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PREVENTION AND EARLY DETECTION (R BENAMOUZIG, SECTION EDITOR)

Blood Tests for Colorectal Cancer Screening in the Standard Risk Population

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Abstract Barriers to screening for colorectal cancer (CRC) might be circumvented by using a blood test. New blood markers continue to be discovered, comprising RNA, DNA, and protein. On reviewing the literature on biomarkers in blood, many potentially valuable markers have been described. Those based on DNA have been the best evaluated to date and are not subject to the same specificity problems as fecal immunochemical tests (FIT), but as a class have relatively poorer sensitivity for adenomas. Most other markers have not been taken beyond the most rudimentary clinical assessment, and extremely few have been assessed in the screening context relative to proven screening tests such as FIT and colonoscopy. Adoption of blood tests into screening programs is going to depend on clarification of adequate accuracy for targeted neoplastic lesions in the screening environment and their relative performance and acceptability to existing proven tests.

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Introduction

Despite high likelihood of cure when diagnosed at an early stage, colorectal cancer (CRC) remains the second leading cause of death from cancer [1]. Early diagnosis has a major impact on survival and can be achieved with regular screening. However, screening can only be effective if the test is both sufficiently accurate and acceptable to the target population. To address some of these challenges, a plethora of studies addressing the discovery and the potential of new biomarkers for CRC (for screening, surveillance of cases, and prognostication) have appeared in the last decade. Biomarkers for screening have been sought principally in blood (plasma, serum, and particulate components), saliva, urine, and feces [2]. Given that more than 95 % of screening age people are familiar with providing blood for clinical tests and prefer blood as the test specimen [3], it could be predicted that blood tests have the potential to further improve screening program outcomes.

Therefore, we review the literature addressing blood-based biomarkers for early detection in screening, with emphasis on the degree to which they have been taken beyond initial discovery to the necessary levels of evidence required to establish them as a useful screening test.

Current CRC Screening Tests

The original occult blood tests were created for forensic purposes, but in 1893, they were applied to feces ([4] reviewed in [5]). In 1901, the importance of the occult blood test was

emphasized as a diagnostic test for intestinal bleeding. In 1907, it was asserted that fecal occult blood tests (FOBT) required accuracy, delicacy, and simplicity. In 1919, it was written

> It cannot be expected that the examination of the stools for blood will have the currency which it merits in clinical practice, until a method is devised which is certain, appropriately sensitive, as simple and easy to perform as possible and of which the possible sources of error are known. (reviewed in [6])

In 1952, the desirable quality of inoffensiveness was also added [7].

It is now recognized that a screening test requires a much higher level of evaluation compared to a diagnostic test. For *diagnostic* tests, while it is essential that accuracy (i.e., sensitivity and specificity) is established, for *screening* tests, more evidence is demanded, with safety, acceptability, feasibility, and cost-effectiveness also required to justify adoption for mass screening [8•, 9]. To ensure that a test is adopted in practice, it is important to undertake a phased evaluation as proposed by Pepe et al. [10••]. The initial phases ensure that test accuracy is sufficient to justify more extensive and costly evaluation of the new method in a screening context. Accuracy data alone are insufficient for test application in mass organized screening programs funded by public health systems. The subsequent phases [10••] address evaluation of the biomarker in a typical screening target population, including ascertainment of test positivity rates and willingness to participate, and relativity to a proven and accepted screening test.

For simplicity and the purposes of classifying how far evaluation has been taken with the biomarkers identified in the literature search, we use the following four phases of evaluation [11] based on the proposals of Pepe et al. [10••]:

1. Determine if the test detects established disease (i.e., CRC)
   This is achieved through retrospective studies to determine if the test can discriminate healthy controls from cases with CRC.
2. Determine if the test detects early disease before it becomes apparent
   This needs to be performed via prospective studies in subjects prior to undergoing the diagnostic procedure. Several thousand subjects may need to be recruited in order to achieve an appropriate sample size for the disease state being assessed. This also provides performance across the full range of neoplastic stages and benign diseases in the colorectum.
3. Assessment of the test in a single round of a screening setting
   This will establish the test positivity rate, the false-positive rate, the number needed to colonoscope to detect the target lesion, and the acceptability/participation rate. These are ideally ascertained relative to an existing proven test.
4. Assessment of the test within a structured screening program over multiple rounds
   This will allow measures of adverse events as well as impact on disease incidence and mortality.

For the development and initial application of any new CRC screening test, determining the mortality benefit would require lengthy trials; therefore, several writers emphasize that for a new test to be adopted, as a minimum, it is important to compare its performance to another non-invasive screening test technology where there is already evidence of benefit [12, 13•]. It is not essential to proceed to randomized controlled trials (RCTs) with mortality from and/or incidence of CRC as endpoints. The effectiveness of the guaiac-based fecal occult blood test (gFOBT) provides the *minimum* standard to be achieved by a new test but the preferred standard is a fecal immunochemical test (FIT) for hemoglobin [14–20].

Apart from these, there are a number of tests shown to be effective in screening to reduce mortality from CRC or its incidence, on an intention-to-screen basis. These include colonoscopy which is the main screening strategy in the USA and Germany [21], and flexible sigmoidoscopy. RCTs in the general population have shown that early detection by screening, such as with fecal occult blood test (FOBT) or flexible sigmoidoscopy, reduces mortality and may also reduce incidence [22–25]. Trials of the invasive test flexible sigmoidoscopy achieved a 33 % reduction (intention-to-screen basis) in incidence of distal but not proximal cancer [26], while there is a mortality reduction of 15–35 % when using the gFOBT Hemoccult [22, 24, 27]. The original gFOBT have now been largely replaced by the FIT as FIT have better sensitivity [14–20]. Mortality reduction is achieved through early CRC detection, with participation with FIT detecting CRC at an earlier and more treatable stage, compared to cancer detected outside of screening [28].

The mortality reduction achieved in these trials is, however, hindered by limitations in test accuracy (sensitivity) and/or population uptake (willingness to participate). If screening is to achieve its full potential in reducing the population burden of CRC, we must respond to the challenges inherent in improving accuracy and participation, with improvement of participation just as important as improvement of accuracy. Despite evidence of effectiveness, poor participation rates are commonly observed in screening programs, and compliance rates in many of the national programs are modest. In 2010, screening program participation rates across Europe ranged from 1.9 to 54.2 % with FOBT [29], while recent participation rates in the Australian screening program were less than 35 % [30]. Many average risk people are also unwilling to undergo screening colonoscopy, with only 29 % reporting having a colonoscopy in the last decade [31].

Colonoscopy is the most sensitive test for early detection. However, poor uptake may be explained by its invasive nature, high cost, and the fact the testing mode is not readily available, is inconvenient, and has risks of adverse events such as perforation. Whereas poor participation with FOBT may be explained through dislike of fecal sampling and contraindication due to non-neoplastic gastrointestinal bleeding conditions (e.g., hemorrhoids) that can interfere with FIT results [32, 33]. A blood sample-based test might overcome some of the behavioral barriers inherent with colonoscopy and fecal testing [3, 34]. New tests are needed to ensure equity of service for early CRC detection.

New Biomarkers

The development of a non-invasive, accurate, and cost-effective test that can complement or improve upon current screening strategies is a major challenge because of both the cost and time involved. Research indicates that preferred test attributes include non-invasive nature, accuracy, convenience, minimal preparation requirements, and absence of pain [35]. Behavioral research in typical target populations has compared acceptability of a number of hypothetical specimen types for CRC screening [3]. This showed that specimen type, collection location, and cost were important attributes determining test preference. In comparing the idea of providing a blood compared to a fecal sample for screening purposes, the majority of those surveyed preferred blood (78 vs 22 %), considering it a more acceptable specimen based on hygiene, embarrassment, comfort, and pleasantness [3].

It is now clear that many cancer-related molecular markers can be present in blood (see Online Supplementary Table 1). These biomarkers may be tumor-derived or tumor-dependent and may be central to pathogenesis, a by-product of genomic instability or an epiphenomenon reflecting reaction to the tumor. Tumor-derived markers are likely to arise from tumor necrosis, apoptosis, active release, and/or lysis of circulating cancer cells [36]. Although traditional cancer-related antigens, such as carcinoembryonic antigen (CEA), are not useful as markers for early detection [37, 38], a variety of other protein, DNA, epigenetic DNA, messenger RNA (mRNA), and micro RNA (miRNA) biomarkers have now been documented in blood of patients diagnosed with a range of cancers, including CRC [36, 39, 40]. Because we focus on biomarkers for screening, we conducted a review of biomarkers that have been assessed for screening for CRC, by using search terms within PubMed such as "colorectal cancer," "blood," "screening," and "detection." We have excluded studies from review that focused only on later stage CRC, those that did not start with colonoscopy to confirm diagnoses, and those that did not report either sensitivity, specificity, and/or ROC analysis.

In order for a biomarker to be suitable for screening purposes, it needs to be able to detect curable CRC and important pre-invasive lesions (specifically advanced adenomas), have a low false-positive rate with specificity of more than 90 % to avoid unnecessary colonoscopy, and have had a phased evaluation as described above. As will become evident from the following, while many biomarkers have been assessed (see Online Supplementary Table 1), few are appropriate for population screening purposes.

Online Supplementary Table 1 lists the extensive list of markers that have been discovered and subjected to initial analysis. In all, there are 149 markers or panels of markers listed comprising 24 mRNA (only 1 progressed to phase 2), 23 microRNA (none progressed beyond phase 1), 1 DNA mutation (phase 2), 8 DNA (1 progressed to phase 2), 26 methylated DNA (1 to phase 2 and 1 to phase 3), and 67 protein (4 progressed to phase 2). The online table includes summary information on sensitivity/specificity/ROC analysis, whether or not it was compared to another test and what phase of evaluation had been undertaken. To simplify this information, given the focus on application of the biomarkers to screening, Table 1 provides additional performance data on those biomarkers which have been subject to evaluation beyond phase 1.

RNA

Both mRNA and miRNAs have been assessed as biomarkers for early detection of CRC. It has been proposed that RNA assessment is more difficult than DNA due to degradation of RNA by blood RNAses. However, extracellular RNA can still be detected and assessed within the blood of cancer patients using RT-PCR amplification [36, 50].

Promising biomarkers utilizing mRNA include self-renewal stem cell factor (SALL4) and L6 which have been shown with phase 1 evaluations to have sensitivities of 96.1 and 78.6 %, and specificities of 95 and 100 %, respectively [51, 52]. Another study shows that human telomerase reverse transcriptase (hTERT) mRNA is good at discriminating between normal and CRC cases, with a sensitivity of 92 % and a specificity of 100 % [53], but other studies report either a lower sensitivity 69.4 % (at 100 % specificity) [54] or good sensitivity (81 and 92 %) at poor specificity (67 and 64 %) [55, 56] (Supplementary Table 1). B cell-specific moloney murine leukemia virus integration site 1 (Bmi-1) has been assessed with phase 2 evaluation (Table 1) and has high sensitivity and specificity for both CRC and advanced adenoma. However, it should be noted that the comparator marker used in this study was CEA which is generally not informative because CEA has never been proven to be of value for screening for CRC, even though it has some value for surveillance of cases for recurrence. Further evaluation of Bmi-1 is warranted.

MiRNAs are short non-coding RNAs that downregulate gene expression through either mRNA degradation or translational repression. It is thought that miRNAs act as tumor

Table 1 Blood biomarkers that have undergone phase 2 or 3 of evaluation (see text for definition) for the purpose of CRC screening

Biomarker	Sensitivity/specificity/AUC for adenoma	Sensitivity/specificity/AUC for early-stage CRC	Sensitivity/specificity/AUC for all CRC	Comparison to established test?	Study details
KRAS exon 2 mutations	Sensitivity=16 % Specificity=100 % (for adenomas with high-grade dysplasia)	Not stated	Sensitivity=8 % Specificity=100 %	N/A	170 FOBT-positive patients undergoing colonoscopy: 12 CRC, 22 advanced adenoma with high-grade dysplasia, 63 without lesions [41]
Bmi-1 mRNA	Sensitivity=78.3 % Specificity=82.8 % (for advanced adenoma)	Not stated but levels increase with stage	Sensitivity=72.2 % Specificity=94.9 %	CEA protein	500 patients undergoing colonoscopy: 158 CRC and 313 non-CRC (23 advanced adenoma) [42]
Cell-free DNA (assessed with amplified hTERT)	AUC=0.573 (for adenomas with high-grade dysplasia)	Not stated	AUC=0.709	N/A	170 FOBT-positive patients undergoing colonoscopy: 12 CRC, 22 advanced adenoma with high-grade dysplasia, 63 without lesions [41]
Methylated Septin9	(1) Sensitivity=11.2 % Specificity=90.9 % (for advanced adenoma) (2) Not stated (3) Sensitivity=22 % Specificity=78.8 % (for advanced adenoma)	(1) Stage I sensitivity=35.0 % Stage II sensitivity=63.0 % Stage I–II sensitivity=44.7 % (2) Stage I sensitivity=61.5 % Stage II sensitivity=80.0 % Stage I–II sensitivity=69.6 % (3) Stage I–III sensitivity=64 %	(1) Sensitivity=48.2 % Specificity=91.5 % (2) Sensitivity=73.3 % Specificity=81.5 % (3) Sensitivity=68 % Specificity=78.8 %	(1) N/A (2) FIT (3) N/A	(1) 1516 asymptomatic people undergoing colonoscopy: 53 CRC, 314 advanced adenomas, 938 without disease [43••] (2) 102 known CRC cases and 199 undergoing screening colonoscopy: 104 CRC, 26 advanced adenoma, 94 without evidence of disease [13•] (3) 1544 undergoing colonoscopy: 44 CRC, 621 advanced adenoma, 444 without evidence of disease [44]
Methylated *BCAT1* and *IKZF1*	Sensitivity=9 % Specificity=94 % (for advanced adenoma)	Stage I sensitivity=38 % Stage II sensitivity=69 % Stage I–II sensitivity=56 %	Sensitivity=66 % Specificity=94 %	N/A	2105 people undergoing colonoscopy for any purpose: 129 CRC, 338 advanced adenoma, 450 without evidence of disease [45]
CD26 protein	(1) Sensitivity=41.7 % Specificity=75.5 % (for advanced adenoma) (2) Sensitivity=39.6 % Specificity=90.2 % (for advanced adenoma)	(1) Not stated (2) Not calculated as only 4 CRC	(1) Sensitivity=81.8 % Specificity=72.3 % (2) Not calculated as only 4 CRC	(1) N/A (2) FIT	(1) 299 people undergoing colonoscopy for any reason: 33 CRC, 48 advanced adenoma, 68 without evidence of disease [46] (2) 516 asymptomatic people with family history of CRC undergoing colonoscopy: 4 CRC, 53 advanced adenoma, 338 without evidence of disease [47]
Gastrointestinal tract acid-446 (GTA-446) protein	Sensitivity=53.3 % (for adenomas with high-grade dysplasia)	Stage 0/I sensitivity=76.7 % Stage II sensitivity=100 % Stage 0–II sensitivity=86.5 %	Sensitivity=86 % Specificity=53.7 %	N/A	4923 people undergoing colonoscopy: 98 CRC, 108 adenomas with high-grade dysplasia, 482 non-neoplastic; and 964 reference population [48]
	Not stated	Not stated	Sensitivity=88.8 % Specificity=84.2 %	N/A	

Table 1 (continued)

Biomarker	Sensitivity/specificity/AUC for adenoma	Sensitivity/specificity/AUC for early-stage CRC	Sensitivity/specificity/AUC for all CRC	Comparison to established test?	Study details
Colon cancer-specific antigen-2 (CCSA-2) protein					174 people undergoing colonoscopy: 27 CRC, 40 without evidence of disease [38]
Panel: CEA, CYFRA 21-1, ferritin, osteopontin (OPN), anti-p53, and seprase protein	Sensitivity=22.7 % (for advanced adenoma)	Stage I sensitivity=44.0 % Stage II sensitivity=75.4 % Stage 0–III sensitivity=64.6 %	Sensitivity=69.6 % Specificity=95 %	CEA protein	1027 people undergoing colonoscopy or surgery: 301 CRC patients, 143 advanced adenoma, 266 without evidence of disease [49]

suppressors and oncogenes and are therefore important in proliferation and apoptosis. Changes in miRNA expression have been observed in tumors, including CRC. Serum miRNAs may be more suitable for clinical application. They are resistant to RNAse A digestion. One study has shown that miRNAs are stable at prolonged storage at room temperature and are not degraded during freeze-thaw cycles [57]. In addition, expression levels of miRNAs are reproducible and consistent among individuals [58].

Many microRNA biomarkers have emerged from various discovery strategies, followed by validation on just a small set of plasma samples with subsequent independent validation on a larger set. However, none has progressed beyond phase 1 studies. On the basis of these studies, miRNAs that should be evaluated further include miR-21, miR-23, and miR-1246 with current sensitivities reported at 82.8, 92, and 95.5 %, and with specificities of 90.6 % [59], 100 % [60], and 91 % [60]. In addition, miR-21 is reported to show reasonable detection of advanced adenoma (sensitivity=76.8 %, specificity=81.1 % [59]) (Online Supplementary Table 1). Creating panels of biomarkers may also provide opportunities to improve test performance. The panel of miR-21, let-7g, miR-31, miR-181b, miR-92a, and miR-203 has a sensitivity of 96.4 % and a specificity of 88.1 % [61].

DNA

The blood of cancer cases contains higher concentrations of DNA than the blood of healthy individuals [62]. The mechanism(s) by which circulating tumor DNA is released into circulation is unclear, but it is thought that it might originate from lytic, apoptotic, or necrotic tumor cells [63]. Measurement of circulating DNA is a promising tool for CRC detection, with several techniques assessed for measurement purposes. Free DNA assessment is promising for early-stage CRC detection (80.3 % sensitivity) as well as for polyp detection (levels significantly higher than control [64]). The specificity in this study was also appropriate for screening purposes (92.3 %) [64] (Online Supplementary Table 1). However, only a small phase 2 study with limited reporting of accuracy has been undertaken (Table 1).

KRAS has also been investigated as a DNA biomarker as the majority of CRCs and advanced adenomas can harbor this mutation. However, it has been shown that *KRAS* is a poor blood biomarker, even if the tissue is positive for the mutations (Table 1) [41].

Dysfunctional regulation of gene methylation plays an important role in oncogenesis [38, 65–69]. Epigenetic changes are believed to occur early in tumor development, preceding genetic changes, indicating that biomarkers based on epigenetic changes will permit early detection and even prevention of cancer [70]. Promoter hypermethylation is associated with silencing of gene expression including that of tumor

suppressor genes and other genes involved in cancer development [65–67, 71]. Studies investigating the use of these epigenetic changes as molecular markers show that these changes are reflected in cell-free circulating tumor DNA extracted from the plasma of CRC patients [72]. An advantage of DNA methylation as a biomarker for cancerous cells is that it can be consistently measured, as it tends to occur in specific regions of the DNA (CpG islands). This change is also appropriate for clinical detection as only short segments of DNA need to be isolated to detect cancer-specific changes [73].

Methylated Septin9 (*SEPT9*) is the most assessed blood biomarker of this class. It has been compared to other screening tests (FIT and the stool DNA test), with a recent prospective study in subjects being screened for CRC by colonoscopy (in effect, a phase 3 study but not with an intention-to-screen element), demonstrating a sensitivity of 48 % for CRC with a specificity of 91 % (Table 1) [43••]. Similar test performance has been found for the methylated panel of *BCAT1* and *IKZF1* [45] when assessed in phase 1 and phase 2 studies (Table 1). While adenoma tissue have been shown to be positive for these three markers (*SEPT9*, *BCAT1*, and *IKZF1)* [74], appearance of the methylated DNA in blood does not provide a sensitive test for advanced adenomas. Further improvements of these tests (or usage of additional methylation DNA biomarkers) would be desirable for population screening purposes. Nonetheless, *SEPT9* performance has been considered acceptable for screening, and it is currently commercially available [75].

Methylated DNA biomarkers, and other blood-based biomarkers, have the potential for less false-positive results compared to that found with population screening using FOBT. As FOBT depend on bleeding from neoplasia, any other condition that causes gastrointestinal bleeding might trigger a positive test. This is a significant issue as almost 50 % of people older than 50 years experience overt rectal bleeding from symptomatic hemorrhoids [76], and the incidence of these conditions rises with age. To avoid the high likelihood of false-positive FOBT results and unnecessary colonoscopy, screening with fecal testing is contraindicated for people with benign bleeding conditions, which is a substantial proportion of the community. This problem inherent with occult blood testing could be overcome by the use of a test that depends on a marker other than bleeding. An initial study that is reported in abstract form indicates that the specificity of the *BCAT1/IKZF1* blood test is not compromised in people that have overt rectal bleeding in the same way that FIT tests are [77].

Protein

Protein biomarkers have been assessed for CRC detection for many years, with carcinoembryonic antigen (CEA) being the first blood marker linked to CRC presence [78]. Protein classes commonly assessed include glycoproteins, antibodies, cytokines, and angiogenesis factors [79]. Protein detection, particularly immunoassay, are well established, and with their stable nature in blood makes them promising markers for CRC screening tests. The techniques for protein detection have included ELISAs and mass spectrometry.

While CEA is one of the most assessed biomarkers, it remains a poor marker for early disease. Specificity for CEA is usually reported at levels acceptable for population screening purposes, but sensitivity can be as low as 22 % when assessing all stages [80]. Circulating CEA concentrations increase with stage, with the sensitivity for early-stage CRC being low (e.g., 6.9 % [81]). CEA is, therefore, not suitable for screening and is an inadequate comparator by which to judge new biomarkers.

The protein biomarkers that have the highest test performance and that have also undergone phase 2 evaluation are dipeptidyl peptidase-4 (CD26) and colon cancer-specific antigen-2 (CCSA-2) (see Table 1). For CD26, several studies have reported a sensitivity greater than 80 % for CRC [46, 82] (Table 1). CD26 also shows promise for early-stage CRCs and adenomas. Advanced adenoma sensitivity is around 40 % [46, 47]. In a phase 1 evaluation, it was shown that sensitivity of the CD26 test is enhanced in stages I–III, but impaired in stage IV [82]. By combining CD26 with CEA measurement (which has high sensitivity for stage IV), overall detection can be improved. Another protein that could benefit through combining with CEA is alpha-L-fucosidase which has sensitivities of 69 % (stage I), 70 % (stage II), and 72 % (stage III), but a lower sensitivity for stage IV (53 %) [83]. Gastrointestinal tract acid-446 (GTA-446) levels in the circulation appear to be stage independent, with all stages having good sensitivity (0/I=76.7 %, II=100 %, III=88.2 %, IV=75.0 %). The appearance in the blood is likely not to be the result of tumor burden as reduced GTA-446 levels in CRC patients are not restored after surgery or therapy. While the sensitivity is promising as a biomarker, GTA-446 levels increase with age, and the false-positive rate needs to be decreased to prevent a high colonoscopy workload and unnecessary anxiety for patients [48].

Proteomics utilizing mass spectrometry also may hold promise for future screening tools. Measurement in the circulation of low mass ion blood metabolites has a sensitivity of 94.79 % and a specificity of 97.96 % [84], while a panel with SELDI-TOF mass spectrometry has a sensitivity for CRC of 87.5 % and a specificity of 93.8 % [85].

Limitations with Studies

As can be seen from above, most CRC biomarkers have only been subject to phase 1 evaluation, comprising retrospective studies that seek to determine if a particular biomarker has the potential to discriminate between CRC cases and healthy controls. Phase 2 evaluation is important because this provides

clearer information, in a prospective manner, concerning test performance across the spectrum of neoplastic lesions from diminutive adenomas to late-stage cancer and then the issues of specificity affected by benign disorders. Of the total of 148 biomarkers identified in this review, only 9 as individuals or panels have proceeded to phase 2 evaluation and only 1 of these has been assessed in true screening cases.

SEPT9 has been evaluated in screening subjects (i.e., it has been subjected to some aspects of phase 3 evaluation), but this has not been combined with an intention-to-screen analysis where subject acceptance is also factored in. For most of the markers, we do not have information about whether they may be affected by age, gender, or a range of environmental factors such as diet and smoking. Phase 3 evaluations are essential to clarify what these confounding factors might be and how they would be evaluated.

Another issue not addressed in most of the studies is whether the biomarker under investigation is detecting cancers other than CRC. Few have assessed biomarker levels pre- and post-resection to determine if the biomarker is tightly associated with the presence of the CRC. There are some blood biomarkers, such as methylated *SEPT9*, that have been shown to be detectable in blood from patients with other tumors [86–89]. It has been also suggested that CRC patients share a large number of serum miRNAs with lung cancer patients [58]. This does not necessarily mean that a blood test based on a methylated DNA will pick up multiple cancer types as it will depend on what sub-region of methylation that the assay is targeting and its specificity for a particular cancer. This will need to be considered in assay design and how much this issue of specificity for different organ cancers will impinge upon screening programs has yet to be clarified.

Biomarker Considerations

Technical Issues

The different chemical nature of the biomarkers, and the associated challenges inherent in blood as a sample, raises a range of complex technical issues that must be managed carefully when setting up assays for application in the clinical setting, for example, quality assurance and regulatory approvals, as they vary considerably between countries. In addition, technical consideration for each biomarker class include sample collection processes, sample preparation, biomarker stability during collection and preparation, transport to the assay laboratory, and maintenance of quality assurance processes in the laboratory.

Different biomarkers, both within and between chemical classes, vary in their stability. As a general rule, DNA tends to be the most stable and RNA the least stable, and so assay performance when subject to all the uncertainties inherent in clinical practice need to be carefully managed. Sample preparation can be a critical issue for those assays where plasma is the preferred biospecimen, since contamination of plasma with protein, DNA or RNA from cellular elements can give rise to specificity issues, but this impact can be minimized depending on choice of assay technology.

Combining Biomarkers

It will be apparent from the discussions above that combinations of biomarkers can be more informative with the use of a single biomarker. This is not surprising, given the different pathogenic processes by which CRC develops and the associated differences in DNA lesions, gene expression, and so on. In combining biomarkers, one seeks to improve sensitivity, but this can be at the cost of deteriorating specificity. This has been demonstrated for the fecal multitarget test [90] and is apparent for a few of the biomarkers discussed above, including the methylated DNA biomarkers in plasma. Evaluation processes require more extensive further consideration of how the above biomarkers might be combined to improve test performance. It remains to be demonstrated that a single molecular biomarker in blood will be more accurate than existing proven screening tests such as FIT.

Quantification

It is now well established that quantification using FIT provides great flexibility in establishing screening programs. Fecal hemoglobin concentration chosen as the threshold for defining test positivity can be adjusted to either maximize sensitivity, achieve high specificity, or control the test positivity rate (which determines the colonoscopic work load) [19]. Many of the biomarker assays provided above can be applied in quantitative mode but with the exception of *SEPT9*; there is insufficient evidence concerning any of the others to inform the choice of the positivity cutoff in different screening environments.

Interval Between Testing

To date, there is no information available on how frequently any of these blood biomarker tests need to be undertaken in screening subjects. This can be modeled in part based on accuracy characteristics and the perceived windows of opportunity for colorectal oncogenesis.

False Positives

Further work also needs to be done to look into the management of "false-positive" results as well as other considerations such as laboratory challenges in automation and quality control. In addition, new screening tests might detect a different

neoplasia-dependent biology, and as a consequence, the value of treatment and benefit to survival might not be the same.

Biomarker Accuracy

Given the preliminary state of evaluation of the vast majority of these biomarkers, it is difficult to reach firm conclusions about their adequacy for use in screening.

The crucial issue is whether a biomarker is of sufficient sensitivity for curable cancer to be useful in the screening environment. Of the biomarkers that have clearly progressed beyond phase 1 evaluation, it can be concluded that *SEPT9* has better sensitivity for CRC than the original gFOBT, although it may not be quite as sensitive as FIT for CRC. Two markers in the same family, methylated *BCAT1* and *IKZ1*, are progressing through phase 2 evaluations, and while not yet evaluated in unbiased screening subjects, indications are that sensitivity for cancer is at least comparable to that of *SEPT9* [45]. From the studies undertaken with these three markers to this point, it seems clear, however, that none of these markers matches the sensitivity of FIT for advanced adenomas.

Is High Sensitivity for Detection of Adenoma Essential?

How crucial is it that these new biomarkers are sensitive for adenomas, especially advanced adenomas? The American Cancer Society guideline highlights the importance of access to screening tests to facilitate cancer prevention through early detection of cancer and the removal of polyps [91]. Indeed, a major advantage of FIT over gFOBT was considered to be the ability of the former to detect a higher proportion of advanced adenomas. While detection of advanced adenomas will lead to reduction in CRC incidence, this detection requires a higher colonoscopy rate and modeling shows that adenoma detection is not crucial to effectiveness. Guaiac-FOBT has a low sensitivity for adenomas, yet reduces cancer mortality [22, 25]. Modeling shows that tests that only detect cancer (and not adenoma) reduce mortality by 71 % [92]. While higher detection rates of adenomas and stage I cancer are intuitively likely to reduce mortality and incidence further, this is at a greater program work load and cost. It should be noted that with the recent advances in treatment, even detection of CRC with regional distribution remains beneficial given a 5-year survival rate of 70.4 % [93]. Furthermore, estimates of the natural history of the adenoma-carcinoma sequence indicate <10 % of the adenomas become cancer, with a dwell time of 10–26 years [94].

Finally, it needs to be emphasized that the detection of target lesions in the screening program is the product of test acceptability and accuracy [19]. Therefore, if a participatory advantage for a blood biomarker bears out in practice in intention-to-screen studies, this might counter-balance any perceived disadvantages with adenoma detection.

What Remains to Be Done?

There is potential for biomarkers in blood to provide a useful test for colorectal cancer screening. Until we have better information on application of the many potentially interesting biomarkers in the screening environment and more extensive comparisons to the existing proven screening tests, it will be difficult to decide which is going to find its place. A blood test may be useful to improve screening participation rates but this cannot be undertaken formally unless adequate accuracy has been demonstrated. In terms of accuracy, many of the investigated biomarkers still need to be compared to a proven comparator test such as FIT, flexible sigmoidoscopy, or colonoscopy.

The key studies demonstrating that population screening can reduce CRC mortality are based on annual or biennial offers over multiple rounds [22, 27], and therefore, ongoing participation must be maintained. This has been highlighted in several cost-effectiveness analyses [95]. We have shown that overall percentage participation in FIT-based screening is maintained over multiple rounds as those who drop out are replaced by previous non-participants who enter the program [96]. To date, there are no available data on whether participation will be maintained in subsequent rounds of offers when a blood test is included in a screening program.

It must also be acknowledged that a CRC screening program using a blood test necessitates a new set of logistic requirements with different potential facilitators and barriers compared to fecal-based programs, such as done in Europe, Asia, and Australia and colonoscopy or flexible sigmoidoscopy programs taken in various places around the world.

Behavioral studies are also crucial because it cannot be assumed, without evidence, that blood tests will be more readily accepted than the current fecal sampling approaches. Despite research indicating that the majority of people prefer the idea of a blood test over a fecal test [3], we cannot assume that this will translate to actual participation as different, potentially significant, barriers arise for blood collection when screening is undertaken in a centrally organized public health program (the context for many countries). Converting a blood-based biomarker into a simple and inexpensive screening test is a costly process and cannot be justified without knowing both the likely accuracy and acceptability to participants. To date, there is limited published research investigating sample preference, although there are some data on attitudes to blood tests [3, 34, 97, 98]. Even if a blood test was found to increase screening participation and adherence, to enable its inclusion within a nation-wide program, it must be proven to be a cost-effective strategy, as cost of molecular markers could exceed 10 times the cost of fecal testing [99]. Cost-effectiveness modeling for the *SEPT9* blood test suggests that an additional screening uptake of 10 % is cost effective as it results in reductions in CRC incidence and mortality at a reasonable cost [100].

An additional benefit is that the blood test introduces the opportunity to more meaningfully incorporate the primary care practitioner into the screening process. More than 90 % of older Australians attend their primary care practitioner once per year [101]. Formal structuring of CRC screening through primary care practices gives the opportunity for the practitioners to assess a person's suitability, to advocate for screening, and to order the test with a likely capacity to sample blood on site. Our previous recruitment of patients in CRC screening trials through primary care practices showed that offering screening with doctor endorsement augments participation by about 10 % compared to an offer from a central screening service [102]. Improving participation in those people that do not participate with fecal testing in spite of endorsement would be an additional indication of the power of blood-based tests to change screening behavior.

Conclusion

Blood-based biomarkers of colorectal neoplasia have the potential to improve the effectiveness of screening programs for CRC through both increased population participation by overcoming barriers to screening and greater clinical accuracy by reducing false positives that are inherent with gFOBT/FIT. However, until full evaluation in screening subjects is undertaken on an intention-to-screen basis that includes assessment of accuracy and detection relative to proven screening tests, it is not possible to advocate strongly for any particular biomarker at this time. The biomarker family most thoroughly evaluated at this point is the methylated DNA markers, specifically *SEPT9*, *BCAT1*, and *IKZ1*. But these lack sensitivity for advanced adenomas, and it is not yet clear if there is a participatory advantage in practice and whether this counterbalances the issue of adenoma sensitivity in cost-effectiveness studies.

Compliance with Ethics Guidelines

Conflict of Interest Erin L. Symonds has received an institutional in-kind donation from Eiken Chemical Company and has received research funding through a grant from Clinical Genomics. Graeme P. Young has received research funding through grants from the National Health and Medical Research Council of Australia to fund studies evaluating methylated DNA biomarkers and from Eiken Chemical Company to his employing university for research into FIT, has received compensation from Clinical Genomics for service as a consultant, and is an inventor on an issued patent for *BCAT1* and *IKZF1* markers.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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