



Research Article

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The Combined Value of Faecal Haemoglobin and Calprotectin in Diagnosis of Colorectal Cancer in Symptomatic Patients Referred to Colonoscopy

Vincy Eklöf¹, David Lundgren², Pontus Karling², Maria L Wikberg¹, Sofia Edin¹, Anna Löfgren-Burström¹, Jörgen Rutegård³ and Richard Palmqvist^{1*}

¹Department of Medical Biosciences/Pathology, Umeå University, Sweden

²Department of Public Health and Clinical Medicine, Umeå University, Sweden

³Department of Surgical and Perioperative Sciences, Umeå University, Sweden

*Corresponding author: Richard Palmqvist, Department of Medical Biosciences/Pathology, Umeå University, Sweden.

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Abstract

Aim: To investigate the diagnostic value of a combined analyses of faecal immunological haemoglobin (FIT) and faecal calprotectin (FC) in detection of colorectal cancer (CRC).

Methods: Out-patients (n=1440) referred to the endoscopy unit were analysed for FIT and FC in stool samples collected before the colonoscopy bowel preparation. The samples were collected from one defecation by the patients at home. Patients with IBD were excluded leaving stool samples from 1133 patients for further analyses. FIT was analysed using the immunological Analyse F.O.B Test and FC was analysed using the CALPRO® Calprotectin Elisa Test. Sensitivity and specificity to detect CRC was calculated for the individual tests, as well as for combined FIT/FC tests.

Results: Out of the included patients, 38 were diagnosed with CRC, 9 with high grade dysplasia (HGD), and 133 with low grade dysplasia (LGD). FIT was analysed in 673 (59.4%), FC in 1021 (90.1%) and both FIT and FC in 561 (49.5%) patients. A ROC curve analysis showed that the most accurate cut-off level for FC in detecting CRC in our study was 105.5 µg/g. The sensitivity for CRC when using FIT, FC (cut-off > 100 µg/g) and the combination of FIT and FC (at least one positive test) was 65.5%, 74.1% and 94.4%, respectively. The corresponding specificity was 84.8%, 74.9% and 68.3%, respectively.

Conclusion: Combined analyses of FIT and FC improved sensitivity for detection of CRC. Further studies in larger cohorts are required to find the optimal cut-off levels for different combinations of tests.

Keywords: Colorectal cancer; F-Calprotectin; F-Hb; Faecal markers; Screening markers

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and has a considerable impact on both the individual and the health care system. The majority of patients with CRC have their initial consultation in primary care [1]. However, the symptoms of CRC often present late. In addition, a vast majority of patients seeking primary care for symptoms associated with CRC (rectal bleeding, a change in bowel habits, diarrhoea, constipation and abdominal pain) are not diagnosed with CRC [2-4]. Therefore, general screening programs among individuals at average risk for CRC, along with guidelines for urgent referral, are implemented widely to reduce mortality of the disease [5,6]. However, there is still a need for improved screening strategies for CRC [4,7,3]. The

recommended "gold standard" screening tool for CRC today is endoscopic examination, such as sigmoidoscopy or colonoscopy, but such examinations are resource-demanding, highly costly and inconvenient for the patients [8-11]. The most important factor in screening is patient adherence, and therefore annual faecal occult blood tests have been suggested as an alternative to endoscopy in CRC screening [7]. Analysis of faecal haemoglobin (F-Hb) using either guaiac-based (gFOBT) tests or, more recently, immunological (FIT) tests [12] is commonly used as a primary screening tool, since it requires no preparation, is cost-effective, and relatively convenient for the patient [13]. A positive F-Hb test is an indication of bleeding in the gastrointestinal (GI) tract, which could be caused

by a premalignant adenoma or an adenocarcinoma. A healthy individual loses in average 1-2 ml of blood per day in the GI-tract. A test for F-Hb therefore needs to be adjusted for this normal blood loss. Another limitation of F-Hb tests, in the ability to discriminate between blood loss associated with a neoplastic lesion from that of normal blood loss in the GI-tract, is that the neoplastic lesions often bleed intermittently [14-16].

The sensitivity of different F-Hb tests vary depending on method and number of repeated tests [17,18]. FIT is more specific than gFOBT in detecting bleeding from colon, since it can distinguish human haemoglobin [17,16,18]. Dependent on the analysis method and the cut-off used, a single FIT test shows a sensitivity of 66-82% and a specificity of 92-98% [19-21]. No additional diagnostic value has been found for repeated testing using FIT [22,23]. FIT tests are thus highly specific but have limited sensitivity in detecting neoplastic lesions in the colon, therefore combined tests using additional faecal markers have been suggested [24,7,25]. One interesting marker is faecal calprotectin (FC). Calprotectin is a Ca- and Zn-binding protein, which is located in the neutrophil leucocytes. FC is protease resistant, stable in the GI-tract [26] and widely used in diagnosis and evaluation of inflammatory bowel disease (IBD). Recently it has also been evaluated as a potential marker in the context of cancer screening, since it has been shown that the level of FC is increased in stool of patients with CRC [26-31] and is decreased after CRC surgery [32].

The rationale of combining analyses of F-Hb and FC is that a combined test can detect bleeding in the GI-tract as well as inflammatory changes associated with CRC. In addition, FC exhibit less variation within patients with CRC than F-Hb [33]. The aim of the present investigation was to assess the value of a combined FIT and FC test in detection of colorectal neoplasia. We hypothesized that a combination of a single FIT and FC test can improve sensitivity in detection of CRC in comparison to using FIT alone. The prospective study was performed in patients referred for colonoscopy to the endoscopy unit on suspicion of CRC. At the time of the study, there was no general screening program for CRC in the catchment area.

Materials and Methods

Study cohort

This prospective study is based on a patient cohort recruited during 2008-2013 at the University Hospital, Umeå, Sweden. Out-patients scheduled for colonoscopy were invited to participate in the study. Indications were changes of bowel habits and/or alarm features (e.g. weight loss) and/or signs of gastrointestinal bleeding (anemia, iron deficiency, positive FIT or visible bleeding). Exclusion criteria were planned colonoscopy less than one week ahead and low performance status and/or cognitive dysfunction. The study protocol was approved by the Research Ethics Committee of Umeå university, Umeå, Sweden (Dnr 08-184M and 07-045M) and the study has been performed in accordance with the ethical standards laid down in the Declaration of Helsinki. All patients gave their informed consent prior to their inclusion in the study. Study information, informed consent form and tubes for stool sample

collection were sent to the patients together with the invitation for the clinical colonoscopy examination. All participants were asked to leave faecal samples before the colonoscopy cleansing procedure started. The endoscopy was performed in clinical routine. Lesions found were biopsied or if indicated removed. The outcome of the endoscopy was routinely recorded in the patient medical records. All clinical findings were retrospectively verified by studying the patient medical records, including the pathologist reports. For patients with several lesions, the most severe lesion was recorded. All patients and all medical personal, including endoscopists and pathologists, were blinded for the results of the faecal tests.

Selection of study patients

Of the out-patients referred for colonoscopy during the study period, 1440 signed an informed consent. One hundred and fifteen patients were excluded due to missing faecal samples and 192 patients diagnosed with IBD were also excluded in the present study. After inclusion and exclusion criteria were fulfilled, 1133 patients remained in the final analysis.

Stool collection and analyses

Three tubes were sent to the patients, one for FC analysis, one for FIT analysis, and one containing 5 ml stabilization buffer, RNAlater® (Applied Biosystems, Life Technology, Stockholm, Sweden). The collected samples were stored in room temperature in maximally seven days before taken care of. The tubes for FC were sent directly to the accredited Clinical Chemistry Laboratory at the University Hospital in Umeå for analysis. FIT was analysed as described below.

Faecal markers

FIT was analysed using the immunological Analyse F.O.B Test (FIT) (ANL products AB, Sweden), according to manufacturer's instructions. The FIT test was scored as positive or negative, with a positive test indicating > 40 ng/ml of human haemoglobin. The FC samples were sent to the accredited Department of Laboratory Medicine, Clinical Chemistry, Umeå University Hospital, and analysed using the CALPRO® Calprotectin ELISA Test (ALP) according to the manufacturer's instructions (Calpro AS, Norway). The measuring range for FC is between 20 µg/g and 10000 µg/g. Values out of range were recorded as < 20 µg/g and > 10000 µg/g.

Statistical analysis

Differences in baseline characteristics and study variables were compared by Fischer's test or χ^2 tests. Sensitivity and specificity for continuous FC were calculated using ROC-curve analysis, giving area under curve (AUC). Sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) at group level for FIT and FC were calculated. All statistics was calculated using SPSS version 23.0 (Chicago, Illinois, USA).

Results

Patient characteristics

Out-patients referred to the endoscopy unit were analysed for FIT and FC in stool samples collected in parallel before the bowel

preparation. Of the 1133 patients included in the study, FIT was analysed in 673 (59.4%), FC in 1021 (90.1%) and both FIT and FC in 561 (49.5%) patients (Table 1). The included patients had an equal gender distribution and a median age of 65.4 years. Overall, 827 (73.0%) of the patients were recorded as normal with no significant findings. Adenocarcinoma and high-grade dysplasia were recorded in 47 patients (4.1%). The distribution of the pathological findings is shown in (Table 1).

Table 1: Clinical characteristics of the study participants (n=1133).

n (%)	
Age	
<59	374 (33.0)
60-69	382 (33.7)
70-79	307 (27.1)
>80	70 (6.2)
Sex	
Men	542 (47.8)
Women	591 (52.2)
Indications for referral*	
Occult blood	205 (20.7)
Rectal bleeding	174 (17.6)
Anaemia	353 (35.7)
Weight loss	145 (14.7)
Change in bowel habits	44 (4.4)
Abdominal pain	148 (15.0)
Family history of colorectal cancer	67 (6.8)
Not specified	82 (8.3)
Pathological classification	
Normal	827 (73.0)
Hyperplastic polyp	78 (6.9)
LGD	133 (11.7)
HGD	9 (0.8)
Adenocarcinoma	38 (3.4)
Other	48 (4.2)
FC 100 µg/g (n=1021)	
Negative	752 (73.7)
Positive	269 (26.3)
FIT (n=673)	
Negative	556 (82.6)
Positive	117 (17.4)

FC, faecal calprotectin; FIT, faecal immunological test for hemoglobin; LGD, low grade dysplasia; HGD, high grade dysplasia. *Multiple indications possible.

FIT

In total, 17.4% of the patients had a positive FIT result (Table 1 and 2). There was no significant difference in gender distribution in the outcome of FIT, but a weak correlation was found to age (Table 2). Patients with CRC had significantly more often a positive FIT than patients without CRC ($P > 0.0001$). However, in 34% (10 out of 29) of the patients with CRC the FIT was negative. Also, 67% (4 out of 6) of the patients with high grade dysplasia had a negative FIT.

The sensitivity and specificity for FIT in detecting CRC and CRC/HGD are shown in (Table 3).

FC

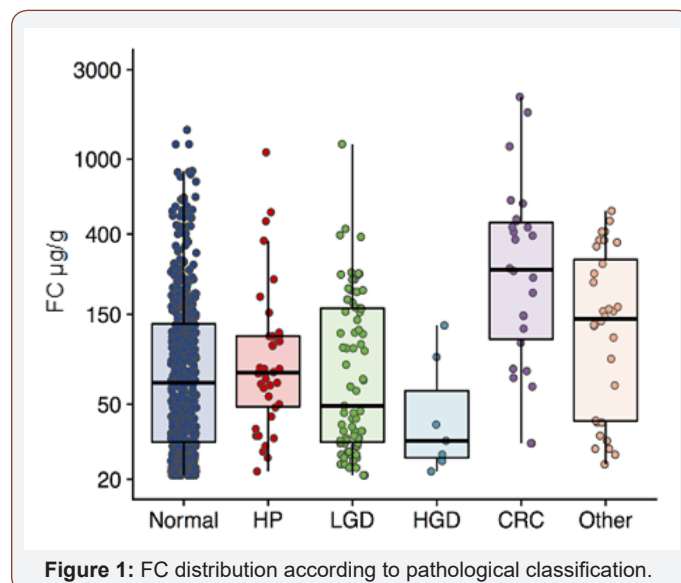


Figure 1: FC distribution according to pathological classification.

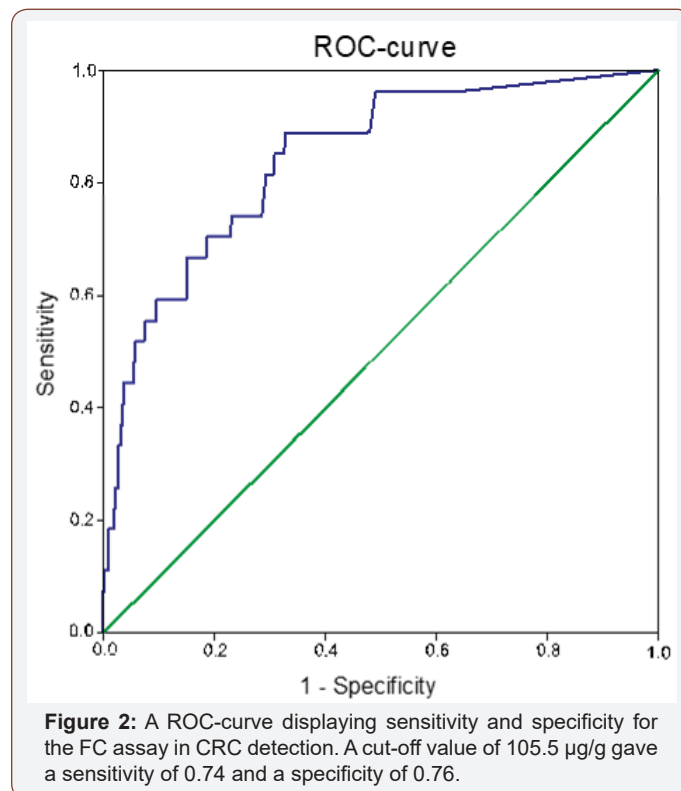


Figure 2: A ROC-curve displaying sensitivity and specificity for the FC assay in CRC detection. A cut-off value of 105.5 µg/g gave a sensitivity of 0.74 and a specificity of 0.76.

The distribution of FC concentrations according to pathological classifications can be found in (Figure 1). A ROC curve analysis revealed that the most accurate cut-off level for FC in detecting CRC in our study was 105.5 µg/g (Figure 2), which is close to the commonly used cut-off value (≤ 100 µg/g) in clinic today. The proportion of subjects with a FC > 100 µg/g was significantly higher in older patients and in patients with CRC (Table 2). A cut-off of > 100 µg/g resulted in a sensitivity of 74.1% and a specificity of 74.9% (Table 3). Seventy-four percent (20 out of 27) of the patients with CRC had an FC > 100 µg/g, while only 11% (1 out of 9) with high grade dysplasia had elevated FC levels (Table 2). When using

a lower cut-off for FC (> 50 µg/g) the sensitivity to detect CRC increased to 88.9 %, but specificity was reduced to 61.8% (Table 3). Using a higher cut-off (> 200 µg/g) instead decreased sensitivity

to 55.6%, but increased specificity to 89.7%. The sensitivity and specificity for FC to detect CRC/HGD is shown in (Table 3).

Table 2: Characteristics of study patients according to FIT and FC, and the combination of FIT and FC (FIT/FC).

	FIT n (%)			FC n (%)			FIT/FC n (%)			
	Negative	Positive	P	Negative ≤100 µg/g	Positive >100 µg/g	P	Both tests negative	At least one test positive	Both tests positive	P
Age category (%)			0.026			<0.0001				<0.0001
<59	195 (35.1)	28 (23.9)		303 (40.3)	48 (17.8)		158 (42.5)	29 (19.7)	13 (31.0)	
60-69	190 (34.2)	37 (31.6)		254 (33.8)	90 (33.5)		129 (34.7)	51 (34.7)	9 (21.4)	
70-79	140 (25.2)	43 (36.8)		159 (21.1)	105 (39.0)		69 (18.5)	53 (36.1)	18 (42.9)	
>80	31 (5.6)	9 (7.7)		36 (4.8)	26 (9.7)		16 (4.3)	14 (9.5)	2 (4.8)	
Gender (%)			0.153			0.829				0.663
Men	259 (46.6)	63 (53.8)		358 (47.6)	126 (46.8)		170 (45.7)	73 (49.7)	21 (50.0)	
Women	297 (53.4)	54 (46.2)		394 (52.4)	143 (53.2)		202 (54.3)	74 (50.3)	21 (50.0)	
Pathological classification (%)			<0.0001			<0.0001				<0.0001
Normal	381 (68.5)	55 (47.0)		581 (77.3)	184 (68.4)		268 (72.0)	84 (57.1)	22 (52.4)	
Hyperplastic polyp	47 (8.5)	10 (8.5)		50 (6.6)	15 (5.6)		29 (7.8)	13 (8.8)	2 (4.8)	
LGD	88 (15.8)	25 (21.4)		83 (11.0)	28 (10.4)		57 (15.3)	30 (20.4)	4 (9.5)	
HGD	4 (0.7)	2 (1.7)		8 (1.1)	1 (0.4)		3 (0.8)	3 (2.0)	0 (0.0)	
CRC	10 (1.8)	19 (16.2)		7 (0.9)	20 (7.4)		1 (0.3)	7 (4.8)	10 (23.8)	
Other	26 (4.7)	6 (5.1)		23 (3.1)	21 (7.8)		14 (3.8)	10 (6.8)	4 (9.5)	

CRC, colorectal cancer; FC, faecal calprotectin; FIT, faecal immunological test for hemoglobin; LGD, low grade dysplasia; HGD, high grade dysplasia. Statistics: The χ^2 test or Fisher's exact test was used for categorical variables. P-value <0.05 was considered statistically significant.

Table 3: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for CRC and CRC/HGD in study patients according to FIT, FC or combinations of FIT/FC.

	Sensitivity (%)		Specificity (%)		PPV (%)		NPV (%)	
	CRC	CRC/HGD	CRC	CRC/HGD	CRC	CRC/HGD	CRC	CRC/HGD
FIT	65.5	60	84.8	85	16.2	17.9	98.2	97.5
FC > 50 µg/g	88.9	72.2	61.8	61.6	5.9	6.4	99.5	98.4
FC > 100 µg/g	74.1	58.3	74.9	74.8	7.4	7.8	99.1	98
FC > 200 µg/g	55.6	41.7	89.7	89.6	12.8	12.8	98.7	97.7
At least one positive test								
FIT/FC > 50 µg/g	94.4	83.3	58.6	58.7	7	8.3	99.7	98.7
FIT/FC > 100 µg/g	94.4	83.3	68.3	68.5	9	10.6	99.7	98.9
FIT/FC > 200 µg/g	94.4	79.2	79.4	79.5	13.2	14.7	99.8	98.8
Both tests positive								
FIT/FC > 50 µg/g	72.2	54.2	92.4	92.4	24.1	24.1	99	97.8
FIT/FC > 100 µg/g	55.6	41.7	94.1	94	23.8	23.8	98.5	97.3
FIT/FC > 200 µg/g	38.9	29.2	97.1	97	30.4	30.4	98	96.8

CRC, colorectal cancer; HGD, high grade dysplasia.

The combined FIT and FC test

The sensitivity and negative predictive value (NPV) improved when combining the FIT and FC test (at least one positive test) (Table 3). Ninety-four percent (17 out of 18) of the patients with CRC had at least one positive test for the two markers. A test combining FIT and FC (at least one positive test) had a sensitivity of 94.4% and a specificity of 68.3%. The sensitivity and NPV did

not differ for the different "FC cut-offs" but specificity and positive predictive value (PPV) was higher for FC > 200 µg/g. Instead, a test combining FIT and FC (both tests positive) for CRC had a higher specificity (94.1%), but the sensitivity was decreased to 55.6% (Table 3). For this test, an FC cut-off of > 50 µg/g gave the highest sensitivity. Again, the sensitivity for detecting both CRC and HGD was generally lower than for detecting CRC alone (Table 3).

Discussion

This study aimed to test the combination of FC and FIT in the detection of CRC in out-patients referred for colonoscopy. The FC test using the cut-off > 100 µg/g, showed a slightly better sensitivity (74%) in detecting CRC than FIT (66%), but at the cost of a poorer specificity (75% compared to 85%). In addition, the proportion of patients with FC > 100 µg/g was low in subjects with precancerous colonic lesions. When creating a combined model of FIT and FC (cut off > 100 µg/g), where a positive score was given to patients positive for at least one of the markers, the sensitivity for CRC increased to 94%. The specificity for the combined model (at least one positive test) was acceptable (68%). The accuracy of a test as an adequate marker of disease depends on the used detection level (cut-off). A low cut-off level improves sensitivity but often at the cost of a lower specificity [34]. Also, in our study, a low cut-off level for calprotectin (> 50 µg/g), when used as a single test, improved sensitivity but decreased specificity for CRC. In general, there are two strategies for detecting CRC using faecal markers. One option is using a single sensitive marker with a high NPV, but often such a test also will have a low specificity and a low PPV [34]. An alternative is to select markers that alone are insufficiently sensitive but in combination have improved sensitivity. The rationale of combining F-Hb and FC is that CRCs that due to intermittent bleeding are not detected by F-Hb tests, may still be detected by the combined test due to a possible low-grade inflammation at the tumour. In (Table 4), we present data

on studies that have evaluated different methods for combining F-Hb tests and FC tests in detection of CRC. In three of these studies no additional benefit was found for the combined test [31,34,35], but in our study and three others the sensitivity increased when combining F-Hb and FC tests [36,37,24]. The challenge is to find the most accurate cut-off level for the specific method used. Combining the right mix of markers with adequate cut-off levels may lead to a highly sensitive test with retained specificity. There are several FC tests on the market using different methods (ELISA, fluorescence enzyme immunoassay, immunochromatography), different antibodies (monoclonal or polyclonal) and different measuring ranges that makes it hazardous to translate test results between different methods [38]. For example, the Bühlmann method shows higher values of calprotectin than the other FC methods [39]. There are also other factors that influence the outcome of a faecal test, such as accidental dilution by water/urine [38] and the transit time in colon [40]. There is still uncertainty of the most accurate cut-off value for FC and how to interpret intermediate values [38]. It is therefore, important to generate functional cut-off levels for FC methods in clinical practice. For the CALPRO® Calprotectin ELISA Test used in the present study the most accurate cut-off level of FC to detect CRC was 105.5 µg/g. For a combined test of FIT and FC (at least one positive marker), a cut-off for FC of > 200 µg/g gave a more specific test, while for the combined FIT/FC test (both markers positive) a reduced cut-off for FC of > 50 µg/g gave a more sensitive test.

Table 4: The sensitivity and specificity for the combination (at least one positive test) of F-Hb and FC in patients with CRC.

Faecal marker method and cut-off			CRC cases (n)	Control subjects (n)	Sensitivity (%)			Specificity (%)			Comments
	FC	F-Hb			FC	F-Hb	FC/F-Hb	FC	F-Hb	FC/F-Hb	
Tibble 2001	ELISA test Quantitative test cut-off >50 µg/g	gFOBT 3 samples	62	229	90	58	90	72	92	NA	Two different groups included (known CRC cases and patients referred to endoscopy)
Hoff 2004	PhiCal test Quantitative test cut-off >50 µg/g	gFOBT (Hemocult II) 3 samples	12	654	67	75	92	75	88	NA	CRC colonoscopy screening cohort
Parente 2012	Bühlman ELISA Quantitative test cut-off >50 µg/g	FIT cut-off ≥ 100 ng/mL single sample	47	233	86	62	91	40	89	36	Patients referred to colonoscopy Patients with IBD excluded
Kok 2012	Bühlman ELISA Quantitative test cut-off >50 µg/g	FIT Clearview > 6 µg/g single sample	19	363	95	84	95	NA	NA	NA	Primary care Patients with IBD included
Mowat 2016	Bühlman Quantitative test cut-off >50 µg/g	FIT OC- sensor (Eiken chemical co.) cut-off detectable Hb single sample	28	727	82	100	100	39	43	20	Patients referred from primary care. Patients with IBD included
Högberg 2017	CALPRO ELISA Quantitative test cut off >100 µg/g	FIT cut-off > 50 ng/mL 3 samples	8	365	50	88	88	85	67	61	Primary care Patients with IBD included
Widlak 2017	EliA immunoassay Quantitative test cut-off >50 µg/g	FIT HM-JACKarc analyzer (Kyowa Medex) > 7 µg/g single sample	25	405	68	84	84	84	93	93	Patients referred to colonoscopy Patients with IBD included
Eklöf (present study)	CALPRO ELISA Quantitative test cut off >100 µg/g	FIT cut off > 40 ng/mL single sample	18	561	74	66	94	75	85	68	Patients referred to colonoscopy Patients with IBD excluded

CRC, colorectal cancer; FC, faecal calprotectin; F-Hb, faecal hemoglobin; gFOBT, guaiac based faecal occult blood tests; FIT, faecal immunological test for hemoglobin; IBD, inflammatory bowel disease; NA, not available; FC/F-Hb, combined test with at least at least one marker positive.

In our study, FIT was only analysed once, which follows the European guidelines. Repeated tests have not shown better sensitivity or specificity than one single test [41,42,23,43,44]. In addition, in our setup FIT and FC were analysed from stool samples collected from the same defecation. A limitation of our study was that a significant number of patients only collected a single tube before the bowel preparation. Therefore, the number of patients who had data on both FC and FIT was lower than we expected. The study cohort represents patients referred to colonoscopy from primary care. The decision of referral was made for various reasons influenced by both patients and physicians. The data for specificity and PPV must therefore be interpreted with caution and cannot directly be translated to a general population, for example in the setting of CRC screening. A major and important challenge is to find markers that could detect premalignant lesions in the colon. Unfortunately, neither FIT nor FC can successfully discriminate dysplastic adenomas from benign findings with acceptable specificity and PPV. With very low cut-off for F-Hb and FC, good sensitivity for adenomas with high grade dysplasia can be achieved but with poor specificity and PPV values [34]. Adding additional markers to F-Hb and FC may improve specificity. For example, the tumour marker M2-PK (dimeric form of pyruvate kinase) was used in combination with FIT and FC with acceptable sensitivity and specificity [24,45]. Also, other evolving markers have been tested with promising results [46,25].

Conclusion

The combination of FIT and the FC test improves the detection of CRC. However, there is still a need for further studies using larger patient cohorts to find the optimal cut-off levels for different combination of tests.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical Approval: All procedures involving human participants were approved by the Research Ethics Committee of Umeå University, Umeå, Sweden and in accordance with the Helsinki declaration.

Informed Consent: Informed consent was obtained from all individual participants included in the study.

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