Survival of the FI-Test

Oort, F.A.

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SURVIVAL OF THE FI-TEST

Past, present and future of Fecal Immunochemical Tests in colorectal cancer screening

Frank Oort

Thesis: VU University Amsterdam

The work described in this thesis was performed at the Department of Gastroenterology & Hepatology of the VU University medical center, Amsterdam (Head: prof.dr. C.J.J. Mulder, MD, PhD) in collaboration with the Tumor Profiling Unit of the department of Pathology of the VU University medical center, Amsterdam (Head: prof.dr. G.A. Meijer, MD, PhD).

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SURVIVAL OF THE FI-TEST

Past, present and future of Fecal Immunochemical Tests in colorectal cancer screening

ACADEMISCH PROEFSCHRIFT

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de Vrije Universiteit Amsterdam,
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in het openbaar te verdedigen
ten overstaan van de promotiecommissie
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door

Frank Albert Oort

geboren te Heemskerk

promotoren:	prof.dr. C.J.J. Mulder prof.dr. G.A. Meijer
copromotor:	dr. R.W.M. van der Hulst

Voor mijn ouders, Tanja, Thijs en Cleo

CONTENTS

1

General introduction

The leading title of this thesis may suggest that only the fittest of individuals diagnosed with colorectal cancer can survive this disease, in analogy with the theory of 'natural selection' proposed by Charles Darwin.[1] However, the FI-test in the title refers to the Fecal Immunochemical Test (FIT), a stool test used in Colorectal Cancer (CRC) screening for tracing malignant or pre-malignant lesions in the large intestine of humans. In this thesis, the performance of FIT is investigated.

In individuals diagnosed with CRC, the primary predictor of survival is the stage of disease at time of diagnosis.[2] Asymptomatic patients, who seem to be in the best physical shape and thereby "the fittest" according to Darwin, can nevertheless suffer from CRC at an advanced stage and consequently their survival rates are only modest.

Given the natural course of disease of CRC, with symptoms such as abdominal pain or hematochezia typically signaling the presence of advanced disease, screening the asymptomatic population is the only realistic approach to influencing mortality. This is one of the main arguments for screening asymptomatic individuals by means of mass screening. More than 50 years ago the World Health Organization defined screening criteria to direct the selection of conditions suitable for mass screening.[3] In their manuscript the authors describe the main requirements for a successful screening program. One of the main criteria is that the targeted condition poses an important health problem.

Colorectal cancer (CRC) is one of the most common cancers worldwide and thereby forms a major health care problem. In the USA, CRC is the third leading cause of cancer death and in the Netherlands CRC ranks second in cancer related deaths among women and third amongst men.[4,5] In addition, due to the aging population the incidence of CRC is expected to rise up to 14,000 cases a year in the Netherlands in 2015.[6]

A second requirement for a successful screening program as defined by the WHO, is that a pre-malignant stage of the disease exists and that appropriate treatment available. As previously mentioned, CRC can remain asymptomatic for a long period of time. The chance of survival, however, decreases with an increasing stage of disease at the time of diagnosis. Secondary prevention, which is defined as detection and treatment of a disease which has yet to become symptomatic, aims to diagnose and treat existing disease in its early stages before it results in significant morbidity. Its high prevalence and natural history make CRC a prime candidate for secondary prevention by means of population screening.

Another main requirement for a successful screening program is the availability of a reliable test or examination to detect the condition. A screening test discriminates individuals who probably have a disease from those who probably have not. For mass screening, the methods available are either structural examinations or fecal tests.[7] The structural examinations are radiologic exams like double-contrast barium enema, CT-colonography or endoscopic examinations, e.g. sigmoidoscopy and colonoscopy.

Colonoscopy enables the direct visualization of the entire colon and is currently considered the gold standard for detecting colorectal cancer. Furthermore, it is the main therapeutic tool to disrupt the progression of precancerous polyps to cancer by polypectomy.[8] However, colonoscopy can be quite troublesome and uncomfortable for patients and there is an inherent risk for complications.[9] Using the gold standard colonoscopy for screening, would lead to a large additional burden of endoscopic work. The already overstretched endoscopic capacity in several countries, hampers a colonoscopy screening program at such a large scale.[6,10] Therefore, a preselecting screening tool is needed to decrease the total number of colonoscopies performed in a colorectal cancer screening program and for this purpose, fecal tests can be used. Moreover, formal cost effectiveness analyses favor screening with FIT over colonoscopy.

The fecal tests can be divided in fecal occult blood tests (FOBTs) and biomarker based tests like stool DNA tests. There are two types of FOBT, namely the guaiac based test, or g-FOBT, and the Fecal Immunochemical Test, or FIT. In the present thesis, the test performance of FIT will be described and its applicability discussed.

AIMS AND OUTLINE

The aim of this thesis was to assess test characteristics of fecal occult blood tests for the detection of precursor lesions of CRC, thereby contributing to the basis of an organized population screening program for CRC. To this end, a large, multicenter, cohort of colonoscopy controlled individuals was investigated. Test characteristics of FIT were compared to the gold standard colonoscopy. In this cohort several research questions could be addressed concerning test performance of FIT.

The chapters of this thesis address three main themes. The first theme is comparing FIT to g-FOBT, which was the standard test in colorectal cancer screening, but in the mean time has become outdated. The second theme deals with different strategies for

optimization of the diagnostic accuracy of FIT. In addition, several factors that influence the test performance of FIT have been identified. The third theme focuses on the future; FIT is combined with a DNA methylation marker. Biomarkers will certainly play a role in the future of fecal tests in the detection of colorectal cancer, and the added value of a methylation marker to FIT is assessed.

"The past"

Guaiac-based fecal occult blood tests (g-FOBTs) were already proposed for use in colorectal cancer screening in the early 1960s.[11] Blood, shed into the colonic lumen by colorectal adenomas and carcinomas, yields a positive g-FOBT due to the peroxidase-like activity of heme in human stool.[12]

Historically stool screening has always relied on the detection of occult blood, which is non-specific and has a limited sensitivity as a marker for colonic neoplasia. Consequently, many physicians and their patients have been hesitant to embrace the use of FOBTs, and a relatively small minority of adults adheres regularly to this type of stool screening.[13] Nevertheless, with this test formal evidence has been generated that occult blood testing can reduce death rates from CRC.[14-16]

In **chapter 2** sensitivity and specificity is assessed of g-FOBT (Hemoccult-II©) and FIT (OC-sensor©) for adenomas and CRC in eligible subjects who underwent complete colonoscopy. At the time of publication, this study was one of the few studies that head to head evaluated FIT and g-FOBT, with all individuals performing both tests at the same time. In addition, because all individuals underwent colonoscopy, specificity could be calculated directly.

The present: pitfalls in use of FIT and improvement strategies

One of the major advantages of FIT is its non-dichotomous but quantitative outcome. A cut-off value can be set to define the threshold for test positivity. By adjusting this cut-off value, the positivity rate can be influenced. When applied in a CRC screening program, a lower cut-off level for FIT will result in more screenees being referred for colonoscopy, and due to lower specificity, a higher number of futile colonoscopies. Higher FIT cut-off levels will decrease the strain on colonoscopy resources, but might also leave more curable CRCs undetected. In **chapter 3** the influence of using higher cut-off values for a FIT on the detection rates of screen-relevant neoplasia was assessed.

By lowering the cut-off value of FIT, sensitivity is increased at the cost of specificity. One approach for improving the sensitivity of FIT based screening could be to increase the number of samples tested, which is common practice for g-FOBTs. **Chapter 4** focuses on repeated FIT sampling as another strategy for improving the test sensitivity of FIT for detection of CRC and its precursors. Test positivity of double FIT sampling was evaluated for three different strategies. First "one of two FITs positive" when at least one out of two measurements exceeded the cut-off value. Second, "both FITs positive" when both measurements exceeded the cut-off value, and third "the mean of two FITs positive" when the geometric mean of two FITs exceeded the cut-off value. These strategies were evaluated at fixed specificities.

In **chapter 5** the focus lies on false positive FITs. In population-based screening with FIT, participants with a positive test result are referred for colonoscopy. In about half of all screenees with a positive FIT result, no advanced neoplasia is found at colonoscopy. These false positives could lead to unnecessary colonoscopy-related complications, pointless strain on endoscopic resources, psychological stress for the screenee, and diminished trust in the screening program. Therefore, the number of false positive results should be kept as low as possible. In this chapter, the contribution of hemorrhoids to the frequency of false positive FITs was determined. In a univariate unadjusted analysis, subjects with hemorrhoids as single abnormality were compared with subjects without any abnormalities.

Recent literature suggests that FITs have a higher sensitivity and lower specificity for advanced colorectal neoplasia in males compared to females.[17] The uptake of a screening program is not solely dependent on test performance of FIT, but a true difference in FIT characteristics between males and females could lead to disparities in the expected benefit of CRC screening between both sexes. Gender disparities could necessitate more tailored screening, i.e. sex-specific screening guidelines. In **chapter 6** potential inequalities in test performance of FIT between sexes are assessed. The aim of this chapter was to compare males and females with respect to the sensitivity and specificity of FIT, at different cut-off values. Outcome measures were CRC and advanced adenomas.

"The future"

The future is the final theme of this thesis, and in **chapter 7** a potential option for improvement of test performance of FIT is described. As mentioned previously, FIT detects hemoglobin, which is a non-specific, blood derived marker, leaked from colonic neoplastic lesions into the stool. The products of cell exfoliation form another type of stool marker[18] Detection of molecular markers for colorectal neoplasia in feces has the potential to improve performance of simple noninvasive screening tests for colorectal cancer. DNA methylation markers are a good option for CRC screening even more because DNA methylation is an early event in colorectal development, preceding even chromosomal abnormalities and mutations. In chapter 7, a new methylation marker, namely Phosphatase and Actin Regulator 3 (PHACTR3), was identified from a pool of potential candidates. This biomarker for the early detection of colorectal cancer in stool was subsequently evaluated for its potential complementary value to a Fecal Immunochemical Test (FIT).

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2

Colonoscopy-controlled intra-individual comparisons to screen relevant neoplasia: faecal immunochemical test vs. guaiac-based faecal occult blood test

F.A. Oort*, J.S. Terhaar sive Droste*, R.W.M. van der Hulst, H.A. van Heukelem, R.J.L.F. Loffeld, I.C.E. Wesdorp, R.L.J. van Wanrooij, L. de Baaij, E.R. Mutsaers, S. van der Reijt, V.M.H. Coupe, J. Berkhof, A.A. Bouman, G.A. Meijer, C.J.J. Mulder

*These authors have contributed equally to the work.

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ABSTRACT

Background: Guaiac-based faecal occult blood tests (g-FOBTs) are most commonly used in colorectal cancer (CRC) screening programmes. Faecal immunochemical tests (FITs) are thought to be superior.

Aim: To compare performance of a g-FOBT and a quantitative FIT for detection of CRCs and advanced adenomas in a colonoscopy-controlled population.

Methods: We assessed sensitivity and specificity of both FIT (OC-sensor) and g-FOBT (Hemoccult-II) prior to patients' scheduled colonoscopies.

Results: Of the 62 invasive cancers detected in 1821 individuals, g-FOBT was positive in 46 and FIT in 54 (74.2% vs. 87.1%, P=0.02). Among 194 patients with advanced adenomas, g-FOBT was positive in 35 and FIT in 69 (18.0% vs. 35.6%, P<0.001). Sensitivity for screen relevant tumours (197 advanced adenomas and 28 stage I or II cancers) was 23.0% for g-FOBT and 40.5% for FIT (P<0.001). Specificity of g-FOBT compared to FIT for the detection of cancer was 95.7% vs. 91.0%, P<0.001) and for advanced adenomas (97.4% vs. 94.2%, P<0.001).

Conclusions: Faecal immunochemical test is more sensitive for CRC and advanced adenomas. Sensitivity of FIT for screen relevant tumours, early-stage cancers and advanced adenomas, is significantly higher. Specificity of g-FOBT is higher compared with FIT.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer death worldwide. Early detection is one of the most realistic approaches to reduce CRC-related death. Guaiac-based faecal occult blood tests (g-FOBTs) were already proposed for this purpose in the early 1970s.[1] Blood, shed into the colonic lumen by colorectal adenomas and carcinomas yields a positive g-FOBT because of the peroxidase-like activity of haeme in stool.[2] Screening programmes using g-FOBT have proven to reduce both incidence and mortality of CRC.[3-7] Yet, both clinical sensitivity (i.e. the percentage of tumours detected in a series of tumour positive patients that perform the test) and programme sensitivity (i.e. the percentage of tumours present in a population intended to screen that actually is detected) are suboptimal.[8,9]

More recently, the faecal immunochemical test (or FIT), has been introduced as an alternative to g-FOBT. The FIT selectively detects the human globin-protein in stool, making it specific to colonic blood loss, while globin from blood lost proximal to the colon will be degraded before entering the colon.[10,11] Several variants of FIT exist, some of which come with automated analysis and have quantitative outcomes, like the one used in the present study.[12]

Comparisons of different techniques to detect occult blood in stool have been performed since 1953.[13] Recent studies that compared g-FOBT and FIT in screening populations indicated superiority of FIT for the detection of both cancers and advanced adenomas.[8,14,15] To evaluate whether FIT can replace the most commonly used test (g-FOBT) in CRC screening, a comparative study design is needed. Both g-FOBT and FIT should be performed in parallel on the same stool samples.[14-16]

In addition, to appraise specificity of a test directly, all test negative individuals should undergo the test that is considered the gold standard, colonoscopy. Inherent to the design of screening studies, only FOBT-positive individuals underwent colonoscopy.[8,14,15,17] A large scale comparison of g-FOBT and FIT in a colonoscopy controlled population is still lacking. In the present study, test characteristics of both tests could be determined directly, as colonoscopy was performed in all included participants.

While population-based screening studies yield crucial information on programme sensitivity and acceptance of a test in the target population, often only small numbers of CRCs are detected.[18] Consequently, the power to stratify these cancers by stage is insufficient.[8,19] In a referral population, like in the present study, a higher prevalence of

CRC and its precursor lesions will allow for stratification of FOBT result for different phases of the natural history of the disease.

AIM

The aim of the present study was an intraindividual comparison of test performance of a g-FOBT and a quantitative FIT for detection of CRCs of all stages and advanced adenomas in a colonoscopy controlled population. In addition, a specific aim was to compare test performance of both tests for the detection of early-stage cancers and advanced adenomas taken together, as these lesions are most relevant for screening.

METHODS

Study population

Five hospitals in the Amsterdam area in the Netherlands participated in this study. From June 2006 to March 2008, all ambulatory patients ≥18 years, scheduled for colonoscopy were invited to participate in this study regardless of the indication for colonoscopy. Two of these five participating hospitals are situated in rural areas, another two are large teaching hospitals with an urban population. One of the centres is an academic medical centre with a predominantly urban population. In all centres, local Medical Ethics Review Board approval was obtained prior to the start of the study, and informed consent was obtained from the participants.

Inclusion and exclusion criteria

All eligible individuals were asked to perform both types of FOBT in the week preceding colonoscopy. Neither patients who presented at an emergency room nor institutionalized patients were enrolled. All indications for colonoscopy, as stated by the referring physicians, were recorded. Patients with a documented history of inflammatory bowel disease (IBD) or with an incomplete colonoscopy were excluded from further analysis as were patients who failed to complete both tests.

Study design

Elective patients were invited to participate either by their gastroenterologist when visiting the outpatient clinic or by telephone by one of five researchers based at each of the participating centres. Once a patient consented in participation, an envelop was sent to his/her home address containing background information on the study, both FOBTs with extensive instructions and an informed consent form. When a person could not be reached over the telephone, the same package was sent but with an additional explanatory letter.

FOBTs

The tests compared were the widely used guaiac-based FOBT; Hemoccult-II (Beckman Coulter Inc., Fullerton, CA, USA) and an automated quantitative FIT: OC-sensor (Eiken Chemical Co., Tokyo, Japan).

Faecal sampling

The three test-cards of the g-FOBT were sampled with stool from three separate bowel movements over a period of 1 week and 7, 3 and 1 day(s) prior to colonoscopy respectively (Figure 2.1). On the g-FOBT test cards, two separate samples from different parts of the stool had to be applied using the enclosed cardboard sticks. The final test card for g-FOBT and the FIT were sampled with stool taken from the day before colonoscopy, and before bowel preparation had started. Illustrated instructions guided the participants to sample their stool ensuring that contact with water or urine was prevented. No restrictions were made with regard to either diet or use of medication during the week in which stool samples were taken.[20]

Test analysis

On the day of colonoscopy, both the completed FOBTs and the informed consent form were handed over to the nursing staff at the endoscopy-department. All FITs were stored at minus 5 degrees Celsius on arrival and all g-FOBTs were stored at room temperature according to the manufacturer's recommendations. Tests were analysed according to the manufacturer's instructions within 1 week by two experienced technicians, who were unaware of the clinical data.[21] Both technicians were tested negative for colour blindness and received special training for reading the test cards.

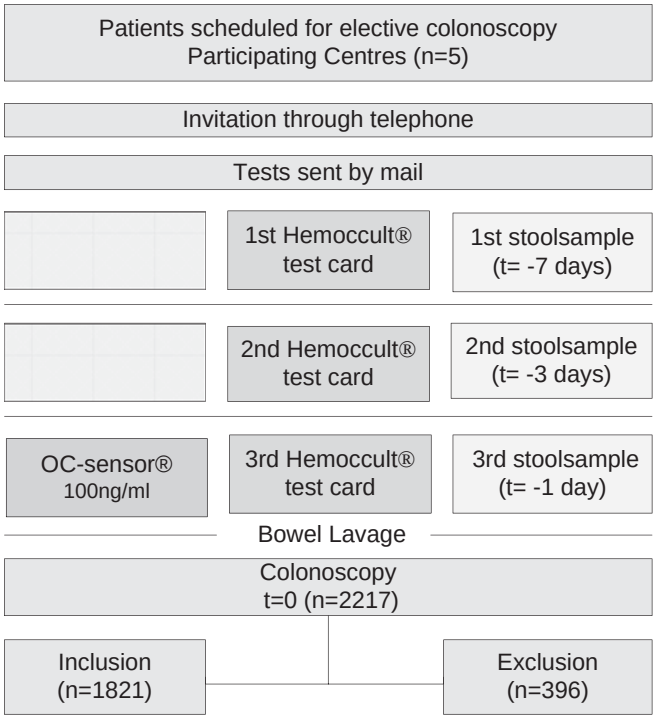

Figure 2.1 Study design.

Faecal immunochemical test samples were processed with the OC-sensor MICRO desktop analyser (Eiken Chemical Co., Tokyo, Japan).[21] A haemoglobin concentration of ≥100 ng /mL was taken as cut off, according to the manufacturer's recommendations. G-FOBT samples were considered positive when a blue colour appeared in at least one out of three test cards, following application of the reagent. Test cards were not rehydrated prior to analysis.

Colonoscopy

All participants underwent complete colonoscopy in one of five participating centres. Colonoscopies were performed, or supervised, by experienced gastroenterologists. Conscious sedation using Midazolam was offered to all patients. Endoscopists were blinded to the results of both tests. A complete colonoscopy was defined as intubation of the caecum with identification of the ileocecal valve or appendiceal orifice, or intubation up to an obstructing neoplasm. In addition, patients with inadequate bowel cleansing, as judged by the individual endoscopist, were excluded as well. Patients were classified

based on the most advanced lesion detected in their colon. Estimated size of all lesions, as reported in the colonoscopy report, were categorized.

Detected lesions

Histology of tissue samples obtained during colonoscopy was evaluated according to standard procedures. Pathology reports were collected centrally and entered into the database. Adenomas ≥1.0 cm, with any villous features (i.e. tubulovillous or villous adenoma) or highgrade dysplasia, were considered advanced adenomas.[22,23] Advanced neoplasia included all cases of CRC and all advanced adenomas. Colorectal carcinomas were staged according to the AJCC cancer staging manual.[24]

Statistical analysis

Defining colonoscopy outcome as gold standard, sensitivity and specificity of both FOBTs were calculated, for advanced adenomas and for CRC separately. Furthermore, these test characteristics were calculated for all participants with either one or more advanced adenoma(s) or an early-stage carcinoma (AJCC stages I and II). Since both FOBTs were performed in parallel on the same stool samples, McNemar's test could be used for comparison of correlated proportions.

The sample size was predetermined, based on an anticipated difference in sensitivity for detection of CRC of 20% between Hemoccult-II and OC-sensor. The enrolment of 1650 participants was required, assuming a prevalence of CRC of at least 2.5% in this study population, to provide the study with a statistical power of 80% to detect a significant difference at a two-sided alpha level of 0.05 with the use of McNemar's test. All analyses were performed using SPSS for Windows Version 15 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographics

Overall, 2,217 individuals who underwent colonoscopy performed at least one of both FOBTs. In total, 396 individuals were excluded for reasons listed in Table 2.1. The mean age of 1,821 individuals that were included for final analysis was 59.6 years (range 18–86 years, 95% CI 59.0–60.1). A majority of the subjects (56.9%) were women.

Table 2.1 Reasons for exclusion of 396 patients out of 2,221 consecutive patients undergoing colonoscopy in five hospitals in the Amsterdam area

Reason for exclusion	N=396 (100%)
Incomplete colonoscopy	
Caecum not reached	102 (25.8)
Insufficient bowel lavage	27 (6.8)
Documented history of IBD	77 (19.4)
FIT not sampled	87 (22.0)
g-FOBT not sampled	98 (24.7)
Colostomy	5 (1.3)

IBD, inflammatory bowel disease; FIT, faecal immunochemical test; g-FOBT, guaiac-based faecal occult blood tests.

Indications for colonoscopy

The primary indication for colonoscopy was available for 1,742 out of 1,821 individuals. In 79 patients (4.3%), the primary indication remained unspecified. Indications were classified in four categories listed in Table 2.2. Almost half of the population (N=897, 49.3%) was referred for colonoscopy because of gastrointestinal symptoms and 44.3% of patients was asymptomatic and had an indication for screening or surveillance colonoscopy (N=807).

Colonoscopy results

In 194 of 1,821 patients (10.7%), at least one advanced adenoma was found on colonoscopy. Adenocarcinomas were found in 62 of 1,821 patients (3.4%). Of these, 28 (45.2%) were classified as early-stage CRC (AJCC stages I and II) and 31 patients (50.0%) as late stage (AJCC stages III and IV). Three rectal cancers could not be accurately staged due to the effects of neo-adjuvant radiotherapy.

Test results

Overall positivity rate for the g-FOBT was 6.7% (122⁄1821) and for the FIT 11.8% (214⁄1,821). The g-FOBT detected 46 of 62 cancers for a sensitivity of 74.2%, whereas the FIT detected 54 of 62 cancers, for a sensitivity of 87.1% (Table 2.3). FIT detected seven

Table 2.2 Primary indications for colonoscopy among 1,821 consecutive patients in five hospitals in the Amsterdam area enrolled in a study comparing faecal immunochemical test vs. guaiac-based faecal occult blood tests

Indication group	Indication for colonoscopy	N
Symptomatic/suspect	Weight loss	15
	Clinical suspicion of diverticulitis	14
	Clinical suspicion of IBD	17
	Abdominal pain	280
	Anaemia	77
	Haematochezia	190
	Altered bowel habits	304
	Clinical suspicion of CRC (inconclusive pathology)	1
	Colonoscopy for polypectomy	37
	Total	935
Screening and surveillance	Average risk	32
	Familial history of CRC	288
	Lynch syndrome	29
	Polyp surveillance	314
	Post CRC surveillance	120
	Radiological suspicion of malignancy	21
	Total	804
Other	Not specified	82
	Grand total	1,821

IBD, inflammatory bowel disease; CRC, colorectal cancer.

cancers that did not score a positive g-FOBT. Only one cancer scored a positive g-FOBT but not a positive FIT. The observed difference in sensitivities between the two tests was significant (P=0.02). Sensitivity for screen relevant lesions (advanced adenomas and earlystage cancers) was 23.0% for g-FOBT and 40.5% for FIT (P<0.001).[25] Yet, g-FOBT was found to have a higher specificity compared to FIT for cancers of all stages (95.7% vs. 91.0%, P<0.001) and for advanced adenomas (97.4% vs. 94.2%, P<0.001).

The difference between sensitivities of g-FOBT and FIT for the detection of early or late-stage CRCs respectively showed a similar trend but did not reach statistical significance. The sensitivities of g-FOBT and FIT for early-stage cancers was 57.1% and FIT 75.0% respectively (Table 2.4). For late-stage cancers, g-FOBT had a sensitivity of 87.1% and FIT 96.8%.

Table 2.3 Sensitivity and specificity of guaiac-based faecal occult blood tests (Hemoccult-II) and faecal immunochemical test (OC-sensor) for detection of advanced adenomas, advanced neoplasia, colorectal cancer (CRC), in a consecutive series of 1,821 patients referred for colonoscopy

	Advanced adenomas (194/1,821)	Early stage CRC (stages I and II) + advanced adenomas[a] (222/1,821)	CRC (62/1,821)	Advanced neoplasia (256/1,821)
Sensitivity (%) Hemoccult-II (95% CI)	18.0 35/194 (12.9–24.2)	23.0 51/222 (17.6–29.1)	74.2 46/62 (61.5–84.5)	31.6 81/256 (26.0–37.7)
Sensitivity (%) OC-sensor (95% CI)	35.6 69/194 (28.9–42.7)	40.5 90/222 (34.1–47.3)	87.1 54/62 (76.2–94.3)	48.1 123/256 (41.8–54.4)
P-value[b]	<0.001	<0.001	0.02	<0.001
Sensitivity (%) Hemoccult-II (95% CI)	97.4 1,524/1,565 (96.5–98.1)	97.4 1,524/1,565 (96.5–98.1)	95.7 1,683/1,759 (94.6–96.6)	97.4 1,524/1,565 (96.5–98.1)
Sensitivity (%) OC-sensor (95% CI)	94.2 1,474/1,565 (92.9–95.3)	94.2 1,474/1,565 (92.9–95.3)	91.0 1,599/1,759 (89.5–92.2)	94.2 1,474/1,565 (92.9–95.3)
P-value[b]	<0.001	<0.001	<0.001	<0.001

[a] Of three rectal tumours, the oncological stage of disease could not be assessed because of the effects of neo-adjuvant radiotherapy.
[b] McNemar's test was used to compare paired proportions.

Table 2.4 Test outcome of guaiac-based faecal occult blood tests (Hemoccult-II) and faecal immunochemical test (OC-sensor) in 62 patients diagnosed with colorectal cancer at an early stage (N=28) and at a late stage (N=31)

	Hemoccult-II		OC-sensor		
	Positive	Negative	Positive	Negative	Total
Early stage disease (stages I and II)	16 (57.1)	12 (42.9)	21 (75.0)	7 (25.0)	28
Late stage disease (stages III and IV)	27 (87.1)	4 (12.9)	30 (96.8)	1 (3.0)	31
AJCC stage unknown[a]	3	0	3	0	3
Total	46 (74.2)	16 (25.8)	54 (87.1)	8 (12.9)	62 (100)

Values within parenthesis represent percentages.
[a] Three rectal tumours could not be accurately staged because of the effects of neo-adjuvant radiotherapy.

DISCUSSION

In this study, performance characteristics of both g-FOBT and FIT were evaluated in parallel in a referral population of 1,821 patients who underwent complete colonoscopy. This design allowed to deal with two major issues that, thus far, have remained unaddressed. First, in most studies so far on FIT, only individuals with a positive test underwent subsequent colonoscopy, which precluded the determination of false negativity rates and thus specificity of the investigated tests. Secondly, previous studies comparing g-FOBT and FIT did so mainly in separate patient groups, which limit determination of exactly which tumours are missed by one test but are detected by the other.

Overall, results of the present study are consistent with earlier observations in screening studies and confirm a significantly higher sensitivity of FIT for CRC as well as advanced adenomas. The referral population that was evaluated in the present study consisted of more individuals with CRC or advanced adenomas than an average risk screening population. For reasons of the relatively high tumour yield in the referral population in the present study, test results of FIT and g-FOBT could be stratified for early-stage cancers (AJCC stages I and II) and late-stage cancers (stages III and IV). Importantly, for the screen relevant neoplasia (i.e. advanced adenomas and early-stage cancers taken together), FIT significantly outperforms g-FOBT in terms of sensitivity with 40.5% vs. 23.0% respectively. This is of special importance as population-based screening programmes for CRC aim to detect this category of neoplasia specifically. A trend in the same direction was observed in early-stage cancers alone, with detection rates of 75.0% vs. 57.1% for FIT and g-FOBT respectively, but this did not reach statistical significance.

In a screening-naive population, both early and late-stage CRCs, the so called prevalent cancers, will be detected in a range consistent with their respective prevalence. Therefore, in a first round of a screening programme, overall CRC detection rates will be inflated by the prevalent advanced stage cancers. However, given the nature of the disease, with usual annual or bi-annual screening programmes, in a second round of screening, less advanced cancers will be left in the population.[26] Therefore, the performance characteristics of the screening programme will largely depend on the potential to detect early-stage CRCs, i.e. incident cancers. In this respect, it is highly relevant that the present study allowed to analyse performance of both tests separately for early and late-stage CRC. Preferably, these results need to be validated in a screening population, but the small numbers of CRCs detected in screening studies will hamper such a study design.

Positive and negative predictive values of a test are influenced by the prevalence of a disorder in the population that is tested. Therefore, in this referral population, these values were not calculated as they would not reflect predictive values in a screening population. However, sensitivity and specificity are characteristics of a diagnostic test and are not influenced by the prevalence of a disease in population.[27]

With respect to whether the present findings are generalizable to a screening population, it could be argued that preclinical (i.e. screen detected lesions) may be different (e.g. in their tendency to bleed) from symptomatic lesions. However, so far, there is no evidence that either supports or falsifies this hypothesis.

The present study shows that the higher sensitivity of FIT goes at the cost of a somewhat lower specificity (advanced neoplasia; 97.4% vs. 94.2%, overall cancer; 95.7% vs. 91.0%, for g-FOBT and FIT, respectively). The higher specificity of g-FOBT compared to FIT, was recently described in another study comparing exactly the same g-FOBT and FIT, but in two separate populations.[8] In this study that used a population screening design, specificity could not be calculated directly, as only FIT or g-FOBT positive individuals were offered colonoscopy. Hence, specificities were calculated based on rare disease assumptions. This may explain the small differences in specificity between their study and the current one in which all individuals underwent colonoscopy. Moreover, not all guaiac-based FOBTs have the same test characteristics. The Hemoccult-II used in the present study is a low sensitivity FOBT. The US only endorse the use of sensitive g-FOBTs (e.g. Hemoccult Sensa) for screening and therefore the results might be different if an FOBT with better sensitivity was used.[28]

Apart from the higher sensitivity for screen relevant tumours, FIT has several other advantages. The technical characteristics of the FIT test used in the present study allow for automated analysis, unlike g-FOBT, which makes it suitable for high throughput application. Furthermore, patient acceptance of a test is a major determinant of compliance and consequently success of a screening programme. The OC-sensor has been found to be significantly better accepted by the average risk target population for CRC screening than the Hemoccult-II.[8] Finally, the reproducibility and quality control of FIT are good, where g-FOBT was not optimal.[29,30]

Secondary prevention of CRC is a major health care issue, and several countries already have introduced g-FOBT in large pilot-studies or nationwide CRC screening programmes.[31,32] A growing body of literature lends support to the notion that FIT is superior to g-FOBT in CRC screening.[8,15,17,33,34] The present study adds to this the observation that FIT has a significantly higher sensitivity for screen relevant tumours than g-FOBT. In addition, the present study allows for a more precise estimation of the specificity of FIT for colorectal tumours.

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3

Higher Fecal Immunochemical Test cut-off levels: lower positivity rates but still acceptable detection rates for early-stage colorectal cancers

F.A. Oort*, J.S. Terhaar sive Droste*, R.W.M. van der Hulst, H.A. van Heukelem, R.J.L.F. Loffeld, S.T. van Turenhout, I. Ben Larbi, S.L. Kanis, M. Neerincx, M. Räkers, V.M.H. Coupe, M.A. Blankenstein, G.A. Meijer, C.J.J. Mulder

*These authors have contributed equally to the work.

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ABSTRACT

Background: Adjusting the threshold for positivity of quantitative fecal immunochemical tests (FIT) allows for controlling the number of follow-up colonoscopies in a screening program. However, it is unknown to what extent higher cut-off levels affect detection rates of screen-relevant neoplasia. This study aimed to assess the effect of higher cut-off levels of a quantitative FIT on test positivity rate and detection rate of early-stage colorectal cancers (CRC).

Methods: Subjects above 40 years old scheduled for colonoscopy in 5 hospitals were asked to sample a single FIT (OC sensor) before colonoscopy. Screen-relevant neoplasia were defined as advanced adenoma or early-stage cancer (stage I and II). Positivity rate, sensitivity, and specificity were evaluated at increasing cut-off levels of 50 to 200 ng/mL.

Results: In 2,145 individuals who underwent total colonoscopy, 79 patients were diagnosed with CRC, 38 of which were with early-stage disease. Advanced adenomas were found in 236 patients. When varying cut-off levels from ≥50 to ≥200 ng/mL, positivity rates ranged from 16.5% to 10.2%. With increasing cut-off levels, sensitivity for early-stage CRCs and for screen-relevant neoplasia ranged from 84.2% to 78.9% and 47.1% to 37.2%, respectively.

Conclusions: Higher FIT cut-off levels substantially decrease test positivity rates with only limited effects on detection rates of early-stage CRCs. However, spectrum bias resulting in higher estimates of sensitivity than would be expected in a screening population may be present.

INTRODUCTION

Screening for colorectal cancer (CRC) using guaiacbased fecal occult blood tests (G-FOBT) has been shown to reduce CRC-related mortality.[1-3] In recent years, a growing body of literature lends support to the notion that fecal immunochemical tests (FIT) are superior to G-FOBT in CRC screening.[4-8] This superiority does not only imply higher participation rates and sensitivity for advanced neoplasia, but also better reproducibility and quality control due to the automated analysis and quantitative test output.[9] The quantitative test output allows for adjusting the threshold for the definition of a positive test. This is important because several recent studies comparing G-FOBT and FIT have reported a lower specificity of FIT when a cut-off level of 50 to 100 ng hemoglobin/mL was used.[5-8] Once this test is applied in a CRC screening program, a lower cut-off level will result in more screenees being referred for colonoscopy, and due to lower specificity, a higher number of futile colonoscopies. Higher FIT cut-off levels will decrease strain on colonoscopy resources, but might also be associated with more curable CRCs being undetected. To test this hypothesis, a study design is needed in which all FIT-negative individuals undergo the reference test, that is, complete colonoscopy. However, in most populationbased screening studies, only FIT-positive individuals undergo colonoscopy.[4-7,10,11] Although these screening studies reflect the target population for screening, sensitivity cannot be calculated. Specificity can be calculated, but only indirectly and based on less accurate rare disease assumptions.[6] Moreover, these studies often have a low yield of CRCs, which restricts the power to stratify these cancers by stage.[6,7,12,13] When aiming at CRC mortality reduction, detection of early-stage cancers is much more relevant than detecting late-stage cancers. In a referral population, like in the present study, a higher prevalence of CRC and its precursors will allow for stratification of quantitative FIT results for different phases of the natural history of the disease. We therefore assessed the effect of a higher cut-off level of a quantitative FIT on positivity rates and on detection rates of curable, early-stage CRCs and advanced adenomas in a colonoscopycontrolled population.

PATIENTS AND METHODS

Study population and study design

Details of study design and of most materials and methods relevant for this study have been published previously in a report on the direct comparison of a FIT and a G-FOBT.[8]

All ambulatory subjects over the age of 40 years scheduled to undergo elective colonoscopy from June 2006 to January 2009 at 1 of the 5 participating hospitals were invited to participate in this study. Invitation was either in person by the referring gastroenterologist or through telephone by 1 of 5 research workers stationed at each of the participating centers. Once subjects consented in participation, they received an envelope containing background information on the study, the FIT with extensive instructions, and an informed consent form. When an individual could not be reached by telephone, the same package was sent but with an additional explanatory letter. Two of these 5 participating hospitals are situated in rural areas, another two are large teaching hospitals with an urban population. One of the centers is an academic medical center with a predominantly urban population. In all centers, local Medical Ethics Review Board approval was obtained prior to the start of the study.

All eligible individuals were asked to sample one FIT on stool from a bowel movement on the day prior to colonoscopy. Patients with a documented history of inflammatory bowel disease (IBD), subjects who failed to complete the test and subjects in whom no written informed consent was obtained were excluded from further analysis. We also excluded subjects with incomplete colonoscopies and subjects with inadequate bowel cleansing, as judged by the endoscopist.

Fecal immunochemical tests

The FIT used in the present study is the automated quantitative OC-sensor test (Eiken Chemical Co.). The FIT was sampled from stool produced the day before colonoscopy and bowel preparation had started. Subjects were excludedwhen the FITwas sampled after initiation of bowel preparation. Illustrated instructions guided the participants to sample their stool ensuring that contact with water and urine was prevented. Norestrictions weremade with regard to diet during the week in which the stool sample was taken.[14] Participants were asked to discontinue anticoagulants and NSAIDs (nonsteroidal antiinflammatory drugs) 5 days prior to colonoscopy. On the day of colonoscopy, the completed test and the signed informed consent form were handed over to the nursing staff at the endoscopy department. All FITs were stored at -5°C on arrival. Tests were analyzed using the OC sensor MICRO desktop analyzer (Eiken Chemical Co.) according to the manufacturer's instructions.[15] Tests were analyzed within 1 week by 1 of the 2 experienced technicians who were unaware of the clinical data. Both technicians received special training for analyzing the tests.

Standards of reference

Colonoscopy was the standard of reference for the presence, size, and location of colorectal neoplasia. Colonoscopies were performed or supervised by experienced gastroenterologists. Endoscopists were blinded to the FIT result. Conscious sedation using midazolam was offered to all patients. A complete colonoscopy was defined as intubation of the cecum with identification of the ileocecal valve or appendiceal orifice, or intubation up to an obstructing neoplasm. The results of histopathologic analysis of tissue samples obtained during colonoscopy were the standard of reference for the diagnosis of adenoma or cancer. Surgical resection specimens were used for the standard of reference for CRC staging. If no surgical resection had been performed, the results of histopathologic biopsy specimens were used instead. Adenomas of size 1.0 cm or greater, with any villous features (i.e., tubulovillous or villous adenoma) or high-grade dysplasia, were considered advanced adenomas.[16,17] Advanced neoplasia included all cases of CRC and all advanced adenomas. Colorectal carcinomas were staged according to the AJCC (American Joint Committee on Cancer) cancer staging manual.[18] Early-stage CRC was defined as AJCC stage I or II, whereas late-stage CRC was defined as AJCC stage III or IV. Because the ultimate goal of screening is the detection of early stages of diseases, we defined screen-relevant neoplasia as one or more advanced adenoma(s) or early-stage CRC.[8,19] If multiple lesions were present, classification was based on the most advanced lesion found.

Statistical analysis

Taking colonoscopy as the reference test, sensitivities and specificities of FIT at 6 cut-off levels were calculated for the following colonoscopy outcomes: (i) the presence of CRC; (ii) the presence of early-stage CRC; (iii) the presence of advanced adenoma; (iv) the presence of screen-relevant neoplasia; and (v) the presence of advanced neoplasia. The sensitivity is calculated as the proportion of positive test results in patients with the colonoscopy outcome under consideration. The specificity is calculated as the proportion of negative test results in patients with an outcome less severe than the colonoscopy outcome under consideration. Note that, therefore, the same specificity results from choosing either outcome 1 (all stages of CRC) or outcome 2 (early-stage CRC), and from choosing either outcome 3, 4, or 5. For dichotomizing the FIT results, we used cut-off levels of 50, 75, 100, 125, 150, and 200 ng hemoglobin/mL, which are levels frequently used in FIT studies (7, 20, 21). The calculations were repeated for the subgroup of patients that are considered at low risk for colonic neoplasia (procedure indications: abdominal

pain, constipation, and screening colonoscopy in average risk individuals) as well as for the high-risk subgroup separately.

We used receiver operator characteristic (ROC) curve analysis, including calculation of the area under the curve (AUC) with 95% CIs to evaluate the relation between the quantitative FIT outcome and (i) the presence of CRC; (ii) the presence of early-stage CRC; (iii) the presence of advanced adenoma; and (iv) the presence of screen-relevant neoplasia. All analyses were performed with SPSS for Windows Version 15 (SPSS Inc.).

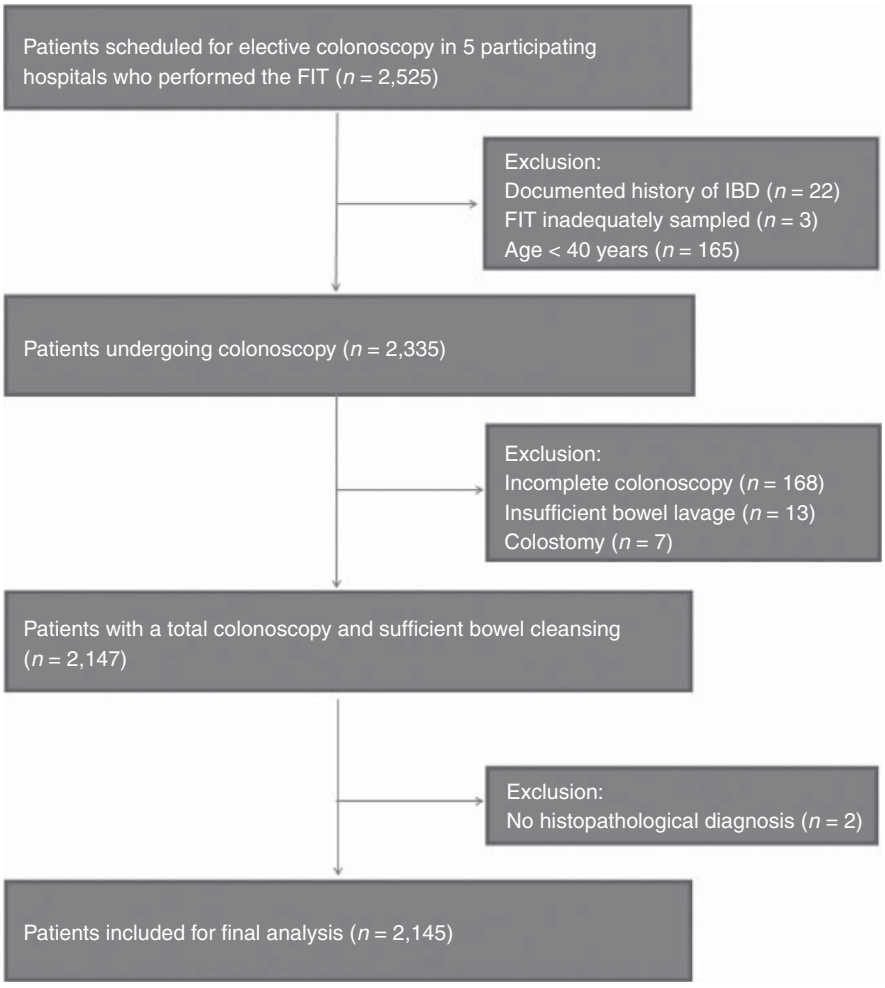

Figure 3.1 Study flow diagram. FIT, faecal immunochemical test; IBD, inflammatory bowel disease.

RESULTS

Characteristics of the study population

Overall 2,525 individuals who underwent colonoscopy sampled a FIT. In total, 380 individuals were excluded (Figure 3.1). The mean age of the 2,145 individuals that were included for final analysis was 61.8 years (range = 40–89 years) and 53.8% of these were female. Colonoscopy was performed because of gastrointestinal symptoms in 1,109 individuals (51.7%), whereas screening or surveillance for CRC was the indication for colonoscopy in 955 asymptomatic individuals (44.5%). Of 81 individuals (3.8%), the primary indication remained unspecified (Table 3.1).

Table 3.1 Primary indications for colonoscopy among 2,145 consecutive patients in 5 hospitals in the Amsterdam area enrolled in a study comparing sensitivities for early-stage cancers at different FIT cut-off levels

Indication group	Indication for colonoscopy	N
Symptomatic/suspect		
	Weight loss	20
	Clinical suspicion of diverticulitis	22
	Clinical suspicion of IBD	17
	Abdominal pain	297
	Anaemia	97
	Hematochezia	237
	Altered bowel habits	253
	Constipation	35
	Diarrhea	81
	Clinical suspicion of CRC (inconclusive histology)	1
	Colonoscopy for polypectomy	49
	Total	1,109
Screening and surveillance		
	Average risk	42
	Familial history of CRC	319
	Lynch syndrome	24
	Polyp surveillance	396
	Post CRC surveillance	147
	Radiological suspicion of malignancy	25
	Screening for CRC in celiac disease	2
	Total	955
Other		
	Not specified	81
	Grand total	2,145

Table 3.2 Sensitivity and specificity of FIT (OC-sensor) for CRC, early- and late-stage CRC, advanced neoplasia, screen-relevant neoplasia, and advanced adenomas in a consecutive series of 2,145 patients referred for colonoscopy

	≥50 ng/ml	≥75 ng/ml	≥100 ng/ml	≥125 ng/ml	≥150ng/ml	≥200 ng/ml
CRC						
Sensitivity, %						
All stages	92.40	91.10	89.90	84.80	82.30	81.00
N=79	73	72	71	67	65	64
95% CI	84.2–97.2	82.6–96.4	81.0–95.5	75.0–91.9	72.1–90.0	70.6–89.0
Early stage[a]	84.20	81.60	81.60	78.90	78.90	78.90
N=38	32	31	31	30	30	30
95% CI	68.8–94.0	65.7–92.3	65.7–92.3	62.7–90.5	62.7–90.5	62.7–90.5
Late stage[b]	100	100	97.20	91.70	86.10	83.30
N=36	36	36	35	33	31	30
95% CI	92.0–100	92.0–100	85.5–99.9	77.5–98.3	70.5–95.3	67.2–93.6
Specificity,[e] %	86.40	88.60	90.00	90.90	91.80	92.80
N=2,231	1,785	1,831	1,859	1,877	1,897	1,918
95% CI	84.8–87.9	87.2–90.0	88.6–91.2	89.5–92.1	90.6–93.0	91.6–93.9

Advanced neoplasia						
Sensitivity, %						
All advanced neoplasia[c]	54.00	52.40	50.50	48.30	46.00	43.20
N=318	170	165	159	152	145	136
95% CI	48.3–59.6	46.7–58.0	44.8–56.1	42.6–53.9	40.4–51.7	37.6–48.9
Screen-relevant neoplasia[d]	47.10	45.30	43.40	42.00	40.10	37.20
N=277	129	124	119	115	110	102
95% CI	41.1–53.2	39.3–51.4	37.5–49.5	36.1–48.1	34.3–46.2	31.5–43.3
Advanced adenoma	41.10	39.40	37.30	36.00	33.90	30.50
N=239	97	93	88	85	80	72
95% CI	34.8–47.7	33.1–46.0	31.1–43.8	29.9–42.5	27.9–40.3	24.7–36.8
Specificity,[f] %	89.90	92.20	93.50	94.30	95.10	95.80
N=1,992	1,646	1,688	1,711	1,726	1,741	1,754
95% CI	88.5–91.3	90.9–93.4	92.3–94.6	93.2–95.3	94.1–96.1	94.8–96.7

Of 5 rectal cancers, the oncological stage of disease at diagnosis could not be assessed due to the effects of neoadjuvant radiotherapy.

[a] Early-stage CRC is defined as AJCC stage I or II.

[b] Late-stage CRC is defined as AJCC stage III or IV.

[c] Advanced neoplasia is defined as either one or more advanced adenoma(s) or a CRC.

[d] Screen-relevant neoplasia is defined as either one or more advanced adenoma(s) or an early-stage carcinoma (AJCC stage I or II).

[e] Specificity in patients without CRC.

[f] Specificity in patients without CRC or advanced adenoma (less severe outcome than either advanced adenoma, screen-relevant neoplasia, or advanced neoplasia).

Colonoscopy results

CRCs were found in 79 individuals (3.7%). Of these CRCs, 38 (48.1%) were classified as early stage (AJCC stage I or II) and 36 (45.6%) were classified late stage (AJCC stage III or IV). For 5 rectal cancers (6.3%), stage could not be determined accurately due to the effects of neoadjuvant radiotherapy. In 236 individuals (11.0%), at least one advanced adenoma was found. This resulted in 315 individuals with advanced neoplasia (either advanced adenoma or CRC) and 274 individuals with screen-relevant neoplasia (either advanced adenoma or early-stage CRC).

FIT results

The overall FIT positivity rates at the different cut-off levels varied from 16.5% (N=354 at cut-off ≥50 ng/ml) to 10.2% (N=218 at cut off ≥200 ng/ml). Table 3.2 shows the test characteristics of FIT at different cut-off levels to detect CRC, early-stage CRC, late-stage CRC, advanced neoplasia, screen-relevant neoplasia, and advanced adenomas. Table 3.3 summarizes the sensitivities and positivity rates for early-stage CRC and for screen-relevant neoplasia.

ROC curves for FIT

The AUC of the ROC curve for the detection of CRC (N=79) was 0.93 (95% CI: 0.89–0.96). For the detection of early-stage CRC (N=38), an AUC of 0.89 was found (95% CI: 0.82–0.95). When all screen-relevant neoplasia were considered (N=274), the AUC was 0.72 (95% CI: 0.68–0.76). The AUC for the detection of advanced adenomas separately (N=236) was 0.69 (95% CI: 0.65–0.73).

Sensitivities in high- versus low-risk populations

Sensitivities of FIT for CRC, early-stage CRC, and screen-relevant neoplasia were compared between indication groups that were considered at low risk for colonic neoplasia versus at high risk. Patients with procedure indications like abdominal pain, constipation, and screening colonoscopy in average risk individuals were considered to belong to a low-risk population (N=374). The remaining procedure indications were considered to reflect a high risk population (N=1,771; Table 3.1). Sensitivity and the yield of screen-relevant lesions in these 2 populations are shown in Table 3.4.

Table 3.3 Positivity rates and sensitivity for early-stage CRCs and screen-relevant neoplasia at different cut-off levels of FIT in a consecutive series of 2,145 patients referred for colonoscopy

	≥50 ng/ml	≥75 ng/ml	≥100 ng/ml	≥125 ng/ml	≥150ng/ml	≥200 ng/ml
Positivity rate, % N=2,145	16.50 354	14.30 307	13.00 279	12.10 259	11.10 239	10.20 218
Detection rate of early stage CRC,[a] % N=38	84.20 32	81.60 31	81.60 31	78.90 30	78.90 30	78.90 30
Detection rate of screen relevant neoplasia,[b] % N=274	47.10 129	45.30 124	43.40 119	42.00 115	40.10 110	37.20 102

Of 5 rectal cancers, the oncological stage of disease at diagnosis could not be assessed due to the effects of neoadjuvant radiotherapy.

[a] Early-stage CRC is defined as AJCC stage I or II.

[b] Screen-relevant neoplasia is defined as either one or more advanced adenoma(s) or an early-stage carcinoma (AJCC stage I or II).

Table 3.4 Sensitivity of FIT (OC-sensor) for CRC, early-stage CRC, and screen-relevant neoplasia in a lowversus high-risk population based on procedure indication in a consecutive series of 2,145 patients referred for colonoscopy

	≥50 ng/ml	≥75 ng/ml	≥100 ng/ml	≥125 ng/ml	≥150ng/ml	≥200 ng/ml
Low risk (N=374)[a]						
Sensitivity, %						
CRC: all stages	85.70	85.70	85.70	71.40	71.40	71.40
N=7	6	6	6	5	5	5
95% CI	42.1–99.6	42.1–99.6	42.1–99.6	29.0–96.3	29.0–96.3	29.0–96.3
CRC: early stage[b]	75.00	75.00	75.00	75.00	75.00	75.00
N=4	3	3	3	3	3	3
95% CI	19.4–99.4	19.4–99.4	19.4–99.4	19.4–99.4	19.4–99.4	19.4–99.4
Screen-relevant neoplasia[d]	45	45	42.10	42.10	42.10	36.80
N=38	17	17	16	16	16	14
95% CI	28.6–61.7	28.6–61.7	26.3–59.2	26.3–59.2	26.3–59.2	21.8–54.0
High risk N=1,771[c]						
Sensitivity, %						
CRC: all stages	93.10	91.70	90.30	86.10	83.30	81.90
N=72	67	66	65	62	60	59
95% CI	84.5–97.7	82.7–96.9	81.0–96.0	75.9–93.1	72.7–91.1	71.1–90.0
CRC: early stage[b]	85.30	82.40	82.40	79.40	79.40	79.40
N=34	29	28	28	27	27	27
95% CI	68.9–95.1	65.5–93.2	65.5–93.2	62.1–91.3	62.1–91.3	62.1–91.3
Screen-relevant neoplasia[d]	47.50	45.30	43.60	41.90	39.80	37.30
N=236	112	107	103	99	94	88
95% CI	40.9–54.0	38.9–51.9	37.2–50.2	35.6–48.5	33.5–46.4	31.1–43.8

Of 5 rectal cancers, the oncological stage of disease at diagnosis could not be assessed due to the effects of neoadjuvant radiotherapy.

[a] Early-stage CRC is defined as AJCC stage I or II.

[b] Late-stage CRC is defined as AJCC stage III or IV.

[c] Advanced neoplasia is defined as either one or more advanced adenoma(s) or a CRC.

[d] Screen-relevant neoplasia is defined as either one or more advanced adenoma(s) or an early-stage carcinoma (AJCC stage I or II).

[e] Specificity in patients without CRC.

[f] Specificity in patients without CRC or advanced adenoma (less severe outcome than either advanced adenoma, screen-relevant neoplasia, or advanced neoplasia).

DISCUSSION

In the present study, test performance of one of the most commonly used FITs was evaluated at different cut-off levels in a large cohort of individuals undergoing colonoscopy. It was found that by increasing the cut-off level specificity increased substantially, whereas the effects on detection rates of curable, early-stage CRCs were only limited.

Although many other aspects have to be taken into account when deciding on the most suiting cut-off level, this study has its focus on sensitivity and specificity. In general, the FIT showed to have good test characteristics for detecting both CRC and early-stage CRC, as reflected by the AUC in the ROC curves. Adjusting the cut-off level from ≥50 to ≥200 ng/mL resulted in a substantial decrease in the number of positive tests (16.5%–10.2%). Compared with a cut-off level of ≥50 ng/mL, 2 early-stage cancers would have been missed at a cut-off level of greater than 200 ng/mL. In fact, from a cut-off level of ≥125 ng/mL upward, no further decrease in sensitivity was found. Specificity, however, increased from 86.4% to 92.8% with increasing cut-off levels. Focusing on all screen-relevant neoplasia, 47.1% were detected with the lowest cut-off level of 50 ng/mL or greater, whereas the highest cut-off level of 200 ng/mL or greater yielded only 37.2% of all screen-relevant lesions.

Consequences of these findings depend on the setting in which FIT is applied. The choice for a higher FIT threshold may be particularly relevant when a screening program is to be implemented, like is planned for the Netherlands.[22] The Dutch Health Council advised to start screening at a cut-off level of 75 ng/mL, even though using 50 ng/mL might be more cost-effective.[22] However, current colonoscopy capacity is insufficient to cope with positive screenees at this cut-off level. A higher FIT cut-off level will limit the number of colonoscopy referrals. In the first round of a screening program, both early- and late-stage CRCs, so called prevalent cancers, will be detected in a range consistent with their respective prevalences in a screening-naive population. So, in the first round, yield and thus strain on the health care system, will be inflated by the prevalent advanced-stage CRCs. In later rounds of screening, however, less advanced CRCs will be left in the population and the performance characteristics of the screening program will largely depend on the potential to detect early-stage CRCs, that is, incident cancers. In this respect, it is highly relevant to know that increasing the cut-off level to 200 ng/mL or greater has a relatively small effect on the sensitivity to detect early-stage cancers when starting a CRC screening program. The lower positivity rate and the higher specificity will result in less referrals for colonoscopy with an acceptable decrease in detection rates. When

the prevalence of target lesions would decrease in later screening rounds, cut-off levels can easily be adjusted to lower values to achieve a more sensitive program. The miss rate for advanced adenomas at a higher cut-off level is somewhat higher (70% at >200 ng/mL vs. 59% at 50 ng/mL). Given the natural history of the disease, an advanced adenoma that would be missed in the initial screening round would have multiple opportunities to be detected in a consecutive round, either still at the stage of an advanced adenoma or as an early-stage cancer.

The colonoscopy-controlled referral population used in the present study has 2 advantages compared with a screening population. Firstly, in most FIT studies to date, only individuals with a positive test underwent subsequent colonoscopy. This precluded the determination of sensitivity, false-negative rates and thus specificity of the investigated tests. The present study design provides accurate data on direct sensitivity of the FIT at different cut-off levels. Secondly, the referral population contained a higher number of individuals with CRC or advanced adenomas compared with an average risk screening population. Consequently, FIT results could be stratified by stage of the disease. More precise data on sensitivity and specificity, that is with smaller CIs than data from screening studies in which there is a lower prevalence of target lesions, could be calculated.[4,6,7,20,23] In screening studies, specificities are calculated on the basis of rare disease assumptions. This may lead to overestimation of specificity.[24] Although sensitivity and specificity are characteristics of a diagnostic test and are not dependent on the prevalence of disease, specificity can still be underestimated in the present study because the subjects are at higher risk of other potentially bleeding disorders than the general population.[25] When comparing data on specificity for advanced neoplasia of the present study with those reported in screening study designs using the same FIT, the present series shows lower specificities (89.9%–95.8% in the present study compared with 95.5%–98.8% in other studies with cut-off levels increasing from ≥50 ng/ml to ≥200 ng/ml [7,20]). Interestingly, in another colonoscopy-controlled study performed in a referral population with a smaller sample size, specificities were comparable with the present findings.[21] These different specificities probably reflect the range of true specificity of the OC Sensor.

The higher positivity rate of the FIT and higher prevalence of advanced neoplasia in the present referral population make it impossible to extrapolate the positive and negative predictive values (PPV and NPV) of this study to a screening population. However, by applying Bayes' theorem, the sensitivity and specificity from this study can be combined with observed prevalences of CRC and advanced adenomas found in the general population

to estimate the NPV and PPV in the general population.[26] Although these computed values for NPV and PPV should be interpreted with caution, it allows us to explore the effect of increasing cut-off levels. The prevalence of CRC found in Dutch screening studies is 0.8% and advanced adenomas are found in 6.7%.[22] In the time period from 2003 to 2007, 54% of all newly diagnosed CRC patients presented with early-stage disease.[27] Increasing the cut-off level of FIT from ≥50 ng/mL to ≥200 ng/mL hardly affected NPV for early-stage CRC (99.9%). The PPV for detection of early-stage cancer, however, increases substantially from 2.6% to 4.5%. The number needed to scope reduced significantly by increasing the cut-off level to 200 ng/mL or greater, resulting in a 42% decrease in required colonoscopies and only a 6% reduction in detection rates of early-stage CRCs.

An important issue is whether the present findings can be generalized to a screening population. The use of a referral population to evaluate a screening test carries the risk of introducing spectrum bias. Spectrum bias refers to the situation that the spectrum of the disease phenotype differs from that in the population in which the test ultimately will be applied.[28] This might lead to overestimation of sensitivity. An ultimate answer to this question can only come from a colonoscopy-controlled screening population. To accrue a similar number of cancers in such a study design as in the present study would require a very large sample size which might frustrate such a study design. According to the number of CRCs found in the 2 large screening trials in the Netherlands, such a study should invite 30,275 average risk individuals for FIT screening to obtain the same CRC yield as in the present study.[6,7] This sample size is based on the assumption of a 60% to 62% participation rate for FIT and 84% to 95% compliance to colonoscopy after a positive FIT.[6,7]

Three other lines of evidence provide indications that the effect of spectrum bias in the present study may be limited. Firstly, when comparing sensitivities of FIT for screen-relevant neoplasia in patients from the present study population who could be considered to have a low risk for colonic neoplasia to those that would be at higher risk, only minor differences in sensitivity were found.[29,30] Secondly, spectrum bias could also be explained by a different tumor stage distribution in the referral population compared with those in a screening population. A comparison of CRCs from a referral and a screening population indeed revealed a higher prevalence of advanced cancers. After stratifying for T stage, however, no differences in FIT results were found between the screening and referral population.[31] Thirdly, test characteristics found in screening studies remain debatable, as 70% of screen detected CRCs in the British NHS National Bowel Cancer Screening Program appeared to be symptomatic.[32]

In conclusion, in the present study higher cut-off levels turned out to result in only a 5.3% decrease in detection rate for early-stage CRC, whereas at the sametime substantially reducing the number of positive FITs with 6.3%. Overestimation of sensitivity, however, due to potential spectrum bias can not be ruled out completely. When a higher cut-off level would be used as a first step preceding colonoscopy in a CRC screening program, lower numbers of colonoscopies would be required. This may facilitate the appropriate allocation of available resources. The lower detection rates of advancedadenomasmay be overcomeby the fact that these lesions are likely to be detected in a later screening round while probably still being at a stage of disease at which death from CRC can be prevented.

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4

Double sampling of a faecal immunochemical test is not superior to single sampling for detection of colorectal neoplasia: a colonoscopy controlled prospective cohort study

F.A. Oort*, S.T. van Turenhout*, V.M.H. Coupé, R.W.M. van der Hulst, E.I.C. Wesdorp, J.S. Terhaar sive Droste, I. Ben Larbi, S.L. Kanis, E. van Hengel, A.A. Bouman, G.A. Meijer, C.J.J. Mulder

*These authors have contributed equally to the work.

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ABSTRACT

Background: A single sampled faecal immunochemical test (FIT) has moderate sensitivity for colorectal cancer and advanced adenomas. Repeated FIT sampling could improve test sensitivity. The aim of the present study is to determine whether any of three different strategies of double FIT sampling has a better combination of sensitivity and specificity than single FIT sampling.

Methods: Test performance of single FIT sampling in subjects scheduled for colonoscopy was compared to double FIT sampling intra-individually. Test positivity of double FIT sampling was evaluated in three different ways: 1) "one of two FITs+" when at least one out of two measurements exceeded the cut-off value, 2) "two of two FITs+" when both measurements exceeded the cut-off value, 3) "mean of two FITs+" when the geometric mean of two FITs exceeded the cut-off value. Receiver operator curves were calculated and sensitivity of single and the three strategies of double FIT sampling were compared at a fixed level of specificity.

Results: In 124 of 1,096 subjects, screen relevant neoplasia (SRN) were found (i.e. early stage CRC or advanced adenomas). At any cut-off, "two of two FITs+" resulted in the lowest and "one of two FITs+" in the highest sensitivity for SRN (range 35–44% and 42–54% respectively). ROCs of double FIT sampling were similar to single FIT sampling. At specificities of 85/90/95%, sensitivity of any double FIT sampling strategy did not differ significantly from single FIT (p-values 0.07–1).

Conclusion: At any cut-off, "one of two FITs+" is the most sensitive double FIT sampling strategy. However, at a given specificity level, sensitivity of any double FIT sampling strategy for SRN is comparable to single FIT sampling at a different cut-off value. None of the double FIT strategies has a superior combination of sensitivity and specificity over single FIT.

BACKGROUND

In the United States of America and in Europe, colorectal cancer (CRC) ranks second as cause of cancer related death.[1,2] Screening is the most realistic approach to decrease CRC related mortality. Screening with guaiacbased faecal occult blood tests (g-FOBTs) has been shown to decrease disease specific mortality.[3-5] Faecal immunochemical tests (FITs or i-FOBTs) have been shown to be superior to g-FOBTs.[6-9] A major benefit of (semi-) quantitative FITs is that by adjustment of the threshold for positivity, test characteristics and number of follow-up colonoscopies can be tuned to local resources.[10,11] Since sensitivity of FIT for CRC is in the range of 66–87%,[8,12,13] and sensitivity for advanced adenomas is even lower (27–38% depending on the cut-off value),[8,13,14] there is still room for improvement. One approach for improving the sensitivity of FIT based screening could be to increase the number of samples tested, which is common practice for gFOBTs.[3-5]

So far, most studies on double FIT sampling either did not perform colonoscopy in FIT negative individuals,[15,16] did not evaluate different definitions of positivity for double FIT sampling,[14,17,19] or did not assess the effect of different cut-off values.[17,20] In addition, none of these studies evaluated the effect of multiple sampling on specificity.

This prospective, multi-centre cohort study aims to investigate whether sensitivity for the detection of screen relevant neoplasia (CRC stage I, II or advanced adenomas) of single FIT sampling can be increased by double FIT sampling, without substantially affecting specificity. Primary goal is to compare sensitivity and specificity of single FIT sampling and different strategies of double FIT sampling, at a predefined range of cut-off values, in a colonoscopy controlled population. In this study, we report that double and single FIT sampling have a comparable combination of sensitivity and specificity, at a different cut-off value.

METHODS

Study population

From June 2008 to October 2009, all ambulatory patients (≥18 years) scheduled for elective colonoscopy in three participating medical centres in and around Amsterdam, were invited to participate in this study irrespective of their indication for colonoscopy (i.e. screening, surveillance, or presence of symptoms). Exclusion criteria were either

hospitalization, age below 18 years, colostomy, total colectomy, colitis with ulcer(s), or a documented history or subsequent diagnosis of inflammatory bowel disease (IBD). In addition, individuals in which colonoscopic examination remained incomplete due to insufficient bowel lavage or technical difficulties, who did not adhere to the instructions on FIT sampling (e.g. failed to provide the dates of FIT sampling), or could not provide informed consent, were excluded from analysis. The local Medical Ethics Review Boards of each of the hospitals approved this study.

Study design

All eligible individuals were asked to perform a FIT on two subsequent days prior to colonoscopy. Elective patients were invited to participate in this study by telephone. Individuals interested in the study received a more detailed information package by mail, including two FITs, sampling instructions and an informed consent form. Subjects who could repetitively not be reached by telephone were send the same package with an additional explanatory letter.

An automated FIT was used (OC-sensor®, Eiken Chemical Co., Tokyo, Japan). This semi-quantitative test is considered positive when the haemoglobin concentration in the test tube exceeds the pre-determined cut-off value. Patients were instructed to perform this test on two separate days, before bowel preparation by laxatives was started, and write the date of performance on the FIT container.

The baseline FIT was defined as the sample taken from a bowel movement one day prior to colonoscopy (t = -1), whereas the additional FIT for double sampling was performed on stool produced two days before colonoscopy (t = -2; see Figure 4.1). Illustrated and written instructions explained participants to sample their stool without contamination with water or urine. All FITs were sampled at home and there were no restrictions in diet or medication during the week in which stool was sampled. Participants were instructed to obtain FIT samples at a maximum of 72 hours prior to colonoscopy, and to put the FIT samples in the zip lock bags that were included in the mail package. Participants were requested to store the zip lock bags in the refrigerator until departure for the endoscopy department.

Completed FITs and informed consent forms were collected at the endoscopy-department at the day of colonoscopy. All FITs were stored at minus 20 degrees Celsius on arrival. Tests were analyzed according to the manufacturer's instructions by an experienced

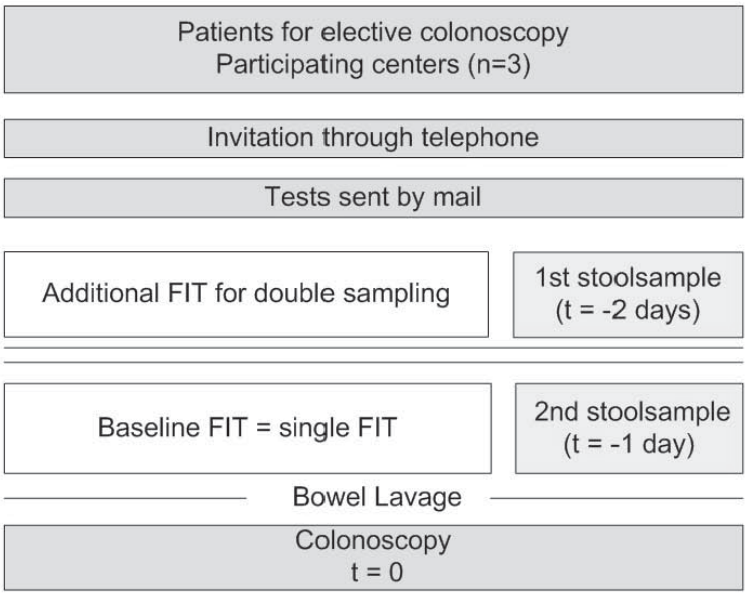

Figure 4.1 Study design. FIT, faecal immunochemical test.

technician, who was unaware of the clinical data, using the OC sensor MICRO desktop analyzer (Eiken Chemical co., Tokyo, Japan).[21]

Colonoscopy and lesions

All colonoscopies were performed or supervised by experienced gastroenterologists, who were unaware of the FIT results. Patients were offered to take conscious sedation by Midazolam. A complete colonoscopy was defined as intubation of the caecum with identification of the ileocaecal valve or appendiceal orifice, or intubation up to CRC (irrespective of the location and visualisation of the whole colon). Incomplete colonoscopies or colonoscopies with insufficient bowel preparation, as judged by the individual endoscopist, were excluded unless CRC was found. However, if a barium enema, virtual colonography or second colonoscopy was performed within six months, evaluation of the colon was considered complete and the subject was included in analysis. Patients were classified based on the most advanced lesion detected.

Histology of tissue samples obtained was evaluated routinely. Lesion size was estimated by the endoscopist. Adenomas ≥1.0 cm, adenomas with a villous component (i.e.

tubulovillous or villous adenoma) or adenomas with high-grade dysplasia were defined as advanced adenomas.[22] Colorectal carcinoma was staged according to the AJCC cancer and TNM staging manual.[23] Screen relevant neoplasia were defined as advanced adenoma and/or early stage cancer (i.e. stage I and II).

Statistical analysis

Primary outcome measures were sensitivity and specificity of the baseline FIT (t=-1; henceforth single FIT) and three strategies for double FIT sampling (results of t=-1 and t=-2) for the detection of screen relevant neoplasia. Results of single and double sampling were compared intra-individually and colonoscopy and histopathology were considered as gold standard. This study did not have the intention to determine the cut-off value with optimal sensitivity and specificity for screening. Instead, we evaluated whether a combination of sensitivity and specificity for double FIT sampling exists that is superior to single FIT sampling.

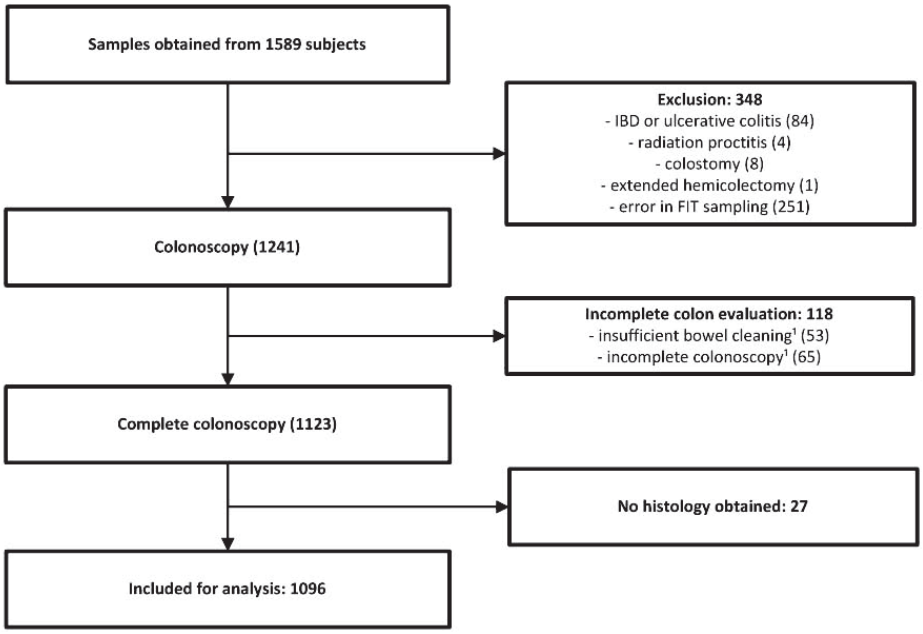

Figure 4.2 Study flow diagram of 1,589 subjects who participated in FIT sampling and subsequently underwent colonoscopy. [1]Incomplete colon evaluation in spite of possible additional evaluation by repeated colonoscopy, barium enema or virtual colonography. FIT, faecal immunochemical test; IBD, inflammatory bowel disease.

Three different strategies for positive reading of double FIT sampling were used:

1. "one of two FITs+": haemoglobin concentrations exceed the cut-off value in at least one out of two samples.
2. "two of two FITs+": haemoglobin concentrations exceed the cut-off value in both samples.
3. "mean of two FITs+": the geometric mean of haemoglobin concentrations from both samples exceeds the cut-off value.

Test sensitivities and specificities were assessed at cut-off values of 50, 75, 100, 150, and 200 ng/ml. The Exact method was used to calculate 95% confidence intervals. Receiver operator curves (ROCs) for detecting screen relevant neoplasia were calculated for single FIT and all three strategies of double FIT sampling. In addition, sensitivities of all three strategies for double FIT sampling were compared to single FIT sampling at a specificity of 85%, 90% and 95% using McNemar's test for correlated proportions. All analyses were performed with SPSS for Windows Version 15.0 (SPSS Inc., Chicago, USA).

RESULTS

Participants

Samples were returned by 1,589 patients, 493 of which were excluded from further analysis because of reasons listed in Figure 4.2. In 33 cases repeated colonoscopy or radiology was performed. Mean age of the participants included was 60, 0 years (range 19–91 yrs, SD 12.5) and 48% of the study cohort was male.

Table 4.1 shows the primary indications for colonoscopy in individuals eligible for analysis. In this cohort 59% (N=646) of individuals were referred for colonoscopy because of symptoms, whereas 37% (N=408) of subjects were referred for screening or surveillance colonoscopy. In 4% (N=42) of all individuals the indication remained unspecified.

Colonoscopy results

Colorectal cancer was found in 35 (3, 2%) of 1,096 included individuals. Malignancies were classified as stage I in 7 (20%), stage II in 13 (37%), stage III in 6 (17%) and stage IV in 3 (9%) patients. Six rectal cancers (17%) could not be staged accurately due to the effects

Table 4.1 Primary indications for colonoscopy among 1,096 consecutive patients enrolled for evaluation of double FIT sampling

Indication group	Indication for colonoscopy	N
Symptomatic/suspect		
	Weight loss	11
	Clinical suspicion of diverticulitis	7
	Clinical suspicion of IBD	8
	Abdominal pain	110
	Anaemia	71
	Hematochezia	156
	Altered bowel habits	182
	Clinical or radiological suspicion of CRC	25
	Colonoscopy for polypectomy	21
	Diarrhea	31
	Constipation	24
	Total	646
Screening and surveillance		
	Average risk	39
	Familial history of CRC	111
	Lynch syndrome	17
	Polyp surveillance	196
	Post CRC surveillance	45
	Total	408
Other		
	Not specified/others	42
	Grand total	1,096

FIT, faecal immunochemical test; IBD, inflammatory bowel disease; CRC, colorectal cancer.

of preoperative radiation. In 104 (9, 5%) individuals, one or more advanced adenomas were found. Consequently, screen relevant neoplasia were found in 124 (11, 3%) subjects.

Colorectal neoplasia detection and positivity rates

At a cut-off value of 50 ng/ml, the positivity rate of single FIT was 17%, resulting in detection of 91, 4% (32/35) of CRCs and 60, 6% (63/104) of all advanced adenomas found at colonoscopy. In subjects who tested negative for occult blood on single FIT, the additional FIT detected 2 more CRCs and 7 additional advanced adenomas.

Positivity rates ranged from 17–10% (with increasing cut-off values) for single FIT, from 22–12% for "one of two FITs+", from 12–7% for "two of two FITs+", and from 17–9% for "mean of two FITs+".

Sensitivity and specificity of single and double FIT strategies

Performance characteristics of single FIT and different strategies of double FIT sampling for detecting screen relevant neoplasia, at different cut-off values, are shown in Table 4.2.

At each cut-off value, maximum sensitivity for screen relevant neoplasia was obtained with "one of two FITs+". Compared to single FIT, the highest increase in sensitivity was obtained with "one of two FITs+" at either 50, 75 or 100 ng/ml (6.4% increase over single FIT). However, the confidence intervals of the sensitivity of single FIT and "one of two FITs+" overlapped, and the specificity of "one of two FITs+" (83.0, 87.4 and 89.8% at 50, 75, and 100 ng/ml, respectively) was lower than for single FIT (88.2, 90.5, and 92.5% at 50, 75, and 100 ng/ml, respectively).

At each cut-off value, maximum specificity was found with "two of two FITs+". The highest specificity (97, 5%) of all double FIT strategies was observed for "two of two FITs+" at the highest cut-off value (200 ng/ml). However, "two of two FITs+" resulted in lower sensitivities than single FIT. Moreover, by using single FIT, comparable specificities as for "two of two FITs+" could be reached (up to 95%) by using higher cut-off values (see Table 4.2).

Test characteristics of double FIT sampling strategies were comparable to single FIT sampling at a different cut-off value. For example at 75 ng/ml, the sensitivity of "one of two FITs+" (52%) was higher than the sensitivity of single FIT (46%). However, when the cut-off value of single FIT was decreased to 50 ng/ml, sensitivity became 48% (CI 39–57) which is close to sensitivity of "one of two FITs+" (52%; CI 43–61). The accompanying specificity of single FIT at 50 ng/ml (88, 2%) was virtually equivalent to the specificity of "one of two FITs+" (87, 4%). As shown in Table 4.2, test characteristics of "mean of two FITs+" were comparable to single FIT.

Receiver operator curves

For single FIT and the three double FIT strategies, ROCs were constructed (see Figure 4.3). Highest sensitivities were reached with "one of two FITs+" and "mean of two FITs+", whereas the highest specificities were reached with "two of two FITs+". For all double FIT strategies, ROCs and area under the curves (AUCs) either overlapped or were very close to each other (see Figure 4.3). Although the highest AUC was found for "mean of two FITs+", all AUCs were within the 95% confidence interval of the AUC of single FIT.

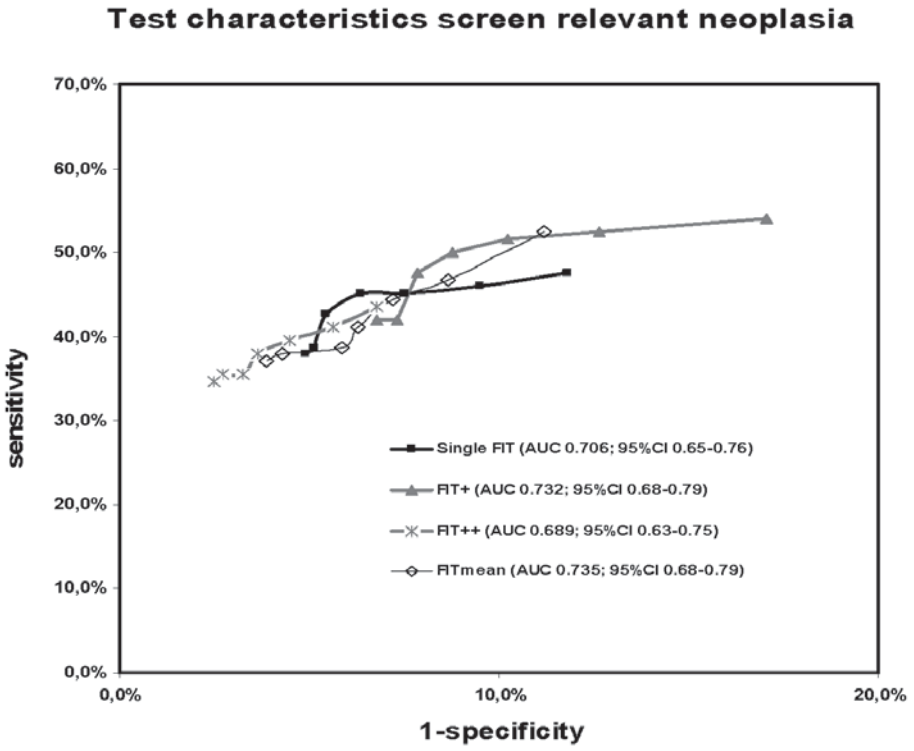

Figure 4.2 ROC curves of single and double FIT sampling strategies for the detection of screen relevant neoplasia. FIT, faecal immunochemical test; "one of two FITs+", at least one of both FITs above the cut-off value; "two of two FITs+", both FITs above the cut-off value; "mean of two FITs+", geometric mean of both FITs above the cut-off value; AUC, area under the curve; CI, confidence interval.

Comparison at fixed specificities

To evaluate to what extent an increase in sensitivity by double FIT sampling went at the cost of decreased specificity, single FIT and the three double FIT strategies were analyzed at equal specificities. Table 4.3 shows cut-off values and sensitivities at 85%, 90% and 95% specificity, for each strategy. At any of these specificities, no strategy for double FIT sampling yielded a sensitivity that differed significantly from the sensitivity of single FIT.

Table 4.2 Test characteristics of single and double FIT sampling for detection of screen relevant neoplasia

	Single FIT		"one of two FITs+"		"two of two FITs+"		"mean of two FITs+"	
Cut-off value	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
Cut-off 50	47.6%	88.2%	54.0%	83.0%	43.5%	93.2%	52.4%	88.8%
N	59/124	844/957	67/124	794/957	54/124	892/957	65/124	850/957
(CI)	(39–57)	(86–90)	(45–63)	(80–85)	(35–53)	(91–95)	(43–61)	(87–91)
Cut-off 75	46.0%	90.5%	52.4%	87.4%	41.1%	94.4%	46.8%	91.3%
N	57/124	866/957	65/124	836/957	51/124	903/957	58/124	874/957
(CI)	(37–55)	(88–92)	(43–61)	(85–89)	(32–50)	(93–96)	(38–56)	(89–93)
Cut-off 100	45.2%	92.5%	51.6%	89.8%	39.5%	95.5%	44.4%	92.8%
N	56/124	885/957	64/124	859/957	49/124	914/957	55/124	888/957
(CI)	(36–54)	(91–94)	(42–61)	(88–92)	(31–49)	(94–97)	(35–54)	(91–94)
Cut-off 150	42.7%	94.6%	47.6%	92.2%	35.5%	96.8%	38.7%	94.1%
N	53/124	905/957	59/124	882/957	44/124	926/957	48/124	901/957
(CI)	(34–52)	(93–96)	(39–57)	(89–93)	(27–45)	(95–98)	(30–48)	(92–96)
Cut-off 200	37.9%	95.1%	41.9%	93.2%	34.7%	97.5%	37.1%	96.1%
N	47/124	910/957	52/124	892/957	43/124	933/957	46/124	920/957
(CI)	(29–47)	(94–96)	(39–57)	(91–95)	(26–44)	(96–98)	(29–46)	(95–97)

Test characteristics at different cut-off values (ng/ml) of single and three strategies of double FIT sampling for detection of screen relevant neoplasia in 1,081* individuals referred for colonoscopy (SRN in 124, no AA nor CRC in 957). *15 cases of late stage CRC were excluded since they were not considered screen relevant. FIT, faecal immunochemical test; SRN, screen relevant neoplasia; AA, advanced adenoma; CRC, colorectal cancer; "one of two FITs+", at least one of both FITs above the cut-off value; "two of two FITs+", both FITs above the cut-off value; "mean of two FITs+", geometric mean of both FITs above the cut-off value; CI, confidence interval; sens, sensitivity; spec, specificity.

Table 4.3 Comparison of sensitivity of single and double FIT sampling for screen relevant neoplasia, at fixed specificities

	Single FIT		"one of two FITs+"			"two of two FITs+"			"mean of two FITs+"		
Spec	Sens	Cut-off	Sens	Cut-off	p-value	Sens	Cut-off	p-value	Sens	Cut-off	p-value
85%	51.6%	34	53.2%	59	1	n.a.*	n.a.*	n.a.*	53.2%	40	1
90%	46.0%	73	51.6%	103	0.07	44.4%	27	0.687	50.8%	60	0.125
95%	38.7%	184	37.9%	371	1	41.1%	91	0.453	37.9%	159	1

Corresponding cut-off values (ng/ml) and sensitivities for screen relevant neoplasia of single FIT sampling and three different strategies of double FIT sampling at fixed specificities of 85%, 90% and 95%. FIT, faecal immunochemical test; "one of two FITs+", at least one of both FITs above the cut-off value; "two of two FITs+", both FITs above the cut-off value; "mean of two FITs+", geometric mean of both FITs above the cut-off value; spec, specificity; sens, sensitivity; cut-off, cut-off value; n.a.*, no corresponding sensitivity and cut-off value found in the range 50–200 ng/ml.

Additional analysis

All analyses described above were repeated for the outcomes advanced adenomas and CRC. Results are shown in Supplementary Tables S4.1–S4.4 and Supplementary Figures S4.1 and S4.2. The results found were very similar to those for screen relevant neoplasia.

In total 251 cases were excluded because of an error in FIT sampling. The majority of 155 cases was excluded as the date of sampling of one or both of the tests was unsure. These cases were included in additional analysis, to evaluate if exclusion of these cases would cause bias. As shown in the Additional file 1, the results of these analysis were similar. The remaining 96 sampling errors were due to sampling on or after the day of colonoscopy, performance of only one test, or failure in FIT analysis.

DISCUSSION

In the present study three different strategies of double FIT sampling were compared to single FIT sampling. In total, 1096 subjects were included and evaluated by colonoscopy. None of the double FIT strategies proved to have a superior combination of sensitivity and specificity compared to single FIT sampling, as is clear from the comparable ROCs and similar AUCs found for all strategies. When comparing sensitivities of single FIT and the three double FIT strategies at fixed specificities of 85%, 90% and 95%, no relevant differences were observed. In fact, at every level of specificity, a comparable sensitivity as observed for "one of two FITs+" could be obtained by single FIT by simply lowering the cut off value.

A priori expectations were that double FIT sampling would increase sensitivity, as this has been observed previously for g-FOBT and FIT.[18,19,24] Accordingly, it was shown in the present study that the highest sensitivity was obtained for "one of two FITs+". However, this strategy resulted in the lowest specificity.

Our findings are in line with a recent study in a population with an increased risk for CRC, in which AUCs for the highest out of one, two or three FITs did not differ.[18] Although a direct comparison with a recent Italian screening study is difficult due to different methodology, the authors could also not find a clear superior performance of double over single FIT sampling either.[15] Two other studies on double FIT sampling lacked calculation of direct sensitivity and specificity, as colonoscopy was not performed in FIT negative individuals.[15,16] These characteristics are needed to determine how an increase in

sensitivity is counterbalanced by a decrease in specificity. Less recent studies did not use quantitative FITs or did not evaluate test characteristics at different cut-off values.[17,20] In a recent study with a high CRC prevalence, average risk individuals sampled stool before screening colonoscopy. The authors found that the sensitivity increased and specificity decreased when a lower cut-off value or multiple tests were used. However, no comparison was made at an equal specificity. The AUCs for advanced neoplasia for one, two or three FITs did not differ.[19] In the present study, the full potential of double FIT sampling was further studied by evaluation of several definitions of positivity. The present study adds important information as it is the first to determine if any of three strategies of double FIT sampling could increase sensitivity for screen relevant neoplasia, without substantially affecting specificity, at different cut-off values and in a colonoscopy controlled population.

A limitation of the present study is that not a screening population was tested but a referral population, partially containing high risk individuals. Therefore, test characteristics that depend on the prevalence of disease, i.e. positive and negative predictive values, cannot be generalized from this study to the screening population. However, the present study focused on sensitivity and specificity, test characteristics that are not influenced by the prevalence of the disease.[25] Still, in this referral population, sensitivity may be overestimated and specificity underestimated due to work-up bias.[26] This may occur as symptomatic participants have an increased likelihood for having both a positive FIT and a colorectal neoplasm. In particular, it should be noted that lower sensitivities for FIT in a screening population have been reported.[19,27] On the other hand, we carried out a formal comparison of FIT results in CRC cases from a screening and referral cohort and found similar FIT results after correcting for tumour stage.[28] Since for screening, only early stage cancers are relevant, in the present study late stage cancers were excluded from the analysis. Although possible differences in FIT results between referral populations, like in the present study, and screening populations cannot fully be excluded, the present study design still allows for comparing the sensitivities of different sampling schemes for FIT for early stage colorectal cancer. However, a complete correction of work-up bias cannot be ascertained. One should keep in mind that alternative study designs also have limitations like absence of a gold standard because no colonoscopies were performed, or in case colonoscopies were performed, relatively low numbers of cancers found.[15,16,27] In addition, in many studies different FITs, different endpoints (advanced adenoma, advanced neoplasia, screen relevant neoplasia), a different amount of cases, and a different selection of participants (e.g. subjects participating in colonoscopy screening) are used.

To evaluate the effect of work-up bias, analyses were repeated after exclusion of subjects with rectal blood loss, anaemia and clinical suspicion of CRC (data not shown). Although the sensitivities for advanced adenomas found were 4.5–10% lower, our results were similar in the sense that double FIT sampling did not yield any superior combination of sensitivity and specificity compared to single FIT. For CRC data were similar, although too few cases remained to draw firm conclusions (data not shown).

In the current study the number of excluded participants was relatively high. This was mainly due to our stringent protocol on FIT sampling. Of the 251 individuals that were excluded from further analysis, in the majority of cases this was because date of sampling was not registered correctly on the FIT container, as described in the study protocol. Additional analysis including these cases showed similar results. The percentage of incomplete colonoscopies in the present study is in line with previous studies.[7,29]

According to our study protocol all FITs should be stored in the refrigerator close to the moment of handing in. In addition, both FITs are sampled maximum 72 hours prior to colonoscopy. As such, the time that the tests are at room temperature is kept as limited as possible. FITs kept at higher temperatures, are more susceptible to a decrease in sensitivity as a result of haemoglobin degradation. When compared to at least one of the screening studies[7] this is still a relative short period of time. Therefore, only a slight decrease in haemoglobin concentration is to be expected.[30]

An important asset of the present study is the relatively high tumour yield, which allowed analyzing FIT performance for early and late stage CRC separately. As the potential health gain is highest for individuals with early stage cancer,[31] this is relevant for population based screening programs. A second strength of this study is the fact that colonoscopy results were available for all participants, allowing the direct calculation of sensitivities and specificities.

CONCLUSION

In conclusion, this study strongly suggests that double FIT sampling, regardless of the definition of test positivity, does not provide a superior combination of sensitivity and specificity compared to single FIT sampling. Moreover, if it is aimed to increase sensitivity at the cost of specificity, this can be achieved equally well by lowering the cut-off value of single FIT sampling rather than by double FIT sampling. To what extent these findings pertain to the general population awaits confirmation in a screening setting.

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5

Hemorrhoids detected at colonoscopy: an infrequent cause of false-positive fecal immunochemical test results

S.T. van Turenhout, F.A. Oort, J.S. Terhaar sive Droste, V.M.H. Coupé, R.W.M. van der Hulst, R.J. Loffeld, P. Scholten, A.C.T.M. Depla, A.A. Bouman, G.A. Meijer, C.J.J. Mulder, L.G.M. van Rossum

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ABSTRACT

Background: Colorectal cancer screening by fecal immunochemical tests (FITs) is hampered by frequent false-positive (FP) results and thereby the risk of complications and strain on colonoscopy capacity. Hemorrhoids might be a plausible cause of FP results.

Objective: To determine the contribution of hemorrhoids to the frequency of FP FIT results.

Design: Retrospective analysis from prospective cohort study.

Setting: Five large teaching hospitals, including 1 academic hospital.

Patients: All subjects scheduled for elective colonoscopy.

Interventions: FIT before bowel preparation.

Main outcome measurements: Frequency of FP FIT results in subjects with hemorrhoids as the only relevant abnormality compared with FP FIT results in subjects with no relevant abnormalities. Logistic regression analysis to determine colonic abnormalities influencing FP results.

Results: In 2,855 patients, 434 had positive FIT results: 213 had advanced neoplasia, and 221 had FP results. In 9 individuals (4.1%; 95% CI, 1.4–6.8) with an FP FIT result, hemorrhoids were the only abnormality. In univariate unadjusted analysis, subjects with hemorrhoids as the only abnormality did not have more positive results (9/134; 6.7%) compared with subjects without any abnormalities (43/886; 4.9%; P=.396). Logistic regression identified hemorrhoids, nonadvanced polyps, and a group of miscellaneous abnormalities, all significantly influencing false positivity. Of 1000 subjects with hemorrhoids, 67 would have FP results, of whom 18 would have FP results because of hemorrhoids only.

Limitations: Potential underreporting of hemorrhoids; high-risk individuals.

Conclusions: Hemorrhoids in individuals participating in colorectal cancer screening will probably not lead to a substantial number of false positive test results.

BACKGROUND

Colorectal cancer (CRC) ranks in top 3 of malignancyrelated mortality in Europe and the United States.[1,2] Survival is closely related to stage of disease,[3] and population-based screening is advocated in many countries.

The screening strategy with the highest sensitivity for detection of colonic neoplastic lesions is high-quality screening colonoscopy. Yet, because screening colonoscopy has drawbacks including low participation rates and complications,[4-7] others advocate preselection by fecal occult blood tests (FOBTs). In population-based screening by fecal immunochemical tests (FITs or immunochemical FOBTs), only participants with a positive test result require colonoscopy. However, in almost half of all screenees with a positive FIT result, no advanced neoplasia is found at colonoscopy.[8,9] These false positive (FP) results could lead to unnecessary colonoscopy-related complications, futile strain on endoscopic resources, psychological stress for the screenee, and a decrease in confidence in the screening program. Therefore, the number of FP FIT results should be as low as possible.

Because of their natural history, hemorrhoids are a plausible explanation for (both visible and occult) rectal bleeding. Although still unknown, hemorrhoids may be an important explanation for FP FIT results. Therefore, the aim of this study was to determine the association between hemorrhoids and FIT results.

METHODS

Study population

For this study, the data set that was used was selected from an ongoing study on FIT performance that was designed to answer several research questions.[10-12] The data were collected between June 2006 and October 2009. In 5 medical centers in and around Amsterdam, the Netherlands, ambulatory subjects 18 years of age and older scheduled for elective colonoscopy were invited regardless of the indication for colonoscopy (screening, surveillance, or symptoms). Exclusion criteria used were no informed consent, hospitalization, age younger than 18 years, colostomy, total colectomy, colitis with ulcer(s), and a documented history or subsequent diagnosis of inflammatory bowel disease. The study was approved by the local medical ethics review board of each of the 5 hospitals.

Study design

Participants were requested to perform at least 1 FIT 1 or 2 days before bowel preparation and colonoscopy. Eligible subjects were invited to participate by telephone. When interested, a detailed information package was sent by mail, containing a FIT, sampling instructions, and an informed consent form. If an individual could not be reached by telephone on multiple occasions, the same package was sent with an additional explanatory letter. The instructions indicated that contact of stool with water and urine should be prevented, and the tests should be kept refrigerated until transport to the hospital. The FIT was performed at maximum 72 hours before colonoscopy. All subjects who were requested to perform only 1 FIT performed the test 1 day before undergoing colonoscopy. For comparability, in participants who performed more than 1 FIT, only the FIT performed 1 day before colonoscopy was selected for analysis in this study.

The test used in this study is the automated OC-sensor (Eiken Chemical Co, Tokyo, Japan), which has a quantitative outcome. Completed FITs and informed consent forms were handed in at the endoscopy department on the day of the colonoscopy. All tests were frozen at -20°C on arrival. Two experienced technicians who were unaware of the clinical data analyzed all tests according to the manufacturer's instructions. The OC sensor MICRO desktop analyzer (Eiken Chemical Co) was used for all analyses.[13]

Colonoscopy and histology

Experienced gastroenterologists performed or supervised all colonoscopies and were unaware of the FIT results. All participants were offered conscious sedation with midazolam. Assessment of bowel preparation was judged by the individual endoscopist. Colonoscopy was considered complete when the cecum was intubated with identification of the appendiceal orifice or the ileocecal valve. The presence of hemorrhoids was classified retrospectively as reported in the endoscopy report. When available, grading of hemorrhoids according to the degree of prolapse was scored.[14] The size of polyps detected was estimated by the endoscopist by using biopsy forceps.

All tissue samples obtained during colonoscopy were evaluated according to routine procedures. Adenomas with high-grade dysplasia, villous components, and/or at least 1 cm in size were considered advanced adenomas.[15] The presence of CRC and/or 1 or more advanced adenomas was classified as the presence of advanced neoplasia.

Statistical analysis

The primary endpoints of this study were the prevalence of hemorrhoids in individuals with a positive FIT result and negative findings on colonoscopy (i.e., FP tests) and the relative frequency of FP test results in subjects with and without hemorrhoids. Multiple colonic abnormalities in 1 patient might influence test result positivity. The frequency of FP FIT results in subjects with hemorrhoids as the only abnormality was compared with the frequency of FP FIT results in subjects without any colonic abnormalities. Logistic regression analysis was used to study which colonic abnormalities are risk factors for FP FIT results.

Colonoscopy and histology were considered the criterion standard for the presence of advanced neoplasia and hemorrhoids. Subjects with an incomplete colonoscopy or insufficient bowel preparation were excluded from analyses unless CRC was found. Subjects with an incomplete colonoscopy were included in analyses if it was followed by a second complete colonoscopy within 6 months. Subjects with 1 or more polyps from which no material was obtained for histological examination (N=147) were excluded. This includes polyps that could not be retrieved after polypectomy or polyps that were not sent for histopathological evaluation because no clinical consequences of a histopathological diagnosis were anticipated (e.g., because of comorbidity). These subjects were excluded because it is unknown whether the polyp was an advanced adenoma and therefore whether the FIT result was true or false positive/negative. In addition, subjects in whom the significance of hemorrhoids was uncertain were excluded (i.e., subjects in whom hemorrhoids reported were described as only 1 hemorrhoid or fibrotic hemorrhoids).

False positivity was defined as a FIT result of 50 ng or more of hemoglobin per milliliter of buffer solution and no advanced neoplasia (either advanced adenomas or CRC) at colonoscopy. All individuals with a FIT FP result were evaluated for the presence of different colonic abnormalities, particularly the frequency of hemorrhoids as the only abnormality detected.

To study the association between hemorrhoids and FP test results in more detail, only subjects without advanced neoplasia were selected. These subjects are by definition at risk of FP FIT results. The Fisher exact test was used to compare the number of FP test results in individuals with and without hemorrhoids. To avoid possible heterogeneity in occult blood loss caused by the presence of other abnormalities 2 additional groups were selected: (1) the group of subjects without any abnormalities at colonoscopy and

(2) the group of individuals in whom hemorrhoids were the only abnormality found at colonoscopy. Groups 1 and 2 were compared for the frequency of test FP results. Finally, logistic regression analysis was used to study the effect of different colonic risk factors that could influence FP FIT results. False positivity (FIT 50, 75, and 100 ng/mL) was used as a dependent variable and the presence of hemorrhoids, the presence of 1 or more diverticula, the presence of 1 or more nonadvanced polyps, and finally the presence of 1 or more other abnormalities (i.e., abnormalities not included in the other variables) were independent variables. The variable other abnormalities was scored positive when abnormalities such as angioectasia, aphthous lesions, lymphoma, and lipoma, were present. In multivariate analysis, the influence of the these variables on false positivity was corrected for age and gender.

All analyses were performed with SPSS for Windows version 15.0 (SPSS Inc, Chicago, Ill).

RESULTS

Participants

In this study, 3,339 subjects underwent colonoscopy and performed an FIT. In 2,893 individuals, a total colonoscopy was performed and histological analysis was complete when applicable (Figure 5.1). Thirty-eight subjects were excluded because the clinical relevance of the hemorrhoids that were reported was not clear. The main indication for colonoscopy was surveillance and screening in 1,021, the presence of symptoms in 1,712, and unspecified in 122 (Table 5.1). Of the 2,855 subjects included in the analysis, 434 (15%) had a positive FIT result (cut-off value of 50 ng/mL) and 371 (13%) had hemorrhoids. Of all individuals with hematochezia as the indication for colonoscopy, 44 (11%) had FP results. This accounts for 44 of 221 (20%) of all FP results. Of all individuals with polypectomy as the indication for colonoscopy, 4 (6%) had FP results. This accounts for 4 of 221 (2%) of all FP results. These subjects were not excluded because the frequency of FP FIT results was studied in subjects with hemorrhoids as the only abnormality, irrespective of the indication for colonoscopy.

Table 5.2 shows the age and sex distribution of the total population and true and FP results.

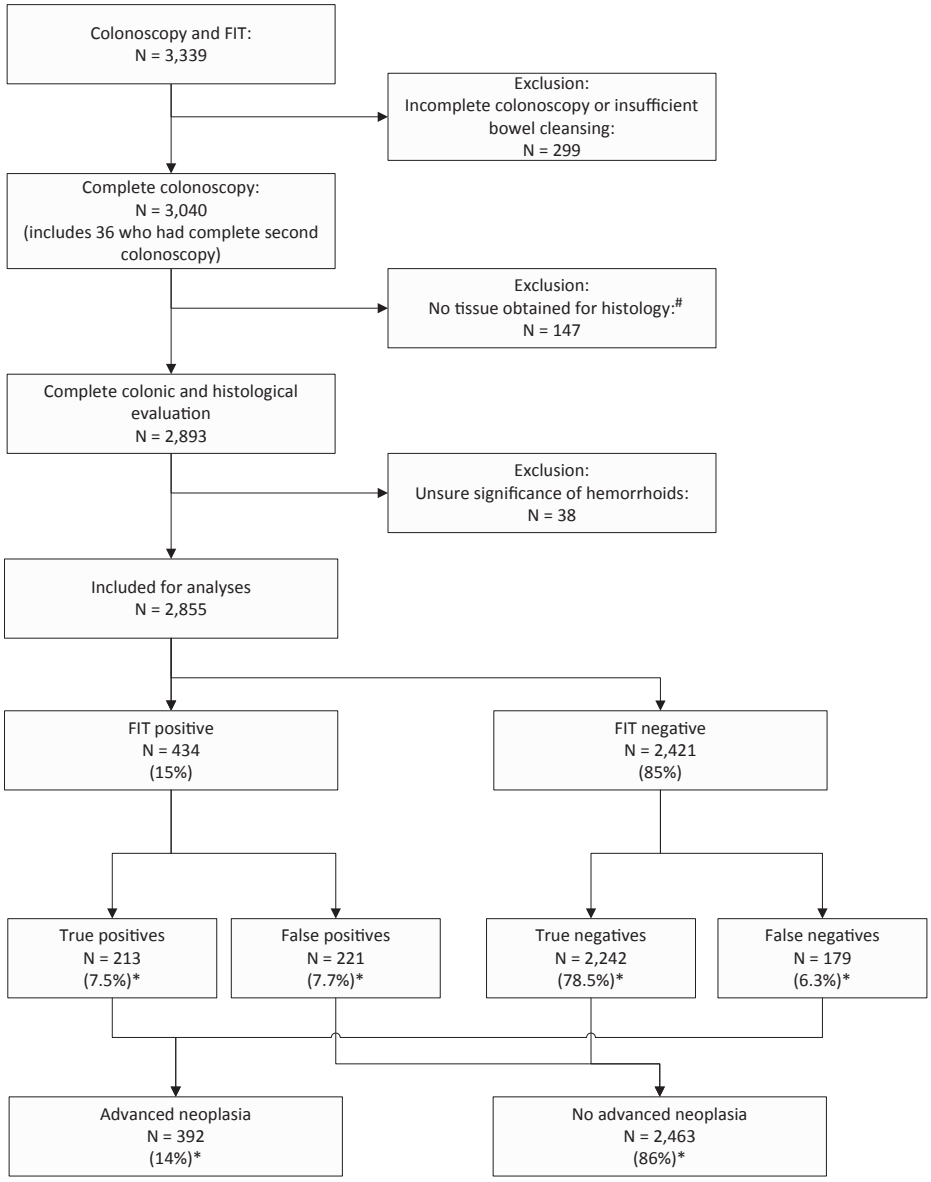

Figure 5.1 Study flow diagram. #No histology obtained: no material obtained for histological examination. This concerns polyps that could not be retrieved after polypectomy or polyps that were not sent for histopathological evaluation because no clinical consequences of a histopathological diagnosis were anticipated. *Percentage of total study population (2,855 subjects). FIT, fecal immunochemical test.

Table 5.1 Primary indications for colonoscopy among 2,855 consecutive patients included in a study of the influence of hemorrhoids on false-positive FIT results

Indication group	Indication for colonoscopy	N
Symptomatic/suspect	Weight loss	29
	Clinical suspicion of diverticulitis	28
	Clinical suspicion of IBD	23
	Abdominal pain	388
	Anemia	164
	Hematochezia	403
	Altered bowel habits	422
	Clinical or radiological suspicion of CRC	47
	Colonoscopy for polypectomy	64
	Diarrhea	93
	Constipation	51
	Total	1,712
Screening and surveillance	Average risk	64
	Familial history of CRC	362
	Lynch syndrome	44
	Polyp surveillance	404
	Post-CRC surveillance	147
	Total	1,021
Other	Not specified/others	122

FIT, fecal immunochemical test; IBD, inflammatory bowel disease; CRC, colorectal cancer.

FP results

The frequency of hemorrhoids and abnormalities other than hemorrhoids detected in FP result cases are shown in Figure 5.2. Of the positives, the results of 221 FITs (51%) were found to be FP (i.e., no advanced neoplasia was detected at colonoscopy) (Figure 5.1). In all cases of FP results, it was observed that in 4.1% (9/221; 95% CI, 1.4–6.8) hemorrhoids were the only colonic abnormality that could have caused a positive test result.

Hemorrhoids

As stated previously, in 371 of 2,855 (13%) subjects, hemorrhoids were detected at colonoscopy. The grade of hemorrhoids was reported in 43%. After retrospective reclassification according to the degree of prolapse into the anal canal,[14] 86% were classified as grade I, 10% as grade II, and 4% were unspecified. From the 2,463 participants without

Table 5.2 Demographics of 2,855 subjects included in a study of the frequency of hemorrhoids as the cause of false-positive FIT results

Group	N	Age, y (SD)	P-value	Females, %	P-value
Total population	2,855	59.5 (12.8)	-	55	-
False positives	221	62.5 (12.2)	<.001	49	.21
True positives	213	66.6 (9.9)	-	43	-
Total group without advanced neoplasia[a]	2,463	58.6 (12.9)	-	56	-
Hemorrhoids present[b]	339	60.0 (11.5)	.03	53	.22
No hemorrhoids[b]	2,124	58.4 (13.1)	-	56	-
Only hemorrhoids present	134	56.8 (13.6)	.004	62	.71
No abnormalities	886	53.2 (11.5)	-	60	-

FIT, fecal immunochemical test; SD, standard deviation.
[a] Susceptible to false-positive results.
[b] Next to hemorrhoids, other abnormalities may be present.

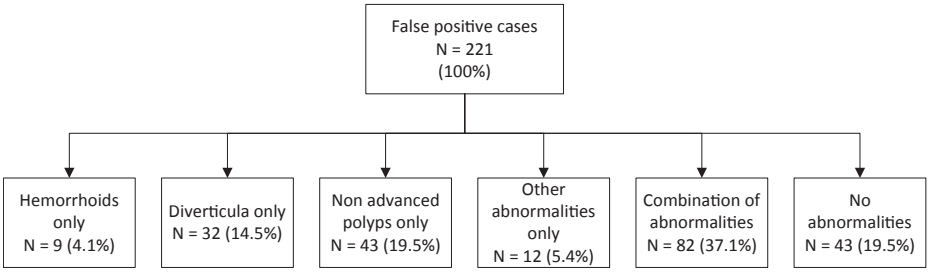

Figure 5.2 Overview of abnormalities and their frequencies in 221 cases with a false-positive fecal immunochemical test result. Note: Due to rounding, percentages add up to 100.1%.

advanced neoplasia, 339 (14%) were found to have hemorrhoids. In Table 5.2, the mean age and gender are presented for participants without advanced neoplasia and with (N=339) or without (N=2,124) hemorrhoids.

Subjects with and without hemorrhoids were compared for FP test results. The number of FP FIT results in all subjects with hemorrhoids (41/339; 12.1%) was significantly higher than the number of FP FIT results in all susceptible subjects without hemorrhoids (180/2124; 8.5%; P=.04; Table 5.3). Individuals with a FP FIT result with hemorrhoids were significantly older compared with individuals without hemorrhoids with FP results

Table 5.3 Frequency of false-positive FIT results in subjects with and without hemorrhoids studied in 2,463 subjects without advanced neoplasia

	Hemorrhoids, N (%)[a]	No. hemorrhoids, N (%)[a]	Total, N
FIT positive results	41 (12.1)	180 (8.5)	221
FIT negative results	298 (87.9)	1,944 (91.5)	2,242
Total	339 (100)	2,124 (100)	2,463

Fisher exact test: P=.04. Cut-off value for FIT results positivity is ≥50 ng/mL.
FIT, fecal immunochemical test.
[a] Other colonic abnormalities may be present in both groups.

(mean age 60.0 vs 58.4 years, respectively; P=.03). In addition, a slight, not statistically significant, gender difference was observed (Table 5.2).

To exclude the potential confounding influence of age and the presence of more than 1 type of abnormality, subjects in whom no abnormalities were found at colonoscopy (N=886), were compared with subjects in whom hemorrhoids were the only abnormality (N=134) detected (groups 1 and 2, respectively). As shown in Table 5.4, groups 1 and 2 did differ, but not statistically significantly, in the frequency of FP FIT results (4.9% vs 6.7%, respectively; odds ratio [OR] 1.41; P=.396). However, the mean age in group 1 was significantly lower (53.2 years vs 56.8 years, respectively; P=.004; Table 5.2), and some statistically nonsignificant gender differences were observed (P=.71). Because these findings might indicate that the absence of a significant difference was caused by confounding, logistic regression analysis was performed.

Logistic regression analysis

Logistic regression analysis was used to study which abnormalities contribute significantly to the probability of an FP FIT result. In the univariate analysis, age, gender, the presence of hemorrhoids, diverticula, nonadvanced polyps, and other abnormalities (including ulcers, angioectasia, aphthous lesions, moderate chronic inflammation, lipoma, and lymphoma) were all significantly associated with FP FIT results.

In multivariate analysis, risk factors for false positivity were corrected for age and gender. It was found that hemorrhoids (OR 1.45), nonadvanced polyps (OR 1.78), and other abnormalities (OR 2.17) influenced the probability of false positivity significantly. Table

Table 5.4 Frequency of false-positive FIT results in subjects with hemorrhoids detected as the only abnormality at colonoscopy and in subjects without any abnormalities at colonoscopy

	Hemorrhoids only, N (%)	No. abnormalities, N (%)	Total, N
FIT positive results	9 (6.7)	43 (4.9)	52
FIT negative results	125 (93.3)	843 (95.1)	968
Total	134 (100)	886 (100)	1,020

Fisher exact test: P=.396. Cut-off value for FIT positivity is ≥50 ng/mL.
FIT, fecal immunochemical test.

5.5 shows the level of significance of the abnormalities and ORs for false positivity at the cut-off values 50, 75, and 100 ng/mL.

Risk of false positivity attributable to hemorrhoids

The risk of FP FIT results exclusively because of hemorrhoids is the difference in risk of FP results in subjects with hemorrhoids only and subjects without any abnormalities (6.7%–4.9%=1.8%). In other words, per 1,000 subjects with hemorrhoids only, 67 FP FIT results will be found, of which 18 are attributable to the presence of hemorrhoids.

DISCUSSION

In this study, a large prospective cohort was used to study the association between hemorrhoids and FP FIT results. In 4.1% of all FP test results, hemorrhoids were the only abnormality detected. After correction for gender, age, and other abnormalities with logistic regression analysis, it was shown that subjects in whom hemorrhoids were the only abnormality found at colonoscopy were found to have a slightly higher rate of FP FIT results. The absolute increase in the risk of false positivity is small, i.e., 1.8% (from 4.9% to 6.7%) and not significant.

Based on the current data, the number of FP FIT results exclusively caused by hemorrhoids seems to be limited. The threshold of 50 ng/mL for positivity was chosen because the number of potential FP test results would be highest at this cut-off value. Because hemolysis is needed before FIT can detect globin, the likelihood of detecting occult blood

Table 5.5 Multivariate logistic regression analysis: abnormalities detected at colonoscopy with their OR for false-positive FIT results at different cut-off values for positivity and corrected for age and gender

	50ng/mL; 221 false positive			75ng/mL; 178 false positive			100ng/mL; 146 false positive		
Cut-off value	P-value	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI
Colonic abnormalities									
Hemorrhoids	.045	1.45	1.01–2.10	.026	1.57	1.06–2.32	.114	1.43	0.92–2.21
Diverticula	.143	1.25	0.93–1.69	.197	1.24	0.89–1.73	.216	1.26	0.88–1.80
Non-advanced polyps	<.001	1.78	1.34–2.37	<.001	1.76	1.29–2.41	.003	1.67	1.19–2.36
Others[a]	<.001	2.17	1.46–3.21	<.001	2.66	1.77–3.21	<.001	2.75	1.77–4.26

OR, odds ratio; FIT, fecal immunochemical test; CI, confidence interval.

[a] Other abnormalities include, eg, angioectasia, ulcers, erosions, aphthous lesions, lipomas, and moderate chronic inflammation.

from hemorrhoids may be low because of their location. By choosing the lowest cut-off value, the chance of detecting hemorrhoidal bleeding would be optimal. The current findings seem to be in line with those of a previous study in which subjects with a positive FIT result were sent for colonoscopy.[17] In this study, the positivity rate in subjects with and without hemorrhoids was found to be similar.[17]

The source of blood loss was not identified for all subjects with positive FIT results. By logistic regression analysis, it was shown that hemorrhoids, nonadvanced polyps, and other abnormalities all contribute to FP test results. In addition, at cut-off values of 75 and 100 ng/mL, which are frequently used in screening,[8,18] the ORs were similar. However, because of fewer subjects with an FP test result at a higher cut-off value, the standard error increased and consequently the level of significance of the associations decreased. In almost 5% of subjects with an FP FIT result, no colonic abnormalities were detected. Because the FIT used in this study detects human globin by specific antibodies, FP test results caused by dietary factors can be excluded.[18] Because the epitope for the antibody reaction of this FIT is within globin, which is degraded through the digestive tract, the likelihood of a more proximally located cause of occult bleeding is low, but cannot be excluded.[19] In addition, the suboptimal sensitivity of colonoscopy for small lesions such as small adenomas is a potential confounder here.[7,20,21]

In population-based screening, even a small percentage of FP test results would cause a large number of unnecessary referrals for colonoscopy. If the current results are extrapolated to a hypothetical screening situation, of all 1,000 subjects with hemorrhoids, 67 FIT results would be FP. Of those FP results, 18 would be exclusively attributed to the presence hemorrhoids. This number could be higher or lower because the prevalence of hemorrhoids in this clinical population is likely to be different from that in average-risk individuals participating in a screening program. A recent study on colonoscopy screening participants in Austria found a prevalence of hemorrhoids of 39%.[22] These individuals might, however, be different from participants in FOBT screening. Unfortunately, the true prevalence of hemorrhoids seems to remain a black box because other studies showed prevalences ranging from 4% to 86%, depending on the population studied and the methodology used.[23,24] We still would expect that this referral population will have a higher prevalence of hemorrhoids (e.g., because of an older population and indications of rectal bleeding). In addition, because the sensitivity of colonoscopy for small lesions such as small adenomas is far from optimal,[7,20,21] a portion of the FP results related to hemorrhoids only might actually be true positives because of 1 or a few missed small

advanced adenomas. However, in general, all potential screenees with an episode of rectal bleeding should contact their physician instead of performing a FIT.

For proper interpretation of these results, some limitations need to be discussed. First, underreporting of hemorrhoids by the endoscopist may have occurred. This might be attributed to less attention to hemorrhoids when significant other abnormalities were found or missing retroflexion of the colonoscope in the rectum. In addition, as description of the appearance of the anus was not a predefined reporting item, other sources of anorectal bleeding such as prolapse with anitis and rhagades may have been unreported. A future prospective study using standardized external anal inspection, rigid anoscopy, and flexible colonoscopy with retroflexion would be superior. Second, colonoscopy may not be the best diagnostic tool to assess the presence of hemorrhoids.[25] However, previous studies suggest the usefulness of flexible colonoscopy for grading of hemorrhoids because the red color, circumference, size, and degree of elevation of rectal columns were found to be associated with rectal bleeding.[26,27] Third, caution should be taken with extrapolation of FP results from this study to the screening setting because subjects from a referral setting were tested, who might well have a higher prevalence and/or different bleeding pattern of hemorrhoids.

The strengths of this study are evaluation of the association of hemorrhoids and the level of FP results of a frequently used FIT with a quantitative outcome. In addition, insight is gained into the number of subjects with hemorrhoids and a negative FIT result in a large sample size.

In conclusion, this study indicates that the number of FP results that can be attributed to hemorrhoids only is small. Therefore, the influence of hemorrhoids on the effectiveness of an FIT-based screening program is likely to be limited.

ACKNOWLEDGEMENTS

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6

Fecal Immunochemical Tests: sex specific cut-off values for equal sensitivity for colorectal cancer?

F.A. Oort*, S.T. van Turenhout*, R.W.M. van der Hulst, A.P. Visscher, J.S. Terhaar sive Droste, P. Scholten, A.A. Bouman, G.A. Meijer, C.J.J. Mulder, L.G.M. van Rossum, V.M.H. Coupé

*These authors have contributed equally to the work.

Submitted for publication

ABSTRACT

Introduction: Faecal immunochemical tests (FITs) are commonly used in screening for colorectal cancer (CRC). Diagnostic accuracy of FIT seems to be different in males and females. This disparity could result in a different benefit from screening. The cause of this disparity and the effect of cut-off values is so far unknown.

Aims & methods: Aim of the present study was to compare the sensitivity and specificity of a frequently used FIT between males and females. Data were used from a large prospective cohort study on FIT performance. All participants in this cohort sampled a FIT and underwent complete colonoscopy. Outcome variables were CRC and advanced adenomas. Areas under the Receiver Operating Characteristic curves (ROC curves) were calculated and compared between males and females. The influence of potential explanatory variables on the relation between sensitivity and gender was evaluated.

Results: At all cut-off values, FITs were found to have a higher sensitivity (range for the difference; 13–23%) and a lower specificity (range 2–4%) for CRC in males compared to females. For advanced adenomas, no statistically significant differences in sensitivity and specificity were observed. ROCs for CRC and advanced adenomas were similar for both sexes, and equal combinations of sensitivity and specificity for both sexes could be achieved by adjusting the cut-off values. For CRC, the difference in sensitivity could not be explained by age or location of the tumour. Although FIT was more sensitive for left sided cancer, this could not explain the gender difference since the anatomical distribution of CRCs over the colon in males and females was similar.

Conclusion: With FIT males have a higher sensitivity and a lower specificity for CRC than females. However, as ROCs were similar, equal accuracy can be achieved by allowing different cut-off values for both sexes. Location and age do not explain the differences in sensitivity observed.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer related death world wide.[1] Early detection by population screening is the most realistic approach to reduce CRC-related death. Faecal immunochemical tests (FITs) are increasingly used as the primary screening test for CRC.[2]

Recently, it was found that FITs have a higher sensitivity and lower specificity for advanced colorectal neoplasia in males compared to females.[3] Although diagnostic accuracy of a screening test is just one of several factors determining uptake of a screening program, a difference in FIT characteristics between males and females could implicate disparities in the expected benefit from CRC screening. Such a difference may require tailored screening. So far, it remains to be resolved whether this difference e.g. reflects a dissimilarity in advanced neoplasia distribution or a difference in the age of onset of CRC.

The aim of the present study is to investigate whether the sensitivity and specificity of a frequently used FIT for the detection of CRC and advanced adenomas differs between males and females, and whether this difference can be explained by age, location, number and size of neoplastic lesions.

MATERIALS AND METHODS

Study population

For the current analysis, data were used from an ongoing study program on FIT performance. This program aims to answer a number of research questions and has been previously described extensively.[4-6] In short, individuals scheduled for elective colonoscopy in 5 participating medical centres were invited to participate and perform a FIT prior to colonoscopy. In addition to the exclusion criteria of these previous studies, individuals with an indication of visible rectal bleeding, anaemia or clinical suspicion of CRC were excluded from the analysis to minimize potential work-up bias. The study was approved by the local medical ethics review board of each of the 5 hospitals.

Study design

The test used was an automated quantitative FIT: OC-sensor® (Eiken Chemical Co., Tokyo, Japan). One experienced technician performed the analyses while being unaware of the

clinical data. All tests were analyzed by using the OC sensor MICRO desktop analyzer (Eiken Chemical co, Tokyo, Japan).[7] Haemoglobin concentrations of ≥50, ≥75, ≥100, and ≥200 ng/ml of buffer solution were taken as cut-off values. These concentrations correspond to respectively ≥10, ≥15, ≥20 and ≥40 milligram of haemoglobin per gram of faeces.

Colonoscopy and lesions

Experienced gastroenterologists performed or supervised all colonoscopies. The endoscopists were unaware of the FIT result, in order to prevent investigator bias. Conscious sedation by Midazolam was offered to all patients.

A complete colonoscopy was defined as intubation of the caecum with identification of the appendiceal orifice or valvula Bauhini, or intubation up to an obstructing neoplasm. Quality control measures included documentation of colonic landmarks. Individuals in whom the bowel cleansing was insufficient and individuals in whom the colonoscopy remained incomplete were excluded from analysis. The right colon was defined as the proximal part of the colon including caecum, ascending colon, right (or hepatic) flexure and transverse colon. The left colon was defined as the distal part of the colon including left (or splenic) flexure, descending colon, sigmoid and rectum.[8,9] In case of multiple neoplasia detected on colonoscopy, patients were classified based on the most advanced lesion found.

Tissue samples obtained at colonoscopy were sent to the department of pathology and evaluated according to current standards. Adenomas ≥1.0 cm, adenomas with a villous component (i.e. tubulovillous or villous adenoma) or severe/high-grade dysplasia were classified as advanced adenomas.[10,11]

Statistical analysis

Using colonoscopy as the reference test, sensitivities and specificities of FIT were calculated for two definitions of colonoscopy outcome: (i) the presence of CRC and (ii) the presence of advanced adenoma. Sensitivity was defined as the proportion of positive test results in patients with the colonoscopy outcome under consideration. Specificity was calculated as the proportion of negative test results in patients with an outcome less severe than the outcome under consideration.

Sensitivity and specificity of FIT at different cut-off values were compared between males and females. Receiver Operating Curves (ROCs) were calculated for both sexes separately and the Areas Under the Curve (AUC) were used to compare accuracies.

To study whether gender dissimilarities are a reflection of differences in age, location and size or number of neoplastic lesions, a stratified analysis of 2x2 tables was done, as well as a multivariate logistic regression analysis. Age was dichotomized into subjects <65 and 65 or older. Location was divided in left and right sided lesions. The number of advanced adenomas was grouped as 1 or >1, and size was grouped <10 mm or ≥10 mm. Stratified analyses and the multivariate analysis were performed for a FIT cut-off value of 50 ng/ml only. In the multivariate analysis, age was used as continuous variable. Subjects with CRC or advanced adenomas on both sides of the colon were excluded from these analyses.

All analyses were performed with SPSS for Windows Version 20 (SPSS Inc., Chicago, USA).

RESULTS

Participants

Between June 2006 and October 2010, 4,704 subjects returned a FIT and underwent colonoscopy. Of these, 1,682 were excluded for different reasons (see Figure 6.1), leaving 3,022 participants for analysis. As the first colonoscopy was incomplete in 107 participants, a second colonoscopy, barium enema or CT-colonography was needed to complete the colonoscopic evaluation. The mean age of the participants was 59.7 years (range 19–91 years, SD 12.6), and 45% was male. The indication for colonoscopy was evaluation of symptoms in 44% (1,331/3,022), screening or surveillance in 47% (1,412/3,022), and unspecified in 9% (279/3,022) (see Table 6.1).

Colonoscopy

In 2.3% of the included subjects, CRC was found (69/3,022) and in another 10.1% one or more advanced adenomas were detected (304/3,022; see Figure 6.1). From all subjects with CRC, 11.6% (8/69) also had one or more advanced adenomas. The distribution of CRC stages according to the TNM classification, is demonstrated in Figure 6.1. The T-stage (Tumour-stage) distribution found was; 16% (11/69) T1, 25% (17/69) T2, 33%

(23/69) T3 and 6% (4/69) T4. In 14 (20%) individuals with CRC, T-stage was unknown due to preoperative radiotherapy or was not determined because of disseminated disease at time of diagnosis.

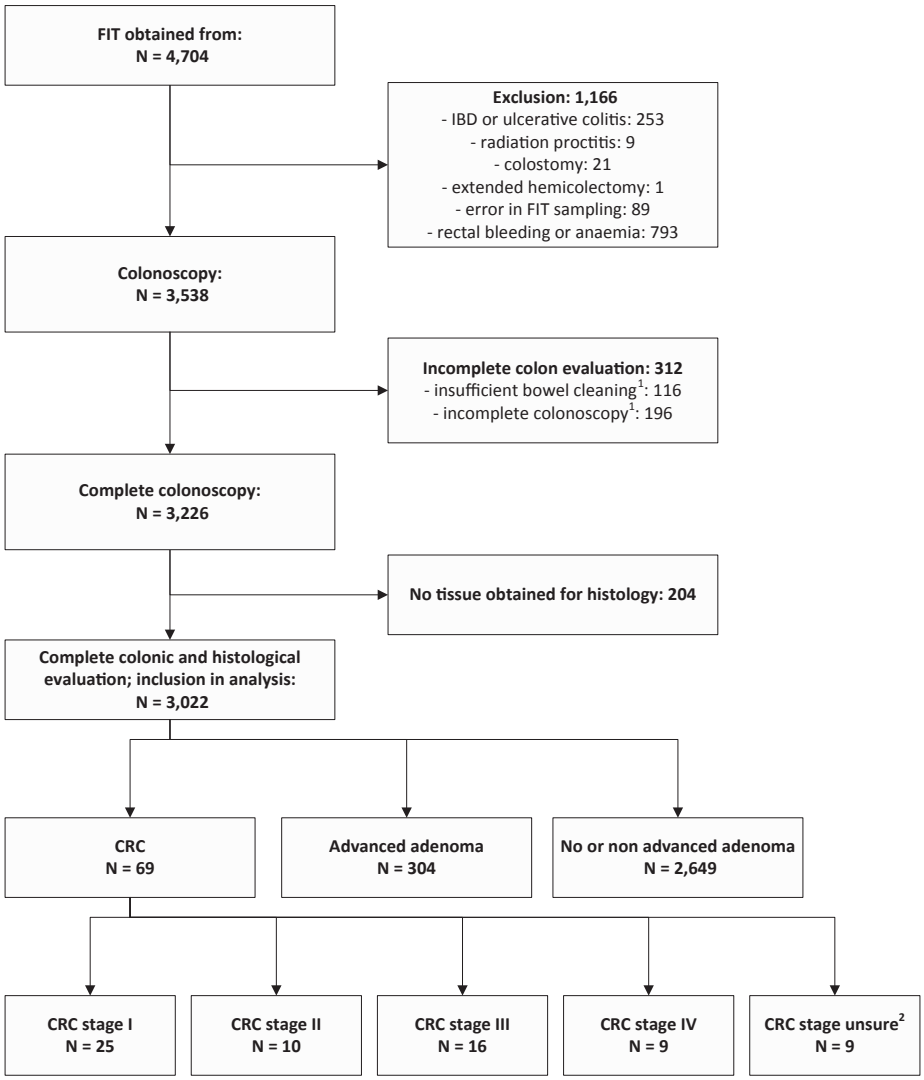

Figure 6.1 Study flow diagram.

[1] Incomplete colon evaluation in spite of possible additional evaluation by repeated colonoscopy, barium enema or virtual colonography within 6 months.

[2] CRC stage remained unsure as these cases received pre-operative radiotherapy or due to disseminated disease exact classification remained unknown.

Table 6.1 Primary indication for colonoscopy among 3,022 consecutive patients included for analysis of FIT characteristics according to gender

Indication group	Indication for colonoscopy	N
Symptomatic	Weight loss	88
	Clinical suspicion of diverticulitis	26
	Clinical suspicion of IBD	40
	Abdominal pain	355
	Altered bowel habits	548
	Clinical or radiological suspicion of CRC	54
	Diarrhoea	128
	Constipation	92
	Total	1,331
Screening & surveillance	Average risk	103
	Familial history of CRC	482
	Lynch syndrome	54
	Polyp surveillance	578
	Post CRC surveillance	195
	Total	1,412
Other	Not specified/others	279
Grand total		3,022

FIT, faecal immunochemical test; IBD, inflammatory bowel disease; CRC, colorectal cancer.

Positivity rates

For the total population, the FIT positivity rate was 12.3% at a cut-off level of 50 ng/ml. Males were found to have a higher positivity rate (15.3%) compared to females (9.8%, P<0.001). At 50 ng/ml, 88% (61/69) of all CRCs and 35% (106/304) of all advanced adenomas tested positive. The positivity rate decreased at increasing cut-off values, to 6.8% at 200 ng/ml.

Sensitivity and specificity

In the total study population, the sensitivity and specificity for CRC at a cut-off value of 50 ng/ml was 88% and 90% respectively. For advanced adenomas, these figures were 35% and 92% respectively (see Tables 6.2 and 6.3). At all cut-off values, males were found to have a higher sensitivity for CRC than females. The difference in sensitivity ranged from 13% to 23% and was significant at the cut-offs 75 and 100 ng/ml. The specificity

Table 6.2 Comparison of sensitivity and specificity of a FIT for detection of CRC in males and females at different cut-off values

Sensitivity					
FIT cut-off	Total N=69*	Males N=45*	Females N=24*	Difference	P-value
50 ng/ml (CI)	88.4% (78–95)	93.3% (82–99)	79.2% (58–93)	14.1%	0.12
75 ng/ml (CI)	85.5% (75–93)	93.3% (82–99)	70.8% (49–87)	22.5%	0.03
100 ng/ml (CI)	85.5% (75–93)	93.3% (82–99)	70.8% (49–87)	22.5%	0.03
200 ng/ml (CI)	75.4% (64–85)	80.0% (65–90)	66.7% (45–84)	13.3%	0.25
Specificity					
FIT cut-off	Total N=2,953#	Males N=1,312#	Females N=1,641#	Difference	P-value
50 ng/ml (CI)	89.5% (88–91)	87.3% (85–89)	91.2% (90–93)	-3.9%	<0.05
75 ng/ml (CI)	91.5% (90–95)	90.1% (88–92)	92.6% (91–94)	-2.5%	<0.05
100 ng/ml (CI)	92.6% (92–94)	91.2% (90–93)	93.7% (92–95)	-2.5%	<0.05
200 ng/ml (CI)	94.8% (94–96)	93.6% (21–95)	95.8% (95–97)	-2.2%	<0.05

FIT, faecal immunochemical test; CRC, colorectal cancer; CI, confidence interval.
* This concerns the total of subjects with CRC from which the sensitivity was calculated. # This concerns the total of subjects without CRC from which the specificity was calculated.

for CRC was significantly lower in males compared to females, but the difference was small (between 2.2 and 3.9%). For advanced adenomas, the differences in sensitivity and specificity between males and females were small and mostly not significant (Table 6.3).

Receiver Operator Curves (ROCs)

The test characteristics for males and females at each cut-off value are visualised in the ROCs in Figures 6.2 and 6.3. The AUCs for CRC for males and females were 0.95 (95% CI 0.909–0.985) and 0.90 (95% CI 0.819–0.981) respectively. The ROCs and AUCs for advanced adenoma were very similar between males and females (see Figure 6.3).

Table 6.3 Comparison of sensitivity and specificity of a FIT for detection of advanced adenomas in males and females at different cut-off values

Sensitivity					
FIT cut-off	Total N=304*	Males N=164*	Females N=140*	Difference	P-value
50 ng/ml (CI)	34.9% (30–41)	36.6% (39–45)	32.9% (25–41)	3.7%	0.55
75 ng/ml (CI)	30.9% (26–37)	32.9% (26–41)	28.6% (21–37)	4.3%	0.46
100 ng/ml (CI)	28.6% (24–34)	31.7% (25–39)	25.0% (18–33)	6.7%	0.21
200 ng/ml (CI)	21.1% (17–26)	24.4% (18–32)	17.1% (11–24)	7.3%	0.16
Specificity					
FIT cut-off	Total N=2,649#	Males N=1,148#	Females N=1,501#	Difference	P-value
50 ng/ml (CI)	92.3% (91–93)	90.8% (89–92)	93.4% (92–95)	-2.6%	<0.05
75 ng/ml (CI)	94.0% (93–95)	93.4% (92–95)	94.5% (93–96)	-1.1%	0.22
100 ng/ml (CI)	95.0% (94–96)	94.4% (93–96)	95.5% (94–97)	-1.1%	0.24
200 ng/ml (CI)	96.6% (96–97)	96.2% (95–97)	97.0% (96–98)	-0.8%	0.28

FIT, faecal immunochemical test; CI, confidence interval.
* This concerns the total of subjects with advanced adenoma from which the sensitivity was calculated. # This concerns the total of subjects without CRC and without advanced adenomas from which the specificity was calculated.

The influence of potential explanatory variables

In patients with CRC, males were found to have a higher FIT positivity rate than females (93% vs 79%, P=0.08). This difference persisted when stratifying CRC by location (i.e. left versus right; 100% vs 88% and 79% vs 51%, respectively). Overall for males and females, left sided CRC was found to have a higher positivity rate (96%) than right sided CRC (71%, P<0.05). However, within males and females with CRC an equal proportion of cancers was left sided. In the multivariate analysis, location was significantly associated with FIT sensitivity for CRC (see Table 6.4), but the univariate odds ratio for gender (OR 3.7, 95% CI 0.8–17.0) was not substantially affected by the inclusion

of location (and age) in the analysis (OR 4.9, 95% CI 0.9–26.8). As such, the relation between gender and FIT sensitivity cannot be explained by either age or the location of CRC.

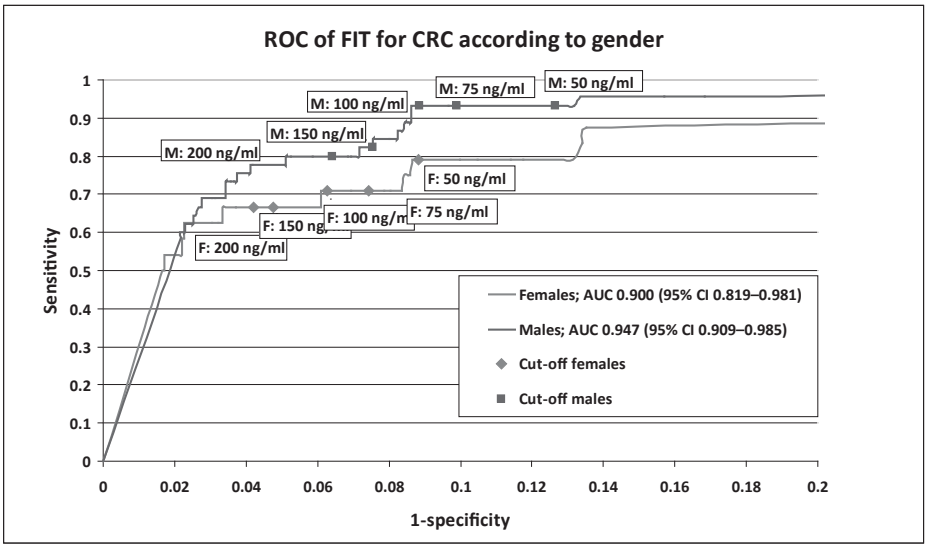

Figure 6.2 Receiver operator curve of FIT for detection of CRC. ROC, receiver operator curve; FIT, faecal immunochemical test; CRC, colorectal cancer; AUC, area under the curve.

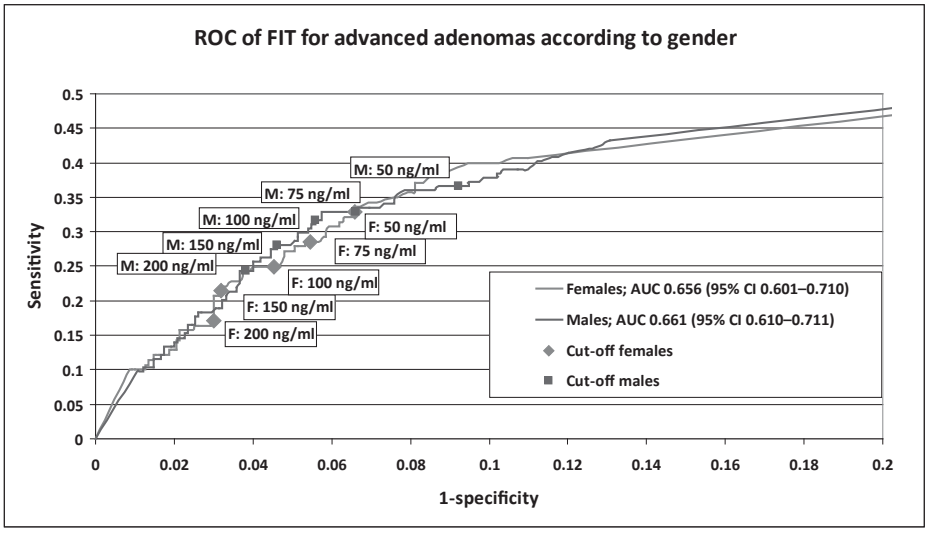

Figure 6.3 Receiver operator curve of FIT for detection of advanced adenoma. ROC, receiver operator curve; FIT, faecal immunochemical test; AUC, area under the curve.

Table 6.4 Level of significance and odds ratios for different variables used in a multivariate logistic regression model predicting sensitivity of a FIT at cut-off of 50 ng/ml for detection of CRC

	Univariate model		Multivariate model	
CRC	OR (95% CI)	P-value	OR (95% CI)	P-value
Gender	3.7 (0.80–17.02)	0.10	4.9 (0.89–26.83)	0.07
Age	1.0 (0.94–1.10)	0.70	1.0 (0.91–1.11)	0.90
Location	**9.0 (1.64–49.45)**	**<0.05**	**11.0 (1.79–67.36)**	**<0.05**
Number of cases included in the model: 68				

FIT, faecal immunochemical test; CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

In people with advanced adenomas, males were found to have a slightly higher positivity rate than females (37% vs. 33%, n.s.). Left sided advanced adenomas were more frequently positive (38%) than right sided lesions (24%, P=0.02). Also, subjects with more than one advanced adenoma were found to have a higher FIT positivity rate (63%) than subjects with one advanced adenoma (27%, P<0.001). The same was found for subjects with an advanced adenoma >9 mm (45%) compared to advanced adenomas <10 mm (12%, P<0.001).

Table 6.5 Level of significance and odds ratios for different variables used in a multivariate logistic regression model predicting sensitivity of a FIT at cut-off of 50 ng/ml for detection of CRC

	Univariate model		Multivariate model	
Advanced adenoma	OR (95% CI)	P-value	OR (95% CI)	P-value
Gender	1.2 (0.73–1.90)	0.50	0.9 (0.54–1.62)	0.82
Age	1.0 (0.99–1.04)	0.27	1.0 (0.98–1.04)	0.49
Location	**2.0 (1.34–3.11)**	**<0.05**	1.4 (0.84–2.33)	0.19
Adv adenoma size	**6.1 (2.87–12.79)**	**<0.05**	**5.7 (2.60–12.30)**	**<0.05**
Number of advanced adenomas	**4.6 (2.50–8.44)**	**<0.05**	**4.0 (1.95–8.16)**	**<0.05**
Number of cases included in the model: 281				

FIT, faecal immunochemical test; CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

In males, a slightly higher proportion of advanced adenomas was right-sided (40% in males versus 32% in females), large in size (76% in males versus 71% in females), or consisted of multiple advanced adenomas (21% in males versus 17% in females). The distribution of these explanatory variables caused a shift in the OR for gender from 1.2 in the univariate analysis to 0.9 in the multivariate analysis, but either way the relation remained non-significant (see Table 6.5). In conclusion, the size and the number of advanced adenomas were important predictors of FIT positivity in advanced adenomas, but gender was not.

DISCUSSION

In this study sensitivity and specificity of FIT was assessed in males and females in a large cohort of subjects referred for colonoscopy. FIT was found to be more sensitive and less specific for CRC in men than in women. The ROC analysis revealed similar AUCs for males and females and a similar accuracy can be reached by adjusting the cut-off value in females. The difference in sensitivity between the sexes, could not be explained by age or location or the lesion. The sensitivity of FIT for advanced adenomas was unrelated to gender but strongly related to size and the number of advanced adenomas.

In males, FIT was found to have 13–23% higher sensitivity for CRC than in females. Previous studies already suggested a difference in test performance for the detection of advanced neoplasia in males and females.[3] To evaluate the relation between gender and FIT sensitivity for CRC, the influence of the potential explanatory variables ´location of colonic lesions´ and ´age of the participant´ was assessed. These variables were chosen because it is known that CRC develops earlier in the lifetime of a man then of a woman.[12] In addition, left sided neoplasia have a higher likelihood for a positive FIT.[9,13]

It is also known that females have a higher prevalence of right sided CRC.[14] In multivariate analysis of the present study, location was significantly associated with FIT sensitivity for CRC, but the univariate odds ratio for gender was not substantially affected by the inclusion of location and age in the analysis. By stratified analysis of 2x2 tables, it was found that the observed relation between gender and FIT sensitivity for CRC could not be explained by either location or age. It can only be speculated on whether the gender difference is due to other confounders as e.g. tumour size or tumour biology (e.g. blood vessel density). Other authors hypothesized that the higher serum concentration of haemoglobin in male blood would cause higher FIT positivity when blood is lost in the colon.[3]

For advanced adenomas, no significant difference in sensitivity was found between the genders. In multivariate analysis and stratified 2x2 tables it was found that the number and size of lesions are predictive for test sensitivity. This last observation is in line with other studies that showed that the number and size of polyps influence test accuracy for detection of advanced adenomas.[15]

Currently, CRC-screening with preselection by means of FIT sampling is a one size fits all approach. Gender specific screening guidelines could be considered, in order to optimize the effectiveness of a screening program in both males and females.[3,16] However, test accuracy is only one of the factors that determine the efficiency of a CRC screening programme, and participation of the target population in the screening programme is an important other factor. In the English and Scottish screening programmes, it was found that participation was higher in women and in older age groups.[17,18] Moreover, in the present study, we found only a difference between males and females in FIT accuracy for CRC and not for advanced adenomas. The same sensitivity for CRC as in males could be reached by lowering the cut-off value in females. Presumably, prevalent cancers will be detected in the first screening round, after which the focus of screening will be to detect advanced adenomas. In the absence of a gender difference in the sensitivity for advanced adenomas, sex specific cut-off values in screening seem unnecessary. In addition, individualizing screening guidelines adds to the complexity of a screening programme and should only be adopted if the expected benefits are substantial. Individualized screening guidelines may confuse providers and consumers to the point of decreasing adherence.[19]

The current study provides insight into the relation between gender and the diagnostic accuracy of FIT for detection of CRC and advanced adenomas. To the best of our knowledge, this is the first study to observe that gender specific differences in FIT sensitivity are only present in CRC and not in advanced adenomas. This difference can be easily overcome by adjusting the cut-off value. Each participant underwent complete colonoscopy regardless of FIT outcome. This enabled direct calculation of not only sensitivity but specificity as well. The high number of advanced colonic neoplasia in the referral population that was used, enabled us to stratify for CRC, which was not possible before.[3]

For proper interpretation of the results, some limitations need to be discussed. Firstly, location of lesions was assessed by the endoscopists by recognition of colonic landmarks, which may limit the precision with which the location of the neoplastic lesions can be called. Furthermore, there may be other explanatory variables for FIT performance that were not included, like for instance the use of NSAIDs.[9] Another limitation is that

we tested a referral population rather than a screening population. Consequently, it cannot be excluded that, even after exclusion of subjects with anaemia en hematochezia, sensitivity may be overestimated and specificity underestimated due to work-up bias.[20] This may occur as symptomatic participants have an increased likelihood for having both a positive FIT and a colorectal neoplasm. However, in a recent comparison of the current study cohort with a screening population, similar faecal immunochemical test results in screening and referral CRC were found.[21] In addition, true screening populations may be biased with symptomatic subjects as well, as reported by two recent studies.[22,23]

In conclusion, males were found to have a higher FIT sensitivity and a lower specificity for CRC compared to females. However, as ROCs are similar, equal characteristics can be achieved by allowing different cut-off values for both sexes. Location of CRC and age of the individuals are not responsible for the observed differences in sensitivity. Whether the difference is relevant in screening remains questionable. No significant difference in test accuracy for advanced adenomas was found between the sexes.

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7

DNA methylation of phosphatase and actin regulator 3 detects colorectal cancer in stool and complements FIT

L.J.W. Bosch, F.A. Oort, M. Neerincx, C.A.J. Khalid-de Bakker, J.S. Terhaar sive Droste, V. Melotte, D.M.A.E. Jonkers, A.A.M. Masclee, S. Mongera, M. Grooteclaes, J. Louwagie, W. van Criekinge, V.M.H. Coupe, C.J. Mulder, M. van Engeland, B. Carvalho, G.A. Meijer

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ABSTRACT

Using a bioinformatics-based strategy, we set out to identify hypermethylated genes that could serve as biomarkers for early detection of colorectal cancer (CRC) in stool. In addition, the complementary value to a Fecal Immunochemical Test (FIT) was evaluated. Candidate genes were selected by applying cluster alignment and computational analysis of promoter regions to microarray-expression data of colorectal adenomas and carcinomas. DNA methylation was measured by quantitative methylation-specific PCR on 34 normal colon mucosa, 71 advanced adenoma, and 64 CRC tissues. The performance as biomarker was tested in whole stool samples from in total 193 subjects, including 19 with advanced adenoma and 66 with CRC. For a large proportion of these series, methylation data for *GATA4* and *OSMR* were available for comparison. The complementary value to FIT was measured in stool subsamples from 92 subjects including 44 with advanced adenoma or CRC. Phosphatase and Actin Regulator 3 (*PHACTR3*) was identified as a novel hypermethylated gene showing more than 70-fold increased DNA methylation levels in advanced neoplasia compared with normal colon mucosa. In a stool training set, *PHACTR3* methylation showed a sensitivity of 55% (95% CI: 33–75) for CRC and a specificity of 95% (95% CI: 87–98). In a stool validation set, sensitivity reached 66% (95% CI: 50–79) for CRC and 32% (95% CI: 14–57) for advanced adenomas at a specificity of 100% (95% CI: 86–100). Adding *PHACTR3* methylation to FIT increased sensitivity for CRC up to 15%. *PHACTR3* is a new hypermethylated gene in CRC with a good performance in stool DNA testing and has complementary value to FIT.

INTRODUCTION

Screening for colorectal cancer (CRC) is the most efficient strategy for reducing death from this devastating disease. Colonoscopy is the gold standard for the detection and removal of early lesions and is highly sensitive, but also invasive and costly.[1,2] For population-wide screening simple and noninvasive procedures like stool testing are preferred.[3] In follow-up to the guaiac-based Faecal Occult Blood Test (FOBT), the more sensitive immunochemical fecal occult blood test (Fecal Immunochemical Test or FIT[4-6]) is now widely used in screening programs in Europe and Japan, and is expected to reduce CRC mortality by around 30%.[7] This test performance though, still leaves room for improvement which could come from molecular stool tests like those testing for tumor DNA in stool. Multiple assays have been developed and evaluated for this purpose, but sensitivities still remain suboptimal.[8-14] The recently introduced combination of mutation markers with DNA methylation markers has yielded substantially improved test performance.[13,15,16] Methylation markers on their own, either alone or combined, have also yielded promising results, whereas the assays are technically less demanding.[17-23] Methylation markers are appealing for CRC screening even more because DNA methylation is an early event in colorectal development, preceding chromosomal abnormalities, and mutations.[24]

Yet, the ultimate marker, or combinations of markers, for stool DNA testing still remains to be determined. To this end, in this study, we aimed to identify new hypermethylated genes in CRC by applying dedicated bioinformatics to microarray expression data of colorectal adenomas and carcinomas and to explore their potential in whole stool DNA testing for CRC.[25,26] Furthermore, we set out to evaluate complementary value of such markers to FIT in a series of stool subsamples.

MATERIALS AND METHODS

Cell line authentication

The CRC cell line HT29 was obtained from the American Type Culture Collection (LGC Standards GmbH). COLO205, Colo320, HCT 116, and RKO cell lines were kindly provided by Dr. G.J. Peters, Department of Oncology, VU University Medical Center, Amsterdam, the Netherlands. The method for authentication was by array comparative genomic hybridization (aCGH, 244 k Agilent oligonucleotide platform), conducted at the VU University Medical Center, Amsterdam, the Netherlands, most recently in

October 2008. The patterns of chromosomal changes observed were in concordance to the previously described chromosomal changes in these cell lines.[27] LS513 was kindly provided by Dr. F. Praz, Centre de Recherche Saint-Antoine, Paris, France. Array CGH confirmed the genomic profile as described in literature (the Wellcome Trust Sanger Institute Cancer Genome Project web site[28]).

Strategy for methylation marker selection

The strategy used for identification and validation of new colon cancer-specific methylation markers included both bioinformatics analysis of microarray-based mRNA expression data and experimental validations of methylation levels as outlined in Supplementary Figure S7.1. First, genes downregulated in CRC compared with adenomas were subjected to a bioinformatics strategy for predicting cancer-specific methylation.[25] In the experimental validation, the presence of DNA methylation was tested by methylation-specific PCR (MSP) using the BioTrove OpenArray platform[29] (BioTrove, Inc.), and high-throughput LightCycler assays. Full details are provided in the Supplementary Methods.

Strategy for evaluating *PHACTR3* methylation as a marker in CRC tissue and stool

Cell lines. For validating *PHACTR3* methylation in CRC 2 different regions around the Transcription Start Site (TSS) were investigated for the presence of methylation in 6 CRC cell lines; regions -149 to -63 bp and +411 to +526 relative to the TSS. Next, HT29 and HCT116 cells were treated with 5-aza-20-deoxycytidine (5-AZA) to evaluate the effect of demethylation on mRNA expression.

Tissue. *PHACTR3* methylation levels in the region +411 to +526 relative to the TSS and mRNA expression were measured in cancer and matched normal tissue samples from 9 CRC patients by quantitative MSP and quantitative RT-PCR, respectively. Then, *PHACTR3* methylation levels were evaluated in an independent series of 34 normal colon mucosa tissue samples from cancer-free patients, 71 advanced adenomas, and 64 carcinomas. Finally, to evaluate the specificity of *PHACTR3* hypermethylation for CRC, methylation levels in other tumor types were analyzed, being tumor tissue samples from 44 breast, 20 cervix, 20 lung, 11 esophagus (5 adenocarcinomas and 6 squamous cell carcinomas), 20 gliomas, 19 pancreas, and 15 stomach.

Stool. To test the performance of *PHACTR3* in a stool-based methylation test for CRC, a collection of 193 whole stool samples was split in a training set and a validation set of equal sizes. The training set consisted of a total of 100 stool samples, of which 66 from patients without colorectal neoplasia (58 healthy controls, 4 patients with colonic diverticula and 4 patients with hemorrhoids), 9 from patients with hyperplastic polyps, 3 from patients with nonadvanced adenoma, and 22 from CRC patients. The training set was designed with more controls then cases, to better assess specificity. The validation set consisted of a total of 93 stool samples, 30 of which from healthy individuals, 19 from patients with advanced adenoma and 44 from CRC patients. The validation set contained more advanced neoplasia, including advanced adenomas, to better assess sensitivity. (Patient characteristics are described in Supplementary Table S7.3). In addition, methylation data for 2 other markers, that is, *GATA4* and *OSMR* were available for comparison in 95% of cases and 91% of controls from the training set and in all stool samples from the validation set tested for *PHACTR3*.[18,20]

Moreover, to examine the complementary value to FIT, an independent series of 92 stool subsamples was analyzed for both FIT and *PHACTR3* methylation. This stool series originated from a retrospective collection from referral subjects, and included 48 stool samples from subjects without colon neoplasia, 24 from patients with advanced adenomas and 20 from patients with carcinomas. All details on tissue and stool collection, sample processing, and methodologies used, are presented in the Supplementary Methods.

Statistical analysis of DNA methylation in tissues and stool

Mean differences in methylation or mRNA expression levels in tissue samples were analyzed with the Mann–Whitney test or ANOVA. The relation between methylation levels in stool and the presence or absence of an advanced lesion was studied by receiver operator characteristic (ROC) analysis. The Area under the curve (AUC) was used as a measure of the test performance. CIs of proportions were calculated using the Wilson score method. To test whether age or gender were confounders in the relation between methylation levels and the presence or absence of a lesion, linear regression was used. For the combination of FIT and *PHACTR3* methylation, we used a distribution-free rankbased method[30] to calculate linear combination of the 2 markers giving highest diagnostic accuracy. Sensitivities were compared with FIT or *PHACTR3* methylation alone at fixed specificities of 92%, 96%, and 98% using McNemar's test. Calculations for

the combination of FIT and *PHACTR3* methylation was carried out in the R package (version 2.8.1.). All other analyses were carried out using SPSS software (version 15.0; SPSS Inc.). Values of P≤0.05 were considered statistically significant.

RESULTS

Bioinformatics for discovery of candidate genes

Bioinformatics for discovery of candidate genes A total of 397 genes were downregulated in carcinomas compared with adenomas as determined by mRNA expression microarray analysis (Wilcoxon rank test P<1e–5 and Thas P<0.05; FDR<0.05).[26] In 284 of these genes, a reported transcription start site (TSS) could be identified. These were subsequently subjected to a bioinformatics approach to predict cancer-specific methylation.[25] This yielded 18 candidate genes, of which *Phosphatase and actin regulator 3* (*PHACTR3*, NM_080672) was the only one that passed all further steps of experimental validation (see Supplementary Figure S7.1 and Supplementary Table S7.1).

PHACTR3 methylation and mRNA expression analysis in CRC cell lines and cancer and matched normal tissues

PHACTR3 harbors a dense CpG island located -678 and +1,353 bp relative to the TSS (GC content: 65%, CpG(obs)/CpG(exp): 1.17). We designed conventional MSP primers in a region -149 to -63 bp and +411 to +526 relative to the TSS (see Supplementary Methods and Supplementary Figure S7.2). The region +411 to +526 relative to the TSS is located in exon 1 and is the same region as analyzed at the initial screen and validation of methylation status (see Supplementary Figure S7.1 and Supplementary Figure S7.2A). Of 6 cell lines tested, only HCT116 showed methylation in the region -149 to -63 bp relative to the TSS. For the region +411 to +526 relative to the TSS, methylation was found in all 6 cell lines tested (see Supplementary Figure S7.2). At the mRNA level these 6 CRC cell lines only showed marginal *PHACTR3* expression levels compared with the positive control (brain tissue; see Supplementary Figure S7.2). Treatment of HT29 and HCT116 with the demethylating agent 5-AZA resulted in reexpression of the gene (see Supplementary Figure S7.2), consistent with *PHACTR3* expression being downregulated by methylation in these cell lines.

Next, *PHACTR3* methylation levels and mRNA expression were measured in cancer tissue and matched normal tissue from 9 CRC patients. All 9 CRC tissue samples showed significantly increased methylation levels compared with their normal counterparts. Although mRNA expression levels were low in both normal and tumor tissues, still in 6 of 9 tumors *PHACTR3* was significantly downregulated compared with expression levels in their normal counterparts (see Figure 7.1).

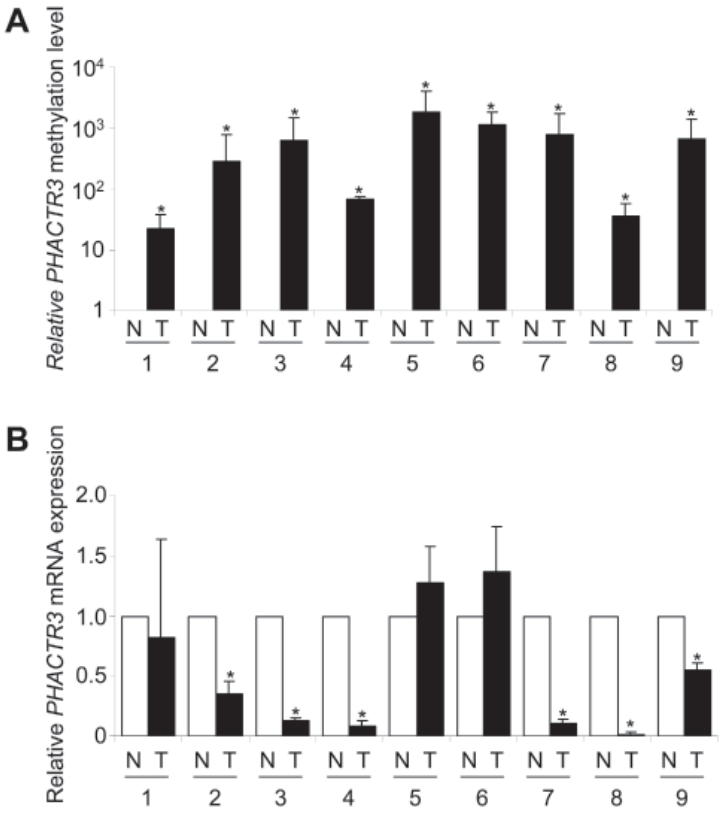

Figure 7.1 *PHACTR3* methylation levels and mRNA expression in cancer and matched normal tissues. **(A)** *PHACTR3* methylation levels in tumor (T) and matched normal (N) tissues from 9 CRC patients. Quantifications represent mean methylation levels (error bars correspond to SD) relative to methylation levels in the normal tissues from 3 independent experiments. Methylation levels are calculated as [relative quantity of methylated *PHACTR3*/relative quantity of unmethylated *Beta-actin* (*ACTB*)] ratio x 1,000. Asterisks mark significant differences ($P<0.05$). **(B)** mRNA expression analysis of *PHACTR3* by RT-PCR in tumor (T) and matched normal (N) tissues from 9 CRC patients. Quantifications represent mean expression values (error bars correspond to SD) relative to expression levels in the normal tissue from 3 independent experiments. Asterisks mark significant differences ($P<0.05$).

PHACTR3 methylation analysis in colorectal advanced adenoma and carcinoma tissues

To confirm the differential levels of *PHACTR3* methylation in CRC compared with normal mucosa, we tested an independent series of tissue samples and included a set of advanced adenomas as well. *PHACTR3* methylation levels were 72-fold and 71-fold

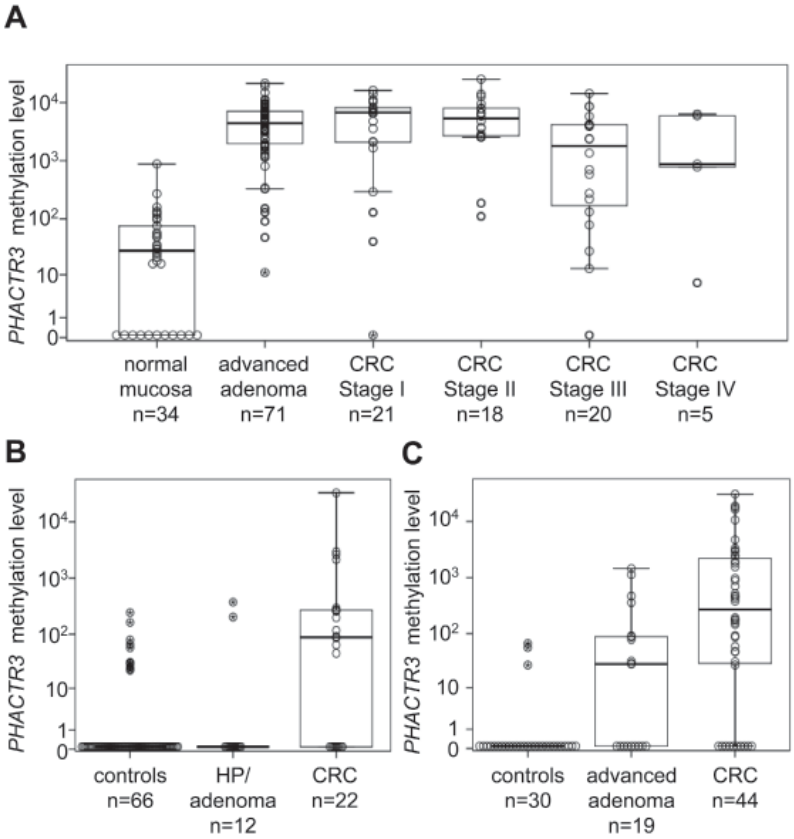

Figure 7.2 *PHACTR3* methylation levels in tissue and stool. **(A)** PHACTR3 methylation levels in tissue samples of 34 normal colon mucosa, 71 advanced adenomas and 64 carcinomas (UICC stage I to IV). **(B)** *PHACTR3* methylation levels in stool samples from 66 patients without colon neoplasia, 12 patients with hyperplastic polyps (HP) or nonadvanced adenoma and 22 CRC patients (training set). Methylation levels are shown as relative quantity of methylated *PHACTR3* x 1,000. **(C)** *PHACTR3* methylation levels in stool samples from 30 patients without colon neoplasia, 19 patients with advanced adenomaand 44CRC patients (validation set). Methylation levels are shown as relative quantity of methylated *PHACTR3* x 1,000. Box plots show first quartile, median, third quartile, and range of methylation levels. Dots represent individual data points, asterisks represent extremes.

higher in advanced adenoma and carcinoma samples, respectively, compared with normal mucosa samples (see Figure 7.2A, P<0.01). ROC analysis yielded an AUC of 0.93 (95% CI: 0.87–0.98) for CRC and 0.95 (95% CI: 0.93–0.98, see Figure 7.3A) for advanced neoplasia (advanced adenomas and carcinomas; see Figure 7.3). When fixing the cut-off for specificity at 100%, 72% of CRC and 81% of advanced neoplasia could be discriminated from normal mucosa. No significant differences in methylation levels were observed between advanced adenoma and carcinoma tissue samples or between carcinomas of different UICC stages (P=0.5 and P=.07, respectively). Age or gender was no confounding factors (P=0.1 and P=0.9, respectively). Methylation levels in other tumor types showed high levels of methylation in tissue samples from tumors of the intestinal tract (pancreatic, gastric, and esophageal cancer) and in cervical cancer, whereas lower levels of methylation were seen in cancers of lung and bladder, and little or no methylation was seen in cancers of breast, prostate, and brain (glioma; Supplementary Figure S7.3).

Sensitivity and specificity of *PHACTR3* methylation in stool for detecting colorectal cancer

To investigate the performance of *PHACTR3* methylation as a biomarker for CRC detection in stool, we measured methylation levels in 2 independent series of stool-derived DNA samples (see Figure 7.2B and C). A training set was used to determine the optimal cut-off to detect CRC patients compared with controls and nonadvanced adenoma patients. The validation set was used to validate the results from the training set and to test the detection rate of advanced adenomas.

Training set. The training set consisted of 100 stool samples from individuals who all had undergone complete colonoscopy. ROC analysis of CRC (N=22) compared with nonadvanced adenomas and control samples (N=78) yielded an AUC of 0.77 (95% CI: 0.64–0.90, see Figure 7.3B). Maximum sensitivity at a fixed specificity of 95% (95% CI: 87–98) was reached at a cut-off value of 82.5 relative copies. At that cut-off, sensitivity was 55% (95% CI: 33–75) for detecting CRC.

Validation set. The validation set consisted of 93 stool samples from individuals who all had undergone complete colonoscopy. ROC analysis with advanced neoplasia (44 CRC and 19 advanced adenoma) compared with healthy controls (N=30) resulted in an AUC of 0.83 (95% CI: 0.75–0.91, see Figure 7.3C). Using a cut-off of value of 82.5 relative copies as defined with the training set yielded a specificity of 100% (95% CI:

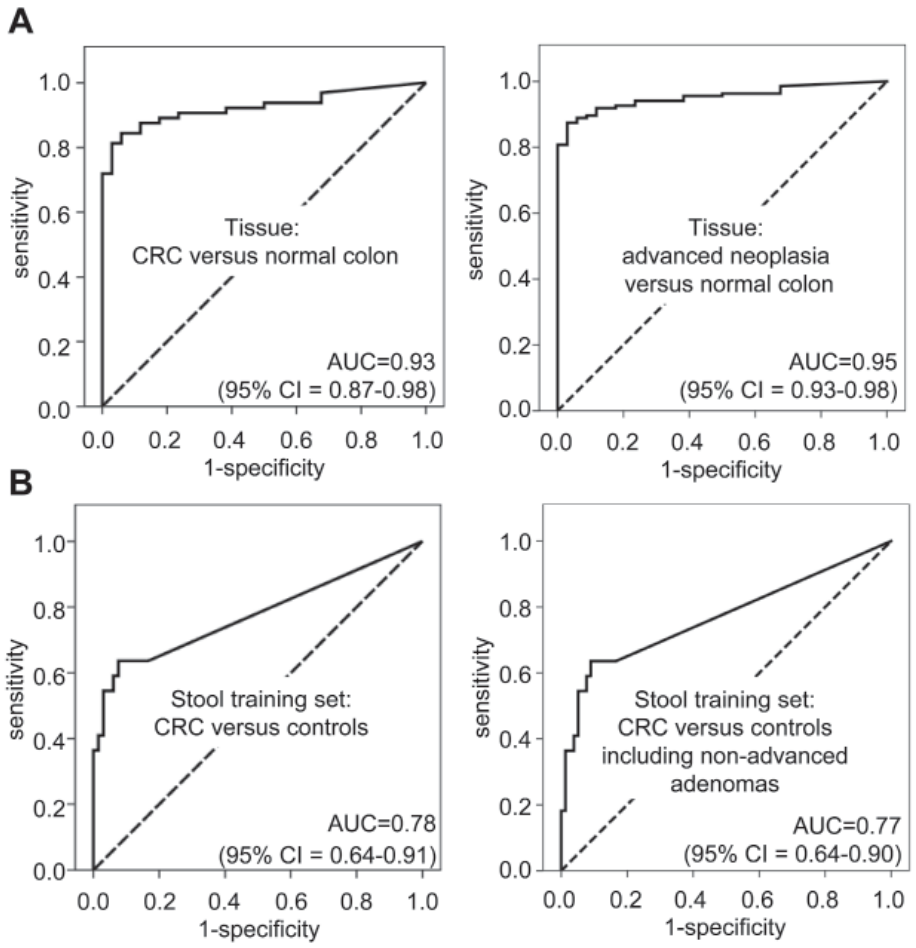

Figure 7.3 Receiver operator characteristic analysis of *PHACTR3* methylation in tissue and stool. **(A)** ROC curves of tissue samples. Sensitivity and specificity at various cutoff values of *PHACTR3* methylation in tissue samples from 64 carcinomas versus 34 normal colon mucosas (left) and 135 advanced neoplasia versus 34 normal colon mucosas (right). The areas under the curves are 0.93 and 0.95, respectively. **(B)** ROC curves of stool samples (training set). Sensitivity and specificity at various cutoff values of whole stool samples from 22 CRC patients versus 66 control patients without colon neoplasia (left) and 22 CRC patients versus 78 control patients (66 patients without colon neoplasia and 12 patients with hyperplastic polyps (HP) or nonadvanced adenoma; right). The areas under the curves are 0.78 and 0.77, respectively. **(C)** ROC curves of stool samples (validation set). Sensitivity and specificity at various cutoff values of whole stool samples from 44 CRC patients versus 30 control patients without colon neoplasia (left) and 63 patients with advanced neoplasia versus 30 control patients without colon neoplasia (right). The areas under the curves are 0.87 and 0.83, respectively. The solid line represents the ROC curve, the dashed line shows the reference line of no discrimination.

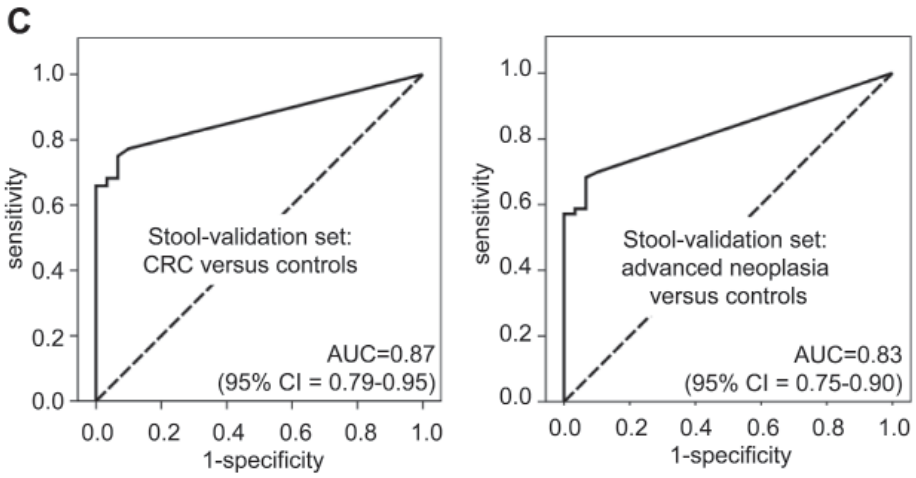

Figure 7.3 *Continued.*

86–100), a sensitivity of 66% (95% CI: 50–79) to detect CRC, and a sensitivity of 32% (95% CI: 14–57) to detect advanced adenomas. Using a cut-off of 28 relative copies, the highest possible sensitivity for advanced adenomas was 53% (95% CI: 32–73), at a specificity of 93% (95% CI: 79–98). Age or gender was no confounding factor (P=1.0 and P=0.4, respectively).

Performance of *PHACTR3* in comparison with other stool methylation markers

To compare the test performance of *PHACTR3* methylation to other stool methylation markers, detection rates were compared with those obtained with the previously published markers, *GATA4*[18] and *OSMR*,[20] which were tested by QMSP in a large proportion of the current training and validation series of stool samples. With the stool training set, the cut-offs for *GATA4* and *OSMR* were determined to detect CRC at equal specificities as *PHACTR3*. Table 7.1 shows the AUC, the cut-offs, sensitivities and specificities of these 3 markers. Compared with *OSMR*, in the training set, *PHACTR3* showed a higher AUC and sensitivity (52% vs. 29%) for carcinomas, and in the validation set *PHACTR3* showed a higher sensitivity for both advanced adenomas (32% vs. 21%) and carcinomas (66% vs. 43%) with higher specificity (100% vs. 90%). *PHACTR3* and *GATA4* showed equal AUCs and comparable sensitivities (52% vs. 57%) for detecting carcinomas in the training set. In the validation set, *PHACTR3* showed a higher sensitivity for both

Table 7.1 Test performances of *PHACTR3*, *GATA4*, and *OSMR*

	PHACTR3	*GATA4*	*OSMR*
Training set			
AUC (95% CI)	0.76 (0.62–0.89)	0.76 (0.62–0.90)	0.60 (0.45–0.75)
Cut-off	82.5	16.5	3.6
Sensitivity CRC (N=21)	52%	57%	29%
Specificity (N=71)	94%	94%	94%
Validation set			
Cut-off	82.5	16.5	3.6
Sensitivity AA (N=19)	32%	16%	21%
Sensitivity CRC (N=44)	66%	39%	43%
Specificity (N=30)	100%	93%	90%

AA, Advanced adenoma; *GATA4*, GATA-binding protein 4; *OSMR*, oncostatin M receptor.

advanced adenomas (32% vs. 16%) and carcinomas (66% vs. 39%) than *GATA4*, with higher specificity (100% vs. 93%).

The combination of these 3 markers, calling positive when at least 1 of the 3 markers would be positive, increased the sensitivity to detect advanced adenoma from 32% to 42% and the sensitivity to detect carcinoma from 52% to 62% (training set) and from 66% to 68% (validation set), however, at cost of specificity (decreased from 94% to 87% and from 100% to 83% for the training and validation set, respectively).

Combination of FIT and *PHACTR3* methylation in stool

To investigate whether FIT and *PHACTR3* methylation would have complementary value for detecting colorectal cancer and advanced neoplasia, both tests were evaluated in an independent series of stool subsamples. Because for DNA methylation analysis, the stool subsamples were processed in a different way compared with whole stool samples, different cut-offs were used, based on ROC analysis, to determine sensitivity and specificity (see Supplementary Methods). The sensitivities to detect advanced adenomas were 21% (5/24, 95% CI: 9–40) for *PHACTR3* and 21% (5/24, 95% CI: 9–40) for FIT. Combining *PHACTR3* with FIT, meaning positive for at least 1 of the 2 measurements, increased the sensitivity to 33% (7/24, 95% CI: 18–53). The sensitivities to detect CRC were 50% (10/20, 95% CI: 30–70) for *PHACTR3* and 65% (13/20, 95% CI: 43–82) for FIT. Combining *PHACTR3* with FIT increased the sensitivity to 95% (19/20, 95% CI: 76–99). The specificity of the

combination remained high [94% (45/48, 95% CI: 83–98) compared with 96% (47/48, 95% CI: 86–99) for *PHACTR3* alone and 98% (47/48, 95% CI: 89–100) for FIT alone]. In addition, a positive test for both FIT and *PHACTR3*, occurring in 7 of the 44 advanced neoplasia cases (see Supplementary Figure S7.4), revealed 100% specificity.

To evaluate whether the sensitivity of the combination of FIT and *PHACTR3* was significantly higher than the sensitivity of either FIT or *PHACTR3* methylation alone, we compared the sensitivities at equal specificity of 92%, 96%, and 98% (see Supplementary Methods). ROC analysis resulted in an AUC of 0.97 (95% CI: 0.93–1.0) for CRC and 0.79 (95% CI: 0.69–0.92) for advanced neoplasia (see Figure 7.4), with sensitivity/specificity combinations of 61%/92%, 55%/96%, and 48%/98% (see Table 7.2 for advanced adenoma and cancer separately). At these specificities, sensitivities for detecting CRC increased up to 15% using the combination of FIT and *PHACTR3* methylation compared with FIT alone, although statistical significance was not reached.

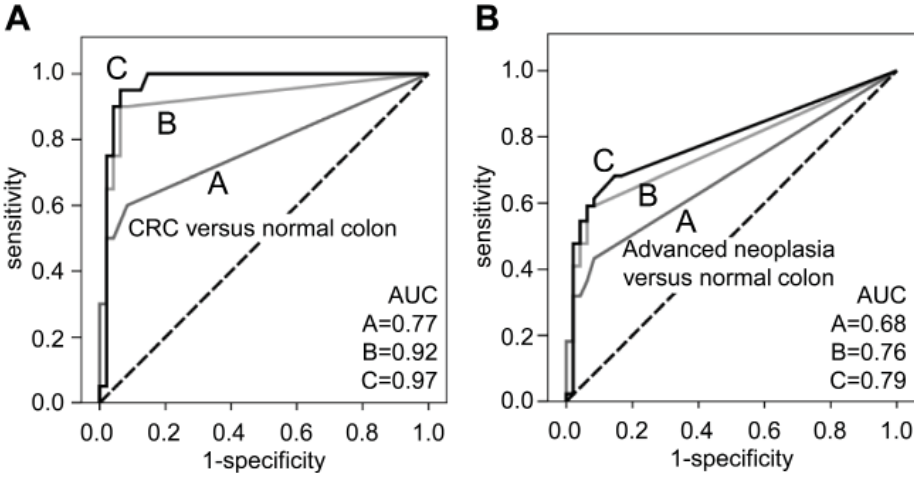

Figure 7.4 Receiver operator characteristic analysis of FIT and *PHACTR3* methylation in stool. **(A)** sensitivity and specificity at various cutoff values of stool subsamples from 20CRC patients versus 48 control patients without colon neoplasia for FIT, *PHACTR3* methylation and for the combination of both tests. The areas under the curves are 0.92, 0.77, and 0.97, respectively. **(B)** sensitivity and specificity at various cutoff values of partial stool samples from 44 patients with advanced neoplasia versus 48 control patients without colon neoplasia for FIT, *PHACTR3* methylation and for the combination of both tests. "A" represents the ROC curve for *PHACTR3* methylation only, "B" represents the ROC curve for FIT only, "C" represents the ROC curve for the combination of FIT and *PHACTR3* methylation. The areas under the curves are 0.76, 0.68, and 0.79, respectively.

Table 7.2 Cut-offs and sensitivities of FIT, *PHACTR3* methylation, and their combination (Y) at equal specificities

		FIT		*PHACTR3*			FIT+*PHACTR3* (Y)		
	Specificity	Cut-off	Sensitivity	Cut-off	Sensitivity	P	Cut-off	Sensitivity	P
AA	92%	1.0	33%	0.2	29%	1.0	16.0	33%	1.0
CRC	92%	1.0	90%	0.2	60%	0.2	19.5	95%	1.0
Advanced neoplasia	92%	1.0	59%	0.2	43%	0.2	16.0	61%	1.0
AA	96%	49.5	25%	0.7	17%	0.7	55.5	25%	1.0
CRC	96%	30.5	75%	0.7	50%	0.3	38.0	90%	0.3
Advanced neoplasia	96%	30.5	48%	0.7	32%	0.2	38.0	55%	0.3
AA	98%	99.5	21%	1.5	17%	1.0	81.5	25%	1.0
CRC	98%	86.5	65%	1.1	50%	0.6	78.0	75%	0.5
Advanced neoplasia	98%	86.5	41%	1.1	32%	0.5	78.0	48%	0.3

P-values are based on the comparison with FIT.
AA, Advanced adenoma.

DISCUSSION

Stool-based DNA testing is an appealing approach for noninvasive early detection of CRC. Both DNA mutations and DNA promoter hypermethylation have been investigated as targets of potential screening assays, and especially panels of markers have shown promising performance in initial studies. In this study, we applied a bioinformatics approach for identifying novel hypermethylated genes in CRC. This resulted in the identification of *PHACTR3* as a new hypermethylated gene in CRC, which showed to have complementary diagnostic power to FIT in a pilot series.

PHACTR3 was first described in 2003 as a protein associated with the nuclear scaffold in human promyelocytic HL-60 leukemia cells. It was found to bind to actin and to the catalytic subunit of Protein Phosphatase 1 (PP1), thereby inhibiting the function of PP1,[31] which in turn can trigger apoptosis and can inhibit oncogenic signaling due to its interaction with pRb.[32] Induced expression of *PHACTR3* in HeLa cervical cancer cells stimulated cell spreading and motility.[33] Finally, in a small study of non–small cell lung cancer patients gene mutations were found in 6/20 patients, which was associated with shortened overall survival.[34] Yet, looking at *PHACTR3* mRNA expression, abundant expression has been found in adult normal human brain and to a lesser extent in ovary, but not in other organs.[31] Accordingly, in this study, we observed high *PHACTR3* mRNA expression in human brain tissue, but much lower expression levels in normal colon tissues and again even lower expression in 6 of 9 matched tumor tissues. Although *PHACTR3* showed high methylation levels in these 6 tumor tissue samples, methylation levels were equally high in the 3 tumor tissues from patients in which mRNA expression was not decreased compared with their normal counterparts. In addition, a direct correlation between the level of methylation and level ofmRNA expression in all 9 tumor tissues could not be shown (Pearson correlation of -0.3, P=0.5, data not shown). Treatment of HT29 and HCT116 cells with the demethylating agent 5-aza did result in reexpression of *PHACTR3*, however, consistent with the recent observation that methylation in the region of the first exon, which is the case for *PHACTR3*, is tightly linked to transcriptional silencing.[35] Yet, the possibility that the observed reexpression of *PHACTR3* could be secondary to demethylation of another gene or locus then *PHACTR3* itself cannot be excluded. Although a direct relationship between *PHACTR3* hypermethylation and silencing of expression in CRC remains to be established, the fact that *PHACTR3* methylation is highly associated with cancer still makes it an interesting candidate biomarker.

Levels of *PHACTR3* methylation in CRC tissues were prominently high and could significantly discriminate advanced adenoma and carcinoma from normal mucosa at the tissue level. High methylation levels are important for obtaining a good signal to noise ratio in a stool-based assay, especially when lesions to be detected are small. Interestingly, advanced adenoma tissue samples showed methylation levels as high as carcinoma tissues, making *PHACTR3* methylation attractive as a biomarker, in compliance with recent guidelines which have stated that the detection of advanced adenomas and not only early carcinoma should be the goal of CRC screening.[36]

When further exploring its potential as a biomarker, in whole stool samples a sensitivity of 55% to 66% for detecting CRC and a sensitivity of 32% for detecting advanced adenoma was observed at a specificity of 95% to 100%. The test performance of *PHACTR3* can be further improved, which is illustrated by the higher AUC in tissues compared with stool, in particular the sensitivity to detect advanced adenomas. With the currently used method higher sensitivities could also be reached, however, at cost of specificity. The highest sensitivity that could be reached to detect advanced adenomas for example was 53%, resulting in a specificity of 93%. With these test performances, *PHACTR3* methylation is one of the best performing single methylation markers described so far.[14] Also in comparison to 2 previously published stool methylation markers *GATA4* and *OSMR*, *PHACTR3* showed superior test performance.

A strategy to improve test performance is to combine multiple markers in a single assay. Especially the high specificity makes *PHACTR3* attractive as a marker for such a panel that would have increased sensitivity without major effects on specificity. The combination of *PHACTR3* with *GATA4* and *OSMR* in this study indeed did increase sensitivity, but at cost of specificity, which mainly is due to the lower individual specificities of *GATA4* and *OSMR*. Nevertheless, it will be difficult to reach 100% sensitivity with methylation markers only, because a portion of CRCs have no or low frequencies of methylated genes, the so-called CpG Island Methylator Phenotype (CIMP)-negative tumors.[37] Therefore, an attractive alternative could be to combine methylation markers with a completely different marker like FIT. Because FIT is already being used in several screening programs throughout the world and logistics have already been put in place, adding a DNA methylation marker would be relatively easy. We therefore carried out a pilot study on the performance of *PHACTR3* methylation in combination with FIT. The combination of FIT and *PHACTR3* methylation increased the sensitivity for advanced neoplasia, that is, adenomas and CRC taken together, while maintaining a high specificity. These

experiments were done in a series of stool subsamples, which would be a good alternative for whole stool samples concerning logistics, stool processing, and storage of samples in large-scale screening programs. Because these stool subsamples were processed in a different manner compared with the whole stool samples described above, systematic differences exist when comparing these results to those from whole stool samples.[4] Still, the results obtained show the power of combining FIT with a DNA methylation marker. This is in line with recent findings that showed an improved test performance of combining other molecular markers (i.e., *APC*, *BAT26*, and *long-DNA*) with FOBT.[12,38]

In conclusion, using a bioinformatics approach, *PHACTR3* was identified as a new hypermethylated gene in CRC. Although we could not unravel the functional effect of *PHACTR3* hypermethylation in CRC, we clearly showed its high potential as a biomarker in stool-based DNA testing. Furthermore, this study suggests that combining *PHACTR3* methylation with FIT could be particularly promising. The full potential of this marker or its combination with FIT awaits validation in a larger, well-controlled cohort study to test its performance in an asymptomatic population.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY METHODS

Bioinformatic strategy for methylation marker selection

In the bioinformatics phase, first, genome-wide microarray based mRNA expression data of 37 colorectal adenomas and 31 adenocarcinomas were analyzed, as described by Carvalho et al.[1] (GEO accession number GSE8067). Since promoter hypermethylation can influence gene expression by reducing or silencing transcription, we focused on genes downregulated in CRC compared to adenomas. Reason for this is that most colorectal adenomas never progress to cancer (~95%),[2] and in order to find markers that are specific for high risk lesions, it makes sense to focus on those molecular alterations that take place during adenoma to carcinoma progression. Genes were selected based on median expression values and considered as differentially downregulated when a Wilcoxon Rank p-value <1e-5, or a Thas[1] p-value <0.05 corresponding to a FDR <0.05. Then, from these genes, those with a reported transcription start site (TSS) were subjected to a bioinformatics approach to select genes with a high potential to be methylated using BROAD and DEEP analysis, as described before.[3]

OpenArray™ and Lightcycler® experiments

A first screen for the presence of methylation was done by methylation specific PCR (MSP) on a BioTrove OpenArrayTM platform[4] (BioTrove, Inc. Woburn, MA, USA) on 83 normal mucosa and 80 carcinoma tissues. Whenever possible, we designed primers covering different transcript variants. MSP reactions were performed in 33 nL reactions based on SYBR® Green I chemistry. A sample was considered to be methylated if the Ct value was less than 42 and Tm fell within an automatically derived marker-specific Tm interval (personal communication Wim van Criekinge). To validate the presence of methylation, we selected genes that were methylated in >25% of CRC samples and in <15% of normal mucosa samples (see Supplementary Table S7.1). Two genes, *Fucokinase* (FUK, NM_145059) and *Phosphatase and actin regulator 3* (*PHACTR3*, NM_080672), met these criteria and presence of methylation was validated by means of high through-put LightCycler MSP in a subset of the series used for OpenArrayTM experiments, consisting of 48 normal mucosa and 42 CRC tissues samples. MSP reactions on the LightCycler® were based on SYBR® Green I chemistry using a total reaction volume of 10 µl. Further analysis of FUK was hampered by technical issues. For *PHACTR3* we were able to create robust MSP assays to study the methylation status in more detail.

Cell lines

All CRC cell lines (HT29, COLO205, Colo320, HCT 116, RKO, and LS513) were cultured under standard conditions in Dulbecco's modified Eagle's medium (DMEM; BioWitthaker, Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 U/ml penicillin (Astellas Pharma BV, Leiderdorp, The Netherlands), 100 g/L streptomycin (FisioPharma, Palomonte, Italy), and 2 mmol/L Lglutamine. To investigate re-expression of *PHACTR3* after inhibition of DNA methyltransferases and HT29 and HCT116 cells were treated with 1 μM 5-aza-2'-deoxycytidine (5-AZA; Sigma Chemical Co., St. Louis, MO, USA) for 3 days.

Tissue samples and DNA isolation

Tissues from 34 normal colon mucosa from cancer-free patients, 71 advanced colon adenomas and 64 colon carcinomas were collected from the tissue archive of the department of pathology at the VU University medical center, Amsterdam, The Netherlands (for patient characteristics, see Supplementary Table S7.2). Adenomas were defined as advanced when ≥10 mm, harboring any villous features (i.e. tubulovillous or villous adenoma) and/or high-grade dysplasia. DNA from formalin-fixed paraffin-embedded (FFPE) was extracted by a column-based method (QIAamp DNA micro kit, Qiagen, Hilden, Germany) as described before.[5,6] DNA from snap-frozen tissues was isolated using TRIzol (Invitrogen, Breda, NL) following the supplier's instructions. DNA was quantified using the Nanodrop 1000 UV spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE, USA). In addition, independent series of FFPE tissues were collected from the tissue archive from the University Hospital Liège, Belgium and from the Maastricht University Medical Center, The Netherlands. All samples were used in compliance with the institutional regulations for use of patient material. DNA was isolated from 9 CRC tissues and matched distant normal colon tissues, from 163 independent colon tissues [83 normal colon mucosas (41 from cancer-free patients and 42 from resection margins from CRC patients) and 80 CRCs], and from cancer tissues of other origin [44 breast, 20 cervix, 20 lung, 11 esophagus (5 adenocarcinomas and 6 squamous cell carcinomas), 20 gliomas, 19 pancreas and 15 stomach]. Per sample 15 sections of 10μm were deparaffinized and DNA extraction was performed using phenol/chloroform extraction and resuspended in 80 μl of LoTE (3 mmol/L Tris, 0.2 mmol/L EDTA, pH 8.0). DNA was quantified using the PicoGreen dsDNA quantitation kit (Molecular Probes, Invitrogen, CA, USA).

Stool samples and DNA isolation

Whole stool samples

Whole stool samples from subjects with either no colon neoplasia, hyperplastic polyps, or (advanced) adenomas were collected from subjects over 50 years of age who underwent primary colonoscopy screening for CRC within the framework of a workplace-based community colorectal cancer study at the Maastricht University Medical Center and from referral subjects who underwent colonoscopy at the VU University Medical Center in Amsterdam. Stool samples from colonoscopy-confirmed CRC patients who were diagnosed with all stages of CRC were collected at the VU University Medical Center in Amsterdam and from a multicenter prospective trial in Germany, in compliance with the institutional ethical regulations. All stool samples were collected prior to colonoscopy and before the start of laxative treatment preceding colonoscopy. All colonoscopies were performed or supervised by experienced endoscopists. Stool stabilization buffer was added to the stool sample by the subject immediately after defecation (Exact Sciences, Madison, WI, USA), processed in the lab within 48 hours, and stored at -80°C until use. Written informed consent was obtained from all subjects who provided stool samples. Stool samples were split into two independent sets of noncancerous control subjects and colorectal cancer patients. The training set consisted of 66 stool samples from patients without colon neoplasia (58 healthy controls, 4 with colonic diverticula and 4 with hemorrhoids), 9 stool samples from patients with hyperplastic polyps, 3 stool samples from patients with non-advanced adenoma, and 22 stool samples from CRC patients. The validation set consisted of 30 control stool samples from healthy individuals, 19 stool samples from patients with advanced adenoma and 44 stool samples from CRC patients (patient characteristics are described in Supplementary Table S7.3). For recovery of human DNA, whole stool samples were homogenized in a sevenfold excess volume of stool stabilization buffer and aliquoted in 32-mL portions that contained the equivalent of 4 g of stool each. DNA isolation was performed as described before.[4] Tumor tissue DNA of these patients was not available.

Stool sub-samples

Stool sub-samples were obtained from a retrospective collection from referral subjects who underwent colonoscopy at the VU University Medical Center in Amsterdam, The Netherlands. Stool samples were collected between November 2004 and January 2007 from referred subjects 1 day before colonoscopy, immediately stored at 4°C and

transferred to -20°C at the day of the colonoscopy without stabilization buffer. Stool samples from subject subjects with no colon neoplasia (n=48), advanced adenoma (n=24) or carcinoma (n=20) were used in this study. Written informed consent was obtained from all subjects who provided stool samples. This study was approved by the Medical Ethical Committee of the VU University Medical Center. Patient characteristics are described in supplementary table 4. At the start of the experiments, stool samples were thawed and after performing FIT, ~1 g stool was sampled from each stool sample and homogenized in a two-fold excess volume of stool stabilization buffer (Exact Sciences) for DNA extraction. DNA was isolated using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany). Homogenized stool (250 µl) was used as starting material for each DNA extraction. DNA was eluted in a volume of 75 µl and DNA from three separate isolations from the same sample was pooled.

Sodium Bisulfite conversion and Quantitative Methylation Specific PCR (QMSP)

For cell line and tissue DNA, 500 ng was sodium bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions and eluted in 50 µl Tris/HCL pH8.0. For DNA from whole stool samples, 2 µg was subjected to bisulfite modification in 96-wells format on a pipetting robot (Tecan), using the EZ-96DNA Methylation kit (Zymo Research), according to the manufacturer's protocol and eluted in 30 µl Tris/HCL pH8.0. For DNA from stool subsamples, 2 µg of DNA or, when the required volume exceeded the maximum volume according to the protocol, a total of 45 µl was subjected to bisulfite modification using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's protocol and eluted in 25 µl Tris/HCL pH8.0. Conventional MSP was performed on CRC cell lines as described before[7] with 2 µl bisulfite-modified DNA in a 25 µl volume using 0.5 U of JumpStart Red Taq DNA polymerase (Sigma Aldrich, Zwijndrecht, The Netherlands). MSP products were analyzed on a 3% agarose gel containing ethidium bromide.

A molecular beacon-based quantitative MSP (QMSP) was developed directed to the hypermethylated region of *PHACTR3* (+411 to +526 relative to the TSS). QMSP was performed on tissue and stool samples was performed on a 7500 fast PCR system and 7900HT real-time PCR system (Applied Biosystems Foster City, CA) as described before,[8] with 5 µl of bisulfite-modified DNA per reaction. Tissue samples were analyzed in duplicate. Due to their limited amount, stool samples were analyzed in one real-time

experiment. A standard curve of a serial dilution of purified PCR product (*PHACTR3*) or plasmid DNA (ACTB) ($2x10^6$ – 20) containing the target sequence was used to determine the relative quantity of methylation in the unknown samples by interpolating their Ct value to the corresponding quantity. Methylation levels in tissues were calculated as 1000 x (relative quantity of methylated *PHACTR3*/relative quantity of Beta-actin (*ACTB*)). In stool samples, the relative quantities of *PHACTR3* were used as methylation level (no ratio was calculated). Only Ct values <40 were included in the calculation. Primer sequences are listed in Supplementary Table S7.5.

Reverse transcriptase PCR (RT- PCR)

Total RNA from cell lines was isolated using TRIzol reagent (Invitrogen, Breda, NL), and subjected to purification using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). After DNase treatment (RQ1 DNAse, Promega, Leiden, The Netherlands), cDNA was made with the Iscript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative RT-PCR was done using SYBR Green PCR master mix (Applied Biosystems, Nieuwerkerk a/d IJssel, NL) as described before,[1] with 0.25 μM of each primer and 50 ng cDNA. Relative expression levels were determined by calculating the Ct-ratio, using B2M as a reference (Ct ratio = 2^-(Ct PHACTR3 – Ct B2M)). Primer sequences are provided in Supplementary Table S7.5.

FIT and *PHACTR3* methylation analysis in stool sub-samples

FIT's (OC-sensor®, Eiken Chemical Co., Tokyo, Japan) were processed with the OC sensor MICRO desktop analyzer (Eiken Chemical) and analyzed according to the manufacturer's instructions. The commonly used cut-off level of 75 ng/ml was initially used[9] to determine sensitivity and specificity of FIT without combination with *PHACTR3*. Because for DNA methylation analysis, the stool sub-samples were processed in a different way compared to whole stool samples, different cut-offs were used, based on ROC analysis, to determine sensitivity and specificity. A cut-off of 0.5 relative copies was used to determine sensitivity and specificity of *PHACTR3* without combination with FIT. To evaluate whether the sensitivity of the combination of FIT and *PHACTR3* was significantly higher than the sensitivity of either FIT or *PHACTR3* methylation alone, we compared the sensitivities at equal specificity. To this end, we used a distribution-free rank-based method[10] to calculate linear combination of the two markers giving highest diagnostic accuracy. The

optimal model for the combination of FIT and *PHACTR3* methylation was the linear combination FIT + 10***PHACTR3* (denoted by Y). Using this model, ROC analysis was used to determine the cut-offs at specificities of 92%, 96%, and 98%. At these specificities, sensitivities were determined and compared to the sensitivities of *PHACTR3* or FIT alone using the McNemar's test.

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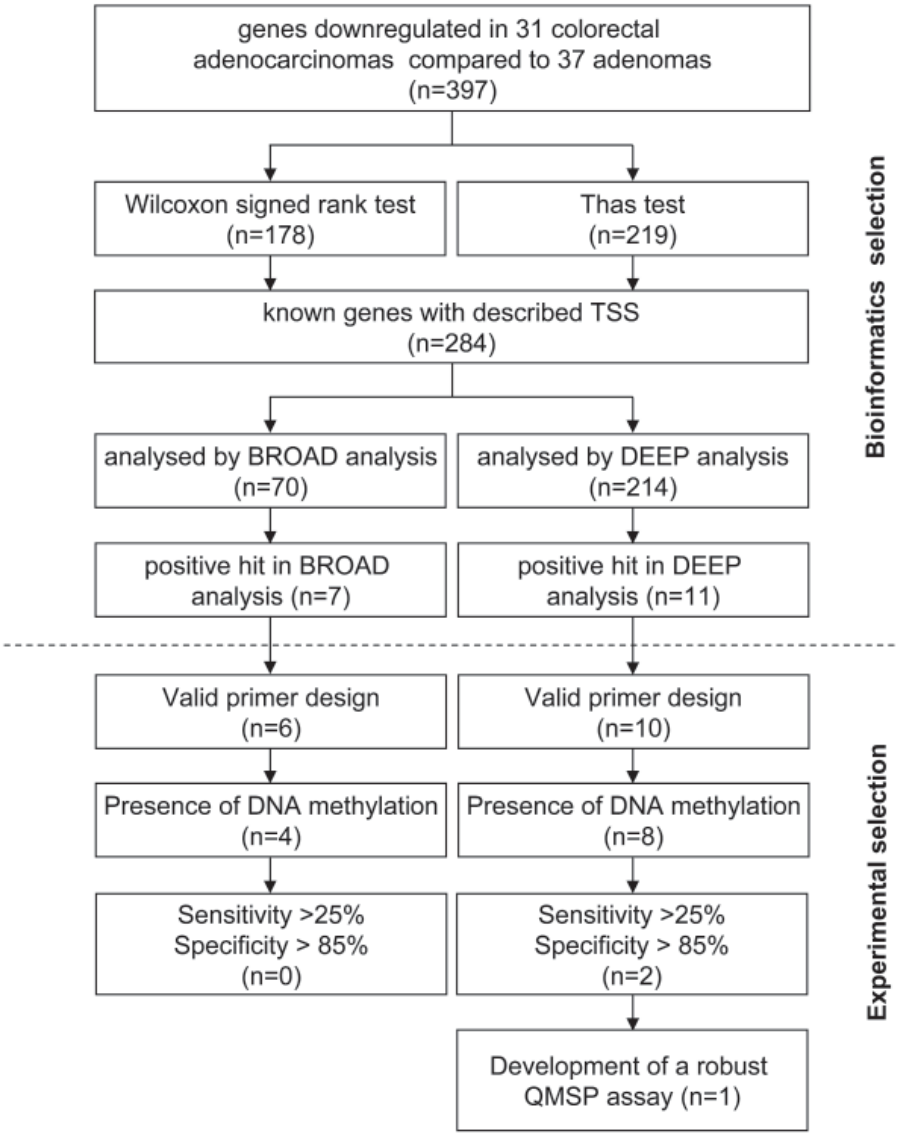
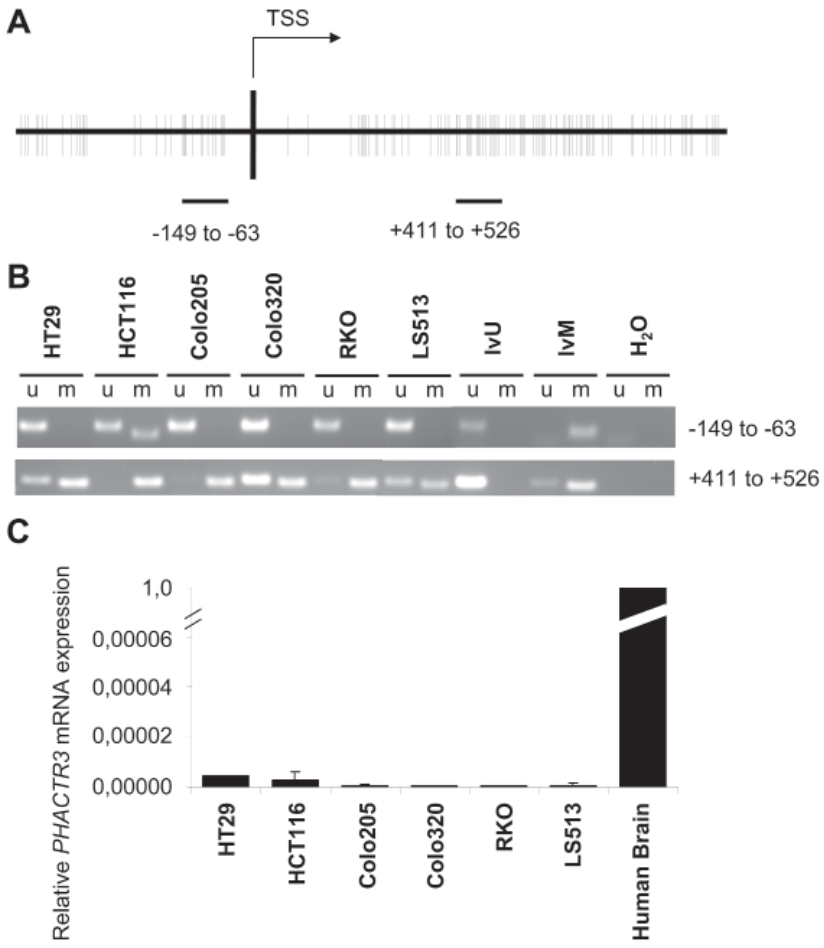
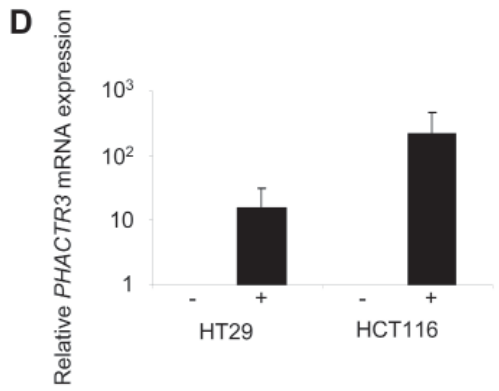


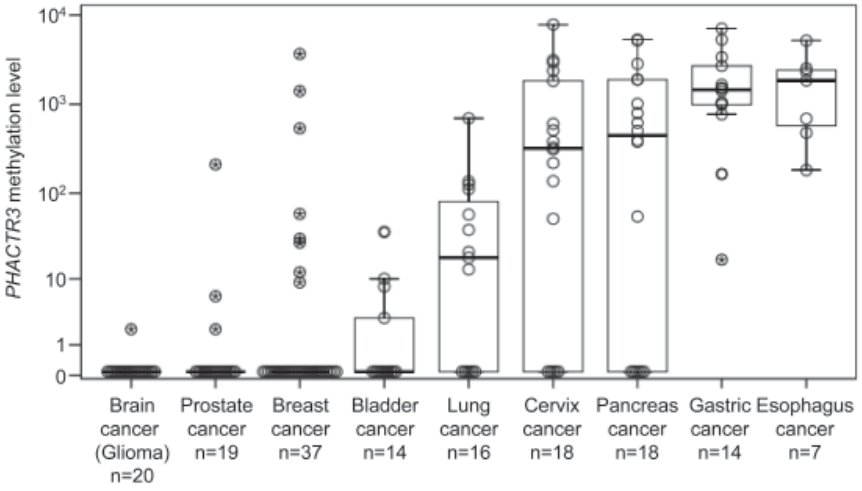
Supplementary Figure S7.1 Flowchart for selection of candidate genes. Candidate genes were selected using microarray expression analysis of 31 colorectal adenomas and 37 colorectal carcinomas, cluster alignment (BROAD analysis) and computational analysis of promoter regions (DEEP analysis) and yielded 18 candidate genes (upper part). Further selection was made based on experimental evidence of the presence of DNA methylation, which resulted in the development of a robust QMSP assay of one candidate gene (lower part).



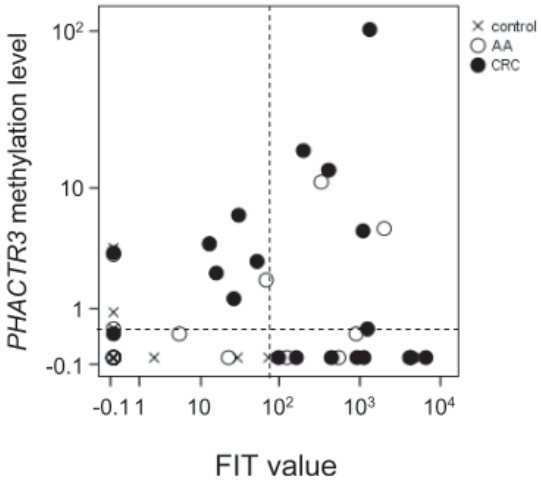
Supplementary Figure S7.2 *PHACTR3* methylation and mRNA expression analysis in CRC cell lines. **(A)** Schematic illustration of the CpG distribution (vertical lines) around the transcription start site (TSS) of *PHACTR3* transcript variant 1. MSP primers were designed -149 to -63 bp and +411 to +526 bp relative to the TSS. **(B)** Methylation status in CRC cell lines -149 to -63 bp and +411 to +526 bp relative to the TSS, analyzed by MSP. U = unmethylated; M = methylated; IvU = unmethylated DNA control IvM = methylated DNA control, H_2O = negative water control). **(C)** mRNA expression analysis of *PHACTR3* in CRC cell lines by RT-PCR. Quantifications represent mean expression values relative to expression in human brain from two independent experiments. **(D)** mRNA expression analysis of *PHACTR3* in CRC cells HT29 and HCT116 by RT-PCR before (-) and after (+) treatment with 1 μM 5-azacytidine. Quantifications represent mean expression values (error bars correspond to standard deviation) relative to untreated cells from two independent experiments.



Supplementary Figure S7.2 *Continued*.



Supplementary Figure S7.3 *PHACTR3* DNA methylation levels in different tumor types. *PHACTR3* methylation levels in carcinomas from brain (glioma, n=20), prostate (n=19), breast (n=37), bladder (n=14), lung (n=16), cervix (n=18), pancreas (n=18), gastric (n=14) and esophagus (n=7) are shown as (relative quantity of methylated *PHACTR3*/relative quantity of unmethylated *Beta-actin* (*ACTB*)) ratio x 1000. Box plots show first quartile, median, third quartile and range of methylation levels. Dots represent individual data points, asterisks represent extremes.



Supplementary Figure S7.4 Scatterplot of *PHACTR3* methylation levels and FIT values in partial stool samples. Scatterplot of *PHACTR3* methylation level (y-axis) and FIT values (x-axis) for individual samples The horizontal and vertical reference lines represent cut-offs of both markers. The left upper quadrant show samples tested positive for *PHACTR3* methylation only. The right lower quadrant show samples tested positive for FIT only. The right upper quadrant show samples tested positive for both *PHACTR3* methylation and FIT. Filled circles represent stool samples from subjects with CRC (n=20), open circles represent stool samples from subjects with advanced adenomas (n=24), crosses represent stool samples from subjects without colon neoplasia (n=48).

Supplementary Table 7.1 Selected candidate genes and the presence of DNA methylation measured by OpenArray platform

RefSeq ID	Gene symbol	Selection method	Percentage of hypermethylated samples in 80 CRC tissues	Percentage of hypermethylated samples in 83 normal colon tissues
NM_145341	PDCD4	BROAD analysis	0	0
NM_001752	CAT	BROAD analysis	4-11	0–12
NM_014142	NUDT5	BROAD analysis	0–4	0–6
NM_001145	ANG	BROAD analysis	0	0
NM_016013	NDUFAF1	BROAD analysis	0–1	0–1
NM_017590	ZC3H7B	BROAD analysis	46	32
NM_152429	FGFBP3	BROAD analysis	NA	NA
NM_138340	ABHD3	DEEP analysis	0	0
NM_052968	APOA5	DEEP analysis	NA	NA
NM_080672	PHACTR3	DEEP analysis	0–36	0–5
NM_015111	N4BP3	DEEP analysis	0–1	0–11
NM_002181	IHH	DEEP analysis	0–4	0–4
NM_005637	SS18	DEEP analysis	1–9	0–15
NM_030790	ITFG1	DEEP analysis	0–3	0–7
NM_004232	SOCS6	DEEP analysis	0	0
NM_030912	TRIM8	DEEP analysis	1–16	0–25
NM_145059	FUK	DEEP analysis	6–39	0–14
NM_033338	CASP7	DEEP analysis	1-3	0–2

The percentages show a range of methylation found in different regions of the same gene based on testing a total of 80 carcinoma tissues and 83 normal colon tissues; NA, not available.

Supplementary Table 7.2 Patient characteristics of colon tissue samples

	Normal mucosa	Advanced adenoma	Carcinoma
Number of samples	34	71	64
Age at diagnosis (years ± SD)	66.4 ± 12.9	69.7 ± 11.5	69.2 ± 10.6
Gender			
Female	23	33	35
Male	11	38	29
Lesion size (mm)			
<10 mm		3	
≥10 mm		64	64
Unknown		4	
Lesion histological type			
Tubular		26	
Tubulovillous		37	
Villous		6	
Unknown		2	
Lesion dysplasia			
Low-grade		55	
High-grade		13	
Unknown		3	
Lesion differentiation			
Well			8
Moderate			47
Poor			8
Mucinous			1
Tumor stage			
UICC I			21
UICC II			18
UICC III			20
UICC IV			5

Supplementary Table 7.3 Patient characteristics of whole stool samples

	Training set				Validation set		
	Controls	Hyperplastic Polyps	Non-advanced adenoma	Carcinoma	Controls	Advanced adenoma	Carcinoma
Number of samples	66	9	3	22	30	19	44
Age at diagnosis (years ± SD)	55.3 ± 10.4	62 ± 8.4	70 ± 8.9	69.7 ± 8.6	51.8 ± 9.9	61.0 ± 9.6	71.0 ± 9.3
Gender							
Female	41	2	2	6	22	11	19
Male	25	7	1	15	8	8	24
Unknown				1			1
Lesion size (mm)							
<10 mm			3			6	
≥10 mm						9	
Unknown				22		4	44
Lesion histological type							
Serrated						1	
Tubular			3			3	
Tubulovillous						15	
Villous						0	
Lesion dysplasia							
Low-grade			3			13	
High-grade						6	
Tumor stage							
Unknown							1
UICC I				7			10
UICC II				8			16
UICC III				6			13
UICC IV				1			4

SD, Standard Deviation; UICC, International Union Against Cancer Classification.

Supplementary Table 7.4 Patient characteristics of partial stool samples

	Controls	Advanced adenoma	Carcinoma
Number of samples	48	24	20
Age at diagnosis (mean [range])	61.6 [50–77]	66.5 [50–82]	68.3 [58–83]
Gender			
Female	25	12	14
Male	23	12	6
Lesion size (mm)			
<10 mm		5	0
≥10 mm		19	19
Unknown		0	1
Lesion histological type			
Tubular		8	
Tubulovillous		14	
Villous		1	
Unknown		1	
Lesion dysplasia			
Low-grade		23	
High-grade		2	
Unknown		1	
Tumor stage			
UICC 0		24	
UICC I			3
UICC II			12
UICC III			3
UICC IV			2

UICC, International Union Against Cancer Classification.

Supplementary Table 7.5 Primer sequences used for (Q)MSP and RT-PCR

MSP primers		
Primer name	Sequence (5' to 3')	Location relative to TSS (bp)
PHACTR3 US 1	TTTATTATGTTGTGTGAGAGGAGTATGATGTTATG	-155 to -51
PHACTR3 UAS 1	CACCCCAACACACAATCCAAATATATACA	
PHACTR3 MS 1	CGCGAGAGGAGTATGACGTTAC	-149 to -63
PHACTR3 MAS 1	GACGCGCGATCCAAATATATACG	
PHACTR3 US 2	GTTATTTTGTGAGTGGTTTTGTGATAT	+407 to +527
PHACTR3 UAS 2	ACCTCAAATACTCTAATTCCACACAACT	
PHACTR3 MS 2	TTATTTTGCGAGCGGTTTC	+411 to +526
PHACTR3 MAS 2	GAATACTCTAATTCCACGCGACT	
PHACTR3 molecular beacon	CGACATGCCCGAACCCATAACCGCGTCGAAGCATGTCG	+459 to +481
RT-PCR primers		
Primer name	Sequence (5' to 3')	
PHACTR3 Fw	CGCTGGCCACGAAGCA	
PHACTR3 Rv	GCTTCTTTGGAGACCCTTT	
B2M Fw	TGACTTTGTCACAGCCCAAGATA	
B2M Rv	AATGCGGCATCTTCAAACCT	

US, unmethylated sense; UAS, unmethylated antisense; MS, methylated sense; MAS, methylated antisense; Fw, Forward; Rv, Reverse; TSS, Transcription Start Site; bp, basepair.

8

Summary, general discussion and future perspectives

SUMMARY

The first section of this thesis deals with the history of fecal occult blood testing. Screening by means of a guaiac based FOBT (g-FOBT) followed by a colonoscopy in case of positivity, has been showed to reduce CRC mortality and incidence.[1-4] However, g-FOBT has been criticized for its fairly low sensitivity and for being non-specific to human hemoglobin. Consequently, as an alternative to g-FOBT, a test specific to human hemoglobin was sought and the Fecal Immunochemical Test (FIT) was introduced.

In **chapter 2** sensitivity and specificity of g-FOBT (Hemoccult-II©) and FIT (OC-sensor©) in advanced adenomas and different stages of CRC was assessed in eligible subjects who underwent complete colonoscopy. G-FOBT was thereafter considered an unreliable test of the past as FIT was found to be more sensitive for CRC and advanced adenomas compared to g-FOBT. In a sub analysis of the neoplastic lesions found in the study cohort, sensitivity of FIT for the screen relevant neoplasia (SRN) as well as for the early stage cancers and advanced adenomas, was proven to be significantly higher compared to g-FOBT. In this direct comparison the more current FIT proved to be superior.

Chapters 3 to 6 form the second section of this thesis and concern different strategies for the use of FIT for the detection of CRC. One of the major advantages of FIT is the non-dichotomous outcome. By adjusting the cut-off value, the test positivity rate can be influenced. The influence of using higher cut-off values for a FIT on the detection rates of SRN was assessed in **chapter 3**. It was found that higher FIT cut-off levels substantially decrease test positivity rates with only limited effects on detection rates of early-stage CRCs. Adjusting the threshold for positivity of quantitative fecal immunochemical tests (FIT) will allow for controlling the number of initial colonoscopies in a screening program.

Chapter 4 focuses on repeated FIT sampling as another strategy, aimed at improving test sensitivity of FIT for detection of early stage CRC and its precursors, defined as SRN. Test positivity and sensitivity of double FIT sampling was evaluated for three different strategies at several cut-off values, "one of two FITs positive" when at least one out of two measurements exceeded the cut-off value, 2) "both FITs positive" when both measurements exceeded the cut-off value, and 3) "the average of two FITs positive" when the geometric mean of two FITs exceeded the cut-off value. Regardless of the cut-off that was used, "two of two FITs+" resulted in the lowest and "one of two FITs+" in the highest sensitivity for SRN (range 35–44% and 42%–54% respectively). Receiver Operator Curves (ROCs) of double FIT sampling were similar to those of single FIT sampling. However,

at fixed levels of specificity of 85/90/95%, sensitivity of any double FIT sampling strategy did not significantly differ from single FIT (p-values 0.07–1.00). In conclusion, none of the double FIT strategies had a superior combination of sensitivity and specificity over single FIT.

In **chapter 5** the focus lies on false positive FITs. In a screening setting, false positive results will result in futile colonoscopies. In this chapter, the contribution of hemorrhoids on the frequency of false positive FITs was determined. In only 9 individuals, out of a cohort of 2855 patients, who had a FP FIT (4.1%; 95% CI 1.4–6.8), hemorrhoids were the only abnormality found. In univariate unadjusted analysis, subjects with hemorrhoids as single abnormality did not have more positive results (9/134; 6.7%) compared with subjects without any abnormalities (43/886; 4.9%; p=0.396). Logistic regression identified hemorrhoids, non advanced polyps and a group of miscellaneous abnormalities(for instance angioectasia) all of significantly influence on false positivity. Still, hemorrhoids detected at colonoscopy remain an infrequent cause of false positive fecal immunochemical tests and therefore, the influence of hemorrhoids on the effectiveness of an FIT-based screening program is likely to be limited.

Recently, FITs were found to have a higher sensitivity and lower specificity for advanced colorectal neoplasia in males compared to females. The aim of **chapter 6** is to compare males and females with respect to the sensitivity and specificity of FIT, at different cut-off values. Outcomes were CRC and advanced adenomas. We studied whether location in the large bowel, number and size of neoplastic lesions were potential explanatory variables in the relation between sex and FIT characteristics.

Using cut-off values between 50–100 ng/ml, a large but non-significant difference of 13%, was found for sensitivity for CRC in favor of men. FIT was significantly more specific for CRC in females than in males. By choosing a lower cut-off value for women, corresponding test characteristics could be reached. For advanced adenomas no sex-specific differences of any clinical relevance were observed. Additionally, FIT proved to be more sensitive for left-sided lesions than for right-sided lesions. However, a difference in distribution of lesions between the sexes proved to not be responsible for the observed gender disparities. Gender specific screening guidelines could be considered in order to optimize or balance the effectiveness of a screening program in males and females. However, since sensitivity for advanced adenomas did not differ between men and women and compliance to screening programs is known to be higher in women, the observed gender difference will probably hold no consequences for the design of a screening program.

Occult gastrointestinal blood loss is unspecific to colonic cancers and large adenomas, while there are several other possible causes.[5] Blood loss from other sources like, for instance, colonic angioectasia will lead to false positive FIT results. Furthermore, false negative results can occur because blood loss from neoplastic lesions is intermittent.[6] False negative FIT's can be a serious problem for the credibility of nationwide screening program. In **chapter 7** the combination of FIT with a methylation marker, in order to improve the FIT for future use, is assessed. Phosphatase and Actin Regulator 3 (PHACTR3) was identified from a pool of potential candidates using a bio-informatics based strategy, as a novel hypermethylated gene that could serve as a biomarker for early detection of colorectal cancer in stool. Subsequently, the complementary value to a Fecal Immunochemical Test (FIT) was evaluated in two series of whole stool samples, which were specifically collected for this purpose. It was found that adding PHACTR3 methylation to FIT increased its sensitivity for CRC up to 15%. This new hypermethylated gene in CRC has a good performance in stool DNA testing and was found to have a additional value to FIT.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Prevention of colorectal cancer (CRC) is the ultimate goal of CRC screening. In order to achieve the maximum benefit in terms of reducing morbidity and mortality, a primary CRC screening tool must be able to effectively detect advanced precursor lesions and cancers at a curable stage throughout the colon.

In September 2013, the much anticipated national screening program for CRC in the Netherlands will finally be initiated.[7] Adequate knowledge on test performance of the primary screening tool is crucial for making estimations of the logistic demands for such a program and is therefore essential for projecting the needed capacity to meet the needs for a nationwide screening program.

Nationwide screening programs are well on their way in several (other) countries. At the time of the initiation of the research reported in this thesis, g-FOBT was the test most used in screening programs. At present, g-FOBT is being replaced by FIT.

The research described in this thesis focuses on the diagnostic accuracy of FITs. Studies of diagnostic test accuracy require the true disease status of each individual to be known. In our population this was determined by colonoscopy, which was considered the gold standard. Here, sensitivity and specificity were calculated in a large cohort of individuals

who were referred for colonoscopy. To avoid bias this gold standard was conducted and interpreted while the endoscopists were blinded to the FIT result.

A limitation of our FIT-cohort is that it is a referral population, partially containing high risk individuals, rather than a true screening population. In a referral population the prevalence of CRC is higher when compared to a true screening population, which consist of asymptomatic individuals. Therefore, test characteristics that depend on the prevalence of disease, like the positive and negative predictive values, could not be generalized from this population. Sensitivity and specificity however, are test characteristics not influenced by the prevalence of the disease.[8] Yet due to work-up bias, in this cohort of referred individuals, sensitivity could still have been overestimated and specificity underestimated.[9] Work-up bias can influence results, as a subset of the individuals has signs or symptoms which increase the likelihood of having both a positive FIT and CRC.[9] Nevertheless, it can be questioned whether a screening population could indeed be free of work-up bias, as it was previously shown that almost half of the subjects with screen detected CRC experienced hematochezia.[10] Similar results are found when comparing sensitivity and specificity of the FIT described in this thesis with results of some of the large screening trials using a FIT.[11,12]

Identifying the most suitable FIT for a screening program and, consequently, reaching the maximum benefit from FIT screening, requires comparability of results between different FITs. FITs new on the market should be evaluated properly and should be compared to the current standard.[13] In order to facilitate adequate comparison of FITs in the future, the concentrations of hemoglobin that one FIT measures should be comparable with the result of a FIT from another manufacturer. For this purpose, uniformity in reporting is required. This analytical comparability could be reached by expressing the quantity of hemoglobin present in the amount of feces in the FIT tube and not in the amount of buffer solution in the test tube.[14]

Compliance to screening and accuracy of the screening tests are the two major determinants of the effectiveness of a screening program.[15,16] With compliance rates around 50%, participation remains poor in population screening programs carried out in Europe. Criticizing the test to be used in a screening program could bear the risk of undermining the faith the general public has in the test and thereby reduce adherence, even before the initiation of the Dutch population screening program. The current debate among professionals concerning the preferred FIT for screening could therefore lead to a reduced uptake of CRC screening.

To date, the Fecal Immunochemical Test seems to be the best available primary screening test for CRC screening as it meets some of the major requirements: it can detect both advanced adenomas and early cancers, it has high specificity to keep the costs of screening low and minimize risks to healthy patients, and it is user-friendly, affordable, and widely available.[17]

FUTURE PERSPECTIVES

Knowledge on test performance and acceptance is required for selection of the FIT best suited to meet pre-specified clinical and logistical requirements of a screening program.

FITs detect blood in stool. Occult blood loss in the colon is non-specific to neoplastic disease and consequently hemoglobin is not an ideal marker for the presence of colonic adenomas and cancers. Additionally, it is unknown whether the adenomas most likely to bleed are indeed those adenomas most likely to progress to malignant disease. As a result, FIT is a rather unspecific tool for detecting (pre)cancerous colonic lesions. Therefore, although it seems to be the best available option at this moment, FIT might not maintain this status in the future.

In order to improve the uptake of a CRC screening program, markers specifically targeted at those adenomatous lesions most likely to progress are needed. These markers could be tumor derived DNA products in stool. However, the ultimate panel of markers for a stool DNA test still has to be developed.[18]

In the Netherlands, the upcoming screening program will create the opportunity to assess the new FITs and other stool markers and compare them to the current standard. This will hopefully facilitate translation of basic research into screening tools ready for implementation into screening programs.

This thesis touched on the past and present use of FITs in CRC screening. As new options will arise in the upcoming years, FIT will probably not hold up as a single marker test in screening programs. The question therefore remains for how long the "FI-test" will survive?

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Samenvatting
(Summary in Dutch)

Dit proefschrift heeft als onderwerp de testkarakteristieken van fecaal occult bloed testen (FOBT's), voor de detectie van het colorectaal carcinoom (CRC) en zijn voorloper laesies, de adenomen. De FOBT's detecteren bloed in de ontlasting, dat vanuit deze neoplastische laesies wordt verloren. Een positieve uitslag van de FIT wordt dan gevolgd door een colonoscopie, waarbij een diagnose gesteld kan worden. De nieuwe generatie FOBT's, die Fecale Immunochemische Test (FIT) wordt genoemd, zal vanaf januari 2014 in Nederland worden gebruikt in het bevolkingsonderzoek darmkanker.

In de verschillende delen van het proefschrift wordt achtereenvolgens de FIT vergeleken met de FOBT uit het verleden, wordt gekeken naar verschillende aspecten van het actuele gebruik van de FIT en wordt tot slot gekeken naar een potentiële verbetering van de test voor toekomstig gebruik.

In het eerste deel van het proefschrift wordt de FIT vergeleken met de zogenaamde guaijak FOBT (g-FOBT). Van het gebruik van de g-FOBT als screeningtest in een bevolkingsonderzoek op darmkanker is bekend dat het de mortaliteit en de incidentie van CRC kan reduceren. Echter, de g-FOBT is berucht vanwege zijn lage sensitiviteit en specificiteit. In **hoofdstuk 2** wordt de sensitiviteit en specificiteit van g-FOBT (Hemoccult-II©) en FIT (OC-sensor©) berekend voor de gevorderde adenomen en de verschillende stadia van CRC. Dit werd gedaan door studiedeelnemers beide testen simultaan te laten uitvoeren. Zij ondergingen vervolgens een volledige colonoscopie, waarbij eventuele CRC of adenomen werden gekoppeld aan uitslagen van de beide testen. De FIT bleek overtuigend sensitiever voor CRC en gevorderde adenomen dan de g-FOBT. In een subanalyse waarbij vroege carcinomen en gevorderde adenomen tezamen werden genomen (de voor screening relevante neoplasia of SRNs genaamd), bleek de sensitiviteit van de FIT voor deze groep significant hoger te zijn dan de sensitiviteit van de g-FOBT.

De hoofdstukken 3 tot en met 6 vormen het tweede deel van dit proefschrift en beschrijven de verschillende strategieën met betrekking tot het actueel gebruik van de FIT voor het opsporen van CRC en zijn voorlopers.

Eén van de grote voordelen van de FIT is dat het een kwantitatieve test is, waarbij een afkapwaarde wordt ingesteld boven welke de test positief wordt genoemd. Door de afkapwaarde aan te passen kan het percentage positieve uitslagen beïnvloed worden. Uiteindelijk zal dit bij het bevolkingsonderzoek relevant zijn voor het aantal verwijzingen voor colonoscopie. Een hogere afkapwaarde zal leiden tot minder verwijzingen voor colonoscopie en is daarmee zeer relevant voor de planning van de logistiek van een

bevolkingsonderzoek. De invloed van het gebruik van hogere afkapwaarden voor de FIT op de detectie van CRC en gevorderde adenomen wordt geëvalueerd in **hoofdstuk 3**. Bij het gebruik van hogere FIT-afkapwaarden bleek dat het percentage gevonden vroegcarcinomen nagenoeg gelijk bleef.

Hoofdstuk 4 is toegespitst op het uitvoeren van niet één maar twee FIT's, teneinde de sensitiviteit en specificiteit van de test voor CRC en zijn voorlopers te verbeteren. De positiviteitsgraad (het percentage testen met positieve uitslag) en sensitiviteit van een dubbele FIT-afname werd geëvalueerd voor drie verschillende strategieën bij verschillende afkapwaarden: 1) "Tenminste 1 van de 2 FIT's positief" wanneer ten minste één van de uitslagen de afkapwaarde overschreed, en 2) "beide FIT's positief" wanneer de uitslagen van beide testen de afkapwaarde overschreden en 3) "het gemiddelde van 2 positieve FIT's" wanneer het meetkundig gemiddelde van de twee FIT's de afkapwaarde overschreed. Ongeacht de gebruikte afkapwaarde leidde "beide FIT's positief" tot de laagste en "Tenminste 1 van de 2 FIT's positief" tot de hoogste sensitiviteit voor SRN, met een respectievelijke spreiding van 35–44% en 42–54% . ROC-curves (Receiver Operator Characteristic curves) van het uitvoeren van twee FIT's waren gelijk aan die van het uitvoeren van een enkele FIT. Echter, bij vaststaande niveaus van specificiteit (85%/90%/95%) bleek de sensitiviteit van dubbele FIT-afname niet significant te verschillen van een enkele FIT-afname (p-waardes 0.07–1). Concluderend blijkt geen van de herhaalde FIT-strategieën een duidelijke meerwaarde te hebben wat betreft sensitiviteit en specificiteit.

In **hoofdstuk 5** ligt het zwaartepunt op foutpositieve FIT-uitslagen. Binnen het kader van een bevolkingsonderzoek leiden foutpositieve FIT's tot onnodig uitgevoerde colonoscopieën. Bloedverlies uit andere bronnen dan CRC en gevorderde adenomen zal immers ook tot een positieve FIT leiden. Hemorroïden zijn een beruchte oorzaak van rectaal bloedverlies. In dit hoofdstuk wordt het aandeel van hemorroïden in het totaal van foutpositieve FIT-uitslagen bepaald. In een cohort van 2855 patiënten vormden hemorroïden bij slechts 9 individuen de enige bij coloscopie gevonden afwijking. In een univariate analyse hadden personen met hemorroïden als enige afwijking niet meer positieve uitslagen (9/134; 6.7%) dan personen waarbij geen enkele afwijking werd gevonden bij colonoscopie (43/886; 49%; p=0.396). Logistische regressieanalyse toonde aan dat hemorroïden, "non-advanced" poliepen en een groep van diverse afwijkingen (zoals intestinale angiodysplasieën) de foutpositiviteit van FIT allen significant beïnvloeden. Desondanks blijven hemorroïden een zeldzame oorzaak van een foutpositieve immunochemische feces occult bloed test en is de invloed van hemorroïden op de ef-

fectiviteit van een FIT bij het gebruik in een bevolkingsonderzoek op CRC waarschijnlijk beperkt.

Uit een eerdere publicatie is gebleken, dat FIT's een hogere sensitiviteit en een lagere specificiteit voor CRC en gevorderde adenomen hebben bij het gebruik door mannen in vergelijking tot het gebruik door vrouwen. Het doel van **hoofdstuk 6** was het vergelijken van sensitiviteit en specificiteit van FIT voor CRC en gevorderde adenomen bij gebruik door mannen en door vrouwen. Onderzocht werd of locatie in de dikke darm, het aantal en de grootte van de gevonden neoplastische laesies potentieel verklarende variabelen zijn in de relatie tussen geslacht en FIT-kenmerken.

Door afkapwaarden tussen 50–100 ng/ml te gebruiken werd een aanzienlijk, doch niet significant, verschil van 13% gevonden voor CRC ten gunste van mannen. Door een lagere afkapwaarde voor vrouwen te kiezen, zouden overeenkomende testkenmerken bereikt kunnen worden. Voor de gevorderde adenomen werden geen klinisch relevante verschillen tussen de seksen gevonden. Tevens bleek FIT sensitiever voor laesies gelokaliseerd in het linker hemicolon dan voor laesies die zich aan de rechterkant van het colon bevonden. Een verschil in distributie van de laesies over het colon bleek echter niet verantwoordelijk voor de gevonden sekseverschillen. Tot slot bleek FIT significant specifieker voor CRC bij het gebruik door vrouwen in vergelijking met gebruik door mannen. Seksespecifieke richtlijnen voor het bevolkingsonderzoek zouden overwogen kunnen worden teneinde de effectiviteit van het programma voor mannen en vrouwen te optimaliseren. Echter, gezien het feit dat de sensitiviteit voor gevorderde adenomen niet verschilt voor mannen en vrouwen en omdat de compliantie aan screeningsprogramma's onder vrouwen aanzienlijker hoger ligt, zal het geobserveerde sekseverschil hoogstwaarschijnlijk geen consequenties hebben voor het ontwerp van een bevolkingsonderzoek.

Occult gastro-intestinaal bloedverlies is niet specifiek voor CRC en gevorderde adenomen. Er zijn meerdere oorzaken voor intestinaal bloedverlies, zoals bijvoorbeeld de eerder genoemde intestinale angiodysplasieën. Derhalve kan een test die bloed als biomarker neemt per definitie slechts beperkt specifiek zijn.

Naast fout-positief kan een FIT ook fout-negatief zijn. In een dergelijk geval is een neoplastische laesie in het colon aanwezig maar is de FIT negatief. Fout-negatieve FIT's kunnen een groot probleem vormen voor de geloofwaardigheid van een bevolkingsonderzoek. **Hoofdstuk 7** onderzoekt of de toevoeging van een zogenaamde methyleringsmarker aan de test, de FIT voor toekomstig gebruik kan optimaliseren.

Gebruikmakend van een strategie uit de bio-informatica werd een nieuw hypergemethyleerd gen in CRC geïdentificeerd, genaamd Fosfatase Actine Regulator 3 (PHACTR3). PHACTR3 kan dienen als biomarker voor de vroege detectie van darmkanker in ontlasting. De potentiële additionele waarde van de PHACTR3 marker aan een FIT werd geëvalueerd in twee series van ontlastingmonsters die specifiek voor dit doel waren verzameld. Vastgesteld werd dat de sensitiviteit van FIT voor CRC tot wel 15% toenam door het toevoegen van de PHACTR3 marker. Deze marker heeft dus veel potentie en lijkt van toegevoegde waarde te zijn bij het gebruik van FIT.

De titel van dit proefschrift luidt: "De overleving van de Fecaal Immunochemische Test". Het beschrijft een vergelijking van FIT met de FOBT uit het verleden en daarnaast verschillende aspecten aangaande het huidige gebruik van de FIT in de context van de screening op CRC. Op dit moment lijkt de FIT de best beschikbare test te zijn voor een bevolkingsonderzoek naar CRC. Echter, gezien de snelheid waarmee nieuwe biomarkers worden ontwikkeld blijft de vraag hoe lang de FIT in zijn huidige vorm zal worden gebruikt.

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About the author

CURRICULUM VITAE

Frank Albert Oort werd op 30 juni 1979 geboren te Heemskerk. Hij behaalde in 1997 zijn atheneumdiploma aan het Jac. P. Thijsse College te Castricum. Vervolgens startte hij zijn studie geneeskunde aan de Vrije Universiteit te Amsterdam, waar hij in 1998 zijn propedeuse behaalde. Tijdens zijn studie werkte hij onder meer als webredacteur voor verschillende medische websites en als practicumbegeleider bij de vakgroep Klinische Chemie van de medische faculteit van de Vrije Universiteit.

Door een onderzoeksstage bij de afdeling Maag-, darm- en leverziekten van het Sint Lucas Andreas Ziekenhuis (begeleiders Dr. N.A.M. van Ooteghem en Dr. I.C.E. Wesdorp), waarvoor hij zijn co-schappen in 2005 onderbrak, raakte zijn interesse in de gastroenterologie gewekt. Met een oudste co-schap bij de afdeling Maag-, Darm- en Leverziekten van het Medisch Centrum Alkmaar, rondde hij zijn opleiding tot arts af. Na het behalen van het artsenbul deed hij klinische ervaring op als assistent-geneeskundige niet in opleiding (AGNIO) op de afdelingen Interne Geneeskunde, Cardiologie en Longziekten van het Kennemer Gasthuis te Haarlem (opleider Prof. Dr. R.W. ten Kate).

In maart 2007 startte hij met promotieonderzoek naar de testkarakteristieken van screeningstesten die in het aanstaande bevolkingsonderzoek darmkanker gebruikt zullen worden, onder begeleiding van promotoren Prof. Dr. C.J.J. Mulder, Prof. Dr. G.A. Meijer en co-promotor Dr. R.W.M. van der Hulst. Na een voltijds onderzoeksperiode van 2 jaar startte hij met de vooropleiding Interne Geneeskunde in het Sint Lucas Andreas Ziekenhuis (opleider Dr. C.E.H. Siegert). In hetzelfde ziekenhuis vervolgde hij zijn opleiding tot maag-, darm- en leverarts (opleider Drs. P. Scholten). Vanaf mei 2012 is hij werkzaam als maag-, darm- en leverarts in opleiding in het VU medisch centrum te Amsterdam (opleider Dr. R.A. de Vries). Begin 2015 zal hij zijn opleiding afronden.

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