC.

Methods: In a total of 221 patients who underwent a colonoscopy, two types of markers were identified (I) classic: carcinoembryonic antigen (CEA), CA19.9, α-fetoprotein, CA125, CA72.4, and ferritin; and (II) experimental: neutrophil gelatinase-associated lipocalin (NGAL), estimated glomerular filtration rate (EGFR), 8-hydroxydeoxyguanosine (8OHdG), calprotectin, and cysteine-rich 61 (Cyr61). We divided the patients into four groups according to colonoscopy results: a control group (n=83) with normal colonoscopy, a polyp group (n=56), a CRC group (n=45), and an inflammatory disease group (n=37). We built an algorithm based on multivariate logistic regression analysis.

Results: A total of 51.6% were males, and the median age was 63 years. We designed an algorithm based on the combination of several markers that discriminated CRC patients from the rest of the patients with a performance of 94%, a sensitivity of 95.6%, and a specificity of 80.6%. Discriminating by sex also resulted in two powerful algorithms, although it performed better in males (97% *vs.* 91%).

Conclusions: Our study has devised a predictive model with high efficacy based on the determination of several biomarkers. We think that it could be incorporated into the set of methods used in CRC screening.

Keywords: Serum biomarkers; noninvasive screening; early detection; proteomics

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Introduction

Colorectal cancer (CRC) is the fourth most common tumor in both sexes worldwide, with a total of more than 1,100,000 cases per year, accounting for 7% of all cancers. Despite having an intermediate prognosis, given its incidence, it results in a large number of deaths, being the second leading cause of cancer mortality with more than 560,000 annual deaths (1). It is a tumor well suited for an effective early diagnosis, since it is a very common tumor, it has a long natural history from the formation of the preneoplastic polyp to invasive cancer, and its prognosis in early phases is very favorable (90% survival).

It is internationally accepted that the general population over 50 years of age is recommended to participate in early

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CRC detection programs (2,3), but controversy exists over the most appropriate method of screening since the sensitivity and specificity are variable (4). Generally, the screen is performed through an annual or biannual fecal occult blood test (FOBT), complemented when necessary with colonoscopy. The results of such programs are favorable and can lead to a decrease in mortality between 16% and 30% (5,6). However, they have limitations such as the high frequency of false positives that entail high costs and the risk of unnecessary colonoscopies, the uncertainty of a low desire to undergo screening, the lack of individualization for high-risk people, the possibility of a false negative if the tumor does not bleed, etc. (6). On the other hand, there are numerous benign colonic pathologies (polyps, chronic intestinal disease, diverticula, etc.) that can cause warning signs indistinguishable from cancer or give positive results in the FOBT test. This non-specificity leads, on the one hand, to many situations of distress for many patients and, on the other hand, to the false reassurance of attributing the alarm symptoms to a benign process in the case of a tumor. Therefore, it is essential to find other complementary methods to optimize the early diagnosis of colon cancer.

The search for a blood marker with sufficient effectiveness is clearly justified (7). If there were a marker or an equation combining several of them, with sufficient sensitivity and specificity to discriminate between benign and malignant pathologies, then colonoscopy, which is an expensive, bloody, and complicated technique, could be reserved for situations with a high probability of cancer, while the rest of the cases could be followed up with periodic controls established by the program. However, studies with various markers in the early phase of colon cancer have not had adequate results, with the most studied being CEA and CA19.9 (8). However, they have shown some utility in various studies when used in combination with other markers or with each other (9). According to international consensus and guidelines, they are useful under other indications, such as periodic monitoring or advanced disease (10,11). Several studies have measured multiple plasma markers to diagnose CRC early (12-15); however, the results and the methods used do not lend themselves to the establishment of a reliable predictive algorithm.

Several genomic molecules (DNA, microRNA, circulating cells, MST1, etc.) have possible roles as biomarkers in early colon cancer (16,17). However, the limited sensitivity or specificity and high cost of these techniques make them unfeasible for wide commercial use (18,19). Another example is the determination of the methylation of Septin9, which despite having 87% sensitivity in early CRC, is to expensive and difficult to detect by PCR for widespread use as a laboratory test (20). Calprotectin is used as a marker mainly in feces, where its concentration is 6 times higher than that found in blood plasma. Its usefulness has been limited to the diagnosis and monitoring of inflammatory bowel disease (IBD), although this marker is also elevated in CRC. Some studies have analyzed it in serum, but as a marker, it has the disadvantage that it is elevated in all processes in which there is leukocytosis (21,22). Sensitivities of 80% and specificities of 70% are associated with occult blood tests (23). Another important marker is Cyr61, which has enough supporting literature to be strongly considered. It is a regulatory protein of the CCN family that has been indicated as a marker of CRC since it is significantly elevated in patients over healthy controls; its value is higher the greater the tumor burden (24). There are other markers that have occasionally been used in colon cancer, but with little specificity, since they can be altered by various situations, such as CA125, ferritin, or CA72.4 (25,26).

Our research group has conducted an extensive study on markers for the early diagnosis of breast cancer (27). We obtained a well-performing algorithm composed of a combination of classic and experimental markers. Based on this experience, we decided to investigate another major disease favoring early diagnosis, CRC. Specifically, colon cancer would be a very suitable target for the experimentation of these types of markers, which have been unexplored in this indication so far. The proposed markers were neutrophil-gelatinase-associated-lipocalin (NGAL), epidermal growth factor receptor (EGFR), and 8-hydrodeoxyguanosine (8OHdG). There are few articles on these as CRC markers, and the few existing ones have studied the expression of these proteins in CRC with high tumor burden and not specifically in the early diagnosis of the disease, thus for the moment leaving the true utility of these molecules unclear (28-34).

The main objective of our study was to establish the utility of the determination of a series of known and experimental tumor markers, either individually or combined into diagnostic algorithms, applied in the early stage of colon cancer. We also aimed to analyze their use to discriminate between different organic diseases of the colon and to assess their potential correlation with the stage or the clinical, endoscopic, and histological activity of these

pathologies. We present the following article in accordance with the STARD reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-747/rc).

Methods

Study design

This was a descriptive, prospective, cross-sectional study evaluating diagnostic tests developed at the Juan Ramón Jiménez Hospital in Huelva, in which the Departments of Digestive, Oncology, Clinical Analysis, and the Research Unit (FABIS, for its initials in Spanish) of the hospital itself and the Department of Integrated Sciences of the University of Huelva participated. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Huelva Provincial Research Ethics Committee (IRB code: PI 018/18, date: May 4th, 2018), and all patients provided oral and written informed consent. All current regulations regarding good research practices have been respected.

Participants

The study included people who underwent routine colonoscopy for any clinical indication from October 2018 to June 2019. In all of them, a complete colonoscopy was performed up to the cecum. According to the colonoscopy results and the corresponding biopsy, the patients were divided into four groups with a minimum expected number of 40, assuming patient losses of 10%. The first group was formed by those patients in whom no organic disease was found in the colon (control group); the second group was formed by patients with benign polyposic lesions; the third group were the patients diagnosed with CRC; the fourth group consisted of patients with IBD, ulcerative colitis, or Crohn's disease in the flare-up phase. Patients with severe cardiopulmonary, liver, or kidney disease; celiac disease; metastatic CRC; a history of previous neoplasia; or other organic processes than those described in the groups above, as well as patients who did not sign the informed consent form, were excluded from the study.

Study methods

The patients were recruited at the endoscopy consultation, where the study was explained to them. They signed the informed consent, and a complete clinical-epidemiological survey was carried out designed for this purpose (age, weight, height, smoking habit, weekly meat intake, weekly intake of vegetables and fruits, alcohol intake, family history, and personal history). Subsequently, a blood test and colonoscopy were performed. In each group, the clinicopathological characteristics of the biopsy were collected. In all patients, hemograms, general biochemistry, and thyroid hormones were studied. The rest of the biopsy sample was processed following established standards and frozen at −80 ℃ under strict conservation and supervision measures. The serum levels of the selected tumor markers were determined. We divided the markers into two groups: (I) classic: CEA, CA19.9, α-fetoprotein, CA125, CA72.4, and ferritin; and (II) experimental: NGAL, EGFR, 8OHdG, calprotectin, and Cyr61. Their serum levels were measured according to the standards established by the manufacturers of the kits, and the quantification was performed by enzyme-linked immunosorbent assay. All authors had access to the study data and reviewed and approved the final manuscript.

Statistical analysis

To verify the normality for quantitative variables, the Shapiro-Wilk test was used. Nonparametric quantitative variables are expressed as the median and interquartile range (IQR, P_{25}–P_{75}), and the qualitative variables are expressed as percentages (%). Pearson's chi-squared test was used for the comparison of percentages, and the chi-squared test for linear trend was used for ordinal variables. Given the nonparametric behavior of the quantitative variables evaluated, the Kruskal-Wallis test was run to detect differences between the four groups, and the Mann-Whitney U test was run to compare the differences between CRC and cancer-free controls. Multivariate logistic regressions were performed through a selection of variables, with the purpose of identifying the independent variables and fitting the relevant clinical covariables. All these analyses were performed with the R Commander 3.6.1 program. The predictor variables were chosen by the backwards step-down method. The calculated sample size was adjusted to that recommended by Peduzzi *et al.* (35), ensuring that the sample size was not lower than n=160 (minimum of 40 per group). We recruited extra patients to compensate for expected losses of 5–10%. The diagnostic feasibility of the algorithm was evaluated using the area under the area under the curve (AUC), the precision, and Nagelkerke's R^2. For the comparison of the different AUCs,

Table 1 Clinical and epidemiological characteristics of the patients included in the study, grouped by pathology

Characteristics	Control (n=83)	Polyps (n=56)	IBD (n=37)	CRC (n=45)	P*
Age (years), median [IQR]	59 [48–68]	65 [60–71]	43 [30.5–50]	72 [66–75]	<0.001
Sex (m/f)	34/49	32/24	20/17	28/17	0.087
Smoker, n (%)	21/83 (25.3%)	13/56 (23.2%)	12/37 (32.4%)	8/45 (17.8%)	0.486
BMI, median [IQR]	25 [24–29]	27 [24–31]	24 [22–37]	27 [24–30]	0.016
Obesity (yes/no)	16/60	14/33	3/33	11/28	0.092
CRC history	34/83 (41%)	26/56 (46.4%)	4/36 (11.1%)	7/45 (15.6%)	<0.001
Tobacco (yes/no)	21/62	13/43	12/25	8/37	0.486
Smoking habit (s/e/n)	21/27/35	12/19/24	12/8/17	7/17/21	0.595
Fruit-vegetable intake (yes/no)	51/32	35/21	14/23	30/15	0.038
Red meat intake (yes/no)	9/74	9/47	0/45	3/34	0.051
Alcohol intake (yes/no)	17/65	18/38	10/27	12/33	0.509
Physical activity (yes/no)	45/38	35/21	24/13	26/19	0.655

Smoking habit: smoker/ex-smoker/non-smoker (s/e/n); Fruit-vegetable intake: yes ≥3 servings/day, no <3 servings/day; Red meat intake: yes ≥3 times/week, no <3 times/week; (*) Kruskal-Wallis test. IQR, interquartile range (P25–P75); m, male; f, female; BMI, body mass index; CRC, colorectal cancer; IBD, inflammatory bowel disease.

the nonparametric Delong test was used with the statistical package R-Commander 3.6.1. Later, a bootstrap resampling method was applied to carry out an internal and unbiased validation of the selected model. We considered a 5% alpha error level to be significant.

Results

Initially, a total of 227 people were included, of whom 6 patients (2.7%) were excluded: four for refusing blood collection and two for insufficient samples. The final cohort was 221 patients. The sex distribution was even (males 51.6%), and the median mean age was 63 years (IQR, 49–77 years). Age did not present significant differences between males (64 years; IQR, 50–72) and females (61; IQR, 47–68) (P=0.07). All patients were distributed as follows: The control group consisted of 83 individuals where no type of histological alteration was found, except in 10 individuals who had colon diverticula. The polyp group was composed of 56 patients (benign n=9, malignant potential without dysplasia n=1, malignant potential with low dysplasia n=38, malignant potential with high dysplasia n=5, unclassified n=3). The CRC group consisted of 45 patients, a majority of whom had early-stage CRC (64% stages 0–I–II and 36% stages III). The IBD group had 37 patients (14 ulcerative colitis, 19 Crohn's disease, four mixed or undefined). Family history of CRC and meat consumption were greater in cancer-free groups, while age was higher in patients with CRC. No significant differences were found in other factors (*Table 1*).

The serum levels of CEA, CA72.4 and calprotectin allowed discriminating between the four study groups, with statistically significant differences (P values <0.01), being higher in patients with CRC. Comparing the significance of the markers, we observed that the high reactivity of patients with IBD led the P values of the Kruskal-Wallis test (post hoc analysis) to be stronger for some inflammatory markers, such as ferritin and CA125 (P<0.01), which hindered the individual validation of these analytes. EGFR, hemoglobin, and serum ferritin were lower in cancer patients than the other groups (P<0.01) (*Figure 1*).

We quantified the statistical significance of the differences between the CRC and the others using the Mann-Whitney U test (*Table 2*). Some markers analyzed discriminated between the two groups. The greatest differences between groups were again found with the markers CEA, CA19.9, calprotectin, and CA72.4 (P<0.001) in patients with CRC, while ferritin, hemoglobin, and EGFR were higher in individuals without cancer (P<0.01) (*Figure 2*).

Finally, we evaluated the diagnostic capacity of each analyte by calculating its AUC (*Figure 3*). No separate

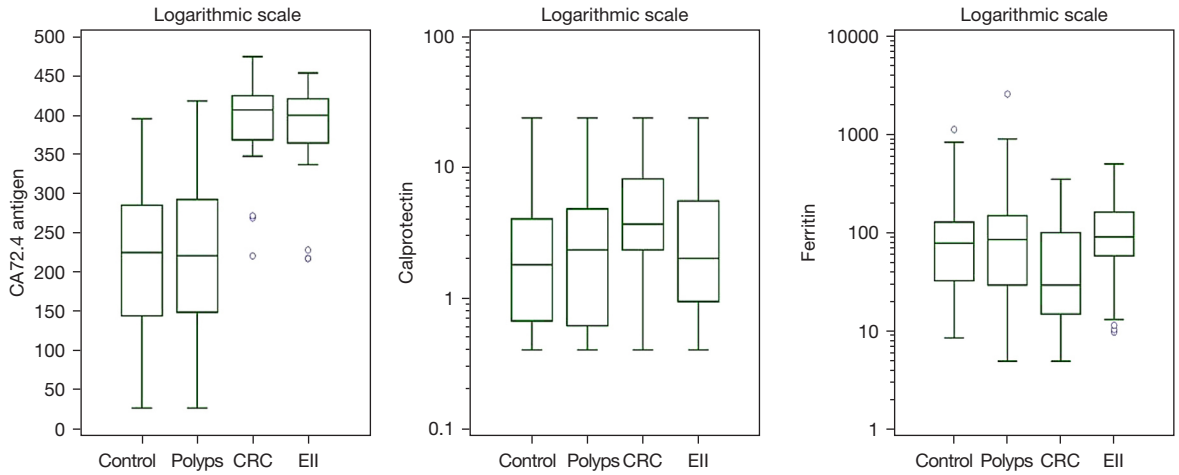

Figure 1 Bivariate analysis of several of the markers studied. CA72.4, calprotectin and ferritin respectively.

Table 2 Bivariate analysis of the different variables using the Mann-Whitney U method

Serum marker	Non-CRC		CRC		P
	Median	SD	Median	SD	
CEA (ng/mL)	1.19	0.05	1.77	0.35	<0.001
CA125 (U/mL)	8.33	0.32	7.19	1.25	0.4323
AFP (ng/mL)	1.10	0.11	1.16	0.18	0.7917
CA19.9 (U/mL)	6.43	0.761	9.55	2.22	0.0243
Hemoglobin (g/dL)	14.20	0.17	12.4	0.15	<0.001
NGAL (ng/mL)	11.38	0.77	12.86	0.99	0.2703
EGFR (ng/mL)	131.65	5.21	96.85	8.50	<0.001
8-OHdG (pg/mL)	2,535.29	112.39	2,677.99	185.91	0.4254
Calprotectin (µg/mL)	2.20	0.47	3.68	1.14	<0.001
Ferritin (mg/dL)	82.70	9.65	29.50	17.16	<0.001
CA72.4 (U/mL)	249.20	20.88	406.69	5.23	<0.001
Cyr-61 (ng/mL)	6.79	0.59	7.29	1.52	0.6325

CRC, colorectal cancer; SD, standard deviation; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; NGAL, neutrophil gelatinase-associated lipocalin; EGFR, estimated glomerular filtration rate; 8-OHdG, 8-hydroxydeoxyguanosine; Cyr-61, cysteine-rich 61.

biochemical marker was sufficient for the correct diagnosis of CRC patients, so we proceeded to combine the most significant covariates form the bivariate analysis (P<0.25) to use them jointly in multivariate diagnostic algorithms. The multivariate analysis allowed the construction of a binary logistic regression model that included certain independent variables. This model presented a Nagelkerke's R^2=0.656 and an AUC =0.94 (*"colonmarker model"*).

The models obtained were the following:

Males ("colonmarker male"):

Logit(P)= –110.034 + 8.116*A* + 2.133*B* + 15.907*C* – 1.393*D*

P: *Probability of having cancer*

Females ("colonmarker female"):

Logit(P)= –33. 71 + 2.1816[a] + 5.6266C – 0.8678D

Note: *The models obtained are subject to intellectual property law and cannot be fully published.*

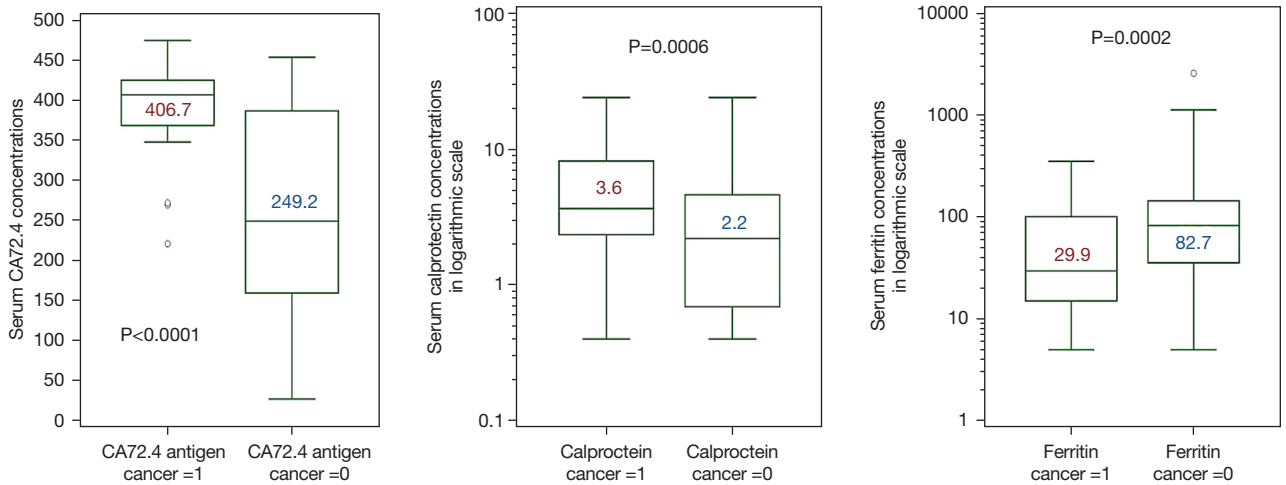

Figure 2 Bivariate analysis between the two groups of several of the markers studied. CA72.4, calprotectin and ferritin respectively.

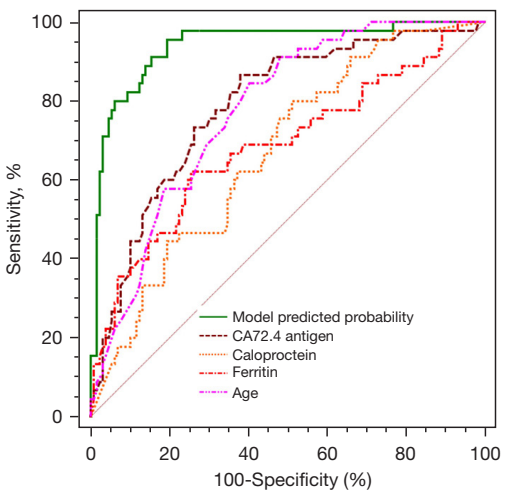

Figure 3 ROC curve colon marker algorithm (model) versus individual markers. ROC, receiver operating characteristic.

A better performance was found in males (0.966, 0.91–0.99) than in females (0.905, 0.84–0.96) (*Table 3*). Using the Youden index, we selected the cutoff point at 0.1937, the algorithm reached a sensitivity of 96.4% with a specificity of 79.7% in males; setting the cutoff at 0.2317, it had 94.1% sensitivity and 83.1% specificity in females (*Figure 4*).

As a complementary approach, we searched for marker combinations with an artificial intelligence algorithm, which achieved interaction terms that resulted in numerous combinations of markers that could trigger a prespecified threshold score, resulting in a precise positive or negative test. Specifically, very high sensitivities and specificities were obtained.

Discussion

CRC screening is a health strategy that reduces mortality and is accepted worldwide as a diagnostic test (5). For this reason, it is important to optimize the quality of the process and improve patient adherence to the programs (36). On the other hand, the need to find a noninvasive method for the early diagnosis of CRC is a public health priority. A simple method such as measuring biomarkers from a simple blood collection could determine the precise indication for colonoscopy, avoiding unnecessary costs and complications. It would also gain accuracy and acceptability, with the ability to repeat frequently if required. Currently, there are very few options for CRC biomarker screening, despite intense research in this area (37-39). In 2014, the U.S. Food and Drug Administration (FDA) approved multitarget stool DNA testing, a test performed on a stool sample where abnormal DNA markers released into the intestinal tract by neoplastic cells are detected (40-42). Its use was included in the main clinical guidelines (43,44) and is one of the most widely used methods along with FOBT and colonoscopy. Despite having high sensitivity, its practical management, three-year frequency of use, limited specificity, low availability for people who refuse colonoscopy, and cost are barriers to its wider implementation (45,46). The only blood test approved by the FDA and other international agencies is the one that detects circulating particles of the methylated *SEPT9* gene, but its limited sensitivity implies

Table 3 Performance of the algorithms obtained in the multivariate study (colon marker/colon marker male/colon marker female)

Sex	R^2 Nagelkerke	Accuracy (%)	AUC (95% CI)	Cut-off point	S (%)	E (%)
Global	0.656	89.7	0.94 (0.90–0.98)*	0.1946	95.6	80.6
Males	0.814	90.2	0.966 (0.91–0.99)	0.1937	96.4	79.7
Females	0.545	91.5	0.905 (0.84–0.96)	0.2317	94.1	83.1

*, P<0.05. AUC, area under the ROC curve; 95% CI, confidence interval at 95% of the area under the ROC curve; S, sensitivity; E, specificity; ROC, receiver operating characteristic.

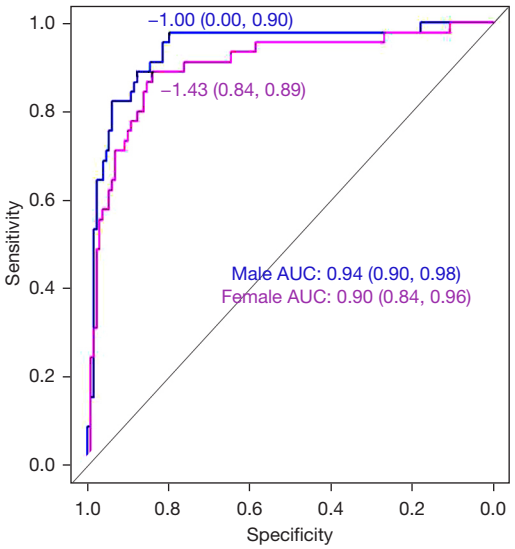

Figure 4 ROC curve male and female colon marker algorithm. AUC, area under the curve; ROC, receiver operating characteristic.

that it is not recommended for routine screening (47,48).

Therefore, our study represents a possible new opportunity in a field with few available alternatives. The logistic regression selected several markers such as those analytes that, when combined, provided the greatest diagnostic capacity, discarding potentially redundant variables. Several predictive equations were obtained that were used to detect cases of CRC in both healthy subjects and patients with related digestive diseases of benign nature. Our algorithms performed very well in the diagnosis of CRC, surpassing other available options, with sensitivities greater than 95% and specificities greater than 80%, making it a potentially valid test to incorporate into screening.

Further, the test has the strength, unlike the rest of the tests, that it is more comfortable for patient, is reproducible, is easily implemented in any laboratory, and is effective, so it can also be repeated when necessary. For this reason and given its safety, it could eventually be used in the management of patients with warning signs, since a positive test result would justify performing an urgent colonoscopy, by which an initial diagnosis of the disease could be made, thereby increasing survival and healing. It would also be useful for close monitoring of patients who refuse to undergo colonoscopy, those with incomplete colonoscopies, or people in the high-risk group even from an early age.

All these relevant aspects are even more crucial in the current era of the COVID-19 pandemic. Global health systems are overburdened with caring for patients with COVID-19, which implies delays in diagnostic testing and cancer prevention programs. In this context, a reliable test that optimizes the diagnosis of CRC could compensate for the foreseeable worsening of the spread of this disease.

We have taken into account the limitations of the study, and we think that they do not decrease its reliability or subtract from the favorable results. On the one hand, it was a single-center study, but the patients included were different clinically and had different diagnostic characteristics. The number recruited was small, but the various commercial tests used have been approved in similar situations with initial pilot studies and then have been validated with larger studies (20). Although there were some data losses in the control group, either due to refusal to extract or due to insufficient sample, the losses were few. In any case, these patients were excluded and were not part of the data analysis. The population included in the study is at risk, which would limit extrapolation to the PDP target population whose prevalence of colon cancer is very low. Due to this potential limitation, the algorithm would support other diagnostic techniques, but we cannot yet confirm that it can replace them as a single test. However, this limitation is common, due to the difficulty of prospective studies of having to include thousands of controls to obtain the necessary number of cases. The groups had similar characteristics, with minimal differences

that did not alter the final results of the study. We also controlled for the most frequent biases in this type of study, such as selection bias, overfitting, and false positive bias, which implies the existence of well-identified nonneoplastic diseases in the subgroups. In our study, a predictive model for dysplastic polyps was not specifically planned since the low number of recruited cases could cause a β error. In fact, our main objective was to discriminate between cancer and other colorectal diseases, including polyps, in order to avoid false positives and overdiagnosis of early benign lesions. Such a situation can involve repeated and unnecessary intervention on benign lesions, which makes screening less effective (49,50). The possibility of repeating the test periodically, for example, every 6 months, and the long natural history of colonic dysplasia, facilitate the early diagnosis of invasive cancer without the need for multiple detections of benign lesions. In any case, a negative test result, even if it was a dysplastic polyp, would always allow the colonoscopy to be deferred according to the availability of the service, thus being a more economical and accessible resource.

Currently, our group has designed a multicenter study to improve and consolidate our algorithm, which will give it greater external validity. In that study, in addition to confirming the diagnosis of CRC, we will define a new specific model for the subgroup of dysplastic polyps with neoplastic potential, independent of the cancer model or other benign lesions. In short, our test could be an effective, fast, automated, reliable, and noninvasive tool for cancer detection and preclinical diagnosis in patients with CRC.

In conclusion, CRC screening has limitations and needs to be optimized by the addition of complementary methods. The determination of blood protein markers is an unbloody, economical, and reproducible method. The model that we have described in our study has high sensitivity and specificity and could be useful as another method to incorporate into CRC screening.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-747/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-747/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-747/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-21-747/coif). JBC's work (research) was financed by Bioprognos SL. as well as the kits of the different markers (material). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Huelva Provincial Research Ethics Committee (IRB code: PI 018/18, date: May 4th, 2018), and all patients provided oral and written informed consent. All current regulations regarding good research practices have been respected.

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