Citation: Clinical and Translational Gastroenterology (2016) 7, e137; doi:10.1038/ctg.2015.67

www.nature.com/ctg

A Blood Test for Methylated *BCAT1* and *IKZF1* vs. a Fecal Immunochemical Test for Detection of Colorectal Neoplasia

Erin L. Symonds, PhD[1,2,4], Susanne K. Pedersen, PhD[3,4], Rohan T. Baker, PhD[3], David H. Murray, BSc[3], Snigdha Gaur, PhD[3], Stephen R. Cole, MPH[1,2], Geetha Gopalsamy, MBBS[1], Dileep Mangira, MBBS[1], Lawrence C. LaPointe, PhD[3] and Graeme P. Young, MD[1]

OBJECTIVES: To compare the performance of a new blood test for colorectal cancer (CRC) to an established fecal immunochemical test (FIT) in a study population with the full range of neoplastic and non-neoplastic pathologies encountered in the colon and rectum.
METHODS: Volunteers were asked to complete a FIT prior to colonoscopy. Blood was collected after bowel preparation but prior to colonoscopy, and plasma was assayed for the presence of methylated *BCAT1* and *IKZF1* DNA using a multiplex real-time PCR assay. Sensitivity and specificity estimates for the blood test were calculated from true- and false-positive rates for neoplasia and compared with FIT at a range of fecal hemoglobin (Hb) concentration positivity thresholds.
RESULTS: In total, 1,381 volunteers (median age 64 years; 49% male) completed both tests prior to colonoscopy. Estimated sensitivity of the *BCAT1/IKZF1* blood test for CRC was 62% (41/66; 95% confidence interval 49–74%) with a specificity of 92% (1207/1315; 90–93%). FIT returned the same specificity at a cutoff of 60 μg Hb/g, at which its corresponding sensitivity for cancer was 64% (42/66; 51–75%). In the range of commonly used FIT cutoffs, respective cancer sensitivity and specificity estimates with FIT were: 59% (46–71%) and 93% (92–95%) at 80 μg Hb/g, and 79% (67–88%) and 81% (78–83%) at 10 μg Hb/g. Although estimated sensitivities were not significantly different between the two tests for any stage of cancer, FIT showed a significantly higher sensitivity for advanced adenoma at the lower cutoffs. Specificity of FIT, but not of the *BCAT1/IKZF1* blood test, deteriorated substantially in people with overt blood in the feces. When combining FIT (cutoff 10 μg Hb/g) with the *BCAT1/IKZF1* blood test, sensitivity for cancer was 89% (79–96%) at 74% (72–77%) specificity.
CONCLUSIONS: A test based on detection of methylated *BCAT1/IKZF1* DNA in blood has comparable sensitivity but better specificity for CRC than FIT at the commonly used positivity threshold of 10 μg Hb/g. Further evaluation of the new test relative to FIT in the population screening context is now required to fully understand the potential advantages and disadvantages of these biomarkers in screening.
Clinical and Translational Gastroenterology (2016) **7,** e137; doi:10.1038/ctg.2015.67; published online 14 January 2016
Subject Category: Colon/Small Bowel

INTRODUCTION

Colorectal cancer (CRC) is a significant cause of mortality, and screening is an important cancer-control tool. The two main types of tests used for CRC screening are endoscopic (flexible sigmoidoscopy or colonoscopy) and non-invasive (e.g., fecal occult blood tests (FOBT). Randomized controlled trials have provided evidence that early detection of colorectal neoplasia achieved by screening with FOBT or flexible sigmoidoscopy reduces mortality and may also reduce incidence of CRC.[1–4] The original guaiac-based FOBT (gFOBT) have now been largely replaced by fecal immunochemical tests (FIT) for hemoglobin (Hb) as FIT have better sensitivity.[5–14]

Reduction in mortality at the population level depends not just on accuracy but also on willingness to do the test.[15,16] Barriers to screening by both endoscopic and non-invasive fecal tests have been well described, and despite aggressive health promotion, participation rates remain suboptimal in organized screening programs.[17–20] For example, screening participation with FIT in the Australian National Bowel Cancer Screening Program is $<35\%$.[21] The need for participants to provide fecal specimens is a behavioral barrier that has been well documented.[22,23] Furthermore, occult bleeding is not exclusively due to colorectal neoplasia, and people with non-neoplastic bleeding return false positives when screened by FOBT. A blood sample-based test might overcome some of the behavioral barriers inherent with fecal-based testing or invasive endoscopy.[24,25]

New screening tests are continually emerging for CRC screening including DNA-based tests. Methylated regions of genes show promise as CRC biomarkers and some have already been incorporated into both fecal and blood tests.[26,27] Methylated *SEPT9* is one such tumor biomarker associated with CRC and is detectable in blood, although its clinical performance as a screening test is considered to be suboptimal.[26]

[1]Flinders Centre for Innovation in Cancer, Flinders University of South Australia, South Australia, Australia; [2]Bowel Health Service, Repatriation General Hospital, South Australia, Australia and [3]Clinical Genomics, North Ryde, New South Wales, Australia
Correspondence: Erin L. Symonds, PhD, Bowel Health Service, Repatriation General Hospital, 202-216 Daws Road, Daw Park, South Australia 5041, Australia. Email: Erin.Symonds@health.sa.gov.au
[4]These authors contributed equally to this work.
Received 15 September 2015; accepted 4 December 2015

Table 1 Reason for colonoscopy, clinical findings, and demographic characteristics for study volunteers completing both tests

	No. cases	Age (years)	Women	Men
	n (%)	Median (min–max)	*n* (%), median age	
Study cohort	1381 (100.0)	64.1 (41.1–85.4)	699 (50.6), 63.4	682 (49.4), 64.7
Colonoscopy indication[a]				
Symptoms	480	63.6 (41.4–85.4)	258 (53.7), 63.0	222 (46.3), 64.0
Positive fecal occult blood test	415	64.2 (41.5–85.1)	190 (45.8), 63.8	225 (54.2), 65.0
Surveillance (family history)	253	62.2 (41.7–84.8)	159 (62.8), 61.3	94 (37.2), 63.7
Surveillance (personal history)	439	66.6 (42.3–85.1)	205 (46.7), 65.9	234 (53.3), 67.6
Screening	17	60.7 (42.0–79.0)	12 (70.6), 60.2	5 (29.4), 63.4
Other[b]	104	62.6 (41.1–83.0)	52 (50.0), 62.9	52 (50.0), 62.3
Principal diagnosis				
Cancer	66 (4.8)	67.4 (42.6–85.4)	25 (37.9), 64.1	41 (62.1), 69.0
Stage I	17 (1.2)	70.1 (49.8–82.5)	8 (47.1), 64.9	9 (52.9), 76.1
Stage II	25 (1.8)	66.2 (45.7–85.4)	8 (32.0), 62.8	17 (68), 69.7
Stage III	17 (1.2)	66.3 (42.6–77.2)	5 (29.4), 68.0	12 (70.6), 66.2
Stage IV	7 (0.5)	69.5 (47.2–83.4)	4 (57.1), 67.8	3 (42.9), 69.5
Advanced adenoma	170 (12.3)	66.2 (42.5–84.4)	63 (37.1), 66.4	107 (62.9), 65.9
Non-advanced adenoma	278 (20.1)	65.3 (41.2–84.8)	130 (46.8), 65.2	148 (53.2), 65.4
No neoplasia[c]	867 (62.8)	62.6 (41.1–85.4)	481 (55.5), 61.6	386 (44.5), 63.2
Non-neoplastic pathologies[d]	574 (41.6)	64.6 (41.5–85.4)	309 (53.8), 65.2	265 (46.2), 64.1
Inflammatory bowel disease	47 (3.4)	53.1 (41.1–83.0)	24 (51.1), 51.3	23 (48.9), 54.1
No evidence of disease	246 (17.8)	59.6 (41.4–84.7)	148 (60.2), 58.9	98 (39.8), 60.2

[a]Some subjects may have more than one indication for colonoscopy referral. [b]Including repeat colonoscopies and surveillance for inflammatory bowel disease, diverticular disease, and radiation proctitis. [c]All non-neoplastic cases, i.e., excluding only cases with adenoma or colorectal cancer. [d]Including polyps (hyperplastic, unspecified, other polyps), angiodysplasia, hemorrhoids, and diverticular disease. Excluding inflammatory bowel disease, which is shown separately.

We have previously reported the identification of a cohort of genes with regions that are methylated with high frequency in colorectal neoplastic tissues,[28] and we have performed an initial evaluation of a blood test for CRC that detects hypermethylated regions in two genes, *BCAT1* and *IKZF1.*[29,30] These genes were chosen following a rigorous, unbiased biomarker discovery and validation program aimed at identifying highly sensitive, and more importantly, extremely specific diagnostic biomarkers, i.e., minimal hypermethylated signal in DNA extracted from blood of healthy donors.[28] Although biological function or likely role in neoplastic transformation was not a selection parameter, it has been demonstrated by other groups that both *BCAT1* and *IKZF1* are involved in tumor growth and invasiveness.[31–37] The blood test that we have now developed appears to have sensitivity and specificity levels that are adequate for population screening for CRC[29] and its implementation in screening programs may overcome barriers to participation associated with tests requiring fecal specimens.

Because the mortality benefit of adopting a new CRC screening test at the population level is unknown and would require lengthy trials, as a preliminary step it is important to compare its performance to another non-invasive screening test technology where there is already evidence of benefit.[27,38] The aim of this study was to compare the sensitivity and specificity of the methylated *BCAT1/IKZF1* blood test with a quantitative FIT set for positivity at fecal Hb concentrations typically used in screening, across the full spectrum of pathology encountered in the colon and rectum.

METHODS

Study overview. This was a prospective study comparing clinical performance of the methylated *BCAT1*/*IKZF1* blood test against a widely used FIT in people with colorectal neoplasia or non-neoplastic pathologies. Findings at colonoscopy were used as the diagnostic standard. Clinical staff audited colonoscopy and clinicopathological reports and verified case classification while blinded to all test results. Feces and blood samples were assayed for Hb and presence of methylated *BCAT1* and *IKZF1* DNA, respectively, by independent staff blinded to clinical diagnosis. Written informed consent was obtained from all study participants prior to any procedures. The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee (4 April 2005). The trial is registered at Australian and New Zealand Clinical Trials Registry, trial registration number 12611000318987.

Population. Any adults (40–85 years of age) scheduled for colonoscopy for standard clinical indications (**Table 1**) were approached about volunteering for the study. Younger age groups were not included as they are considered to be at lower risk for developing CRC. The participating centers were Repatriation General Hospital (Daw Park, South Australia) and Flinders Medical Centre (Bedford Park, South Australia). Following enrollment, subjects were excluded if the scheduled colonoscopy was canceled, if insufficient blood was drawn, or if FIT kits were returned to the processing laboratory >2 weeks after sample collection.

Clinical procedures. Consenting subjects were sent a FIT kit (OC-Sensor, Eiken Chemical Company, Tokyo, Japan) 2 weeks prior to colonoscopy and were instructed to sample from one bowel movement. Samples were returned by mail to the Bowel Health Service Laboratory (Repatriation General Hospital). Participants were asked to record date of fecal

sampling and whether they had observed blood during sampling.

Venous blood (18 ml) was collected into K_3EDTA Vacuette tubes (Greiner Bio-One, Frickenhausen, Germany) from participants prior to being sedated for colonoscopy but after consumption of bowel preparation solution. Blood tubes were kept at 4 °C prior to plasma processing (not >4 h from blood collection). Plasma was prepared by centrifugation at 1,500 g for 10 min at 4 °C (deceleration at lowest setting), followed by retrieval of the plasma fraction and a repeat centrifugation. The resulting plasma was stored at – 80 °C. Frozen plasma samples were shipped on dry ice to Clinical Genomics Technologies (Sydney, Australia) and stored at – 80 °C until testing.

FIT processing. Returned FIT kits were analyzed for Hb using the OC-Sensor DIANA instrument as recommended by manufacturer. Samples not analyzed on the day of receipt were stored at 4 °C until analysis (but analyzed within 7 days). Samples with Hb concentrations above the analytical range (200 μg Hb/g feces) were diluted (1:15 and 1:250) and re-assayed. A sample was considered positive at selected fecal Hb concentration cutoff levels in the range 10–80 μg Hb/g feces, to match with the range of commonly used cutoff levels in population screening programs.[39,40]

Blood DNA methylation testing. All plasma samples of at least 3.9 ml were assayed at Clinical Genomics Technologies for the presence of methylated *BCAT1* and *IKZF1* DNA (see Supplementary Material for further details). Samples were processed and assayed in batches of 22 samples plus two process controls as previously reported,[29] but with the following changes: the bisulphite conversion setup and subsequent purification was automated on a QIAcube HT instrument (Qiagen, Hilden, Germany) and the *IKZF1* component in the methylation-specific PCR assay was modified to enable detection of partially methylated *IKZF1* target regions (Supplementary Material and Supplementary Table 1). Bisulfite-converted DNA from each plasma sample was assayed in triplicate with real-time PCR performed on a Light Cycler 480 II instrument (Roche Diagnostics, IN, USA) (Supplementary Material). A sample was deemed qualitatively positive if at least one PCR replicate was positive for either *BCAT1* or *IKZF1* DNA methylation.

Pathological classification. All colonoscopy procedures were performed by hospital-accredited specialists and so met site-specific standards for sedation, monitoring, imaging, and equipment. Histopathology and staging of neoplasia followed routine procedures at each study site. No study-wide control of colonoscopy or pathology procedures or quality was undertaken as the study aimed to assess test performances relative to outcomes determined in usual clinical practice.

An independent physician assigned diagnosis for all cases used in this study on the basis of colonoscopy and clinicopathological findings. CRC were staged according to AJCC 7th Edition.[41] Advanced adenoma was defined as adenoma with any of the following characteristics: (a) ≥ 10 mm in size, (b) >20% villous change, or (c) high-grade dysplasia. Cases with more than two tubular adenomas or stage 0 cancer were also classified as advanced adenoma. Non-advanced adenoma refers to those not meeting the characteristics of an advanced adenoma. Hyperplastic polyps were classed as non-neoplastic pathologies.

Where multiple pathologies were present, the most advanced neoplasm was used as the principal diagnosis. Location of the principle neoplasm was defined as that of the most advanced lesion in a patient with multiple neoplasms. Where multiple non-neoplastic diagnoses were present, the principal diagnosis was allocated in the following hierarchy (descending): inflammatory bowel disease, hyperplastic polyp, angiodysplasia, hemorrhoids, diverticular disease.

Statistical analyses. The main outcome measure was positivity rate by diagnosis. Binomial distribution was assumed for calculations of 95% confidence interval (95% CI). Differences in paired positivity proportions and concordance analyses were analyzed using McNemar's test, whereas differences in non-paired proportions used a χ^2-test (two-tailed; significant level, 0.05). Potential confounding co-variables (age, gender) were analyzed by multiple logistic regression analysis. Test sensitivity estimates were expressed as the ratio of true positives over the sum of true positives plus false negatives. Specificity was estimated as 1–positivity rate in cases with no CRC. As FIT is quantitative and the cutoff for positivity can be varied, test comparison was facilitated by undertaking receiver operating characteristic curve analysis and estimating relative true-positive rates (and hence sensitivity) at an equivalent specificity to the blood DNA test. The GraphPad online scientific software tool (http://graphpad.com/scientific-software) was used for the statistical analyses described above. P values <0.05 were considered statistically significant.

RESULTS

Population. Study recruitment was from September 2011 to June 2014. **Figure 1** summarizes the disposition of volunteers from initial approach through to diagnosis, including reasons for exclusion or withdrawal. Fecal Hb and blood methylated DNA testing were completed prior to a colonoscopic investigation in 1,381 participants whose clinical findings and demographic characteristics are shown in **Table 1**.

Table 2 summarizes positivity rates for FIT (at selected Hb cutoff levels for positivity) and the *BCAT1/IKZF1* blood test, relative to colonoscopy findings. The overall positivity rates were 22.4 and 9.1% for FIT at Hb cutoffs of 10 and 80 μg Hb/g feces, and 10.8% for the blood DNA test.

Estimates of sensitivity for CRC. Of the 66 cases diagnosed with CRC, 41 (62.1%) were positive for methylated *BCAT1*/*IKZF1* in blood. For FIT, at cutoff levels of 10 and 80 μg Hb/g feces (the most- and least-sensitive criterion values used in screening) 52 (78.8%, $P=0.05$) and 39 (59.1%, $P=0.85$) CRC cases were positive, respectively. There were no significant differences in positivity rates between the blood test and FIT at any stage, or for early (stage I+II) vs. late (III+IV) cancer, regardless of the FIT cutoff used (**Table 2** and **Figure 2a**). Similar results were observed

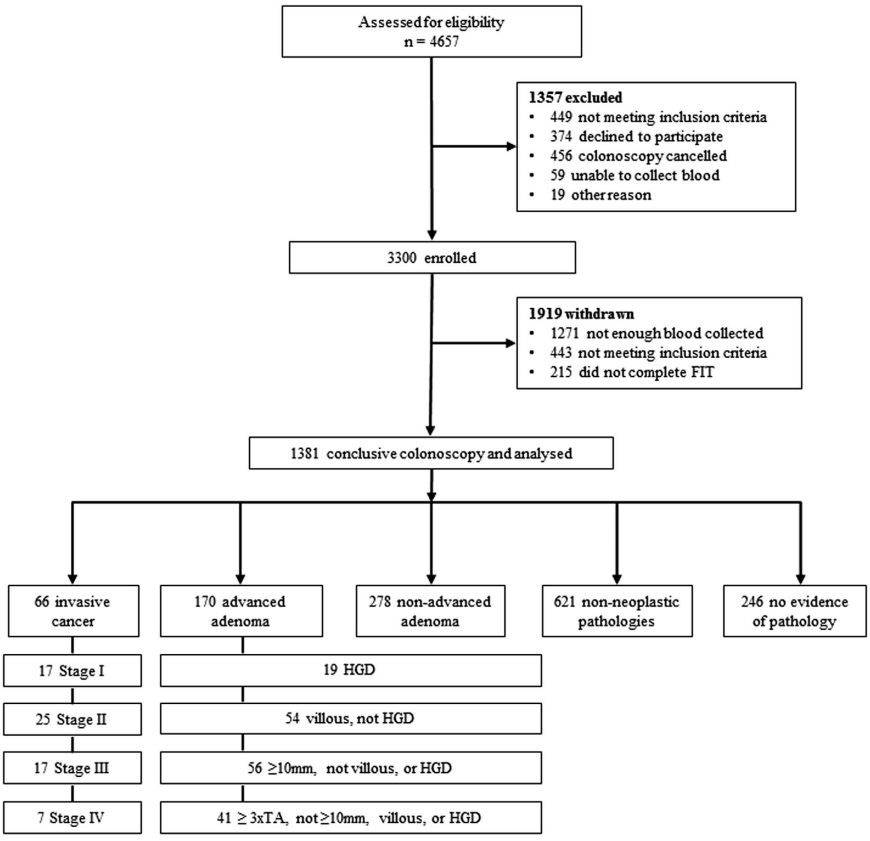

Figure 1 Disposition of study volunteers and clinical findings. HGD, high-grade dysplasia; TA, tubular adenoma.

when the population was limited to just screening age participants (50.0–74.9 years; Supplementary Table 2). Sensitivity for CRC was 63.6% for both the blood test and FIT at the cutoff level of 80 μg Hb/g feces.

Estimates of sensitivity for adenoma. The positivity rate for advanced adenoma was significantly higher with FIT at the Hb cutoff level of 10, but not 80 μg Hb/g feces, compared with the *BCAT1/IKZF1* blood test (10 μg Hb/g feces = 43.5%; blood test = 9.4%; **Table 2**). Similarly, the sensitivity for non-advanced adenoma was significantly higher with FIT at the Hb cutoff level of 10 μg Hb/g feces compared with the blood test (10 μg Hb/g feces = 23.0%; blood test = 9.0%; **Table 2**).

Estimates of specificity. When specificity for CRC was calculated, significant differences between the tests emerged: at a cutoff of 10 μg Hb/g feces, FIT was significantly less specific (80.5% vs. 91.8%, $P < 0.01$) relative to the *BCAT1/IKZF1* blood test (**Figure 2b**).

Given the observed false-positive rate of 8.2% for the blood DNA test, receiver operating characteristic curve analysis showed that the same false-positive rate was seen with FIT at a cutoff of 60 μg Hb/g feces (data not shown). At that cutoff, the sensitivity of FIT for cancer was 63.6% (42/66, $P = 1.00$), no different from the sensitivity of the *BCAT1/IKZF1* blood test (**Table 2** and **Figure 2a**).

Bleeding status during fecal sampling was recorded by 1,124 participants (81.4%). Visible bleeding was noted by 21 participants who did not have cancer. Of these 21 participants, 11 (52.3%) and 10 (47.6%) were positive with FIT at cutoff levels of 10 and 80 μg Hb/g feces, respectively, whereas only two (9.5%) were positive for methylated *BCAT1*/*IKZF1* DNA.

Age and gender effect on positivity. In the subgroup with no neoplastic pathologies, the positivity rate of both the *BCAT1/IKZF1* and FIT tests showed a complex relationship with age (Supplementary Table 3, Supplementary Figure 1). In the youngest age group, the blood test showed significantly fewer false positives than FIT, whereas in the oldest age group there was no difference (Supplementary Figure 1). Positivity of the blood test and FIT in the presence of neoplasia were not affected by gender (Supplementary Figure 2, Supplementary Table 3).

Distal vs. proximal disease. Positivity rates did not differ significantly for either test when comparing cases with proximal ($n = 29$) or distal ($n = 37$) cancer (Supplementary Table 4).

Test concordance. Concordance between the two tests is shown for a subset of selected clinical phenotypes in **Table 3**. Of the 66 CRC cases (using the most sensitive criterion value for FIT of 10 μg Hb/g feces), 34 (51.5%) were positive by both tests, whereas 25 (37.9%) cases were positive by one test but

Table 2 Test positivity rates by diagnostic class

Principal diagnosis		Positive result			
		BCAT1/IKZF1 blood test	Fecal immunochemical test		
Study cohort size, $n=1381$	No.	Counts (%; 95% CI)	Counts (%; 95% CI), McNemar's test P value[a]		
			Cutoff 10 µg Hb/g	Cutoff 60 µg Hb/g	Cutoff 80 µg Hb/g
Neoplasia					
Cancer	66	41 (62.1; 49.3–73.8)	52 (78.8; 67.0–87.9), 0.046	42 (63.6; 50.9–75.1), 1.000	39 (59.1; 46.3–71.0), 0.850
Stage I	17	7 (41.2; 18.4–67.1)	13 (76.5; 50.1–93.2), 0.077	10 (58.8; 32.9–81.6), 0.505	10 (58.8; 32.9–81.6), 0.505
Stage II	25	19 (76.0; 54.9–90.6)	20 (80.0; 59.3–93.2), 1.000	17 (68.0; 46.5–85.1), 0.724	15 (60.0; 38.7–78.9), 0.343
Stage III	17	10 (58.8; 32.9–81.6)	13 (76.0; 50.1–93.2), 0.371	10 (58.8; 32.9–81.6), 0.617	9 (52.9; 27.8–77.0), 1.000
Stage IV	7	5 (71.4; 29.0–96.3)	6 (85.7; 42.1–99.6), 1.000	5 (71.4; 29.0–96.3), 0.617	5 (71.4; 29.0–96.3), 0.617
Early Stage (I+II)	42	26 (61.9; 45.6–76.4)	33 (78.6; 63.2–89.7), 0.146	27 (64.3; 48.0–78.4), 1.000	25 (59.5; 43.3–74.4), 1.000
Late Stage (III+IV)	24	15 (62.5; 40.6–81.2)	19 (79.2; 57.8–92.9), 0.289	15 (62.5; 40.6–81.2), 0.724	14 (58.3; 36.6–77.9), 1.000
Advanced adenoma	170	16 (9.4; 5.5–14.8)	74 (43.5; 36.0–51.3), <0.001	32 (18.8; 13.2–25.5), 0.024	29 (17.1; 11.7–23.6), 0.061
HGD	19	1 (5.3; 0.1–26.0)	11 (57.9; 33.5–79.7), 0.004	4 (21.1; 6.1–45.6), 0371	4 (21.1; 6.1–45.6), 0.371
TVA[b]	54	7 (13.0; 5.4–24.9)	24 (44.4; 30.9–58.6), 0.002	15 (27.8; 16.5–41.6), 0.118	14 (25.9; 15.0–39.7), 0.169
≥ 10 mm[c]	56	3 (5.4; 1.1–14.9)	25 (44.6; 31.3–58.5), <0.001	11 (19.6; 10.2–32.4), 0.043	10 (17.9; 8.9–30.4), 0.070
≥ 3 TAs (<10 mm)[c]	41	5 (12.2; 4.1–26.2)	14 (34.1; 20.1–50.6), 0.027	2 (4.9; 0.6–16.5), 0.450	1 (2.4; 0.1–12.9), 0.221
Non-advanced adenoma	278	25 (9.0; 5.9–13.0)	64 (23.0; 18.2–28.4), <0.001	26 (9.4; 6.2–13.4), 1.000	20 (7.2; 4.4–10.9), 0.522
No neoplasia[d]	867	67 (7.7; 6.0–9.7)	119 (13.7; 11.5–16.2), <0.001	49 (5.7; 4.2–7.4), 0.108	38 (4.4; 3.1–6.0), 0.005
Non-neoplastic pathologies	621	48 (7.7; 5.8–10.1)	94 (15.1; 12.4–18.2), <0.001	37 (6.0; 4.2–8.1), 0.267	29 (4.7; 3.1–6.6), 0.035
Inflammatory bowel disease	47	1 (2.1; 0.1–11.3)	16 (34.0; 20.9–49.3), <0.001	13 (27.7; 15.6–42.6), 0.002	13 (27.7; 15.6–42.6), 0.002
Angiodysplasia	7	1 (14.3; 0.4–57.9)	1 (14.3; 0.4–57.9), 0.480	1 (14.3; 0.4–57.9), 0.480	1 (14.3; 0.4–57.9), 0.480
Hemorrhoids	198	19 (9.6; 5.9–14.6)	26 (13.1; 8.8–18.6), 0.324	7 (3.5; 1.4–7.1), 0.025	3 (1.5; 0.3–4.4), <0.001
Diverticular disease	164	16 (9.8; 5.7–15.4)	19 (11.6; 7.1–17.5), 0.719	8 (4.9; 2.1–9.4), 0.153	6 (3.7; 1.4–7.8), 0.055
Polyps[e] and other lesions	205	11 (5.4; 2.7–9.4)	32 (15.6; 10.9–21.3), 0.001	8 (3.9; 1.7–7.5), 0.646	6 (2.9; 1.1–6.3), 0.332
No evidence of disease	246	19 (7.7; 4.7–11.8)	25 (10.2; 6.7–14.6), 0.440	12 (4.9; 2.5–8.4), 0.281	9 (3.7; 1.7–6.8), 0.089

CI, confidence interval; HGD, high-grade dysplasia; TA, tubular adenoma; TVA, tubulovillous adenoma.
[a]*BCAT1/IKZF1* blood test vs. FIT at designated cutoffs.
[b]No HGD.
[c]No HGD or TVA.
[d]All cases except for cancer and adenoma.
[e]Hyperplastic, unspecified, inflammatory, other polyps.

not the other. The *BCAT1/IKZF1* blood test detected seven CRC cases that were FIT negative (five of which were stage 1 or 2), whereas FIT detected 18 CRC cases that were negative for methylated *BCAT1/IKZF1* DNA in blood ($P=0.05$). The seven cancers positive only by the blood DNA test were somewhat more likely to show lymphovascular invasion ($P=0.08$).

In subjects with no neoplasia ($n=867$), 10 were positive by both tests (1.2%) and 166 (19.1%) positive by one test but not the other, with most of the discordant positive cases being FIT positive (109/166, 65.7%; $P<0.01$, **Table 3**).

Test complementarity. As each test detected a slightly different cancer population, we explored the value of combining the two tests by considering a positive result as one with either test sample being positive (**Table 4**). At a cutoff of 10 µg Hb/g feces for FIT, combining the tests improved sensitivity estimates to 89.4% (95% CI: 79.4–95.6%) for cancer with 74.2% specificity (95% CI: 71.8–76.6%). At a cutoff level of 80 µg Hb/g feces, the combined test results returned estimates of 81.8% sensitivity (95% CI: 70.4–90.2%) and 85.7% specificity (95% CI: 83.7–87.6%).

Quantitative levels of fecal Hb and methylated DNA in circulation. Results of the methylated *BCAT1/IKZF1* test can also be quantitatively reported, for instance, as the fraction of methylated *BCAT1* and *IKZF1* DNA measured in total yield of DNA isolated per blood specimen. Both the mass of fecal Hb and methylated DNA in blood increased as a function of disease severity ($P<0.01$, **Figure 3**).

DISCUSSION

This prospective study, comparing FIT with a blood test detecting methylated *BCAT1* and *IKZF1* DNA was undertaken because our prior retrospective studies showed that these two biomarkers, selected from a larger panel of hypermethylated genes associated with CRC and colorectal adenomas, appeared to be the most discriminatory between colorectal neoplasia and the non-neoplastic state when applied together.[28–30]

Based on the observed true-positivity rate of the *BCAT1/IKZF1* blood test, sensitivity for CRC was 62.1% and for advanced adenoma was 9.4%. Sensitivity for early-stage CRC was 61.9% and for later stage CRC was 62.5%. The sensitivity for CRC of the *BCAT1/IKZF1* blood test is within the upper half of the reported sensitivity range of 37–79% for gFOBT in populations such as studied here or in true screening populations.[42]

We chose to compare the *BCAT1/IKZF1* blood test with FIT because the latter technology has largely replaced gFOBT.[5–14] Describing performance relative to FIT is complex, however, because the best FIT are quantitative and the criterion value chosen to define positivity (i.e., the fecal Hb concentration) varies between the many screening programs around the world.[39,40] In choosing the cutoff value for FIT that returned the same specificity as the *BCAT1/IKZF1* DNA test, the sensitivity of FIT for CRC was 63.6%. In other words, by estimating sensitivity with one operating characteristic (the false-positive rate) set to an equivalent colonoscopy workload required to detect each CRC, the two tests were comparable in sensitivity for CRC. FIT however remained a superior test with regard to detection of advanced adenoma.

A major advantage of FIT over gFOBT is the ability of the former to detect a higher proportion of advanced adenomas. Sensitivity of certain FITs for advanced adenomas falls in the range of 29–45% when applied as a one-time test.[27,43] Although detection of advanced adenomas will lead to reduction in CRC incidence, this detection requires a higher colonoscopy rate and modeling shows that non-invasive tests that detect cancer well, but adenomas poorly, still reduce mortality from CRC in participants by 71%.[44] Given the low sensitivity for advanced adenomas, the *BCAT1/IKZF1* blood test in its current configuration should not be expected to impact significantly on CRC incidence, but will still reduce mortality. Therefore, eventual application of the *BCAT1/IKZF1* blood test to screening will depend in part on the desired operating characteristics for test accuracy of the screening program, and the capacity of the *BCAT1/IKZF1* blood test to overcome participatory barriers.

If the FIT cutoff value was set at 80 μg Hb/g feces, sensitivity for CRC was 59.1%, almost the same as with the *BCAT1/IKZF1* blood test, but sensitivity for advanced adenoma was slightly higher (not significant) at 17.1%. If the cutoff value was set at 10 μg Hb/g feces, sensitivity for CRC increased to 78.8% and for advanced adenoma to 43.5%. When comparing estimated sensitivities for early-stage CRC, there was no significant difference between FIT at any cutoff value compared with the *BCAT1/IKZF1* blood test. The previously reported relationship between assay positivity and depth of tumor invasion[29,30] suggests that there might be a biological limitation to the capacity of blood-based gene tests to detect adenomas, which has also been observed by others.[45] This is despite the observations that both *SEPT9* as well as *BCAT1* and *IKZF1* are all methylated at high frequency in tissue specimens at the earliest onset of colorectal neoplasia.[28,46]

The false-positive rate for FIT observed in our study was slightly higher than reported in screening studies using the same test and cutoff.[47–49] This observation is not surprising because the current study comprised a population undergoing

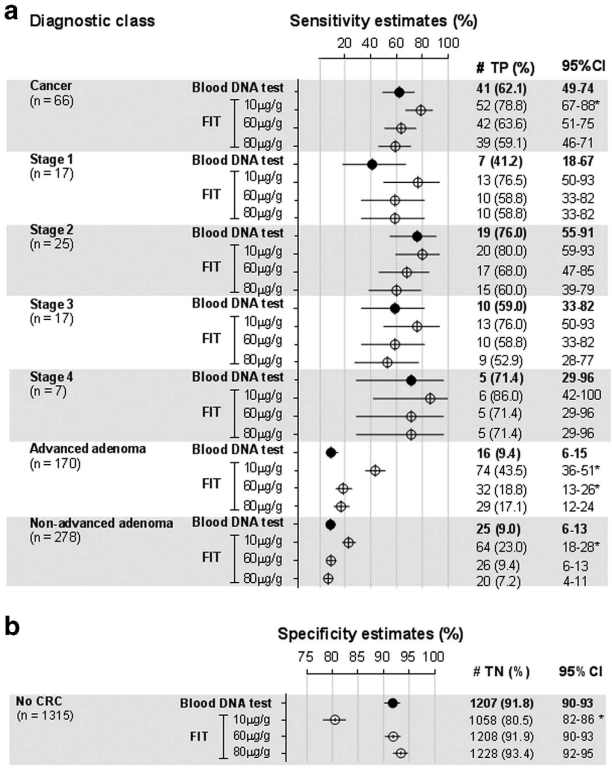

Figure 2 Sensitivity and specificity estimates. The sensitivity (**a**) and specificity (**b**) estimates were calculated using true-positive (TP) and true-negative (TN) rates, respectively, for selected diagnostic classes. Closed and open circles represent estimates for the *BCAT1/IKZF1* blood test and FIT, respectively, at various cutoff values. Bars represent 95% confidence intervals (95% CI). Asterisks: McNemar's Test *P* value < 0.05 (relative to *BCAT1/IKZF1* blood test results in a specific subgroup).

Table 3 Concordance between tests

Primary finding	*BCAT1/*IKZF1 blood test	Fecal immunochemical test								
		Cutoff 10 μg Hb/g			Cutoff 60 μg Hb/g			Cutoff 80 μg Hb/g		
		# Pos	# Neg	*P* value[a]	# Pos	# Neg	*P* value[a]	# Pos	# Neg	*P* value[a]
Cancer (*n* = 66)	# Pos	34	7	0.046	29	12	1.000	26	15	0.850
	# Neg	18	7		13	12		13	12	
Advanced adenoma (*n* = 170)	# Pos	8	8	< 0.001	2	14	0.024	2	14	0.061
	# Neg	88	66		30	124		27	127	
No Neoplasia (*n* = 867)	# Pos	10	57	< 0.001	2	65	0.108	2	65	0.005
	# Neg	109	691		47	753		36	764	
Non-neoplastic pathologies (621)	# Pos	9	39	< 0.001	2	46	0.267	2	46	0.035
	# Neg	85	488		35	538		27	546	
No evidence of diseases (246)	# Pos	1	18	0.440	0	19	0.281	0	19	0.089
	# Neg	24	203		12	215		9	218	

[a]McNemar's test.

Table 4 True positivity rates in selected diagnostic classes for a combination testing strategy where either FIT and/or blood DNA test is positive

Principal diagnosis	No.	Positive result with either test Number of positive cases (%; 95% CI)		
		FIT at 10 μg/g	FIT at 60 μg/g	FIT at 80 μg/g
Neoplasia				
Cancer	66	59 (89.4; 79.4–95.6)	54 (81.8; 70.4–90.2)	54 (81.8; 70.4–90.2)
Stage I	17	14 (82.4; 56.6–96.2)	13 (76.5; 50.1–93.2)	13 (76.5; 50.1–93.2)
Stage II	25	24 (96.0; 79.6–99.9)	22 (88.0; 68.8–97.5)	22 (88.0; 68.8–97.5)
Stage III	17	14 (82.4; 56.6–96.2)	12 (70.6; 44.0–89.7)	12 (70.6; 44.0–89.7)
Stage IV	7	7 (100.0; 59.0–100.0)	7 (100.0; 59.0–100.0)	7 (100.0; 59.0–100.0)
Advanced adenoma	170	82 (48.2; 40.5–56.0)	46 (27.1; 20.5–34.4)	43 (25.3; 19.0–32.5)
Non-advanced adenoma	278	80 (28.8; 23.5–34.5)	48 (17.3; 13.0–22.2)	42 (15.1; 11.1–19.9)
No neoplasia[a]	867	176 (20.3; 17.7–23.1)	114 (13.1; 11.0–15.6)	103 (11.9; 9.8–14.2)
Non-neoplastic pathologies[b]	621	133 (21.4; 18.3–24.9)	83 (13.4; 10.8–16.3)	75 (12.1; 9.6–14.9)
No evidence of disease	246	43 (17.5; 12.9–22.8)	31 (12.6; 8.7–17.4)	28 (11.4; 7.7–16.0)

CI, confidence interval; FIT, immunochemical test.
[a]All cases except for cancer and adenoma.
[b]Including polyps (hyperplastic, unspecified, other polyps), angiodysplasia, hemorrhoids, diverticular disease, inflammatory disease, and other lesions.

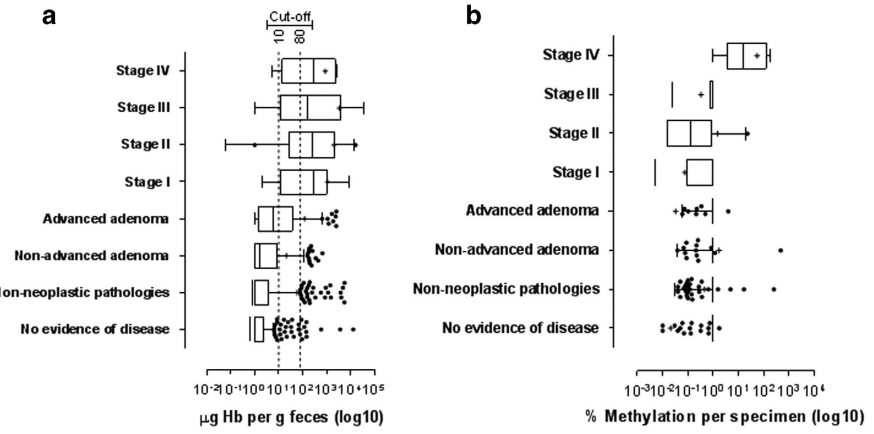

Figure 3 Mass of hemoglobin in feces and methylated *BCAT1* and *IKZF1* in plasma. Box-whisker diagrams showing (**a**) the mass of fecal hemoglobin (μg Hb/g feces) and (**b**) methylated *BCAT1* and *IKZF1* DNA in circulation (%methylation) by clinical findings. Whiskers, 5–95% percentile; vertical lines, median; plus sign, mean; and outliers are indicated as individual points. Average and median mass levels are indicated for the following clinical findings: Cancer (17 Stage I, 25 Stage II, 17 Stage III, 7 Stage IV), advanced adenoma ($n = 170$), non-advanced adenoma ($n = 278$), non-neoplastic pathologies ($n = 621$), and cases with no evidence of disease ($n = 246$). Cutoff level ranges are indicated in **a**.

colonoscopy for all indications, whether for the presence of symptoms (including overt rectal bleeding), high-risk surveillance, or screening. Although this population is appropriate for establishing a new screening test[50] and determining the conditions associated with false-positive results, a limitation is that the study cohort reported here is not a typical screening population, and therefore positivity rates in a true screening population cannot be accurately estimated from this study.

The false-positive rate for the *BCAT1/IKZF1* blood test provides insight into specificity and the factors that might influence it, and hence the cost of screening, especially as a major component of cost is related to the colonoscopy workload. Estimated specificity for the *BCAT1/IKZF1* test for cancer and advanced adenomas was 91.8%. This was similar to the specificity of 91% reported for *SEPT9* in a true screening population,[26] and significantly better than that of FIT at a cutoff level of 10 μg Hb/g feces in the colonoscopy population used in the current study. When the population was limited to screening-aged people without symptoms or overt rectal bleeding, specificity of the blood test remained significantly better (87.5% vs. 93.4%, data not shown). In examining clinical subsets (**Table 2**), it is apparent that specificity of FIT was seriously compromised in people with non-neoplastic pathologies in the colon or rectum and especially those with inflammatory bowel disease, whereas specificity of the *BCAT1/IKZF1* test was not. The same applies in subjects who saw blood in their feces. FIT is not a suitable screening test for subjects who have benign bleeding disorders because of the higher false-positive rate and our data confirm the higher positivity rate in such subjects. Consequently, the *BCAT1/IKZF1* test provides an alternative non-invasive screening test for these subjects.

We have previously concluded that *BCAT1/IKZF1* blood test false positives (as determined by colonoscopy) in subjects with no evidence of disease reflect a true appearance of methylated *BCAT1* and *IKZF1* DNA.[29] There was a trend to an increase in the false-positive rate with ageing but this never exceeded the false positivity rate of the FIT. Longitudinal

follow-up studies are required to understand whether this low blood test false-positive rate in healthy cases reflects chance events of no consequence, or an early indication of colorectal neoplasia and/or other extra-colonic cancers. The presence of these gene markers in blood is not likely to be limited to only CRC,[35,51] which has also been noted with *SEPT9*.[52] One could speculate that the mass of these methylated gene markers in the blood might need to be taken into consideration when dealing with patients who return a positive *BCAT1/IKZF1* test but are negative for neoplasia at colonoscopy, as the likelihood of cancer increases with increased mass in the plasma (as demonstrated in **Figure 3**).

Early detection of CRC by screening using an FOBT is effective based on detection of the bleeding phenotype. However, as not all cancers may bleed, there is interest in including markers that detect a different cancer biology. The most recent demonstration of this phenomenon is the multi-target fecal test that combines FIT with several DNA markers in feces to demonstrate that sensitivity for CRC can be improved.[27] For this reason, we examined our results utilizing a panel comprising the FIT and the *BCAT1*/*IKZF1* blood test. The majority of test-positive CRC cases showed concordance; of the 59 CRC cases positive by either test, 34 were positive for both tests. *BCAT1*/*IKZF1* in blood detected seven CRC cases not detected by FIT, with a trend for these cancers to have a more frequent presence of lymphovascular invasion. Combining the fecal and blood tests into one test panel improved the detection rate for cancer to 89.4%, which was better than either test alone (FIT, 78.8%; *BCAT1/IKZF1*, 62.1%), but unsurprisingly associated with a reduction in specificity. Although participation in CRC screening is modest, surveys have shown that people would prefer to complete a combination screening test involving a FOBT and a blood test if it had better accuracy than the standard tests.[53]

The impact of a screening test on population mortality from CRC is dependent not only on test accuracy but also on participation rates. Many screening programs are shifting from gFOBT to FIT to augment the efficiency of detecting pre-cancerous lesions. However, the uptake rates remain suboptimal.[21] Based on the accuracy we observed for the *BCAT1/IKZF1* blood test, it is justified to proceed to prospective evaluation of accuracy and participation in a true screening population that includes comparison of both test accuracy and population participation rates relative to another simple, proven screening test such as FIT. A likely advantage of a blood test will be its greater acceptability in those contemplating screening given the findings of Adler *et al*. and Osborne *et al*.[24,25] Sensitivity of the *BCAT1/IKZF1* blood test for adenomas might be considered a disadvantage but not if it is counterbalanced by a higher participation rate.

CONFLICT OF INTEREST

Guarantor of the article: Erin L. Symonds, PhD.

Specific author contributions: Erin L. Symonds managed recruitment and collection of clinical data, contributed to data analysis and manuscript preparation. Susanne K. Pedersen coordinated specimen testing, contributed to data analysis, and manuscript preparation. Rohan T. Baker, David H. Murray, and Snigdha Gaur contributed to assay testing and data collation. Stephen R. Cole contributed to conception of the study, sample choice, and provision. Geetha Gopalsamy and Dileep Mangira audited clinical data and verified case classifications. Lawrence C. LaPointe contributed to overall project design and provided input into data interpretation. Graeme P. Young contributed to overall project design, clinical interpretation, sample choice and provision, provided input into data interpretation, and manuscript preparation. All authors read and approved the final manuscript.

Financial support: This study was funded in part by the National Health and Medical Research Council (APP1006242 and APP1017083) and Clinical Genomics. Scientists from Clinical Genomics conducted the blood assays (blinded to all clinical outcomes), assisted with the data analysis and writing of the manuscript. Fecal immunochemical tests were provided by Eiken Chemical Company, Tokyo, Japan, but they had no influence on study design, analysis, or the decision to submit the manuscript for publication.

Potential competing interests: G. Young is a paid consultant of Clinical Genomics. S. Pedersen, L. LaPointe, R. Baker, S. Gaur, and D. Murray are employed by Clinical Genomics.

Acknowledgments. We thank Dawn Bastin for analyzing all the FIT samples; Jane Upton, Libby Bambacas, Susan Byrne, and Kathy Cornthwaite for patient recruitment; and Jo Osborne for database maintenance. We are grateful to John Baron who reviewed the manuscript, and advised regarding the analysis of results and their presentation.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Screening for colorectal cancer (CRC) with fecal occult blood tests (FOBT) or colonoscopy reduces mortality from the disease.
- ✓ Participation rates within screening programs are below target rates.
- ✓ Epigenetic changes including hypermethylation are observed in DNA from CRC.
- ✓ Of the potentially useful tumor-derived biomarkers that appear in blood, a blood test that detects methylated regions of *BCAT1* and/or *IKZF1* has been shown to have a sensitivity for CRC of 77% in a retrospective study.

WHAT IS NEW HERE

- ✓ In a prospective study sensitivity for CRC of the *BCAT1/IKZF1* blood test was 62%. This was not significantly different from the sensitivity of a fecal immunochemical test (FIT), which ranged from 59 to 79% at positivity cutoff values commonly used in screening programs.
- ✓ Specificity of the *BCAT1/IKZF1* blood test was 92%, whereas FIT specificity varied between 81 and 93%.
- ✓ Specificity of FIT but not the blood DNA test was affected by non-neoplastic pathologies that cause colonic bleeding.
- ✓ A CRC test that uses FIT combined with the *BCAT1/IKZF1* blood test has better sensitivity then either used alone.

1. Elmunzer BJ, Hayward RA, Schoenfeld PS *et al.* Effect of flexible sigmoidoscopy-based screening on incidence and mortality of colorectal cancer: a systematic review and meta-analysis of randomized controlled trials. *PLoS Med* 2012; **9**: e1001352.

2. Hardcastle JD, Thomas WM, Chamberlain J *et al.* Randomised, controlled trial of faecal occult blood screening for colorectal cancer. Results for first 107,349 subjects. *Lancet* 1989; **1**: 1160–1164.
3. Kronborg O, Fenger C, Olsen J *et al.* Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet* 1996; **348**: 1467–1471.
4. Mandel JS, Church TR, Bond JH *et al.* The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603–1607.
5. Allison JE, Sakoda LC, Levin TR *et al.* Screening for colorectal neoplasms with new fecal occult blood tests: update on performance characteristics. *J Natl Cancer Inst* 2007; **99**: 1462–1470.
6. Garborg K, Holme O, Loberg M *et al.* Current status of screening for colorectal cancer. *Ann Oncol* 2013; **24**: 1963–1972.
7. Halloran SP, Launoy G, Zappa M *et al.* European guidelines for quality assurance in colorectal cancer screening and diagnosis First Edition–Faecal occult blood testing. *Endoscopy* 2012; **44** (Suppl 3): SE65–SE87.
8. Lansdorp-Vogelaar I, van Ballegooijen M, Boer R *et al.* A novel hypothesis on the sensitivity of the fecal occult blood test: results of a joint analysis of 3 randomized controlled trials. *Cancer* 2009; **115**: 2410–2419.
9. Levi Z, Birkenfeld S, Vilkin A *et al.* A higher detection rate for colorectal cancer and advanced adenomatous polyp for screening with immunochemical fecal occult blood test than guaiac fecal occult blood test, despite lower compliance rate. A prospective, controlled, feasibility study. *Int J Cancer* 2011; **128**: 2415–2424.
10. Park DI, Ryu S, Kim YH *et al.* Comparison of guaiac-based and quantitative immunochemical fecal occult blood testing in a population at average risk undergoing colorectal cancer screening. *Am J Gastroenterol* 2010; **105**: 2017–2025.
11. Smith A, Young GP, Cole SR *et al.* Comparison of a brush-sampling fecal immunochemical test for hemoglobin with a sensitive guaiac-based fecal occult blood test in detection of colorectal neoplasia. *Cancer* 2006; **107**: 2152–2159.
12. Steele RJ, McDonald PJ, Digby J *et al.* Clinical outcomes using a faecal immunochemical test for haemoglobin as a first-line test in a national programme constrained by colonoscopy capacity. *United European Gastroenterol J* 2013; **1**: 198–205.
13. Young GP, Symonds EL, Allison JE *et al.* Advances in fecal occult blood tests: the FIT revolution. *Dig Dis Sci* 2015;**60**:609–622.
14. Rabeneck L, Rumble RB, Thompson F *et al.* Fecal immunochemical tests compared with guaiac fecal occult blood tests for population-based colorectal cancer screening. *Can J Gastroenterol* 2012; **26**: 131–147.
15. Yamada T, Alpers D, Owyang C. *Textbook of Gastroenterology*, 5th edn, Lippincott Williams and Wilkins: Philadelphia, PA, USA, 2009.
16. Young GP. Screening for colorectal cancer- new evidence in the last 10 years. *Cancer Forum* 2014; **38**: 11–14.
17. Cole SR, Gregory T, Whibley A *et al.* Predictors of re-participation in faecal occult blood test-based screening for colorectal cancer. *Asian Pac J Cancer Prev* 2012; **13**: 5989–5994.
18. Gregory T, Cole SR, Wilson CJ *et al.* Exploring the validity of the continuum of resistance model for discriminating early from late and non-uptake of colorectal cancer screening: implications for the design of invitation and reminder letters. *Int J Behav Med* 2013; **20**: 572–581.
19. Potter MB. Strategies and resources to address colorectal cancer screening rates and disparities in the United States and globally. *Annu Rev Public Health* 2013; **34**: 413–429.
20. Quintero E, Castells A, Bujanda L *et al.* Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. *N Engl J Med* 2012; **366**: 697–706.
21. AIHW. *National Bowel Cancer Screening Program monitoring report: 2012–13.* Australian Institute of Health and Welfare Canberra: Canberra, Australia, 2014.
22. Cole SR, Young GP, Esterman A *et al.* A randomised trial of the impact of new faecal haemoglobin test technologies on population participation in screening for colorectal cancer. *J Med Screen* 2003; **10**: 117–122.
23. Cole SR, Zajac I, Gregory T *et al.* Psychosocial variables associated with colorectal cancer screening in South Australia. *Int J Behav Med* 2011; **18**: 302–309.
24. Adler A, Geiger S, Keil A *et al.* Improving compliance to colorectal cancer screening using blood and stool based tests in patients refusing screening colonoscopy in Germany. *BMC Gastroenterol* 2014; **14**: 183.
25. Osborne J, Wilson C, Moore V *et al.* Sample preference for colorectal cancer screening tests: blood or stool? *OJPM* 2012; **2**: 326–331.
26. Church TR, Wandell M, Lofton-Day C *et al.* Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* 2014; **63**: 317–325.
27. Imperiale TF, Ransohoff DF, Itzkowitz SH *et al.* Multitarget stool DNA testing for colorectal-cancer screening. *N Engl J Med* 2014; **370**: 1287–1297.
28. Mitchell SM, Ross JP, Drew HR *et al.* A panel of genes methylated with high frequency in colorectal cancer. *BMC Cancer* 2014; **14**: 54.
29. Pedersen S, Symonds E, Baker R *et al.* Evaluation of an assay for methylated BCAT1 and IKZF1 in plasma for detection of colorectal neoplasia. *BMC Cancer* 2015; **15**: 654.
30. Pedersen SK, Baker RT, McEvoy A *et al.* A two-gene blood test for methylated DNA sensitive for colorectal cancer. *PLoS One* 2015; **10**: e0125041.
31. Javierre BM, Rodriguez-Ubreva J, Al-Shahrour F *et al.* Long-range epigenetic silencing associates with deregulation of Ikaros targets in colorectal cancer cells. *Mol Cancer Res* 2011; **9**: 1139–1151.
32. Kibriya MG, Raza M, Jasmine F *et al.* A genome-wide DNA methylation study in colorectal carcinoma. *BMC Med Genomics* 2011; **4**: 50.
33. Schjerven H, McLaughlin J, Arenzana TL *et al.* Selective regulation of lymphopoiesis and leukemogenesis by individual zinc fingers of Ikaros. *Nat Immunol* 2013; **14**: 1073–1083.
34. Tonjes M, Barbus S, Park YJ *et al.* BCAT1 promotes cell proliferation through amino acid catabolism in gliomas carrying wild-type IDH1. *Nat Med* 2013; **19**: 901–908.
35. Vincent A, Omura N, Hong SM *et al.* Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma. *Clin Cancer Res* 2011; **17**: 4341–4354.
36. Yoshikawa R, Yanagi H, Shen CS *et al.* ECA39 is a novel distant metastasis-related biomarker in colorectal cancer. *World J Gastroenterol* 2006; **12**: 5884–5889.
37. Zhang Z, Xu Z, Wang X *et al.* Ectopic Ikaros expression positively correlates with lung cancer progression. *Anat Rec (Hoboken)* 2013; **296**: 907–913.
38. Johnson DA, Barclay RL, Mergener K *et al.* Plasma septin9 versus fecal immunochemical testing for colorectal cancer screening: a prospective multicenter study. *PLoS One* 2014; **9**: e98238.
39. Benton SC, Seaman HE, Halloran SP. Faecal occult blood testing for colorectal cancer screening: the past or the future. *Curr Gastroenterol Rep* 2015; **17**: 428.
40. Fraser CG, Allison JE, Young GP *et al.* A standard for Faecal Immunochemical TesTs for haemoglobin evaluation reporting (FITTER). *Ann Clin Biochem* 2014; **51**: 301–302.
41. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 2010; **17**: 1471–1474.
42. Whitlock EP, Lin JS, Liles E *et al.* Screening for colorectal cancer: a targeted, updated systematic review for the U.S. Preventive Services Task Force. *Ann Intern Med* 2008; **149**: 638–658.
43. Lane JM, Chow E, Young GP *et al.* Interval fecal immunochemical testing in a colonoscopic surveillance program speeds detection of colorectal neoplasia. *Gastroenterology* 2010; **139**: 1918–1926.
44. Haug U, Knudsen AB, Lansdorp-Vogelaar I *et al.* Development of new non-invasive tests for colorectal cancer screening: the relevance of information on adenoma detection. *Int J Cancer* 2015; **136**: 2864–2874.
45. Betge J, Pollheimer MJ, Lindtner RA *et al.* Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. *Cancer* 2012; **118**: 628–638.
46. Payne SR. From discovery to the clinic: the novel DNA methylation biomarker (m)SEPT9 for the detection of colorectal cancer in blood. *Epigenomics* 2010; **2**: 575–585.
47. Chiu HM, Lee YC, Tu CH *et al.* Association between early stage colon neoplasms and false-negative results from the fecal immunochemical test. *Clin Gastroenterol Hepatol* 2013; **11**: 832–838.
48. Denters MJ, Deutekom M, Bossuyt PM *et al.* Lower risk of advanced neoplasia among patients with a previous negative result from a fecal test for colorectal cancer. *Gastroenterology* 2012; **142**: 497–504.
49. van Roon AH, Goede SL, van Ballegooijen M *et al.* Random comparison of repeated faecal immunochemical testing at different intervals for population-based colorectal cancer screening. *Gut* 2013; **62**: 409–415.
50. Symonds EL, Young GP. Blood tests for colorectal cancer screening in the standard risk population. *Curr Colorec Cancer Rep* 2015,10.1007/s11888-015-0293-2.
51. Zhou W, Feng X, Ren C *et al.* Over-expression of BCAT1, a c-Myc target gene, induces cell proliferation, migration and invasion in nasopharyngeal carcinoma. *Mol Cancer* 2013; **12**: 53.
52. Connolly D, Hoang HG, Adler E *et al.* Septin 9 amplification and isoform-specific expression in peritumoral and tumor breast tissue. *Biol Chem* 2014; **395**: 157–167.
53. Benning TM, Dellaert BG, Dirksen CD *et al.* Preferences for potential innovations in non-invasive colorectal cancer screening: a labeled discrete choice experiment for a Dutch screening campaign. *Acta Oncol* 2014; **53**: 898–908.

Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)