Faecal biomarkers do not always identify pre-cancerous lesions in patients who present in primary care with bowel symptoms

Jacqueline Keenan, Alan Aitchison, Jacquie Leaman, John Pearson, Frank Frizelle

ABSTRACT

AIM: To determine if tumour-derived M2-PK is potentially a more accurate biomarker of pre-cancerous bowel lesions in patients who present in primary care with bowel symptoms than the detection of faecal haemoglobin.

METHODS: Patients requested by their general practitioners (GPs) to provide a stool sample to determine the presence of faecal haemoglobin consented for the same stool samples to be tested for the presence of M2-PK. For comparison M2-PK levels were also measured in stool samples from patients recently identified with colorectal cancer and healthy controls who self-reported no bowel problems at the time of sampling.

RESULTS: M2-PK levels measured in 185 GP-derived samples were comparable to the control cohort (57 healthy controls) and notably lower than those in the 57 patients with CRC (2.6, 3.2 and 18.2U/ml\(^{-1}\), respectively). Sixty-seven of the GP patients were referred for colonoscopy. While 26 of these patients had a positive M2-PK, only 10 were found to have colonic lesions. Conversely, 18 of the 41 patients who had a negative M2-PK were found to have lesions that included one CRC, 13 adenomas and four other polyps. The FIT also failed to identify colonic disease in 19 of 48 patients referred for colonoscopy. There was, however, a significant association between lesions greater than 1cm and a positive FIT (p<0.02) that was not the case with M2-PK. A positive FIT identified one patient in the GP patient cohort subsequently diagnosed with colorectal cancer at follow-up colonoscopy whereas the same stool sample tested negative for M2-PK.

CONCLUSIONS: Measurement of faecal M2-PK levels lacks the specificity and sensitivity (and therefore diagnostic accuracy) to identify the individuals who should be progressed for clinical follow-up. Accordingly, M2-PK is not is not a robust biomarker for identifying pre-cancerous bowel lesions in a primary care setting.

The Piper study showed that in New Zealand 34% of people with colorectal cancer (CRC) present acutely to the emergency department, and 24% of all patients with CRC have metastatic disease at presentation.\(^1\) The prognosis for the patients who present acutely with metastatic disease is generally poor. In contrast, early diagnosis of CRC improves survival. Accordingly, there is a need for accurate screening tools to identify those patients who present in primary care with early stage disease.

The gold standard for early detection of pre-cancerous changes in the bowel is colonoscopy, where adenomas, the potential precursors of cancer, can be detected and removed. Colonoscopy, however, is not used as a population screening tool due to cost (risk)-benefit. Instead, clinicians currently rely on the detection of faecal haemoglobin.
in stool samples. The faecal immunochemical test (FIT) quantifies haemoglobin using antibodies and is shown to provide a higher sensitivity for detecting advanced colorectal neoplasia and CRC than the original guaiac test for faecal occult blood (FOB). However, the FIT is dependent on the presence of blood, meaning that stool samples from patients with non-bleeding (or intermittently bleeding) cancers are likely to be reported as negative. As a result there is a need for other biomarkers to screen for early stage disease that includes adenomas and localised cancers.

Tumour M2-PK is a dimeric isoenzyme of pyruvate kinase expressed by proliferating tumour cells that is released into the faeces of patients with CRC or adenoma. Moreover, this tumour-specific form of the enzyme (hereafter referred to simply as M2-PK) can be measured using a commercially available enzyme-linked immunosorbent assay (ELISA). A meta-analysis of studies using this assay reported the mean faecal M2-PK sensitivity and specificity for diagnosing CRC in asymptomatic individuals as 80.3% and 95.2%, respectively. Additionally, there is evidence suggesting that elevated levels of this faecal protein are a highly sensitive marker for colon cancer and can correlate with tumour staging.

The objective of this study was to determine if measurement of faecal M2-PK is potentially a more accurate screening tool than the FIT test currently used to identify the patients who present in primary care with bowel symptoms, and who are subsequently referred for clinical investigation to identify (or exclude) the presence of pre-cancerous bowel lesions and/or CRC.

Material and methods

Study participants

Stool samples from three patient groups were assessed for M2-PK.

a. Patients who presented to their general practitioners (GPs) with bowel problems and were subsequently referred for a faecal immunochemical test (FIT) to detect the presence of faecal haemoglobin (f-Hb), and who gave informed written consent for their stool sample to also be tested for evidence of M2-PK.

b. Patients with a recent diagnosis of CRC between 2012 and 2014, using standard endoscopic, histological or radiological criteria, were also tested for evidence of faecal M2-PK using a stool sample provided prior to chemo-radiation and/or surgery.

c. Stool samples from a cohort of healthy volunteers who self-reported no evidence of bowel problems at the time of sampling were also tested.

The CRC patient (b) and healthy control (c) cohorts were collected as part of an unrelated study. The three cohorts are described in Table 1. Clinical follow-up on the patients in the GP cohort was monitored for a minimum of 12 months after stool collection.

Faecal immunochemical testing

A qualitative (one-step membrane cassette) immunoassay was used for detecting f-Hb in each stool sample (Ngaio Diagnostics Ltd, Nelson, New Zealand). The samples were collected at home by the participants, who were asked to deliver them to the laboratory as soon as possible. When delivery was delayed (e.g., overnight) patients were asked to store their samples at 4°C. This assay detects human haemoglobin above 50µg of f-Hb per gm of faeces, and is shown to be specific for human haemoglobin.

Faecal M2-PK levels

An ELISA (ScheBo Biotech AG, Giessen, Germany) was used to measure levels of M2-PK in each stool sample following extraction of proteins according to the manufacturer’s instruction. This assay is based on two monoclonal antibodies against the tumour-associated dimeric form of the enzyme. The first recognises and binds M2-PK to the ELISA plate while the second, labelled monoclonal antibody binds to the immobilised M2-PK. The level of secondary antibody binding was measured photometrically and M2-PK values in each sample (tested in duplicate) were calculated against a standard curve.

Statistical analysis

Median and interquartile ranges are reported for age and M2-PK levels as the data is moderately right skewed. T-tests with Satterthwaite’s adjustment for unequal variances were used to compare M2-PK
levels while Fisher's exact test with small sample-adjusted risk ratios were used to compare categories and binary diagnostic tests. Specificity and sensitivity with exact binomial confidence intervals for each diagnostic test were calculated and compared with McNemar's Chi-square test on the non-colonoscopy and colonoscopy groups respectively. ROC analysis was used to assess the cut-off value for the M2-PK diagnostic test. All statistical tests were two-sided, considered significant if \( p < 0.05 \) and performed in the R language for statistical computing version 3.5.0 (Vienna, Austria).

**Ethics**

Patients invited by their GPs to take part in this study all provided written informed consent (as approved by the University of Otago Ethics Committee H14/019). The CRC and healthy control samples were collected as part of a wider study approved by the Upper South A Regional Ethics Committee of New Zealand and again all participants provided written informed consent.

**Results**

One hundred and eighty-nine patients who presented to their GP with bowel problems gave consent for their stool sample to be assayed for evidence of M2-PK as well as f-Hb. Four of these patients were subsequently excluded from the GP-derived cohort because bacterial pathogens (Campylobacter jejuni and Yersinia enterocolitica) and/or parasites (Dientamoeba fragilis, Giardia lamblia and Entamoeba fragilis cysts) were detected in their samples. M2-PK levels were also measured in stool samples from 57 healthy individuals and a similar number of patients with a confirmed diagnosis of colorectal cancer (Table 1).

**FIT results**

The commercial faecal immunohistochemical test (FIT) was positive (>50\( \mu \)g/gm of faeces) in 29 of the 185 samples from the cohort of GP patients (Figure 1). Nineteen of these patients subsequently progressed to further investigation that included real and virtual (CT) colonoscopy. Nine of the 19 (47%) were found to have evidence of colonic lesions that included CRC (n=2), adenomas (n=5) and other polyps (n=2). Apart from the patients identified with CRC, only three of the patients presented with lesions greater than 1cm in size. These included two patients with tubulovillous adenomas and a third patient found to have multiple sessile serrated adenomas. The other four patients with evidence of colonic lesions and a positive FIT test had lesions of less than 1cm, including two patients with tubular adenomas. The remaining 10 FIT-positive patients were reported as having a normal colonoscopy (Figure 1). Patients with diverticulosis were considered normal if no evidence of colonic lesions was found. Our study was observational, not interventional, and the decision not to progress the remaining 10 patients with evidence of f-Hb above the test cut-off to the next level of investigation was made by the respective general practitioners in consultation with the patient.

A further 48 patients in the GP-derived cohort had a colonoscopy, despite having a negative FIT (Figure 1). Colonoscopy (real or virtual) identified abnormal lesions in 19 of these patients. These were histologically identified as adenomas (n=12) and other polyps (n=7). Of these, two lesions were just over 1cm in size while the remaining 17 were less than 1cm. There was a significant association between lesions greater than 1cm and a positive FIT (Figure 1).

**Table 1:** Numbers, average age, M2-PK levels and positivity by cohort.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number</th>
<th>Gender (M:F)</th>
<th>Median age (interquartile range)</th>
<th>Median M2-PK level (U/ml(^{-1})) (interquartile range)</th>
<th>Number (%) M2-PK positive (&gt;4U/ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP patients</td>
<td>185</td>
<td>91:94</td>
<td>59 (51–70)</td>
<td>2.6 (0.5–6.1)</td>
<td>72 (39)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>57</td>
<td>25:32</td>
<td>64 (40–74)</td>
<td>3.2 (0.7–7.0)</td>
<td>21 (37)</td>
</tr>
<tr>
<td>CRC patients</td>
<td>57</td>
<td>33:24</td>
<td>72 (63–76)</td>
<td>18.2 (6.5–31.6)</td>
<td>47 (82)</td>
</tr>
</tbody>
</table>
Samples which tested positive for FIT had over twice the risk of lesions greater than 1cm (risk ratio 2.2, 95% CI 0.7–7.1, p<0.02). The other 29 patients were found to have either a normal colon (n=19) or evidence of diverticular disease (n=10). These findings identified the diagnostic accuracy of the FIT used in this study as a biomarker for clinical follow-up, but also highlighted the lack of sensitivity of this test (Figure 1).

M2PK results
The median M2-PK level in the GP patient stool samples was found to be 2.6U/ml\(^{-1}\) (interquartile range 0.5–6.1). To put this finding into context, M2-PK levels were also measured in stool samples from 57 healthy individuals and a similar number of patients with a confirmed diagnosis of colorectal cancer, as detailed in Table 1. There was little difference in median M2-PK levels across the healthy control and GP patient cohorts (3.2 and 2.6U/ml\(^{-1}\), respectively). In contrast, the level of M2-PK measured in the CRC patients’ stool samples was found to be significantly higher (18.2, interquartile range 6.5–31.6) than the median level in the healthy and GP patient cohorts, a difference that was reflected in the number of CRC patients with an M2-PK level above the threshold of the test (Table 1). Elevated levels of faecal M2-PK (>4U/ml\(^{-1}\)) were detected in 72 of the 185 samples in the GP cohort (Figure 2). While median M2-PK levels were not significantly higher than those in the healthy control cohort (Table 1), maximum levels of 72.4 and 27.8, respectively, suggested differences between the two groups (not shown). This finding, however, did not translate into clinical significance because the presence (or absence) of colonic lesions identified in those patients subsequently progressed to further clinical investigation were similarly distributed, irrespective of their faecal M2-PK levels (Figure 2). Specifically, among the 67 GP patients who subsequently had a colonoscopy, 26 were found to have a positive M2-PK. Ten of these (38%) were found to have evidence of colonic lesions that included CRC (n=1), adenomas (n=6) and other polyps (n=3). Apart from the patient identified with CRC, only two patients with tubulovillous adenomas had lesions reported as being greater than 1cm in size, as did another patient found to have

Table 2: Lesion size versus FIT positivity.

<table>
<thead>
<tr>
<th>Lesion Size</th>
<th>FIT positive</th>
<th>FIT negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion &gt; 1cm</td>
<td>5/9</td>
<td>2/19</td>
</tr>
<tr>
<td>Lesion &lt; 1cm</td>
<td>4/9</td>
<td>17/19</td>
</tr>
</tbody>
</table>

Figure 1: The presence of blood in stool is not an accurate biomarker of clinical follow-up (colonoscopy) or clinical outcome (presence of CRC, adenomas or polyps) in patients presenting to their GP with bowel problems.
multiple sessile serrated adenomas. The other six patients with a positive M2-PK test all had lesions of less than 1cm. This group included three samples histologically characterised as tubular adenomas. The remaining 16 M2-PK-positive patients (62%) reportedly had a normal colon (Figure 2).

A further 41 patients in the GP cohort that had a negative M2-PK were also progressed to clinical follow-up (Figure 2). Colonoscopy (real or virtual) identified abnormal lesions in 18 of these patients, including one patient with an adenocarcinoma in the ascending colon. The remaining 17 patients were histologically identified as having adenomas (n=13) and other polyps (n=4). Of these, two lesions were just over 1cm in size while the remaining 15 were less than 1cm. The other 23 patients were found to have a normal colon.

There was no evidence of different rates of M2-PK positivity between patients who were colonoscoped and those who were not (not shown). Likewise, M2-PK positivity did not reflect lesion size (Table 3).

Collectively, these findings indicate that measurement of an elevated level of faecal M2-PK (>4U/ml) lacks the specificity and sensitivity (and therefore diagnostic accuracy) to identify the individuals who should be progressed for clinical follow-up (Table 4).

Table 3: Lesion size versus M2-PK positivity.

<table>
<thead>
<tr>
<th>Lesion Size</th>
<th>M2-PK positive</th>
<th>M2-PK negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1cm</td>
<td>4/10</td>
<td>3/18</td>
</tr>
<tr>
<td>&lt;1cm</td>
<td>6/10</td>
<td>15/18</td>
</tr>
</tbody>
</table>

Collectively, these findings indicate that measurement of an elevated level of faecal M2-PK (>4U/ml) lacks the specificity and sensitivity (and therefore diagnostic accuracy) to identify the individuals who should be progressed for clinical follow-up (Table 4).

Table 4: Comparison of the diagnostic accuracy of elevated M2-PK and presence of blood in the stool as biomarkers of clinical follow-up.

<table>
<thead>
<tr>
<th></th>
<th>M2-PK &gt;4U/ml¹</th>
<th>Hb &gt;50μg/gm</th>
<th>Difference (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.39</td>
<td>0.28</td>
<td>-0.10 (-0.25,0.04)</td>
<td>0.16</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.61</td>
<td>0.92</td>
<td>0.31 (0.20,0.41)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
M2-PK versus FIT

Individually, FIT and M2-PK failed to identify colonic disease in significant numbers of the GP patients who were progressed to colonoscopy (Figures 1 and 2). The level of M2-PK, however, was found to be significantly higher in those samples that were FIT-positive compared to those that were FIT-negative (mean value 10.3 and 5.1, respectively: p=0.008, unpaired t-test, Figure 3).

Despite this apparently positive association between these two biomarkers, there was no association between M2-PK (cut off of 4U/ml\(^{-1}\)) and a positive FIT using Fisher’s exact test (RR 1.07, 95% CI 0.94–1.23, p=0.30) (Table 5). Moreover, this didn’t change when cut-off for M2-PK was increased to 6, 8 or 10U/ml\(^{-1}\). Half the individuals with evidence of haemoglobin in their stool samples had M2-PK levels below the threshold of the test (Table 5), and a combined positive M2-PK and FIT identified only one of two patients in the GP cohort subsequently diagnosed with colorectal cancer. In the other patient, who was also found to have an ascending colorectal cancer, only the FIT test was positive.

**Table 5:** M2-PK versus FIT positivity.

<table>
<thead>
<tr>
<th></th>
<th>FIT positive</th>
<th>FIT negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2-PK positive</td>
<td>14/29</td>
<td>58/156</td>
</tr>
<tr>
<td>M2-PK negative</td>
<td>15/29</td>
<td>98/156</td>
</tr>
</tbody>
</table>

**Figure 3:** M2-PK levels are significantly increased in FIT-positive samples.

Total versus cut-off values / ROC analysis

ROC analysis of M2-PK with scope (yes/no) as an outcome showed an area under the curve of 0.54 (95% CI 0.45, 0.62). The best threshold for M2-PK by Youden’s criteria was 2.23, which improved specificity to 0.61 (95% CI 0.49, 0.72) at the cost of reducing sensitivity to 0.39 (95% CI 0.42,0.59). The flatness of the ROC curve, demonstrated by the AUC being very close to 0.5, indicates that shifting thresholds does not greatly improve diagnostic accuracy.

**Discussion**

The early identification of patients with colorectal cancer or precancerous lesions leads to better outcomes. This study has found that tumour-derived M2-PK levels in stool samples from patients referred by GPs for faecal haemoglobin testing is not a robust biomarker for identifying pre-cancerous bowel lesions or colorectal cancer. While some might think it would be ideal to colonoscope everyone, the reality of resource limitations and risk of harm from such tests means that tests are used to select those who may proceed. Current best practice (for individuals and/or at-risk populations) is based on the detection of haemoglobin in stool samples. The development of an immunological test that detects the globin moiety of human haemoglobin has increased sensitivity over the original guaiac FOB test, and there is...
evidence to suggest that the overall sensitivity of a one-time FIT for detecting cancer can be as high as 79% with a specificity of 94%. However, important caveats are whether the FIT is quantitative or qualitative and/or the cut-off is reported as the concentration of f-Hb present in the measurement solution rather than the quantity of f-Hb present in the stool. These points need to be taken into consideration when comparing published studies, as discussed in more detail below.

Interest is also growing in the use of assays that can detect elevated levels of other host protein biomarkers in stool samples that might likewise signal increased risk of CRC. Calprotectin has been considered a potential candidate but it is a protein found largely in neutrophils and therefore not cancer-specific. Instead, an increase in the faecal level of the dimeric form of the glycolytic pyruvate kinase isoenzyme M2-PK shows more promise as a biomarker for detecting CRC and larger polyps. The expression of this enzyme, which is upregulated in proliferating tumour cells, is easily quantifiable in stool using a laboratory-based ELISA or point-of-care tests that are reportedly comparable.

Our finding that M2-PK levels were increased in stool samples also found to have evidence of faecal haemoglobin appears to support the idea of screening for this protein biomarker. However, we found no significant association (p=0.30) between detection of f-Hb or M2-PK levels >4U/ml-1 and this did not materially change when we increased the threshold for detecting M2-PK up to 10U/ml-1 (p=0.37). Instead, we found half the individuals with evidence of haemoglobin in their stool samples had M2-PK levels below the threshold of the test. This gains significance with the observation that the FIT used in this study has a threshold of 50µg f-Hb per gram of faeces, a threshold that is increasingly considered comparatively high in a diagnostic setting. To put this into context, a threshold of 40µg of faecal haemoglobin per gram of stool has been set as the threshold for the bowel cancer screening programmes in New Zealand and the Netherlands as a means to reduce the number of patients being triaged for unnecessary colonoscopy.

To date, only 67 of the 185 patients have been referred for colonoscopy (36%) but the results suggest that measurement of M2-PK in stool samples is not more sensitive as a biomarker for detecting pre-cancerous lesions in this group when compared to testing for faecal haemoglobin, despite the high threshold of the FIT. Moreover, the finding of lesions in 18 patients with faecal M2-PK levels less than 4U/ml-1 (including 12 patients found to have dysplastic polyps) supports the meta-analysis by Tonus et al, which shows while the sensitivity of the faecal M2-PK test correlates to the size of colonic lesion, it still detects only 51% of adenomas. This lack of sensitivity is highlighted by our observation that 10 of the 57 CRC patient-derived stool samples had faecal M2-PK levels below the 4U/ml-1 threshold. Conversely, we found patients in the GP cohort who had increased levels of faecal M2-PK, a negative FIT and/or a normal colonoscopy. Collectively our findings are in line with other studies that also find that M2-PK levels in faecal extracts do not always correlate with the detection of faecal haemoglobin and/or the detection of CRC in a diagnostic setting.

An unexpected discovery during this study was the high threshold of the FIT used to screen for evidence of faecal haemoglobin in the Canterbury region of New Zealand. While our study found that samples which tested positive for FIT had over twice the risk of lesions greater than 1cm, setting such a high FIT threshold in assessing symptomatic patients may mean that a number of individuals with smaller (<1cm) pre-cancerous lesions are missed in a primary care setting. The NICE (National Institute for Health and Care Excellence) guidelines recommend a threshold of 10–15µg Hb/gm faeces be used to triage patients presenting in primary care with bowel symptoms. This recommendation is based on evidence showing that optimal FIT performance (maximising sensitivity and specificity) occurs at this lower threshold (10–15µg) whereas increasing the cut-off reduces sensitivity. This is highlighted in our study, where 48 patients were progressed to colonoscopy despite having a negative FIT. Nineteen of these individuals were found to have colorectal lesions, including 12 with...
histological evidence of adenomas with low-grade dysplasia. Reducing the threshold of the FIT may have identified these patients at primary care level. The issue remains, however, that a high rate of false-negative results is still likely in patients presenting with small adenomas.22

In summary, this study finds that while levels of M2-PK were significantly higher in the stools of patients with known CRC than in healthy controls, M2-PK lacks the sensitivity and specificity required of a predictive biomarker, either on its own or in combination with faecal haemoglobin, in a primary care setting.

Competing interests:
Frank Frizelle is the Editor of the New Zealand Medical Journal.

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