**Title:** Biomarker panel predicts survival after resection in pancreatic ductal adenocarcinoma: a multi-institutional cohort study.

**Authors:** Christopher B Nahm MBBS(Hons) FRACS[1,2,3,6], John Turchini MBBS FRCPA[1,4], Nigel Jamieson PhD FRCS[9], Elizabeth Moon BSc(Hons)[3,6], Loretta Sioson BSc[4,6], Malinda Itchins MBBS FRACP[1,3,5,6], Jennifer Arena RN[5,7], Emily Colvin PhD[1,3,6], Viive M Howell PhD[1,3,6], Nick Pavlakis PhD FRACP[1,3,5,6,7], Stephen Clarke PhD FRACP[1,3,5,6,7], Jaswinder S Samra DPhil(Oxon) FRACS[1,2,6,7,8], Anthony J Gill MD FRCPA[1,4,7,8], Anubhav Mittal MBChB PhD FRACS[1,2,7,8]

**Affiliations:**

1. The University of Sydney Northern Clinical School, Sydney NSW Australia
2. Upper Gastrointestinal Surgical Unit, Royal North Shore Hospital, St. Leonards NSW Australia
3. Bill Walsh Translational Cancer Research Laboratory, Kolling Institute, University of Sydney, Sydney NSW Australia
4. Cancer Diagnosis and Pathology, Kolling Institute, University of Sydney, Sydney NSW Australia
5. Department of Medical Oncology, Royal North Shore Hospital, St. Leonards NSW Australia
6. Sydney Vital, Kolling Institute, Sydney NSW, Australia
7. Australian Pancreatic Centre, Royal North Shore Hospital, St. Leonards NSW, Australia
8. Faculty of Medical and Health Sciences, Macquarie University, Sydney NSW, Australia

9. Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, UK.

**Corresponding Author**

Anubhav Mittal

Upper Gastrointestinal Surgical Unit

Level 8A, Acute Services Building, Royal North Shore Hospital

Reserve Road, St. Leonards NSW 2065 AUSTRALIA

P: +61 2 9463 2899 E: anubhav.mittal@sydney.edu.au

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**Running Head**

Prognostic biomarker panel in PDAC

**Declaration of interest:** None

**Abstract**

**Background:** Up to 60% of patients who undergo curative-intent pancreatic ductal adenocarcinoma (PDAC) resection experience disease recurrence within six months. We recently published a systematic review of prognostic immunohistochemical biomarkers in PDAC and shortlisted a panel of those reported with the highest level of evidence, including p53, p16, Ca-125, S100A4, FOXC1, EGFR, mesothelin, CD24 and UPAR. This study aims to discover and validate the prognostic significance of a combinatorial panel of tumor biomarkers in patients with resected PDAC.

**Methods:** Patients who underwent PDAC resection were included from a single institution discovery cohort and a multi-institutional validation cohort. Tumors in the discovery cohort were stained immunohistochemically for all nine shortlisted biomarkers. Biomarkers significantly associated with overall survival (OS) were reevaluated as a combinatorial panel in both discovery and validation cohorts for its prognostic significance.

**Results:** 224 and 191 patients were included in the discovery and validation cohorts, respectively. In both cohorts, S100A4, Ca-125 and mesothelin expression were associated with shorter OS. In both cohorts, the number of these biomarkers expressed was significantly associated with OS (discovery cohort 36.8 vs. 26.4 vs 16.3 vs 12.8 months, $P<0.001$; validation cohort 25.2 vs 18.3 vs 13.6 vs 11.9 months, $P=0.008$ for expression of zero, one, two and three biomarkers, respectively). On multivariable analysis, expression of at least one of three biomarkers was independently associated with shorter OS.

**Conclusion:** Combinations of S100A4, Ca-125 and mesothelin expression stratify survival after resection of localized PDAC. Co-expression of all three biomarkers is associated with the poorest prognostic outcome.

## 1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is projected to be the second most common cause of cancer-related death by 2030.[1] There is mounting evidence that PDAC fails to follow the traditional Halstedian hypothesis of tumor progression from primary tumor to lymph nodes to distant metastases. Such data include the acquisition of epithelial-to-mesenchymal traits and vascular invasion of tumor cells in genetic murine models of PDAC even prior to tumor formation.[2] Furthermore, integrated genomic investigations have determined distinct molecular PDAC subtypes based on transcriptomic profiling corresponding to clinical outcomes.[3-5] These experimental data, coupled with the repeated clinical observation that R0 pancreatic resection is rarely curative even in the absence of nodal metastases, are leading to increasing acceptance that PDAC is a systemic disease even when detected "early".[6] Up to 60% of patients who have curative-intent pancreatic cancer resection will experience recurrence of disease at six months postoperatively,[7] supporting the notion that the majority of patients have clinically inapparent micrometastatic disease at the time of resection. This demonstrates the inadequacy of preoperative imaging modalities and highlights the need to integrate tumor biology assessment within staging protocols.

There has recently been a dramatic increase in the number of potential biomarkers for PDAC. However, except for Ca19-9[8], few have been clinically validated and entered routine clinical practice. The current authors recently published a systematic review of all reported PDAC biomarkers available in blood and/or tissue shown to have prognostic utility.[9] One hundred and fifty-eight studies were included, and 256 biomarkers were identified and ranked according to the quality of the evidence and reporting in individual studies. Of the highest scoring biomarkers, nine were shortlisted such that they represented a range of prognostic

outcome parameters and Gene Ontology (GO) processes of oncogenic significance. These processes include cellular proliferation, cell adhesion, cellular migration, epithelial-to-mesenchymal transition (EMT), and regulation of cell cycle. The nine-biomarker panel comprised S100A4, Ca-125 (MUC16), mesothelin, CD24, p53, p16, FOXC1, EGFR, and UPAR (PLAUR). We hypothesized that assessment of these biomarkers as a combinatorial panel would provide information of prognostic significance.

In this study, we aimed to: (i) validate the prognostic significance of these nine individual biomarkers in PDAC, (ii) identify prognostically significant combinations of biomarker expression in PDAC; and (iii) validate the findings of prognostically significant biomarker combinations in an external cohort of patients.

## 2. Methods

### *2.1 Study design and selection criteria*

This was a cohort study of prospectively collected data and tissue. Separate discovery and validation cohorts were obtained for analysis. The discovery cohort comprised consecutive patients who underwent upfront resection of histopathologically proven PDAC at a tertiary level Australian institution between 1996 and 2016. The validation cohort comprised patients with histopathologically proven PDAC from whom upfront resected tumor tissue was collected from 1992-2010 as part of the multi-institutional Australian Pancreatic Genome Initiative (APGI). Patients from the discovery cohort contained in the validation cohort were excluded from the latter. Patients with 90-day mortality were excluded from analysis. Ethical approval was obtained for this project from the Northern Sydney Local Health District Human Research Ethics Council (ref: HREC/16/HAWKE/105).

### *2.2 Patient treatment*

All patients underwent standard pancreatic resection (pancreatoduodenectomy, distal pancreatectomy and splenectomy, or total pancreatectomy). Patients were routinely offered adjuvant therapy six to eight weeks after surgery. As previously reported, in the period from 2010 to 2016, the rate of commencement of adjuvant chemotherapy in our unit for upfront resectable patients with PDAC was 84%, of whom 94% received gemcitabine alone and 5% received gemcitabine plus capecitabine (eight cycles).[10] In the validation cohort, information regarding adjuvant chemotherapy was available for 181 patients. Fifty-six (30.9%) patients in the validation cohort received adjuvant chemotherapy.

*2.3 Immunohistochemistry*

Tissue microarrays (TMAs) of archived formalin-fixed paraffin embedded (FFPE) PDAC specimens were formed using 1mm tissue cores of tumor taken from each patient in replicates of two to six and re-embedded in paraffin. 4µm-thick sections were taken from each TMA block. Missing cores, or cores where no PDAC tumor could be identified were excluded from the analysis. TMA sections were deparaffinized in xylene, rehydrated in graded ethanol solutions, and quenched in 0.3% hydrogen peroxide. The biomarkers analyzed were: S100A4, Ca-125 (MUC16), mesothelin, CD24, p53, p16, FOXC1, EGFR, and UPAR (PLAUR). Secondary antibody incubation was performed (EnVision mouse/rabbit kit; DAKO, Glostrup, Denmark), followed by chromogen, then hematoxylin counterstain. Details regarding staining methodology are summarized in Supplementary Table 1.

Immunolabelling of all antibodies was scored by a surgical pathologist (JT) who was blinded to all clinical data. With the exception of p53 and S100A4, immunolabelling for all antibodies was determined as either positive or negative according to the intensity of staining and the percentage of PDAC tumor cells stained (Figure 1). p53 immunolabelling was defined as either normal or abnormal, where abnormal staining was defined as either a complete absence of staining or a diffusely strong pattern of staining. Normal p53 staining was defined as a scattered patchy pattern of staining as previously described.[11] S100A4 immunostaining was defined as negative, weakly positive, or strongly positive according to the staining intensity and percentage of PDAC tumor cells stained.

*2.4 Biomarker Combinations*

Individual biomarkers significantly associated with shorter overall survival in the discovery cohort were subsequently evaluated for their capacity to stratify overall survival when

assessed in combination. These prognostically significant individual biomarkers and their combinations were re-evaluated in the validation cohort.

*2.5 Clinicopathological data*

Clinicopathological data including demographic information, tumor stage, tumor grade, perineural invasion, lymphovascular invasion, and survival data, were retrieved from a prospectively maintained database. The survival period was defined as the number of months from the date of surgery to the date of death.

*2.6 Data analysis*

The significance of associations between categorical data were evaluated using Fisher's exact test. Univariable survival analyses were performed using Kaplan-Meier method with log-rank comparison or Cox proportional hazards regression analysis. Clinicopathological factors found on univariable analysis to be significantly associated with survival in the discovery cohort were reevaluated in the validation cohort. Variables associated with overall survival on univariable analysis ($P < 0.1$) in both discovery and validation cohorts were included in a multivariable Cox proportional hazards regression model to identify factors independently associated with overall survival. $P$ values $< 0.05$ were accepted as statistically significant. All statistical analyses were performed using SPSS for Windows v25 (IBM, Armonk, NY, USA).

Where the number of patients analysed did not equate to the number of patients in the entire cohort, the denominator has been noted in the tables.

## 3. Results

### *3.1 Baseline characteristics*

Baseline characteristics are detailed in Table 1. Two hundred and twenty-four patients in the discovery cohort and 191 patients in the validation cohort met inclusion criteria.

### *3.2 Prognostic significance of routine pathological characteristics*

A summary of the prognostic significance of key pathological characteristics is detailed in Table 2. Factors noted to be significantly associated with poor prognosis in both discovery and validation cohorts included: lymph node positivity, lymphovascular invasion, and perineural invasion. High tumor grade was significantly associated with poor prognosis in the discovery but not the validation cohort.

### *3.3 Prognostic significance of immunohistochemically evaluated biomarkers*

The prognostic significance of individual biomarkers is detailed in Table 2. In the discovery cohort, on univariable analysis, biomarkers significantly associated with poorer survival in both discovery and validation cohorts were S100A4, Ca-125, and mesothelin. These three biomarkers were subsequently evaluated as part of a combinatorial panel.

### *3.4 Prognostic significance of combinations of S100A4, Ca-125 and mesothelin*

According to the expression pattern of the three biomarkers within each tumor, patients were categorised as “triple negative”, “single positive”, “double positive”, or “triple positive”. A “triple negative” category corresponded to failure of tumor expression of all three biomarkers. A tumor was “single positive”, “double positive”, and “triple positive” where there was expression of one, two, and three of these biomarkers, respectively.

The pattern of biomarker expression across the cohorts is illustrated in Supplementary Table 2. Combinations of S100A4 (strong positivity), Ca-125 and mesothelin expression were evaluated for their association with overall survival. In both discovery and validation cohorts, there was an incremental increase in hazard ratio and decrease in 2-year survival with the expression of each additional biomarker (Figure 2). Overall survival was significantly different across all four biomarker combinations (discovery cohort, $P < 0.001$; validation cohort, $P = 0.008$). The triple positive group was associated with the shortest median overall survival in both cohorts (discovery 12.8 months, validation 11.9 months), whereas the triple negative group was associated with the longest median overall survival (discovery 36.8 months, validation 25.2 months).

The expression of at least one of three biomarkers was a significant predictor of overall survival on multivariable analysis in both the discovery cohort ($P$=0.020) and the validation cohort ($P$=0.014) (Table 3).

*3.5 Correlation of PDAC histological phenotype and biomarker combinations*

Histological subtype data were available for the discovery cohort, but not in the validation cohort. Six patients in this cohort demonstrated features of the rare adenosquamous histological phenotype of PDAC as defined by morphology. All six of these patients demonstrated at least a double positive combination of biomarker expression. ($P$=0.0031, Fisher's test).

## 4. Discussion

In this study, we demonstrated for the first time in a discovery and validation cohort that a panel of three biomarkers (S100A4, CA-125 and mesothelin) is able to stratify patients into four survival groups after resection of PDAC.

The ability of this biomarker panel to stratify oncological outcome is maintained despite significant differences in baseline characteristics between the discovery and validation cohorts. This strengthens the validity of these findings as they remain applicable to a range of real-world clinical contexts where there is likely to be significant institutional variation in patient characteristics, receipt of adjuvant chemotherapy, and overall survival outcomes. In the present study, the differences in baseline characteristics reflect nationwide referral patterns, where the discovery cohort comprises patients with more complex tumors who have been referred to a high-volume tertiary institution from other surgeons. In addition, there is a more aggressive approach to adjuvant chemotherapy in the discovery cohort.

In the last decade, there have been significant efforts to profile the genomic landscape of PDAC. As a result, gene expression data from 456 PDAC tumors revealed that PDAC comprises four major subtypes, each with a unique transcriptomic signature: (i) squamous; (ii) pancreatic progenitor; (iii) immunogenic; and (iv) aberrantly differentiated endocrine exocrine (ADEX).[5] The squamous subtype in particular was associated with the shortest median overall survival of 13.3 months after pancreatic resection. This subtype was characterized by upregulation of gene programs including those associated with *TP63ΔN* transcriptional targets (responsible for EMT) and Wnt signaling pathways.[2] With such a short postoperative survival interval, patients exhibiting the squamous subtype of PDAC probably do not derive significant oncological benefit from surgical resection, whilst

enduring the significant postoperative recovery period and reduction in quality of life associated with pancreatic resection.[12]

S100A4, Ca-125 and mesothelin are each significantly associated with key biological processes that characterize the squamous PDAC subtype, which may explain their association with poorer prognosis in the present study. S100A4 is one of a family of S100 calcium-binding proteins coded on chromosome 1q21, implicated particularly in EMT[13]. Ca-125 expression is also closely linked with Wnt signaling via promotion of β-catenin gene expression and decrease in cytoplasmic β-catenin degradation.[14] Overexpression of mesothelin has been demonstrated to promote EMT and stemness by upregulating markers such as aldehyde dehydrogenase (ALDH), SNAIL, SLUG and TWIST, and downregulating E-cadherin, caveolin, microphthalmia-associated transcription factor (MITF) and OCLN.[15] Co-expression of Ca-125 and mesothelin has previously been demonstrated to be associated with poor survival outcomes in PDAC patients and has been demonstrated to be associated with worse survival than the expression of either protein alone.[16] Ca-125 and mesothelin undergo N-glycosylation dependent binding to each other, leading to upregulation of matrix-metalloprotease 7 (MMP-7) and subsequent increase in metastatic potential.[17]

These data lead to the hypothesis that co-expression of S100A4, Ca-125 and mesothelin is significantly associated with aggressive tumor biology and potentially the squamous PDAC subtype – thereby reducing the number of genes required to stratify PDAC patients in future studies. In the present study, this association was supported by the finding that all PDAC tumors with the aggressive adenosquamous phenotype expressed at least two of the three biomarkers. The histological adenosquamous phenotype has previously been demonstrated to be significantly associated with the squamous PDAC subtype based on gene expression

data.[5] This association between biomarker expression and the transcriptomic signature remains to be evaluated and confirmed in future integrated studies of gene and protein expression.

Whilst it is possible to preoperatively analyze tumor subtype at the level of gene expression, significant financial and logistic barriers prevent this from being routinely applicable to all patients with resectable PDAC. The difficulties associated with this approach were highlighted by the IMPaCT trial, which suffered significant participant dropout rate due to multiple logistic barriers resulting in an inability to return genetic analysis data to 25% of participants in a timely fashion.[18] Therefore, a more economically viable and practical solution to profiling tumor biology continues to be required, preferably requiring no additional infrastructure and utilizing methodologies already employed in the clinical setting, such as immunohistochemistry.

The validation of the prognostic utility of these biomarker combinations on 1mm tissue cores in the present study suggests it may have clinical utility on similarly sized core biopsy specimens obtained via endoscopic ultrasound (EUS). This should be the subject of future prospective studies, and may lead to improved pre-operative prognostication of the patient with PDAC, where reference to such biomarker combinations would allow the clinician to accurately stratify the risk of early postoperative recurrence and serve as an additional tool in providing informed consent to patients. Whether patients with triple positive biomarker expression, for example, may be better treated with an extended course of neo-adjuvant chemo/chemoradiotherapy instead of earlier resection should also be investigated in future studies.

In future, the three biomarkers investigated here may demonstrate even greater clinical utility as they each represent potential therapeutic targets. Anti-S100A4 antibodies have been demonstrated *in vitro* to have capacity to abolish tumor growth and angiogenesis in pancreatic cancer cell lines,[19] but no trials exist yet for the evaluation of S100A4 inhibition in humans. Novel immunoadhesins to disrupt the interaction between CA-125 and mesothelin have also demonstrated cytotoxicity against Ca-125-expressing cancer cells *in vitro*.[20] Several mesothelin-targeted immunotherapeutic strategies for PDAC have been evaluated in phase I/II clinical trials including tumor vaccines[21], adoptive CAR T-cell therapy (NCT01583686 and NCT02159716) and antibody drug conjugates (e.g. anetumab ravtansine - NCT 03102320, NCT01439152 and NCT02485119).

Due to the method by which biomarkers were chosen for evaluation in the present study, which was based on those identified from a previously published systematic review,[9] the present study has focused on prognostic biomarkers expressed by tumor cells, and has not considered those expressed by stromal elements. Given the mounting evidence for the role of stromal elements such as pancreatic stellate cells[23, 24] in the progression of PDAC, biomarkers related to these factors should also be the subject of future studies.

There are some limitations in the present study. Whilst the biomarker panel was able to stratify survival outcomes after PDAC resection in both discovery and validation cohorts, the absolute values for survival duration should be interpreted with caution as the rates of receipt of adjuvant chemotherapy and overall survival durations differed significantly between the two cohorts. In addition, most patients in this study received single-agent gemcitabine, which is no longer standard of care. The prognostic utility of these biomarkers should therefore be further evaluated in the setting of modern adjuvant chemotherapeutic combinations. With

increasing support for the use of routine neoadjuvant therapy for upfront resectable PDAC[22], changes in biomarker expression also need to be investigated in future studies in patients after neoadjuvant chemotherapy. Also, the multivariable analysis in the present study demonstrating the independent association of the biomarker panel to overall survival is limited by the absence of margin status in the validation cohort, which has led to its exclusion from the Cox regression model. The model nevertheless demonstrates a significant association between the biomarker panel and overall survival independent of the other prognostically significant covariates listed.

## 5. Conclusion

S100A4, Ca-125 and mesothelin are prognostically significant biomarkers in pancreatic cancer. Combinations of these three biomarkers stratify survival after resection of localized pancreatic cancer. Patients co-expressing all three biomarkers appear to gain minimal oncological benefit from pancreatic resection.

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**Figure Legends**

**Figure 1 – Representative images of positive and negative staining of immunohistochemically detected biomarkers in tissue microarrays. Original magnification 10x.** *A and B*, Ca-125 negative and positive (cytoplasmic/membranous) staining. *C and D*, CD24 negative and positive staining (cytoplasmic). *E and F*, EGFR negative and positive (cytoplasmic/membranous) staining. *G and H*, FOXC1 negative and positive (nuclear/cytoplasmic) staining. *I and J*, mesothelin negative and positive (cytoplasmic/membranous) staining. *K and L*, p16 negative and positive (nuclear/cytoplasmic) staining. M *and N*, UPAR negative and positive (cytoplasmic/membranous) staining. *O*, normal focal scattered pattern of p53 (nuclear) expression. *P*, abnormal negative staining for p53 consistent with null mutation. *Q*, abnormal diffuse positive staining for p53 consistent with missense mutation. *R*, S100A4 negative staining with normal staining of stromal and immune cells. *S and T*, S100A4 positive and strong positive (nuclear/cytoplasmic) staining.

**Figure 2 – Cox Proportional Hazards Survival Curve of the prognostic effect of S100A4, Ca-125 and Mesothelin combinations. *A*, Discovery cohort (n=203). *B*, Validation cohort (n=169).**

**Table 1 – Baseline clinicopathological characteristics**

| Variable | Discovery Cohort (n=224) Number of patients (%) Median (range) | Validation Cohort (n=191) Number of patients (%) Median (range) | *P*-value |
|---|---|---|---|
| Age, years | 69 (34-87) | 66 (26-84) | 0.002 |
| Gender, male | 98 (43.8) | 109 (57.1) | 0.008 |
| Follow-up, months | 22 (3-184) | 17 (3-229) | 0.341 |
| Overall survival, months | 25.3 | 18.5 | 0.113 |
| Adjuvant chemotherapy | 116/127 (91.3) | 56/181 (30.9) | <0.001 |
| Tumor size, mm | 35 (3-100) | 28 (8-90) | <0.001 |
| T-stage (AJCC 7th Edition) | | | 0.014 |
| - 1-2 | 15 (6.7) | 26 (13.6) | |
| - 3-4 | 209 (93.3) | 164 (85.9) | |
| Lymph node metastases | | | 0.403 |
| - Negative | 81 (36.2) | 59 (30.9) | |
| - Positive | 143 (63.8) | 126 (66.0) | |
| Tumor grade | | | >0.999 |
| - Low | 156 (69.6) | 133 (69.6) | |
| - High | 66 (29.5) | 57 (29.8) | |
| LVI, present | 116 (51.8) | 77 (40.3) | 0.806 |
| PNI, present | 150 (67.0) | 142 (74.3) | 0.014 |
| R1 resection (margin ≤1mm) | 139/215 (64.7) | - | - |

LVI, lymphovascular invasion; PNI, perineural invasion.

**Table 2 – Prognostic significance of clinico-pathological variables on overall survival in discovery and validation cohorts (univariable analysis)**

| Variable | Discovery Cohort | | | Validation Cohort | | |
|---|---|---|---|---|---|---|
| | **No. of patients (%)** | **Hazard Ratio (95%CI)** | ***P* value** | **No. of patients (%)** | **Hazard Ratio (95%CI)** | ***P* value** |
| T-stage 3-4 | 209/224 (93.3) | 1.934 (0.982-3.809) | 0.056 | 164/190 (86.3) | 1.450 (0.939-2.240) | 0.093 |
| Lymph node positivity | 143/224 (63.8) | 1.471 (1.056-2.051) | **0.023*** | 126/185 (68.1) | 1.597 (1.142-2.235) | **0.006*** |
| Tumor grade, high | 66/224 (29.5) | 1.663 (1.198-2.309) | **0.002*** | 57/190 (30.0) | 1.038 (0.749-1.436) | 0.824 |
| LVI, present | 116/224 (51.8) | 2.149 (1.475-3.130) | **<0.001*** | 77/118 (65.2) | 1.642 (1.090-2.472) | **0.018*** |
| PNI, present | 150/224 (67.0) | 1.507 (1.020-2.225) | **0.039*** | 142/167 (85.0) | 1.628 (1.013-2.617) | **0.044*** |
| R1 resection | 139/215 (64.7) | 1.614 (1.147-2.270) | **0.006*** | - | - | - |
| ≥ 1 of 3 biomarkers positive** | 144/203 (70.9) | 1.904 (1.289-2.812) | **0.001*** | 98/169 (58.0) | 1.666 (1.202-2.308) | **0.002*** |
| S100A4, positive | 151/209 (72.2) | 1.683 (1.166-2.430) | **0.005*** | 125/178 (70.2) | 1.478 (1.053-2.074) | **0.024*** |
| S100A4, strongly positive | 84/209 (40.2) | 1.673 (1.211-2.312) | **0.002*** | 57/178 (32.0) | 1.323 (0.952-1.839) | 0.095 |
| Ca-125, positive | 132/214 (61.7) | 1.932 (1.370-2.723) | **<0.001*** | 77/177 (43.5) | 1.900 (1.374-2.628) | **<0.001*** |
| Mesothelin, positive | 37/215 (17.2) | 1.867 (1.255-2.778) | **0.002*** | 28/174 (16.1) | 1.641 (1.081-2.490) | **0.020*** |
| EGFR, positive | 11/214 (5.1) | 1.960 (0.993-3.869) | 0.052 | - | - | - |
| p53, abnormal | 66/216 (30.6) | 1.195 (0.845-1.691) | 0.314 | - | - | - |
| p16, negative | 146/213 (68.5) | 1.075 (0.768-1.505) | 0.673 | - | - | - |
| CD24, positive | 17/216 (7.9) | 1.345 (0.775-2.334) | 0.292 | - | - | - |
| FOXC1, positive | 56/212 (26.4) | 1.224 (0.856-1.751) | 0.269 | - | - | - |
| UPAR, positive | 103/213 (48.4) | 1.117 (0.814-1.532) | 0.493 | - | - | - |

LVI, lymphovascular invasion. PNI, perineural invasion.

*denotes P-value <0.05 **Biomarker panel includes S100A4, Ca-125, Mesothelin.

**Table 3 – Multivariable Cox proportional hazards analysis of the prognostic significance of pathological factors and biomarker combination on overall survival.**

| Variable | Discovery Cohort (n=161) | | Validation Cohort (n=90) | |
|---|---|---|---|---|
| | **HR (95%CI)** | ***P* value** | **HR (95%CI)** | ***P* value** |
| T-stage 3-4 | 1.429 (0.604-3.381) | 0.417 | 1.152 (0.634-2.092) | 0.642 |
| Node positive | 1.817 (1.180-2.798) | **0.007*** | 1.477 (0.910-2.396) | 0.114 |
| LVI, present | 1.903 (1.229-2.945) | **0.004*** | 1.616 (0.932-2.801) | 0.088 |
| PNI, present | 1.118 (0.707-1.769) | 0.633 | 1.098 (0.572-2.105) | 0.779 |
| ≥ 1 out of 3 biomarkers positive | 1.729 (1.088-2.747) | **0.020*** | 1.750 (1.119-2.734) | **0.014*** |

LVI, lymphovascular invasion. PNI, perineural invasion.

* denotes P-value <0.05

**Supplementary Table 1 – Immunohistochemical antibody details**

| Antibody | Company | Clone | Mouse/Rabbit | HIER | HIER Duration | Dilution | Chromogen | Staining Method |
|---|---|---|---|---|---|---|---|---|
| **UPAR** | Dako* | R4 | Mouse | pH 6 | 15mins | 1:45 | INR | Manual |
| **CD24** | ThermoFisher** | SN3b | Mouse | pH 9 | 20mins | 1:100 | INR | Manual |
| **S100A4** | Dako* | A5114 | Rabbit | pH 6 | 20mins | 1:1000 | INR | Manual |
| **FOXC1** | Atlas++ | HPA040670 | Rabbit | pH 9 | 20mins | 1:100 | INR | Manual |
| **Mesothelin** | Novocastra*** | 5B2 | Mouse | pH 6 | 20mins | 1:20 | DAB | Autostainer |
| **EGFR** | Dako* | H11 | Mouse | pH 6 | 20mins | 1:100 | DAB | Autostainer |
| **p16** | Santa-Cruz+ | JC8 | Mouse | pH 6 | 20mins | 1:200 | DAB | Autostainer |
| **p53** | Dako* | DO7 | Mouse | pH 6 | 20mins | 1:50 | DAB | Autostainer |
| **Ca-125** | Dako* | M11 | Mouse | pH 9 | 20mins | 1:100 | INR | Manual |

HIER, Heat induced epitope retrieval. INR, ImmPact NovaRED (Vector Laboratories, Burlingame, CA, USA). DAB, 3,3'Diaminobenzidine. *Dako/Agilent (Santa Clara, CA, USA). **Thermofisher (Waltham, MA, USA). +Santa-Cruz (Dallas, TX, USA). ++Atlas (Bromma, Sweden). ***Novocastra Laboratories (Newcastle Upon Tyne, UK).

**Supplementary Table 2 – S100A4, Ca-125 and mesothelin biomarker combinations in discovery and validation cohorts**

| | Category | All Negative | S | C | M | S + C | S + M | C + M | S+C+M |
|---|---|---|---|---|---|---|---|---|---|
| **Discovery (n=203)** | **Triple Negative** (n=59) | 59 | | | | | | | |
| | **Single Positive** (n=65) | | 17 | 46 | 2 | | | | |
| | **Double Positive** (n=58) | | | | | 44 | 1 | 13 | |
| | **Triple Positive** (n=21) | | | | | | | | 21 |
| **Validation (n=169)** | **Triple Negative** (n=40) | 40 | | | | | | | |
| | **Single Positive** (n=59) | | 53 | 6 | 0 | | | | |
| | **Double Positive** (n=44) | | | | | 42 | 0 | 2 | |
| | **Triple Positive** (n=26) | | | | | | | | 26 |

S, S100A4; C, Ca-125; M, mesothelin.