Correlation between faecal tumour M2 pyruvate kinase and colonoscopy for the detection of adenomatous neoplasia in a secondary care cohort

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Author Contribution:

AB was responsible for collecting samples, conducting laboratory work, data analysis, writing and editing the manuscript. MB assisted with laboratory work, data analysis, writing and editing the manuscript. DS collected samples and assisted in editing the manuscript. CP and BC devised the study and were responsible for writing and editing the manuscript.

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Introduction

There is a well-described relationship between adenoma detection rates and future mortality from colorectal cancer. Therefore, detecting and removing adenomatous polyps is a pertinent medical issue. Colorectal cancer remains a leading cause of mortality and morbidity worldwide. Colorectal cancer screening programmes have been shown to reduce this mortality: many using faecal occult blood testing (FOBt) prior to colonoscopy as the initial screening tool. Other faecal-based diagnostic tools have been proposed, including, tumour M2-pyruvate kinase (tM2-PK). In the current study, we have used this assay in a novel secondary care cohort of patients derived from (1) the England Bowel Cancer Screening Programme and (2) symptomatic patients in an attempt to determine whether this assay has a role in diverse settings or as an adjunct to existing FOBt based screening.

Method

Patients undergoing colonoscopy in our centre were eligible for inclusion in this prospective study. Patients provided faecal samples immediately prior to bowel preparation. Faecal tM2-PK concentrations were measured using a proprietary ELISA by an investigator blinded to the patients' diagnoses. Sensitivity, specificity, positive predictive value, negative predictive values and ROC analyses were calculated. Kruskal-Wallis and Dunn's post-hoc analyses were applied to numerical data.

Results

96 patients returned faecal samples, of whom, 50 were diagnosed with adenoma and 7 with cancer. Their median age was 68 years. Median faecal tM2-PK concentration in individuals with non-neoplastic colonoscopy results was 3.8 U/mL, in individuals with either adenomatous or malignant disease the faecal tM2-PK concentration was significantly greater (median 7.7 U/mL and 24.4 U/mL, respectively, $p=0.01$). Receiver-operator characteristic (ROC) analysis of the whole population demonstrated that the assay was of limited overall utility, with an area under the curve of 0.66 (95 % CI; 0.55-0.77 %): sensitivity was 72.4 % (95 % CI; 59.1-83.3 %), specificity 48.7 % (95 % CI; 32.4-65.2 %), positive predictive value 67.7 % (95 % CI; 54.7-79.1 %) and negative predictive value 36.7

% (95 % CI; 36.7-71.2). Patients with a prior positive FOBt had more abundant faecal tM2-PK (p=0.03) and its diagnostic accuracy was greater (AUROC 0.82).

Conclusion

The application of faecal tM2-PK ELISA shows clear beneficial outcomes when used as an adjunct to FOBt in a homogenous screening group, such as the BCSP. The interference from a heterogenous group, such as our secondary care cohort, appears to impact upon its diagnostic utility.

There is a correlation between faecal tM2-PK and a finding of neoplasia at colonoscopy. However, whilst in isolation faecal tM2-PK exhibits improved sensitivity compared with FOBt, its lower specificity does not support its use for the investigation or surveillance of colorectal cancer in secondary care. There may be benefits from its use in combination with FOBt as part of national bowel cancer screening, by increasing the likelihood of having colonic neoplasia at colonoscopy.

Introduction

Colorectal cancer is a leading cause of mortality and morbidity worldwide, with an estimated European incidence of 43.5 per 100,000 in 2012 and mortality of 19.5 per 100,000 [1]. UK lifetime risk is 1 in 15 for men or 1 in 19 for women [2]. Across Europe, colorectal cancer is the second most common cause of cancer related mortality [1]. Colorectal cancer carries a significant financial burden for the National Health Service (NHS), with a mean annual cost of £12,000 and £8,800 for those with rectal and non-rectal colon cancer, respectively [3].

A number of well-characterised genetic conditions increase the risk of colorectal cancer, such as Familial Adenomatous Polyposis (FAP), however >90 % of colon cancers arise sporadically from adenomatous polyps. Adenomatous polyps develop over a 10- to 15-year period through the adenoma-carcinoma pathway [4]. The potential for malignant transformation combined with the long lag-time, during which identifiable, treatable, premalignant lesions exist, makes colorectal cancer an excellent candidate for population-based screening. This is supported by evidence that early diagnosis improves outcomes: patients presenting with early, Duke's A stage, disease have a 93.2 % chance of 5-year survival, whilst individuals presenting with advanced, Duke's C or D, disease have only 47.7 % and 6.6 % 5-year survivals, respectively [5].

Europe-wide guidance for bowel cancer screening recommends a faecal occult blood test (FOBt) between the ages of 50-74, followed by colonoscopy if FOBt is positive [6]. Screening programme vary across the UK, for example in Scotland, it is performed from 50 to 74 years of age with optional opt-in after 75 years. Currently in England all people between 60-74 years of age are sent FOBt; there is an optional opt in after 75. Positive FOBt is usually followed by colonoscopy. Two forms of FOBt (immunological, i-FOBt and guaiac, g-FOBt,) are currently used for screening purposes worldwide [7]. Currently in England, g-FOBt is used in the initial part of the screening process.

The g-FOBT uses guaiac (derived from wood resin of Guajacum trees) to detect haem in faeces. This test is particularly prone to false positive results because of ingestion of foods including red meat, carrots, potatoes and figs[8]. The g-FOBt identifies a daily blood loss of approximately 10 mL.

Immunological FOBT can provide both qualitative and quantitative results. Qualitative i-FOBt is an instant and objective test which utilizes immuno-chromatography to detect occult blood loss in faecal. However, most i-FOBts are quantitative measures of faecal haemoglobin using automated immunoturbidimetry. i-FOBt is specific to human haemoglobin and only reacts with the intact haemoglobin molecule. Therefore, i-FOBt requires no dietary restriction. In 2010, a meta-analysis reported a sensitivity (67 %) and specificity (85 %) for immunological FOBt compared with 54 % and 80 % for g-FOBt, for the detection of colorectal cancer and pre-cancerous neoplasia [7]. The low sensitivity of g-FOBt has led to criticism of its use for population-based screening [9].

An alternative faecal-based screening tool is tumour M2-pyruvate kinase (tM2-PK) [9,10]. tM2-PK is an isoenzyme of pyruvate kinase, it is involved in glycolysis and catalyzes the ATP-producing conversion of phosphoenolpyruvate to pyruvate. During tumourigenesis other isoenzymes of pyruvate kinase are lost and there is increased expression of the M2 subtype [11]. In neoplastic tissue, M2-PK is mainly found in the dimeric form, rather than tetrameric, driven by modifications by a number of oncoproteins. Dimeric M2-PK is more abundant in cancerous states, including ovarian, lung and colorectal[12], and has therefore been described as tumour M2-PK (tM2-PK). Abundance of tM2-PK can be quantified by a sandwich enzyme-linked immunosorbent assay (ELISA; ScheBo Biotech AG, Giessen, Germany). A number of studies have demonstrated increased abundance of plasma tM2-PK and correlation with stage of melanoma, thyroid, breast, lung, kidney, oesophageal, gastric, pancreatic, colorectal, ovarian, cervical and renal cell cancer [9,13,14]. Increased plasma concentration of tM2-PK has also been described in other colonic pathology, including diverticulosis and inflammatory bowel disease, however, a meta-analysis, in 2012, concluded that the use of faecal tM2-PK in colorectal cancer screening would "close a clinical gap", because of its high sensitivity and specificity [12].

In the current study, we have assessed the faecal tM2-PK ELISA in a novel secondary care cohort of patients derived from (1) the England Bowel Cancer Screening Programme, being conducted locally, and (2) symptomatic patients attending the Royal Liverpool University Hospital in an attempt to characterise whether this assay may have a role in diverse settings. Such utility may include its use as

a pre-screen to colonoscopy in order to stratify risk and allow for service planning or as an adjunct to the current BCSP.

Materials and Methods

Patient Recruitment

Patients undergoing colonoscopy for the investigation of iron deficiency anaemia, polyp surveillance, intervention for known polyps e.g. endoscopic mucosal resection (EMR), change in bowel habit and investigation of abnormal radiology results were eligible for inclusion. These patients were not part of a dedicated screening programme. Those participating in the England BCSP being conducted locally i.e. after a positive g-FOBT, were also eligible for inclusion. Recruitment was directed at these patients, rather than all patients attending for colonoscopy in order to maximise the burden of adenoma/carcinoma pathology. Collection kits were sent to patients prior to their intended colonoscopy. They were asked to produce their sample within 48 hours of their colonoscopy, but before starting bowel preparation medication. Patients followed the usual dietary modifications required prior to colonoscopy. Samples were returned at the time of colonoscopy: thus, all samples were frozen within 48 hours of egestion. Colonoscopy results, including any histological findings, were recorded. Patients were then categorised as having malignant, adenomatous or non-neoplastic ("normal") results. In those with adenomatous disease location, size and number of polyps were recorded. Polyps were assigned to the adenoma group only when this was confirmed by histology. Hyperplastic polyps were classified as "normal".

Ethical Consideration

Full ethical committee approval was given by NRES Committee South West - Central Bristol, reference 14/SW1162.

Sample Handling and Statistical Analysis

Faecal samples were delivered by patients when they attended for colonoscopy and were immediately transferred to the laboratory and frozen at -20°C in the same glass headspace vials (Supelco, USA) in which they were collected, until analysis. Storage was in line with the ELISA manufacturer's instructions. tM2-PK concentration was measured with a commercially available sandwich ELISA (ScheBo Biotech AG, Giessen, Germany). Assays were performed in our university-based research laboratory by a researcher who had undergone specific training and validation provided by the assay manufacture. This analytical performance of each plate was assessed by interpolated control calculation using a sample of known tM2-PK abundance. If a deviation >15 % from expected concentration was identified assay failed QC and was repeated. All analysis was performed by an investigator blinded to the patients' diagnoses, under standardised conditions. Graphpad Prism 6 was used to compare pathological groups by two tailed t test and Kruskal-Wallis and Dunn's post-hoc analyses (p value significant <0.05), and to determine the area under ROC curves, sensitivity, specificity, positive predictive value and negative predictive values. Further testing was performed using a binomial method McNemar's test.

Results

Patient Characteristics

96 patients returned samples within the allotted time. 36 patients were participating in the BCSP and, therefore, had a prior positive g-FOBt. The remaining 60 were symptomatic patients outside of the BCSP and had an unknown FOBt status: the indications for their procedures were iron deficiency anaemia (n=18), change in bowel habit (n=15), surveillance or intervention for known polyps (n=20), family history of colorectal cancer and abnormal radiology (n=7). 52 of the cohort were male and 44 female. The median age was 68 years. Faecal tM2-PK levels were significantly higher in males (15.37 U/mL (95% CI 10.8-19.8) vs 8.034 U/mL (95% CI 5.01-10.9), p=0.01 by 2-tailed Student's t-test) (Figure 1A). To characterise whether patient's age influenced faecal tM2-PK concentration the cohort was divided into quintiles according to their age at the time of sampling. No significant differences in

mean faecal tM2-PK were identified between any two quintiles (Kruskal-Wallis 1-way ANOVA and Dunn's *post-hoc* analysis; Figure 1B).

tM2-PK concentration is elevated in patients with adenomatous and malignant disease

Median faecal tM2-PK concentration in individuals with non-neoplastic colonoscopy results was 3.8 U/mL, in those with either adenomatous or malignant disease the concentration was significantly greater (median 7.7 U/mL and 24.4 U/mL, respectively: *p*=0.01 by Kruskal Wallis 1-way ANOVA and Dunn's post-hoc analysis, Figure 1C).

Diagnostic Utility

To assess the suitability of the faecal tM2-PK ELISA as a screening test in our cohort, we categorised patients into those with non-neoplastic disease and those with a burden of neoplastic disease by colonoscopy. We performed an ROC analysis on the basis of these two groups and showed that the assay may have limited overall utility with an area under the ROC curve of 0.66 (*p*=0.006) (Figure 2A).

The ELISA manufacturer proposed a cut-off value of 4.0 U/mL. Adopting this as a cut-off to define a positive tM2-PK test, the assay's sensitivity to detect adenoma or carcinoma was 72.4 % (95 % CI; 59.1-83.3 %), specificity 48.7 % (95 % CI; 32.4-65.2 %), positive predictive value 67.7 % (95 % CI; 54.7-79.2 %) and negative predictive value 36.7 % (95 % CI; 36.7-71.2 %). Application of McNemar's test did not show a significant difference between the tM2-PK results and the presence of neoplasia at colonoscopy (*p*=0.73), suggesting a limited relationship between the faecal tM2-PK value and findings at colonoscopy (Appendix 1). There was a false positive rate of 20.6 % across the 96 samples. In total 37 % of samples were incorrectly categorised by tM2-PK testing.

The same statistical analysis was performed using a series of different threshold levels (Table 2 and Figure 2B). The optimal cut-off value identified by the highest McNemar's test was at 4.8: this gave a sensitivity of 69 % and a specificity of 56 %.

Of the 50 patients with adenomatous disease 22 were found to have a single polyp greater than 1cm in size. The median faecal tM2-PK reading for this group was 5.7 U/mL (interquartile range 1.3-19.5). Of these patients, 59 % (n=13) had a faecal tM2-PK result above the 4.0 U/mL cut-off, representing a true positive result by the assay manufacturer's criteria. There was no significant difference in the tM2-PK concentration detectable in the faeces of patients with polyps >1cm or <1cm, p=0.63 (Figure 3A). There was also no significant difference in the tM2-PK concentration in faeces of those with <5 or ≥5 adenomatous polyps (Figure 3B).

Assessment of role as adjunct to FOBt in BCSP

When we isolated those individuals (n=36) referred for colonoscopy as part of the BCSP those with neoplasia (n=25) were found to have significantly greater faecal tM2-PKconcentration than those without neoplastic pathology (Figure 4A, p=0.03). A macroscopically normal colonoscopy was seen in all BCSP patients who did not otherwise have neoplastic disease (n=11). ROC analysis of the BCSP patients alone demonstrated an AUROC of 0.82 (p=0.002) (Figure 4B). Sixty one percent of patients with a prior positive fFBOt also had a positive tM2-PK. Of the BCSP with no neoplasia 72% had a negative tM2-PK, whilst 76% of those with neoplasia had a positive tM2-PK.

This difference was not observed in the non-BCSP group; 29 of whom were classified as non-neoplastic, of these 7 had IBD and/or diverticulosis. Within this subset, 6 had elevated (false positive) tM2-PK concentration. The remaining 22 subjects had a macroscopically normal colonoscopy, of whom 11 received a true negative faecal tM2-PK result, whilst 11 had falsely positive results. Within this subset AUROC was not significantly different to 0.5 (AUROC 0.51, p=0.87)(Figure 4C).

Discussion

The faecal tM2-PK concentration observed in this study demonstrated an increasing trend as pathology progressed along the adenoma-carcinoma pathway, supporting potential utility for the assay to distinguish between absence and presence of neoplastic disease.

Our cohort contained more males, with a median age of 68, therefore representative of a potential colorectal cancer population. The faecal tM2-PK concentration was higher in males, with age appearing to have no impact on the abundance of this enzyme. In a clinical setting, this lack of variation with age could be deemed beneficial.

Based on the manufacturer's threshold value of 4 U/mL, we found that the assay's sensitivity to detect any adenomatous pathology was 72 % with specificity of 48 %. This is consistent with the findings of a meta-analysis, from 2012, which examined seventeen studies and found the quoted sensitivities, for colorectal cancer, to range from 68-97 % and adenoma detection to range from 28-76 %.Within the meta-analysis several of the studies focused solely on the detection of carcinoma, rather than adenomatous polyps.

To optimise the diagnostic accuracy of a faecal tM2-PK assay within a future screening programme the diagnostic threshold for the assay could be adjusted away from the manufacturer's recommended threshold of 4 U/mL. To understand whether this process may have utility we applied a series of thresholds to our data set and calculated binomial McNemar statistics to define the threshold with optimal diagnostic utility for our cohort. Using this methodology we demonstrated optimal utility with a threshold of 4.8 U/mL. This led to an improved sensitivity of 69.0 % and specificity of 56.4 % in our cohort. At this threshold 40.2 % of tests yielded a true positive results, 22.7% a true negative, whilst 17.5 % were false positive results and 19.5 % were false negative results. One of the obvious frailties of this methodology is that with this statistical approach the consequences of false negative and false positive results are conferred equal weighting. This is clearly not the case in this clinical situation where the consequences of a false negative result manifestly outweigh those of a false positive.

Using the manufacturer's recommended threshold of 4 U/mL the faecal tM2-PK assay had a lower false negative rate of 16.5 %, but an increased false positive rate of 20.6 % in our cohort. Whilst this threshold could reduce the number of patients with adenomatous disease that were not detected by the faecal tM2-PK assay, it would lead to an increased number of endoscopic procedures that yielded no

adenomatous pathology being performed. This false positive rate is substantially higher than that reported by Tonus *et al*, but is consistent with a study from 2006 which reported a low specificity with high false positive rate [15], suggesting that it may be within the range expected in a clinical cohort. One study from the USA estimated that a 5–10 % false positive rate during colorectal screening would amount to an expense of US$6 billion if 100 % participation rate is assumed [16]. The current UK Bowel Cancer Screening Program reports a sensitivity of 36.5 % and specificity of 92.2 % for g-FOBT. The literature suggests that if a different screening tool were to be implemented, for example i-FOBT, a higher detection rate could be achieved e.g. a sensitivity of 70.9 % and specificity of 96.3 % [17]. The consequence of a false negative test in bowel cancer screening is grave and thereforethe lower specificity associated with tM2-PK testing will mean it is unlikely to replace either of these methods in colorectal screening or as a preliminary test in a secondary care based population. False positive results have been reported in inflammatory bowel disease, infective disease and diverticulosis[18,19], this is replicated in our cohort as those with non neoplastic pathology and no prior FOBt had a false positive rate of 50 %.

The combination of FOBt with faecal tM2-PK ELISA has been proposed as a strategy to optimise the sensitivity and specificity for a screening programme. When we segregated our cohort into those recruited from the BCSP and those with other indications for colonoscopy we demonstrated greater diagnostic utility for tM2-PK ELISA in patients recruited from the bowel cancer screening population (Figure 4). This was associated with a significantly lower median tM2-PK concentration in the faeces of patients without adenomatous disease in the BCSP cohort compared to those without adenomatous disease in the non-BCSP cohort. This suggests that the utility of faecal tM2-PK as a biomarker for colonic adenomatous disease may be limited to the relatively homogenous asymptomatic screening cohort, rather than the more heterogenous group of symptomatic patients.

A study from 2014 assessed the utility of g-FOBt with faecal tM2-PK ELISA prior to potential colonoscopy. 1800 individuals were invited to participate, with a response rate of 54 % (n=978), culminating in over 800 analysable samples being returned and 186 colonoscopies being performed. Overall, positivity was significantly increased by the addition of tM2-PK (27 %). Had only i-FOBt

been tested, 77 % (n=189) fewer patients would have had a positive faecal test and been identified for a screening colonoscopy and 70 % (n=35) fewer patients would have had polyps detected and removed[20].. This study also reported that 10 % (n=99) of tM2-PK samples could not be analysed, because of a lapse in the 48 hour window required for its processing. This is clearly an issue for a postal based screening programme as is currently in operation in the UK. A further study from Italy assessed the impact of combined testing on 280 samples. For colorectal cancer detection, i-FOBt was the test with the highest specificity and positive predictive value (0.89 and 0.53), whereas tM2-PK had the highest sensitivity and negative predictive value (0.87 and 0.96). It demonstrated a good ability of the combined test to identify colorectal cancer, with patients showing positivity to both markers, the risk of cancer was as high as 79 % [21]. Other assays have been assessed in combination with FOBT, including faecal calprotectin, which demonstrated sensitivity for the detection of colorectal cancer of 79 %, whereas the sensitivity for the combination of i-FOBT and tM2-PK was 93 % [22].

An analysis of 697 patients in the Cleveland Clinic Foundation Adenoma Registry showed that, compared with one to two small adenomas, risk is increased 10-fold after removal of multiple adenomas at least one of which is larger than 1 cm [23], and adenomas of >1cm diameter identify individuals at increased risk of colorectal cancer even if removed [24]. It is therefore clinically important to identify polyps of >1cm diameter. With increasing size, one might predict increased abundance of tM2-PK, however within our cohort this was not the case.

There is a similar increase in risk for those with ≥5 individual polyps, even if they are all <1cm in size. The apparent utility of the tM2-PK assay to detect polyps irrelevant of size makes it appealing for use in a screening or surveillance programme. Another beneficial element of this finding is that polyps <1cm do not tend to bleed and, therefore, may be missed by FOBt [25]. The tM2-PK assay does not have this problem as it does not rely on the presence of blood and, therefore, has the potential to detect non-bleeding adenomatous disease. Moreover, in our cohort no difference in the abundance of tM2-PK was identified in those with 5 or more adenomas, compared to those with fewer.

The application of tM2-PK shows potentially beneficial outcomes when used in a homogenous clinical group, such as the BCSP. The interference from a heterogeneous population, such as our secondary care cohort appears to impact upon the diagnostic utility of faecal tM2-PK analysis. A dedicated randomised controlled study would be required to explore this potential combination further. We must also concede that the relatively small number of patients included in this study is a limitation.

Conclusion

In our secondary care cohort, the faecal tM2-PK assay appears to have a sensitivity that is comparable to, and in some instances, superior to existing faecal markers used as part of colorectal cancer screening programmes. The present study demonstrates a superior sensitivity to that associated with g-FOBt. However, the main issues that will prevent its independent use are its comparatively low specificity, high false positive rate and time constraints for sample collection. Its use with existing FOBt methods does however seem appealing. Combining the two methods appears to rectify some of the flaws of each, and could improve the accuracy of an initial faecal biomarker screening tool. One role may be the sequential use of gFOBt followed by tM2-PK. However, the introduction of this type of screening would represent additional time and financial burdens that may constrain the use of these investigations. Ultimately, in order to justify their use, a reduction in mortality from colorectal cancer with such screening tools would need to be demonstrated.

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Appendix:

Appendix 1: 2x2 table used for McNemar test when using a cut-off of 4.0U/mL.

	Colonoscopy positive	Colonoscopy negative
Faecal tM2-PK positive	42	20
Faecal tM2-PK negative	16	19

Figure 1: A) Bar chart representing concentration of tM2-PK according to gender of patient (*p<.05, by Student's *t*-test). B) Bar chart representing concentration of tM2-PK according to age of patients separated by quintiles. C) Box and whisker plot demonstrating tM2-PK concentration stratified by diagnosis at colonoscopy. (*p<0.05, by Kruskal-Wallis 1-way ANOVA and Dunn's *post-hoc* analysis)

Figure 2: A) ROC curve showing utility of faecal tM2-PK ELISA for detection of adenomatous disease in this cohort, shaded area represents 95 % confidence intervals. B) Surface plot demonstrating the variation in diagnostic accuracy as the diagnostic threshold concentration changes FN: false negative, FP: false positive, TN: true negative, TP: true positive.

Figure 3: A) Box and whisker plot showing faecal tM2-PK concentration stratified by size of largest adenoma. B) Box and whisker plot of faecal tM2-PK concentration stratified by the number of adenomas indentified in each patient.
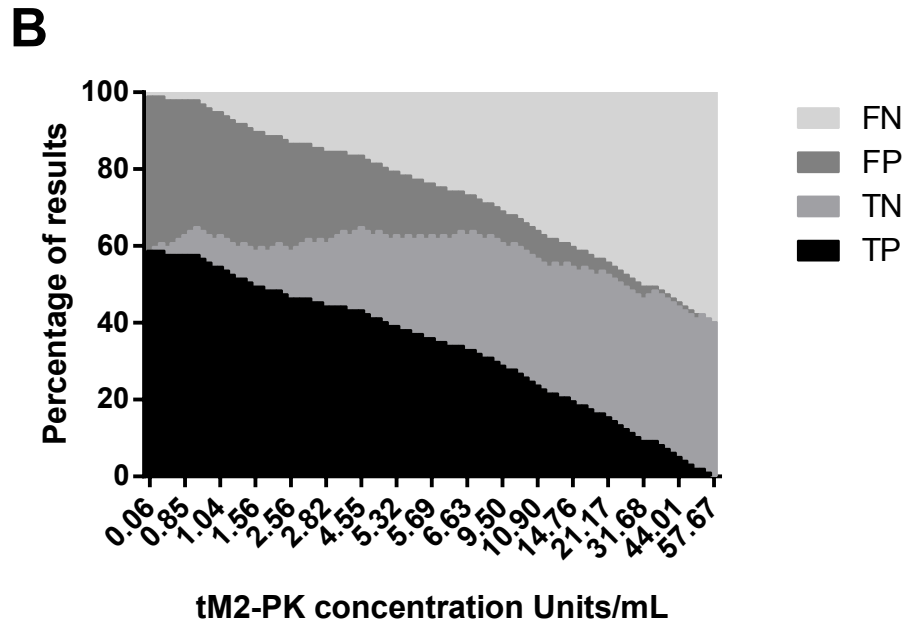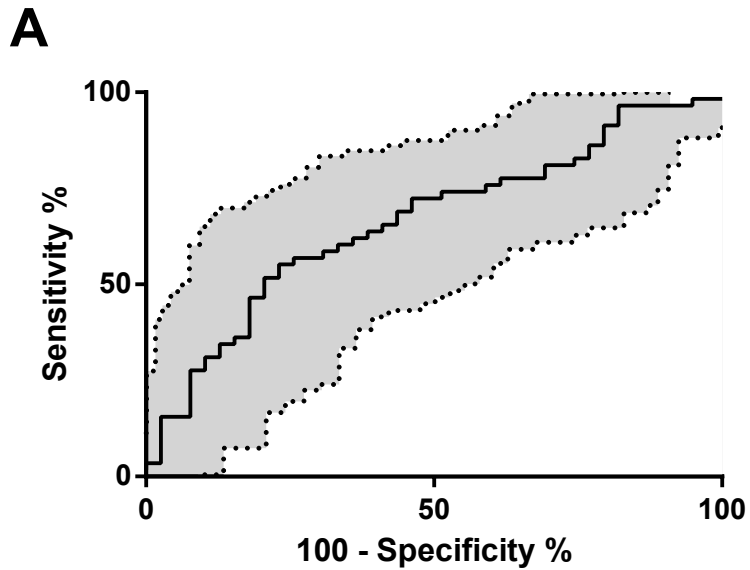
Figure 4: A) Box and whisker plot demonstrating faecal tM2-PK concentration in patients recruited via BCSP or non-BCSP route, and with or without adenomatous pathology (*p<0.05, **p<0.01, by Kruskal Wallis 1-way ANOVA and Dunn's *post-hoc* analysis). B) ROC curve demonstrating the diagnostic accuracy of faecal tM2-PK ELISA in patients recruited following colonoscopy referral from the BCSP. C) ROC curve demonstrating the diagnostic accuracy of faecal tM2-PK ELISA in patients not recruited via the BCSP. Shaded areas represent 95 % CI.
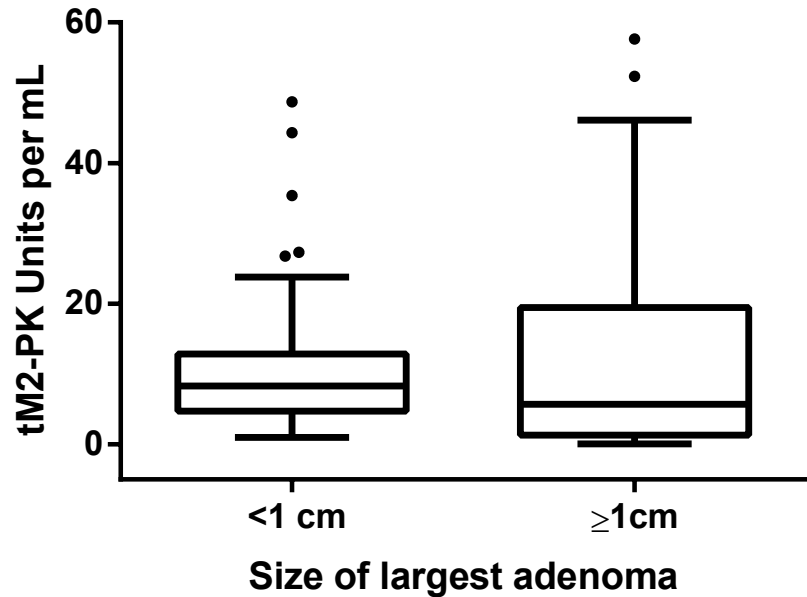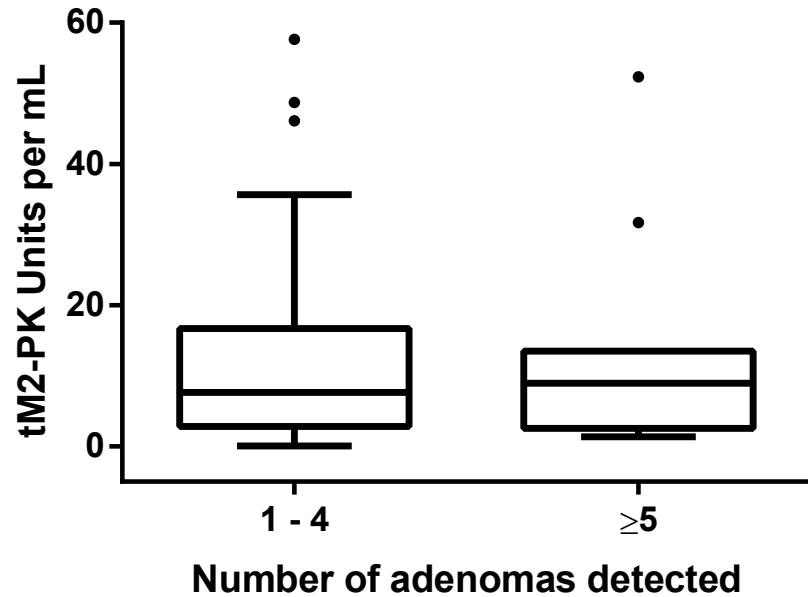
Table 1: Table showing the sensitivity and specificity of different faecal tM2-PK thresholds for the study cohort, with binomial method McNemar values for each of these thresholds.
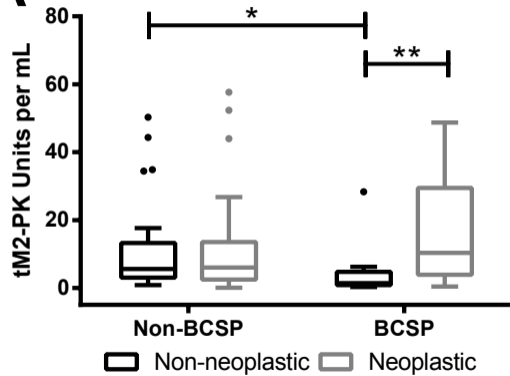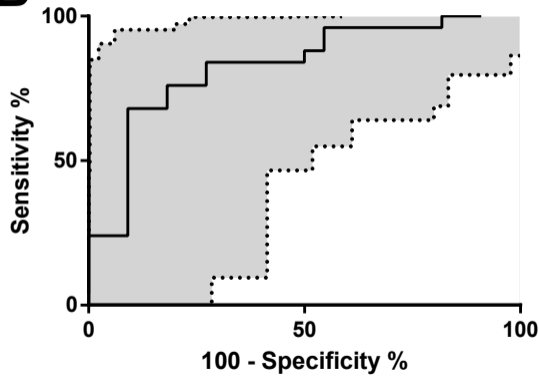
Threshold	Sensitivity %	95 % CI	Specificity %	95 % CI	Likelihood ratio	McNemar Binomial p-value
3	75.86	62.83 to 86.13	41.03	25.57 to 57.90	1.286	0.2559
3.2	74.14	60.96 to 84.75	41.03	25.57 to 57.90	1.257	0.2559
3.4	74.14	60.96 to 84.75	43.59	27.81 to 60.38	1.314	0.324
3.6	74.14	60.96 to 84.75	43.59	27.81 to 60.38	1.314	0.324
3.8	74.14	60.96 to 84.75	48.72	32.42 to 65.22	1.446	0.6177
4	72.41	59.10 to 83.34	48.72	32.42 to 65.22	1.412	0.7359
4.2	72.41	59.10 to 83.34	48.72	32.42 to 65.22	1.412	0.7359
4.4	72.41	59.10 to	51.28	34.78 to	1.486	0.7359

		83.34		67.58		
4.6	72.41	59.10 to 83.34	53.85	37.18 to 69.91	1.569	0.8642
4.8	68.97	55.46 to 80.46	56.41	39.62 to 72.19	1.582	0.8679
5.0	67.24	53.66 to 78.99	56.41	39.62 to 72.19	1.543	0.7428
5.2	65.52	51.88 to 77.51	56.41	39.62 to 72.19	1.503	0.7428