M2-PK as a novel marker in ovarian cancer. A prospective cohort study

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Summary

Background: Pyruvate kinase isoenzyme M2-PK is instrumental to tumour metabolism and hence over-expressed in tumour cells leading to detectable plasma concentrations.

Objectives: To assess the degree of association between M2-PK plasma concentrations and ovarian cancer and to determine the cut-off values for its sensitivity and specificity for differentiating between benign and malignant ovarian disease.

Settings: The Gynaecological Cancer Centre at both King's College and St. Thomas' Hospitals, London, UK.

Methods: Patients with suspected ovarian cancer referred to the above centre were recruited prospectively during the years 2004-2005. Blood samples were collected before surgery for plasma M2-PK assays. Results were assessed with respect to cancer diagnosis, patient and tumour characteristics. Statistical analysis including the receiver operator characteristic (ROC) curve was performed using Analyse-It® and SPSS V13®.

Results: 100 patients with age range 14-88 years and a median of 57 years were recruited in the study. Of whom 52 were diagnosed with invasive ovarian cancer. Of these 35 (67%) were Stage III and above with two secondary tumours. M2-PK was not related to patient age ($p = 0.43$). There was a significant correlation between CA125 and M2-PK ($p < 0.001$). The mean M2-PK concentration in cancer patients was 52 U/ml versus 27 U/ml in patients with benign conditions ($p < 0.001$). At a cut-off value of 22 U/ml the sensitivity of M2-PK for detecting cancer was 70% with a specificity of 65%.

Conclusion: M2-PK was significantly raised in ovarian cancer patients, however its role in clinical practice needs further evaluation.

Key words: Ovarian cancer; Pyruvate Kinase; M2-PK; Tumour Marker, Diagnosis.

Introduction

Ovarian cancer is the fourth commonest cause of cancer deaths in women and the leading cause of mortality from gynaecological malignancies in the Western world. The lifetime risk of developing ovarian cancer is one in 75 with 6,190 new cases and 4,560 deaths recorded in the UK in 2000 [1-3]. Diagnosis at an early stage carries a better prognosis with a five-year survival approaching 90% in Stage I disease versus less than 30% in Stages III and above [4, 5]. Screening for ovarian cancer is fraught with difficulties due to the absence of a recognizable premalignant condition and efforts are focusing on the early detection of small volume disease. However, the majority of ovarian cancer patients are diagnosed at an advanced stage due to the lack of reliable early symptoms or accurate diagnostic tools.

Tumour markers are biologic substances produced by malignant tumour cells that are measurable in the circulation. Ideally, they should be tumour specific and have very high sensitivity, specificity and positive predictive value. They should be detectable in early disease and related to tumour load [6]. The tumour marker CA125 has long been used as a screening and diagnostic tool for ovarian cancer. Nevertheless, its elevated serum concentrations in many benign and non-gynaecological malignant conditions have limited its positive predictive value [7]. Moreover, plasma concentrations of CA125 are only raised in 50% of early-stage disease [8]. Therefore, there is a need to explore other potential useful markers.

Tumour metabolism is characterized by the ability of proliferating cancer cells to survive the unstable nutritional conditions and varying degrees of blood supply. Specific adaptation mechanisms occur via changes in the isoenzyme composition, and share a common pathway during tumourigenesis; regardless of the tumour type or cell lines [9, 10]. Pyruvate kinase is a key enzyme in tumour metabolism and glycolytic pathways. In its active tetrameric form, pyruvate kinase is responsible for ATP synthesis and energy production. Four different isoenzymes (L, R, M1, and M2) are tissue specifically expressed in this active tetrameric form. However, during tumour formation, a shift in the isoenzyme composition consistently occurs whereby the tissue specific form disappears and the isoenzyme pyruvate kinase type M2 (M2-PK) is over-expressed [11]. During the tumour metabolic process, M2-PK switches between two forms, the active tetrameric and the inactive dimeric forms. While the active form is part of the glycolytic pathway responsible for energy production, the inactive dimeric form is over-expressed in tumour tissue and hence often referred to as Tumour M2-PK. This relatively inactive dimeric form, Tumour M2-PK, is responsible for the accumulation and channeling of phosphometabolite sub-

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strates for synthetic processes such as nucleic acid and protein synthesis required by rapidly proliferating cells. Thus, in tumour tissue cells the utilization of metabolic products for synthetic processes occurs at the expense of ATP and energy production. The over-expressed Tumour M2-PK is released by tumour cells into the blood and other body fluids where it can be detected in plasma samples taken from patients with a variety of cancers [11-14]. This study assesses the potential use of Tumour M2-PK in ovarian cancer patients.

Patients and Methods

Study objectives

This study was set out with the aim to a) assess the association between Tumour M2-PK plasma concentrations and ovarian cancer and b) determine the cut-off values for M2-PK sensitivity and specificity to differentiate benign from malignant ovarian disease.

Study design and recruitment

This is a prospective observational cohort study conducted at the gynaecological oncology centre and the gynaecology unit at two University Teaching Hospitals of the South East London Cancer Network, London, UK. Seventy-two patients with suspected ovarian malignancy and 28 with benign gynaecological conditions were recruited. For those with suspected ovarian cancer, the inclusion criteria were the presence of pelvic mass and/or ascites confirmed on imaging together with abnormally elevated serum CA125 concentrations or risk of malignancy index (RMI) exceeding a score of 250 [15].

Patients concomitantly receiving chemotherapy, those with moderate or severe renal failure and those participating in other studies were excluded. The study was approved by the ethics committee and patients received the relevant information leaflet detailing the study rationale and protocol prior to giving their informed consent. This cohort study was conducted under single blind conditions aiming to minimise bias on the part of the subjects, investigators and analysts. Clinical information was collected in a standard and confidential case report form. Results were not used in any clinical decision making or made known to the subjects or investigators until the study was completed.

Preoperative

Patient demographics and details of their clinical condition and preoperative investigations were recorded and stored in a secure database.

Preoperative blood samples for Tumour M2-PK were obtained using venepuncture with minimal stasis. Samples were withdrawn into 5 ml EDTA tubes and centrifuged for 10 min at 3000 rpm. Plasma aliquots were separated and stored in -70°C freezers until analysed.

According to standard management, preoperative blood samples for CA125 measurement were taken into 5 ml tubes without anticoagulant. Serum aliquots were separated by centrifugation as above. CA125 concentrations were measured using an immunometric sandwich assay on the ADVIA Centaur analyzer (Bayer Diagnostics, Newbury, Berks) with a cut-off value for normal of ≤ 35 kU/l.

Laparotomy

Patients underwent staging laparotomy as described in the FIGO surgical staging for ovarian cancer [16].

Tissues removed at laparotomy were histologically examined using conventional and immunohistochemistry methods. Histological diagnoses of ovarian cancer and grading were established according to the WHO and Gynecologic Oncology Group (GOG) criteria [17, 18]. Histological subtypes were documented and surgicopathological staging revised if necessary.

Plasma M2-PK assay

The Tumour M2-PK isoenzyme is released from tumour cells and is quantitatively detectable in body fluids where its concentration can be assessed with respect to a diagnosis of malignancy; its concentration is quantitatively measured in EDTA plasma via a highly sensitive enzyme-linked immunosorbent assay (ELISA) developed by ScheBo-Tec® (Giessen, Germany). The test is based on two monoclonal antibodies which specifically react with Tumour M2-PK and do not cross-react with the other isoenzymes.

The first step comprises binding of the plasma Tumour M2-PK to the first monoclonal antibody whereby it is immobilised on the antibody coated ELISA plate. During the next incubation period, a second monoclonal antibody which is biotinylated binds to the immobilised tumour M2-PK. Then a conjugate of peroxidase and streptavidin binds to the biotin moiety. Subsequently the peroxidase oxidizes 3,3'5,5'-tetra-methyl benzidine (TMB) which turns yellow. Finally, the concentration of the oxidized TMB is determined photometrically. The test kit allows the quantification of Tumour M2-PK within the range of 5 to 100 units/ml (U/ml) in EDTA plasma; however, values above 100 U/ml following sample dilution were accurately recorded for the purpose of this study. The manufacturer average intra-assay coefficient of variance (CV) is 3.5% (2.4-7.0%).

Statistical analysis

The data were recorded on an investigative report form. Statistical analysis was carried out using Analyse-It® and SPSS® V13 software. Descriptive statistics of patient characteristics were calculated using simple methods for the mean, median, standard deviation (SD), minimum and maximum values. Comparison of group means was carried out using both parametric and non-parametric tests. The chi-square test was used to compare categorical data.

The receiver operator characteristic (ROC) curve was used for analysis of the presurgical results of the Tumour M2-PK to determine cut-off values for both its specificity and sensitivity in the diagnosis of ovarian cancer. Negative and positive predictive values are also displayed. Correlation matrix and coefficient of correlation (r) for relationship of different variables were calculated using Spearman rank non-parametric correlation statistics.

Results

Patient characteristics

There were 100 patients recruited for the study; the age range was 14-88 years with a mean of 55.5 and a median of 57 years. Patients with cancer had a mean age of 61 years (95% CI: 57-65 years; $p < 0.05$) and presented mainly with increased abdominal girth and pain (52%) with a duration of symptoms from one to three months in the majority of them (55%). Table 1 demonstrates important patient characteristics. There were 52 patients with histological diagnoses of cancer, 35 with benign conditions and 13 patients had histology results confirming

Table 1. — *Selected patient characteristics*

Patient characteristics		Cancer diagnosis Yes (n = 52)	No (n = 48)	% Total
Menopausal status	Postmenopausal	41%	19%	60%
	Premenopausal	11%	29%	40%
Patients' parity	P0	11%	16%	27%
	P1	12%	6%	18%
	> P1	29%	26%	55%
Past history of cancer	No	41%	42%	83%
	Yes	11%	6%	17%
Family history of cancer	No	41%	42%	83%
	Yes	11%	6%	17%
Presence of ascites	Yes	31%	10%	41%
	No	21%	38%	59%

borderline ovarian tumours. In total, there were 31 patients with Stage I-II disease and 34 in Stage III-IV. The mean preoperative serum creatinine for cancer patients was 69 umol/l (95% CI: 65-74 umol/l) which was not significantly different from that of non cancer patients (mean: 71; 95% CI: 68-74 umol/l; p = 0.33).

Tumour M2-PK results

Discrimination between cancer and non cancer patients: Preoperative Tumour M2-PK results were available for all the 100 patients; however five cases were excluded from the analysis as they had received neo-adjuvant chemotherapy prior to their staging laparotomy. Only patients with histological confirmation of invasive cancer (n = 52) are defined as cancer positive; of whom 47 were entered in the analysis. The median preoperative plasma Tumour M2-PK value for cancer patients was 35 U/ml with a mean of 52 U/ml (95% CI: 38-65 U/ml), and for those without histological confirmation of cancer results were 18 U/ml and 27 U/ml (95% CI: 20-35 U/ml) for the median and mean, respectively; the difference was highly significant (p = 0.001). (Table 2, Figure 1). For patients with histological confirmation of cancer, the median preoperative plasma Tumour M2-PK in the presence of ascites was 44 U/ml with a mean of 65 U/ml (95% CI: 44-86 U/ml). In the absence of ascites, the median and mean values for Tumour M2-PK were 23 U/ml and 32 U/ml (95% CI: 20-44 U/ml) respectively; this was significantly different compared with levels recorded in the presence of ascites (p = 0.015); (Figure 2). This echoed the pattern of change of preoperative serum CA125 concentrations in association with the presence of ascites, albeit with less variation; the median and mean values were 509 kU/l and 717 kU/l (95% CI: 485-949 kU/l) respectively for cancer patients with ascites compared with 64 kU/l and 394 kU/l (95% CI: 5-904 kU/l) for the median and mean, respectively, in the absence of ascites (p < 0.001). Interestingly, when looking at invasive cancer and borderline tumour patients, Tumour M2-PK concentrations were significantly higher in late stage (FIGO III & IV) compared with early stage (FIGO I & II) disease (p < 0.05), (Table 2).

Table 2. — *Plasma tumour M2-PK by cancer diagnosis and FIGO stage.*

Plasma tumour M2-PK (U/ml)	Cancer diagnosis Yes (n = 52)	No (n = 48)	p
Median	35	18	
Mean	52	27	< 0.001
95% CI	38-65	20-35	
Std. deviation	46	25	
Range	186	145	
Interquartile range	52	23	
Plasma tumour M2-PK (U/ml)	**FIGO stage I-II**	**III-IV**	**p**
Median	21	36	
Mean	42	52	< 0.05
95% CI	24-59	36-68	
Std. deviation	43	43	
Range	186	147	
Interquartile range	55	37	

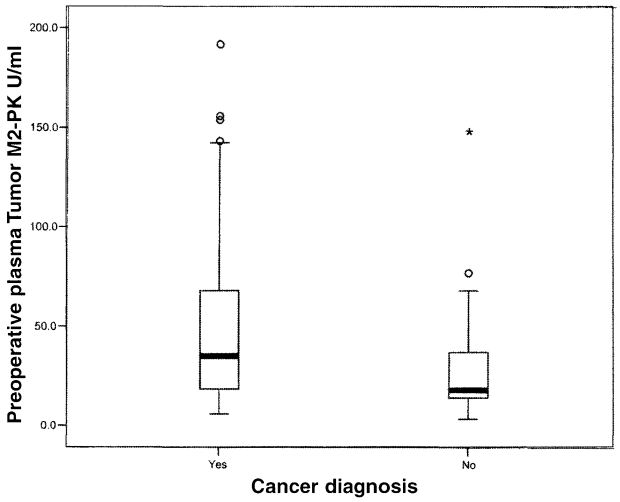
Table 3. — *Diagnostic parameters for Tumour M2-PK and CA125 blood tests*

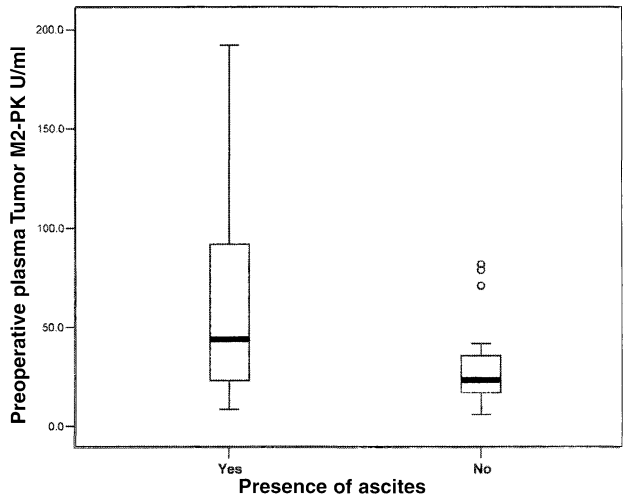
Test	Tumour M2-PK	CA125
Sensitivity %	70	82
Specificity %	65	60
Positive predictive value %	66	68
Negative predictive value %	69	75
Test efficacy % (95% CI)*	67 (57-76)	71 (61-79)
PLR	1.98 (95% CI: 1.3-3)	2 (95% CI: 1.4-3)
NLR	0.46 (95% CI: 0.3-0.8)	0.3 (95% CI: 0.16-0.6)
DOR	4.3 (95% CI: 1.8-10)	6.6 (95% CI: 2.4-17.7)

*p > 0.05; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; DOR: Diagnostic odds ratio

Correlation Statistics: There was strong correlation between the preoperative concentrations of Tumour M2-PK and CA125 (p < 0.001), (Figure 3). Importantly, Tumour M2-PK did not correlate with either patient age (p = 0.37) or serum creatinine (p = 0.27).

ROC for Tumour M2-PK: The ROC curve was performed for Tumour M2-PK values between the observed minimum and maximum records of 3.6 U/ml and 192 U/ml, respectively; all patients were included apart from the five cases who received neo-adjuvant chemotherapy. At a cut-off value of 22 U/ml, the sensitivity of Tumour M2-PK for detecting cancer was 70% with a specificity of 65%; the positive and negative predictive values (PPV, NPV) at this cut-off point were 66% and 69%, respectively. The efficacy of a test is the probability that the test results and the diagnosis agree. It is calculated as: (true positives + true negatives)/(all positives + all negatives) x 100. The calculated efficacy for the Tumour M2-PK at 22 U/ml cut off was 67%; this concentration yielded the best performance with respect to diagnostic accuracy. The equivalent results for the observed values of the preoperative serum CA125 taking 35 kU/l as a cut-off point were calculated, and showed sensitivity, specificity, PPV and NPV of 82%, 60%, 68% and 75%, respectively. The





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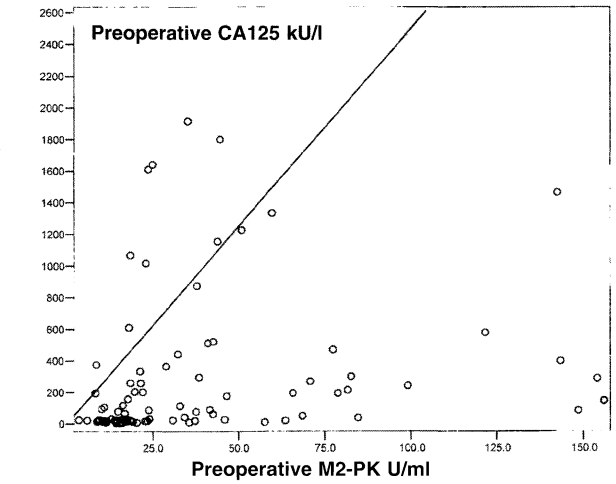

* p < 0.001

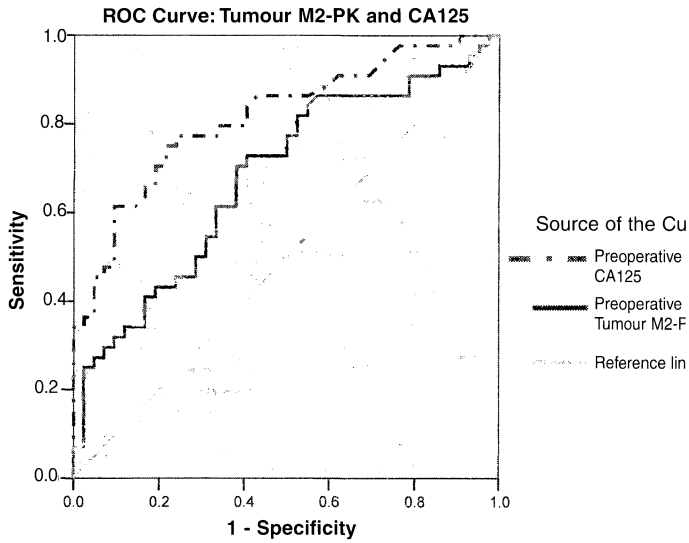

Figure 1. — Plasma Tumour M2-PK in cancer and non cancer patients.
Figure 2. — Plasma Tumour M2-PK levels in ovarian cancer patients with ascites versus those without*.
Figure 3. — Correlation between Tumour M2-PK and CA125*.
Figure 4. — ROC for Tumour M2-PK and CA125.

diagnostic efficacy observed using serum CA125 in our cohort was 71%. Although the standard CA125 test performed generally better than Tumour M2-PK, except for specificity, the odds ratio for a clinical difference in the diagnostic efficacy between both tests was 0.85 (95% CI: 0.45-1.59) which is not statistically significant (Table 3, Figure 4). Another way of looking at diagnostic test performance is the likelihood ratio (LR) and diagnostic odds ratio (DOR). The "positive LR" (> 1) indicates an increased probability of the disease if the test is "positive". Similarly, a "negative LR" (< 1) indicates a decreased probability of the disease if the test is "negative"; whereas a LR of one indicates no change in the likelihood of the disease. The DOR of a test is the ratio of the odds of positivity in a disease condition relative to the odds of positivity in the non-diseased (i.e., the odds of disease in test positives relative to the odds of disease in test negatives); it is not prevalence-dependent and is often used as a single indicator of the discriminative power of a test [19]. The DOR has the value of 1 if the test does not discriminate between disease and non-disease; the higher the values above one the better discrimination power. Values less than one indicates the test application is faulty. Table 3 demonstrates the relevant lR and DOR of both serum CA125 and plasma Tumour M2-PK at the 35 kU/l and 22 U/ml concentrations, respectively.

Table 4. — *Studies for Tumour M2-PK*

Study	Description
Schulze 2000 [20]	Retrospective analysis of 413 patients with a variety of gastrointestinal cancers. The sensitivity of Tumour M2-PK was 73% for pancreatic, 67% for gastric, 59% for oesophageal, and 50% for colorectal cancers. Interestingly, these results outperformed those of the CEA, CA 72-4 and CA19-9, except for the case of CA19-9 in pancreatic cancer.
Roigas *et al.* 2001 [21]	Tumour M2-PK concentrations were compared in 63 patients with renal cell carcinoma (RCC), 36 with bladder transitional cell carcinoma, 58 patients with prostate cancer and 28 with benign prostatic hyperplasia compared with 57 healthy individuals. Tumour M2-PK was only significantly elevated in those with RCC; particularly in metastatic disease (sensitivity 66.7%). It was concluded that Tumour M2-PK could be used as a marker for advanced RCC.
Ventrucci *et al.* 2004 [22]	Tumour M2-PK concentrations were assessed in 265 patients with a variety of malignant and non malignant gastrointestinal diseases as well as healthy individuals. Sixty patients had confirmed pancreatic cancer. Tumour M2-PK sensitivity to detect pancreatic cancer was 85% with specificity of 41%; when combined with CA19-9 the sensitivity improved to 97% but specificity lowered to 38%. Tumour M2-PK concentrations were also elevated in patients with other digestive tract malignancies or neuroendocrine tumours.
Bena-Boupda *et al.* 2003	Tumour M2-PK was studied in 26 patients with metastatic thyroid carcinoma. At a cut-off level of 15 U/ml, Tumour M2-PK detected 50% of patients compared with only 27% at a cut-off value of 20 U/ml. The conclusion was that Tumour M2-PK is less valuable in the detection of malignant thyroid disease than the established thyroglobulin marker.
Ugurel *et al.* 2005	Tumour M2-PK concentrations were studied in 300 melanoma patients and 53 controls. Tumour M2-PK levels were significantly high in melanoma patients and correlated with tumour load and disease stage ($p < 0.001$). The combination with S100beta improved the estimation of prognosis.
Kaura *et al.* 2004	Concentrations of Tumour M2-PK were checked in 50 patients with cervical cancer, 10 patients with chronic cervicitis and 10 healthy individuals. The test sensitivity for malignant cervical disease was 82% for a specificity of 60%. It was concluded that Tumour M2-PK can be used for follow-up of patients with cervical cancer.
Staib *et al.* 2006	Plasma Tumour M2-PK was assessed in 284 patients with a heterogeneous variety of malignant and non malignant conditions. Although Tumour M2-PK concentrations were found to be significantly higher in a group of haematological malignancies; 37% of healthy individuals and 44% of patients with non malignant disease, particularly those with acute inflammatory reaction (67%) had levels above a cut-off value of 15 U/ml. Both the sensitivity and specificity for cancer were poor at 51% and 59%, respectively. The authors concluded that Tumour M2-PK is not a valuable marker for haematological malignancies or solid tumours.

Table 5. — *Conditions that may be associated with a raised serum CA125 > 35 KU/l and ascites ± pelvic mass/masses.*

NON-MALIGNANT DISORDERS		MALIGNANT DIOSRDERS	
Associated with pelvic mass	Not associated with pelvic mass	Primary pelvic tumour	Secondary pelvic inolvement
Meig's and pseudo-Meig's syndrome	Liver cirrhosis	Ovarian cancer	Pancreatic carcinoma
Wide-spread pelvic endometriosis with endometriomas and peritoneal reaction	Pancreatitis and cholecystitis	Advanced fallopian tube cancer	Breast cancer with peritoneal metastases
	Tuberculous peritonitis	Primary peritoneal cancer	
	Uraemia and renal failure	Advanced uterine cancer	Lymphoma with peritoneal involvement
Multivisceral tuberculosis	Nephrotic syndrome	Advanced rectal/bladder cancer	Gastric cancer with peritoneal metastases
Ovarian hyperstimulation syndrome	Significant heart failure		
	Fulminant hepatic Failure		Advanced hepatocellular carcinoma
			Appendiceal cancer and pseudomyxoma peritonii

Discussion

Since rapidly proliferating cells and tumour cells in particular over-express Tumour M2-PK isoenzyme, several attempts have been made to assess its possible use as a diagnostic and prognostic tool in a range of cancers with varying results [20-26] (Table 4). To date, there have been no studies assessing the potential clinical use of Tumour M2-PK in ovarian cancer.

This study was set out to assess the ability of Tumour M2-PK to discriminate between ovarian cancer and non cancer patients; if this was the case, the next step was to find out a suitable cut-off point for the best test performance. Concentrations of Tumour M2-PK were highly elevated in ovarian cancer patients. In addition, no correlation between Tumour M2-PK and patient age was demonstrated. Further analysis indicated that higher concentrations of the tumour marker, Tumour M2-PK, were associated with the presence of ascites and higher stage

disease which is a reflection of the increased metabolic burden associated with carcinogenesis. This process increases the need for enriched metabolic substrates to support tumour growth and/or spread which is achieved by over-expressing the dimeric form of pyruvate kinase isoenzyme namely Tumour M2-PK. The higher concentrations of serum CA125 in association with ascites are thought to be due to different mechanisms. These include serosal involvement and irritation which are not necessarily associated with carcinogenesis [7, 27]; this explains the high variability observed and wider confidence interval (median = 64 kU/l; mean = 394 kU/l; 95% CI: 5 - 904 kU/l). Table 5 summarises conditions associated with raised serum CA125 and ascites.

Plasma Tumour M2-PK concentrations correlated well with those of the serum CA125 with a two-tailed p value of < 0.001. The ROC analysis demonstrated the best cut-off level for Tumour M2-PK of 22 U/ml for sensitivity, specificity, PPV, NPV and efficacy of 70%, 65%, 66% and 69%, respectively. Corresponding figures for serum CA125 were generally better apart from the specificity which was 60%. Nevertheless, test efficacy, positive, and negative lR were not significantly different. The diagnostic odds ratio for serum CA125 was higher than that for Tumour M2-PK (6.6 vs 4.3). So far, the use of the CA125 serum assay as a single diagnostic tool is restricted by the fact that the antigen to CA125 is also produced by normal epithelia (peritoneum, pleura, and pericardium) in addition to many adenocarcinomas [28].

The present study, despite the limitations of the relatively small recruitment, indicates that Tumour M2-PK can discriminate between ovarian cancer and non cancer patients. Moreover, significantly higher values were found in high-stage disease. The shape of the ROC for Tumour M2-PK is not perfect and as for many other diagnostic tests, the test performance is less than ideal and underperforms the CA125 blood test. However its inherent metabolic association with rapid proliferation and carcinogenesis, and hence better specificity, allows for the potential useful combination with other predictors as well as a possible role as a prognostic indicator which needs to be further evaluated.

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