PROFESSOR RAMESH ARASARADNAM (Orcid ID : 0000-0002-2231-3062)

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Original Article

Risk stratification of Symptomatic Patients Suspected of Colorectal Cancer using Faecal and Urinary Markers

Monika M. Widlak[1,4], Matthew Neal[9], Emma Daulton[8], Claire L. Thomas[2], Claudia Tomkins[2], Baljit Singh[3], Christopher Harmston[6], Alfian Wicaksono[8], Charles Evans[6], Steve Smith[2,7], Richard S. Savage[9], James A. Covington[8], Ramesh P. Arasaradnam[1,4,5]

[1] Department of Gastroenterology, University Hospitals Coventry and Warwickshire NHS Trust; [2] Department of Biochemistry, University Hospitals Coventry and Warwickshire NHS Trust; [3] Department of Colorectal Surgery, Leicester General Hospital; [4] Medical School, University of Warwick; [5] Applied Biological and Experimental Sciences, University of Coventry; [6] Department of Colorectal Surgery, University Hospitals Coventry and Warwickshire NHS Trust; [7] Midlands and North West Bowel Cancer Screening Hub, University Hospitals Coventry and Warwickshire; [8] School of Engineering, University of Warwick; [9] Department of Statistics, University of Warwick

Corresponding author**:** Professor Ramesh P Arasaradnam; r.arasaradnam@warwick.ac.uk**.** Clifford Bridge Road, Coventry CV2 2DX. Tel: +44 2476 966087 Fax: +44 2476 966090

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What does this paper add to the literature?

- This novel study has applied both faecal and urinary markers to improve the diagnostic accuracy for CRC from 80% to 97% with a NPV of 100% in those with lower gastrointestinal symptoms.
- Application of low cost (<£50) diagnostic tests could result in changes to the current clinical pathway.

SUMMARY:

BACKGROUND: Faecal markers such as faecal immunochemical test for haemoglobin (FIT) and faecal calprotectin (FCP) have been increasingly used to exclude colorectal cancer (CRC) and colonic inflammation. However, in those with lower gastrointestinal symptoms, there are considerable numbers with cancer but have a negative FIT test (false negative), which, has impeded its use in clinical practice.

AIMS: We undertook a diagnostic accuracy study of CRC using faecal immunochemical test for haemoglobin (FIT), faecal calprotectin (FCP) and urinary volatile organic compounds (VOCs) in patients with lower gastrointestinal symptoms.

METHODS: 1016 symptomatic patients with suspected CRC referred by family physicians were recruited prospectively in accordance with national referring protocol. A total of 562 patients, who completed colonic investigations, in addition to providing stool for FIT, FCP as well as urine samples for urinary VOC measurements, were included in the final outcome measures.

RESULTS: The sensitivity and specificity for CRC using FIT was 0.80 (95% confidence interval (CI): 0.66 – 0.93) and 0.93 (CI: 0.91 – 0.95) respectively. For urinary VOCs, the sensitivity and specificity for CRC was 0.63 (CI:0.46 – 0.79) and 0.63 (CI:0.59 – 0.67) respectively. However, for those who were FIT negative CRC (false negatives), adding urinary VOCs resulted in sensitivity of 0.97 (CI: 0.90 – 1.0) and specificity of 0.72 (CI: 0.68 - 0.76).

CONCLUSIONS: When applied to FIT negative group, urinary VOCs improves CRC detection (sensitivity rises from 0.80 to 0.97) thus showing promise as a second stage test to complement FIT in CRC detection.

INTRODCUTION

Colorectal cancer (CRC) is the second most common cause of cancer related deaths with a strong age relationship. Clinical presentation of CRC is varied, hence the dilemma for the clinician is to distinguish those with significant versus non-significant pathology without recourse to invasive and costly investigations.[1]

Symptoms alone are not sufficiently sensitive to diagnose CRC and up to a third of patients undergoing invasive investigations have normal outcomes: colonoscopy reported macroscopically and microscopically normal.[2] For those with lower gastrointestinal symptoms, there is increasing evidence for the use of faecal immunochemical test for haemoglobin (FIT) as first line testing showing high negative predictive value (NPV) of 0.99, but sensitivity is relatively low (0.80 – 0.90). [2-4]

In symptomatic patients it remains uncertain as to how to interpret a FIT negative result - in other words, can such patients be sufficiently reassured that they do not have cancer, alternative or additional tests are required. For example, a stool marker for inflammation - faecal calprotectin (FCP) or urinary volatile metabolic markers could be utilised to risk stratify those suspected of CRC. Metabolic markers such as urinary, faecal or breath volatile organic compounds (VOCs) have previously been shown to aid in CRC detection.[5-8] To our knowledge this is the first study using a combination of faecal tests (FIT and FCP) as well as urinary VOC testing in a symptomatic population suspected of CRC.

MATERIAL & METHODS

Design and Setting

A single-centre, prospective, blinded study of patients with lower gastrointestinal symptoms referred by family physicians to tertiary care with suspected colorectal cancer. Ethical approval was granted by Coventry & Warwickshire Research Ethics Committee, UK as part of the FAMISHED (Food and Fermentation using Metagenomics in Health and Disease) multicentre study – 09/H1211/38. The study protocol conforms to ethical guidelines of the 1975 Declaration of Helsinki as reflected by the institutions human research committee.

Participants

A total of 1850 patients were approached with criteria for inclusion based on national referral criteria (supplemental document 1). Of these, 834 were excluded due to a combination of reasons including physical frailty, illness, language barriers etc. 1016 patients provided consent and underwent colonic investigations (endoscopic or radiological colonic cross sectional imaging). 16 patients withdrew from the study and 310 failed to provide stool samples (69% return rate). A further 78 patients were excluded as only one stool sample was provided (both FIT sample collection device and stool for FCP were required) . Spot urine samples were received from 762 patients (76% return rate), but 39 were excluded due to insufficient sample volume or failed VOC urine analysis. This left a sample of 562 patients with matching urine and stool samples (FIT & FCP), which were included for final statistical analysis (Figure 1). Those under the age of 18, pregnant, not meeting the referral criteria for urgent review for lower gastrointestinal symptoms, or with incomplete colonic examinations were excluded from the study.

All study participants were given a pack containing a FIT sample collection device (Extel Hemo-auto MC A device; Kyowa Medex, Japan via Alpha Laboratories Ltd., Eastleigh, UK), which holds 2 mg of faeces in 2 ml of buffer, and a Universal Sterilin 30ml stool pot for FCP sample. Written and pictorial instructions for collections were provided, with Fe-Col® sample collection aid (Alpha Laboratories Ltd., Eastleigh, UK). Time of collection and time of receipt of samples at the laboratory were recorded and all samples were stored refrigerated at 2-8^{o}C until analysis. Patients were asked to return the sample prior to colonic investigations. Samples returned more than four days post-collection were excluded from analysis.

All study participants were also provided a Universal Sterilin 30ml pot to collect a spot urine sample at the time of clinic visit. Timing of collection was recorded and urine with a sealed cap was stored in a -80^{o}C freezer. In patients who were unable to provide the sample in the clinic, urine container was provided as well as a return envelope, and the sample was sent via courier from their primary physician and stored at -80^{0}C.

Intervention

Quantitative FIT was performed on automated HM-JACKarc analyser (Kyowa Medex, Tokyo, Japan) by the Midlands and North West Bowel Cancer Screening Hub, Rugby, UK on a weekly basis. Stool samples for FCP analysis were extracted manually by trained laboratory staff alongside the routine calprotectin service. Extracted calprotectin was measured using the EliA Calprotectin fluoroimmunoassay on the automated ThermoFisher ImmunoCap 250 analyser (Thermo Fisher Scientific, Waltham, MA, USA).

In the analyses for CRC, thresholds were determined from the data (*a priori*) to maximise sensitivity under the constraint that the negative predictive value (NPV) was ≥ 0.99. For FCP no threshold achieved an NPV ≥ 0.99. The lowest detection limit of this assay for FIT is 3µg/g faeces.

Samples were initially stored at -80^{0}C. Prior to urinary VOC analysis, they underwent a graded defrost process (based on our established protocol; unpublished). 10mL glass vial aliquots (Fisher Scientific, UK) suitable for use with an auto sampler (MPS, Gerstel, Germany) were used. Crimp caps (Chromacol Ltd., UK) with silicone PTFE (polytetrafluoroethylene) septa were used to seal each sample. Each septa and crimp cap was baked for 6 hours at 200^{0}C prior to use to remove any potential interfering molecules. Control blanks of air were prepared using the same method. A commercial gas analysis instrument (Lonestar (FAIMS), Owlstone UK), based on ion mobility spectroscopy (IMS), was utilised to analyse VOCs emanating from urine samples. Details of the Lonestar and its application in medical diagnostics have been reviewed by Covington et al.[9] The setup was bespoke for this application, to detect unique VOC chemical 'fingerprints'. Our previous study has determined optimal sample capture and storage to minimise diurnal and day to day variation.[10] See supplemental document 2 for further methodological details using FAIMS.

Outcomes

Participating clinicians, endoscopists and radiologists were blinded to the results of the urinary VOCs and faecal tests (FIT/FCP). Diagnosis of colorectal cancer and adenomas was confirmed histologically. High-risk adenomas were defined as lesions with high-grade dysplasia and/or serrated, villous histology, ≥10mm in size or presence of ≥ 3 adenomas. Hyperplastic polyps were excluded. Faecal and urinary VOC results were compared to the outcome of their colonic investigations and divided into clinical groups: colorectal cancer, high-risk adenoma, all adenomas, others and normal.

All analysis was carried out using R 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). To combine FIT and FCP measurements ten-fold cross validation and a Bayesian robust logistic regression model were used to generate predictive scores for each patient using the above criteria.

The VOC data variables were assessed for predictive value using a Mann-Whitney U-test at a significance level of 0.8, with multi-test correction performed using sequential goodness-of-fit (SGoF).[11] This yielded a set of ~10^4 candidate variables. The dimensionality of the selected variables was then reduced using PCA (principal component analysis)[12], with the number of principal components chosen using cross-validation[13] (typically being ~35). For testing using VOCs alone a logistic regression model was trained on the results. For the two-phase test incorporating FIT as a first phase a support vector machine, radial kernel was used on the top 128 variables, without using SGoF for variable selection. Ten-fold cross validation was used to generate predictive scores for each patient, and a threshold was selected to maximise sensitivity.

RESULTS

The main patient characteristics are shown in Table 1. A total of 562 patients, who completed colonic investigations, in addition to providing stool for FIT and FCP as well as urine samples for urinary VOC measurements, were included in the final statistical analysis.

Faecal biomarkers (FIT and FCP)

Using FIT alone, for CRC, revealed a sensitivity of 0.80 (95% confidence interval (CI): 0.66 – 0.93) and specificity of 0.93 (95 % CI: 0.91 – 0.95). The area under the curve (AUC) was 0.90 (95% CI: 0.85- 0.96). The negative predictive value (NPV) was 0.99 (95% CI: 0.98 – 1.0).

For high-risk adenomas, applying threshold of 3µg/g faeces which, is the lowest detection limit of the assay, the sensitivity was 0.70 (CI:0.52 - 0.87), specificity of 0.66 (CI: 0.623 – 0.703) with a negative predictive value (NPV) of 0.98 (CI: 0.96 - 0.99). See Table 2 for FIT diagnostic performance.

FCP alone performed less well than FIT in CRC and adenoma groups. Test performances combining FIT and FCP are shown in Table 2. The respective receiver operator curve characteristics (ROCs) for CRC, high-risk adenomas and all adenomas using FIT are shown in Figure 2.

Urinary VOCs

For urinary VOC analysis, logistic regression using sequential goodness of fit selection and principal component analysis (PCA) was applied to form a unique 'chemical fingerprint'. The sensitivity of urinary VOCs for the detection of CRC was 0.63 (95% CI: 0.46 – 0.79) and specificity 0.63 (95% CI: 0.59 – 0.67). AUC was 0.67 (95% CI: 0.57 – 0.77). The NPV was 0.96 (95% CI: 0.94 – 0.98).

For high-risk adenoma and all adenomas, using urinary VOCs, the sensitivity was 0.93 (0.81 - 1.0) and 0.91 (0.85 - 0.97) respectively with specificity of 0.16 (0.13 – 0.20) and 0.15 (0.12 – 0.19) respectively – (Table 3).

Combining FIT and Urinary VOCs

When used in combination (FIT and urinary VOCs) for CRC, the sensitivity was 0.80 (95% CI: 0.66 – 0.93) and specificity was 0.89 (95% CI: 0.87 – 0.93). AUC was 0.86 (95% CI: 0.77 – 0.94). The NPV was 0.99 (95% CI: 0.97 – 1.0).

Further analysis of urinary VOCs in a setting of FIT negative CRC (missed cancer) improved the sensitivity to 0.97 (95% CI: 0.90 – 1.0) and specificity to 0.72 (95% CI: 0.68 - 0.76) with NPV of 1.0 (95% CI: 0.99 – 1.0). Figure 3 shows a box plot applying the two-stage filter process to depict improvement in CRC detection when urinary VOCs are used in FIT

negative CRC patients compared to FIT alone. The decision threshold line in each case is the value (of either FIT or predicted probability) that divides prediction of cancer. Thus, for any patient above the line the test indicates likelihood of having cancer, and vice versa. Overall, patients are classed as negative for the two-stage test if they are negative for both FIT and VOC screening, and positive if they are positive for either one test - FIT (above the threshold) or VOC screening. We did not observe any differences even after stratifying for age or gender. For all adenomas, urinary VOC did not improve detection in those with false negative FIT.

DISCUSSION

Whilst studies evaluating various faecal markers (methylated genes, miRNA and protein markers) have shown promise for the detection of colorectal cancer and adenomas its application within a clinical setting has been limited due to high cost and poor sensitivity especially when applied in low disease prevalence areas. [14] Thus, the emphasis has been for low cost, non-invasive testing for detection of CRC and adenomas such as FIT. Experience from using FIT in the screening population reveals that it has a relatively high specificity at the expense of sensitivity. Various FIT devices have been trialled and it has become evident that there is considerable heterogeneity in FIT devices for the detection of CRC. This is further compounded by the fact that various threshold levels are applied, and uncertainty surrounding those who test negative with FIT. [15]

In the UK, CRC detection based on symptoms alone is low; ranging from 4-8% [16 17.] This suggests that over 90% of patients undergo negative tests for CRC exclusion. Thus, it is imperative that alternative non-invasive pre-screening markers such as FIT and other faecal or urinary markers are used in those with gastrointestinal symptoms to minimise unnecessary investigations.

In this study, we have shown for the first time the value of dual modality testing using non-invasive markers (FIT and urinary VOCs) as a two-stage process to exclude CRC. In those with lower gastrointestinal symptoms suspicious of CRC, using FIT alone revealed a sensitivity of 80% (meta-analysis suggests 90% (CI: 87 – 92%); unpublished). Thus, there is the potential to miss 1-2 cancers out of every 10 which is not sufficiently robust for everyday clinical use. Urinary VOC on its own was less sensitive (0.63; CI:0.46-0.79) and in combination with FIT did not show improvement in sensitivity (0.80; CI: 0.60-0.93).

However, if utilised as a two stage test, the addition of urinary VOC testing in those who test negative for FIT (i.e. the false negative CRC) increases the combined sensitivity to 97%, which, is more acceptable for clinical use and comparable to colonoscopy performance at a fraction of the cost.

Urinary VOC analysis on its own (VOC 'positive') only provides 63% sensitivity and specificity for CRC detection and performance does not improve when used in combination with FIT or FCP. This may be due to the heterogeneity of the chemical fingerprint that is produced by CRC. However, following pre-selection by FIT, it performs well as there is reduced background 'volatile noise' thus making it more specific to detect either the haemoglobin moiety/ breakdown products or glycation end products. Whilst urinary VOCs demonstrate a high sensitivity for adenoma detection, the lack of specificity and high false positive rate suggests that this marker may not perform as well for adenoma detection.

The VOCs that are detected using our pre-analytical method and FAIMS are unique, as its detection is based on volatiles existing in the gaseous phase rather than in the liquid phase. The disease separation is characterised by mobility of individual ions (i.e. physical rather than chemical properties of the ion), which, have low molecular weights (20 – 200kDa). Unlike conventional gas chromatography and mass spectroscopy (GC-MS), the specific chemicals are not identified but a 'chemical fingerprint' is formed. GC-MS is limited by its high running and labour cost as well as run time. This impedes its use within routine clinical practice where rapid, low cost, and simple (non-skilled) operation would be prferable. The composition of key volatile compounds gives rise to the unique chemical fingerprint identified in this study allowing classification for 'VOC positive or negative' outcomes.

VOCs reflect metabolic cellular changes within the host, for example detection of advanced glycation end products, which has been implicated in colon carcinogenesis.[18] Our previous work[19] (undertaken using a Bruker Scion GCMS, fitted with dynamic head space sampling and solid-phase micro-extraction (SPME) pre-concentration system) has identified three chemicals which are modulated in CRC – 'VOC positive' signature. Specially, we noted a high incidence of 1,3,5,7-Cyclooctatetraene and a low incidence of 1,3-Propanediamine and 4-methylbenzoic acid (dietary metabolite). Methylbenzene has also recently been reported to provide a unique chemical signature in those with CRC through the use of different technology (non-GCMS).[20] A higher incidence of acetone was noted in those with colorectal adenomas, which, has been shown by members of our group (unpublished) to be produced by

C. Difficile and in other *Clostridiales*. Allyl Isothiocyanate was also detected in those with CRC but not at elevated levels; the latter is produced by certain *E. Coli* strains as dietary substrates with can affect gut mucosal integrity.[21]

The use of dual modality testing; initially with FIT followed by urinary VOCs, enables 97% sensitivity with 100% NPV if both tests are negative for the detection of CRC and high grade adenoms. Furthermore, findings from this study suggest that the combination of FIT and VOC offers the option for personalised strategies for CRC detection in those with symptoms and avoids the need for repeat FIT testing (if FIT is negative the test probability is unlikely to improve unless there are pre-analytical errors).

It is envisaged that both these non-invasive tests (FIT and urinary VOCs) can be undertaken within primary care and analysed within a central laboratory (as FIT is currently) at low cost to guide secondary care referral patterns. The FAIMS unit is commercially available and urine VOCs are deemed stable up to 12 months when stored frozen.[10] It has been purported within a simulation model, that for an equivalent biomarker to compare with FIT (£18/test) it should not exceed a 7-fold unit cost of FIT [22] – this is fulfilled in urinary VOCs (£28/test), which, has less than 2-fold unit cost of FIT. A proposed clinical algorithm is outlined highlighting the use of a dual non-invasive diagnostic approach in those with lower gastrointestinal symptoms. (supplemental figure 5).

Specific Author contributions: RPA, initiated the project, led the grant funding application, ethics approval and manuscript writing. MMW recruited patients, performed sample handling, produced documentation and contributed to manuscript writing. ED, AW, JAC developed and performed the VOC analysis and contributed to manuscript writing. MN and RSS performed statistical analysis and contributed to manuscript writing. RPA, JAC, CH, CE, SS and BS reviewed the manuscript for intellectual content. CT and CLT were involved in the study design, performed biochemical analysis and contributed to manuscript writing. SS supervised the biochemical analysis. All authors reviewed the final manuscript.

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Abbreviations used in this paper: AUC, area under the curve; CI, confidence interval; CRC, colorectal cancer; DD, diverticular disease; FAIMS, field asymmetric ion mobility spectrometry; FAMISHED, food and fermentation using metagenomics in health and disease; FCP, faecal calprotectin; FIT, faecal immunochemical test for haemoglobin; GC-MS, gas chromatography and mass spectroscopy; HRA, high-risk adenoma; IBD, inflammatory bowel disease; IMS, ion mobility spectroscopy; MC, microscopic colitis; MPS, multipurpose sample; NPV, negative predictive value; PCA, principal component analysis; PPV, positive predictive value; PTFE, polytetrafluoroethylene; ROC, receiver operator curve; SGoF, sequential goodness-of-fit; SPME, solid-phase micro-extraction; VOCs, volatile organic compounds.

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Table 1. Patient demographic to include clinical characteristics and main diagnostic outcomes

Characteristic	N (%)	Median FIT (µg/g feces)	Median FCP (µg/g feces)
Total	562		
Sex Male	286 (51)		
Median age years; (range)	68; (29 – 89)		
***Presenting symptoms:** **Altered bowel habit** **Weight loss** **Rectal bleeding** **Anaemia** **Iron deficiency anaemia** **Abdominal pain**	 369 (66) 87 (15) 232 (41) 121 (22) 91 (16) 164 (29)		
CRC	35 (6.2)	270	86
High-risk adenoma	27 (4.8)	14	23
All adenomas (low and high risk)	94 (17)	3.5	19
Others ^(DD, IBD, MC etc.)	173 (31)	1.8	21
Normal	233 (41)	1.4	16

^DD – Diverticular disease, IBD – inflammatory bowel disease, MC – microscopic colitis. Normal – no colonic pathology identified.

* Note some patients may present with a constellation of symptoms.

Table 2. Diagnostic performance of FIT alone and in combination with FCP for CRC, high-risk adenoma and all adenomas

Disease group	Sensitivity (CI)	Specificity (CI)	NPV (CI)	PPV (CI)	AUC (CI)
FIT					
CRC (n=35)	0.80 (0.66 - 0.93)	0.93 (0.91 - 0.95)	0.99 (0.98 - 1.0)	0.44 (0.32 - 0.56)	0.90 (0.85 - 0.96)
High-risk adenoma (n= 27)	0.63 (0.44 - 0.81)	0.76 (0.72 - 0.79)	0.98 (0.96 - 0.99)	0.11 (0.07 - 0.17)	0.73 (0.63 - 0.83)
All adenomas (n= 94)	0.53 (0.43 - 0.63)	0.68 (0.63 - 0.72)	0.88 (0.84 - 0.91)	0.25 (0.19 - 0.31)	0.63 (0.58 - 0.69)
FIT+ FCP					
CRC (n=35)	0.80 (0.66 - 0.93)	0.93 (0.91 - 0.95)	0.99 (0.97 - 1.0)	0.43 (0.31 - 0.55)	0.91 (0.86 - 0.96)
High-risk adenoma (n= 27)	0.93 (0.81 - 1.0)	0.25 (0.21 - 0.29)	0.99 (0.96 - 1.0)	0.06 (0.04 - 0.08)	0.69 (0.59 - 0.79)
All adenomas (n= 94)	0.86 (0.79 - 0.93)	0.26 (0.22 - 0.30)	0.90 (0.85 - 0.95)	0.19 (0.15 - 0.23)	0.60 (0.54 - 0.67)

High-risk adenoma (HRA) - adenoma with high-grade dysplasia, villous histology, ≥10mm or ≥ 3 adenomas.

Thresholds applied to achieve the highest sensitivity under the constraint of keeping the negative predictive value >0.99

*Note the lowest limit of detection for the FIT assay is 3µg/g feces.

Table 3. Diagnostic performance of urinary VOCs for CRC, high-risk adenoma and all adenomas

Disease group	Sensitivity (CI)	Specificity (CI)	NPV (CI)	PPV (CI)	AUC (CI)
CRC (n=35)	0.63 (0.46 - 0.79)	0.63 (0.59 - 0.67)	0.96 (0.94 - 0.98)	0.10 (0.06 - 0.14)	0.67 (0.57 - 0.77)
High-risk adenoma (n= 27)	0.93 (0.81 - 1.0)	0.16 (0.13 - 0.20)	0.98 (0.94 - 1.0)	0.05 (0.03 - 0.07)	0.56 (0.45 - 0.68)
All adenomas (n= 94)	0.91 (0.85 - 0.97)	0.15 (0.12 - 0.19)	0.90 (0.83 - 0.96)	0.18 (0.15 - 0.21)	0.55 (0.49 - 0.61)

High-risk adenoma (HRA) - adenoma with high-grade dysplasia, villous histology, ≥10mm or ≥ 3 adenomas.

Figure 1. Study flow diagram of total patients recruited via the urgent colorectal lower gastrointestinal pathway having met the inclusion criteria.

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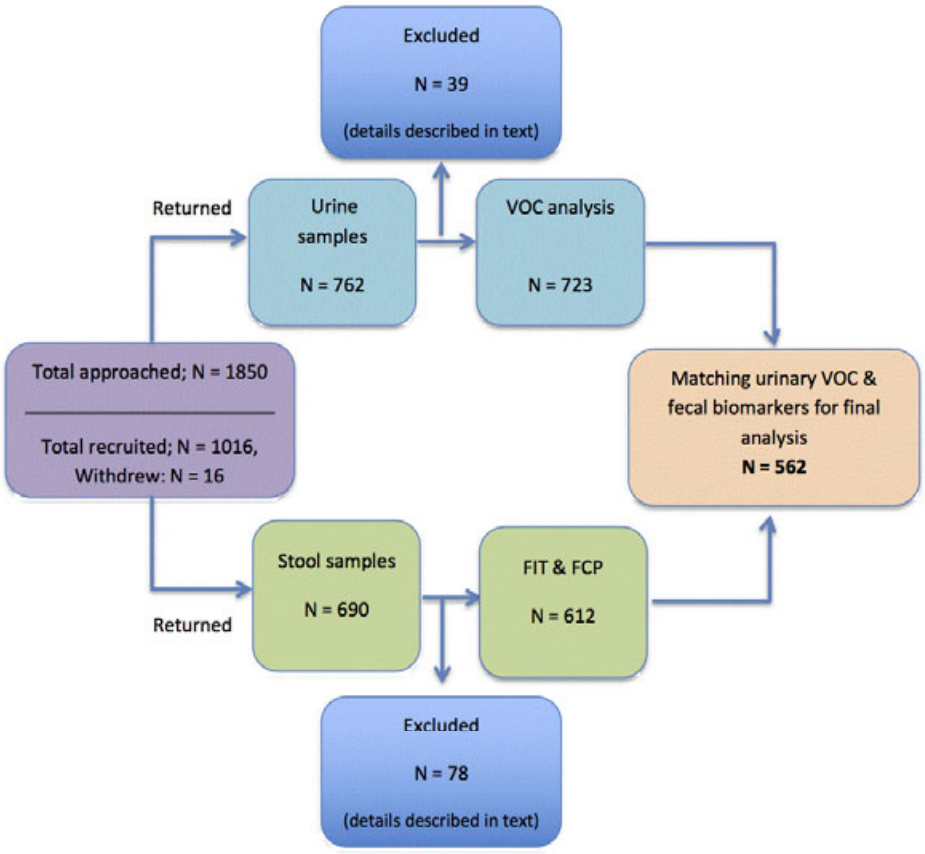

Figure 2. Respective ROC curves for a) CRC, b) high-risk adenoma and c) all adenoma using FIT.

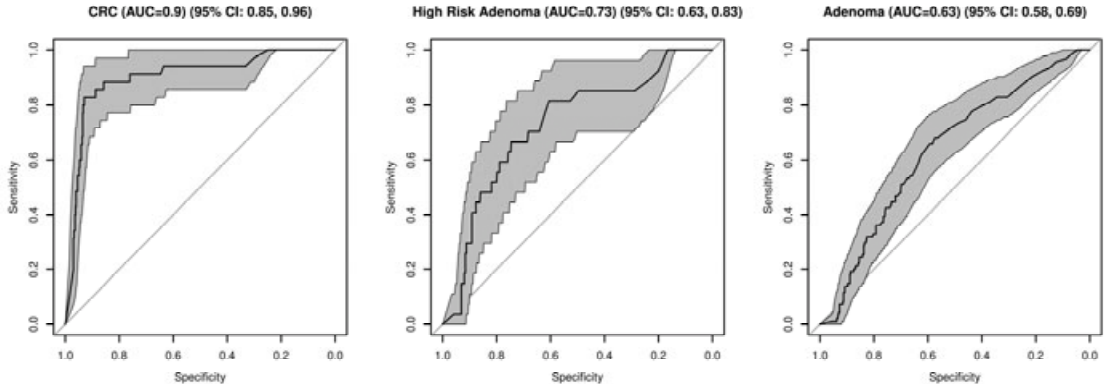

Figure 3. Two plots showing the stages of the FIT/VOC two-stage test. The left-hand plot shows the distribution of FIT measurements for CRC-positive and CRC-negative patients. The dotted line shows the threshold for the first stage test. Patients above this threshold are classed as positive for the test, while patients below this threshold go on to the second stage of VOC screening, shown in the right-hand plot. For patients who are screened using VOCs, the distribution of predicted probabilities of CRC is shown in the right-hand plot, with the decision threshold again shown by a dotted line. Patients above the line are positive for the test, while patients below the line are negative.