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## Precision Oncology in Surgery: Patient Selection for Operable Pancreatic Cancer

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## INTRODUCTION

Whilst molecular markers are being used more frequently to select patients for systemic targeted agents, only imaging modalities are used to stage patients and assess suitability for operative resection. Decisions on primary surgery, or a neoadjuvant approach are made without biological measures of tumor aggressiveness, or the risk of occult metastatic disease. This is exemplified in pancreatic ductal adenocarcinoma (PDAC), which has overtaken breast cancer to become the third most common cause of cancer death in the USA.<sup>1</sup> Surgical resection offers the only chance of cure, with chemotherapy adding modest benefit, but surgery can be associated with significant morbidity and mortality risk. Even with complete resection and adjuvant chemotherapy, the five-year survival rate is only ~20%<sup>2-6</sup>, with ~30% succumbing within the first year (mostly due to distant metastatic disease).<sup>7, 8</sup> This high metastatic recurrence rate indicates current staging modalities for PDAC cannot identify patients with occult metastases and aggressive biology. For these patients, surgical resection brings uncertain benefit. Whipple's pancreaticoduodenectomy can be associated with significant mortality risk and morbidity that leads to long post-operative recovery periods of 3-6 months, which presents significant implications on patients' quality of life<sup>9</sup>. Hence, better selection methods are urgently needed.

Prognosis prediction tools such as nomograms have been developed for many cancer types to better inform treatment decisions. The most widely used tool in resectable PDAC is the prognostic nomogram developed at the Memorial Sloan-Kettering Cancer Centre (MSKCC).<sup>10,11-13</sup> However, these can only be applied after resection as they

include clinicopathological variables only available following assessment of the resected specimen.

Numerous molecular biomarkers with potential clinical utility have been studied in PDAC, but few have been independently validated.<sup>14-16</sup> Our group and others have demonstrated that aberrant expression of S100A2 and S100A4 calcium-binding proteins, both of which function to accentuate tumor aggressiveness and metastasis, are associated with poor survival in PDAC.<sup>17-19</sup> Using RNA sequencing and methylation arrays, we recently reported hypomethylation of S100A2 is associated with the prognostic 'Squamous' subtype of PDAC.<sup>20</sup> This poor prognostic subtype is consistently defined in molecular classifications of PDAC (also termed Quasi-Mesenchymal<sup>21</sup> or Basal<sup>22</sup>) and S100A2 remains a highly significant gene in each classifier.<sup>20-22</sup>

Here, we assess and validate the prognostic value of these two molecules in 1184 patients. Aberrant expression of these biomarkers stratify patients with resectable pancreatic cancer into three distinct prognostic groups in a training set ( $n = 518$ ) and form the basis of a biomarker-based pre-operative nomogram aimed at identifying those at high risk of early recurrence. This nomogram was validated in two further cohorts ( $n = 198$  and  $468$ ), and the proof-of-concept feasibility of its pre-operative use was assessed using pre-operative endoscopic ultrasound fine-needle aspiration biopsies (EUS-FNA).

## **METHODS**

### **Patients and Tissue Specimens**

Detailed clinicopathological and outcome data were obtained for three cohorts of consecutive unselected patients, totaling 1184, with primarily resected PDAC (Figure 1). None of the patients received neoadjuvant chemo- or radiotherapy. The training cohort of 518 patients were accrued prospectively through the Australian Pancreatic Cancer Genome Initiative (APGI) ([www.pancreaticcancer.net.au](http://www.pancreaticcancer.net.au)), for the International Cancer Genome Consortium (ICGC; [www.icgc.org](http://www.icgc.org)).<sup>23</sup> The two independent validation cohorts of 198 and 468 patients were from the West of Scotland Pancreatic unit, Glasgow Royal Infirmary, United Kingdom; and the Technical University of Dresden, Dresden, Germany respectively (Table 1). Some patients from these cohorts were used for previous studies.<sup>19, 20, 24-29</sup> The current study was conducted in accordance with the TRIPOD Type 3 model development approach, and REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria (Supplementary Material).<sup>30</sup> All patients were treated after 1998 with more modern approaches such as multi-modality therapy, and some were part of Phase III randomized-controlled trials such as ESPAC-3.<sup>2</sup> All cohorts displayed clinical and pathological features consistent with the behavior of PDAC and are similar to published PDAC cohorts worldwide.<sup>31, 32</sup> The diagnosis and all pathological features were reviewed centrally by at least one specialist pancreatic histopathologist, and the date and cause of death was obtained from Central Cancer Registries or treating clinicians. RNA sequencing data was generated as part of the APGI's contribution to the ICGC, and sample processing and data analysis was performed as previously described.<sup>20</sup>

## **Ethical Statement**

Ethical approval for the acquisition of data and biological material was obtained from the Human Research Ethics Committee at each participating institution (Supplementary Material).

### **Tissue Microarray & Immunohistochemistry**

Detailed methods for tissue microarray (TMA) construction and immunostaining assessment were described previously.<sup>33</sup> In brief, immunostaining on resection specimens were performed using TMAs constructed from 3 distinct areas per tumor. TMA sections were incubated with anti-S100A2 mouse monoclonal antibody, 1:50 dilution, (clone DAK-S100A2/1; Dako Corporation, Glostrup, Denmark) and anti-S100A4 rabbit polyclonal antibody (NeoMarkers, Cat. #RB-1804, Fremont, CA, USA) with a dilution of 1:100 for 60 minutes. **As part of a standardized biomarker discovery and development process, initial cut-offs were generated using earlier training/discovery cohorts, then validated using independent validation cohorts. S100A2 and S100A4 expression analysis was performed in an early training cohort of PC to define the optimal expression for analysis.**<sup>19</sup> High S100A2 expression was defined as cytoplasmic staining with intensity 3+ in >30% of cells and positive S100A4 expression was defined as either nuclear and/or cytoplasmic staining of any intensity in > 1% of cells.<sup>19</sup> Aberrant expression of S100A2 and S100A4 were correlated to survival after pancreatectomy.

### **Statistical Analysis**

The influence of clinicopathological variables on survival was assessed with Cox proportional hazards regression, and the differences in outcome between predefined subgroups was evaluated using the log-rank test.<sup>34</sup> Where multiple cohorts were



included in a single model, baseline hazard was always stratified by cohort throughout the procedure. On the basis of exploratory analysis, age was modelled with a cohort interaction term in the combined models; no other substantive variable to cohort interactions were identified. *P*-values of less than 0.05 were considered statistically significant. Statistical analysis was performed using SPSS (Version 22.0; IBM SPSS Statistics, IBM Corporation, Armonk, NY). Model fitting and nomogram generation was performed using R 3.4.0 (The R Project for Statistical Computing, Vienna, Austria). Disease-specific survival (DSS) was used as the primary endpoint for the APGI and Glasgow cohorts. Patients succumbing to other causes were right censored in the analysis. As the majority of patients with PDAC unfortunately succumb to disease, even after seemingly curative resection<sup>35</sup>, overall survival (OS) was used for the German cohort, as disease-specific survival was not available.

## **Prognostic Nomograms**

### *MSKCC Nomogram Evaluation*

The published MSKCC nomogram<sup>10</sup> was applied to the APGI, Glasgow, and German cohorts, yielding per-patient estimates of linear risk score and 6-, 12- and 24-month survival probabilities (Figure 1) (Supplementary Material). Some variables in the MSKCC nomogram were not collected in the current cohorts and were imputed to the mean value of that variable reported for the MSKCC nomogram derivation cohort (Table S2).

### *Nomogram Construction*

Two Cox proportional hazard models were fit to the APGI training cohort data (Figure 1), one containing conventional clinicopathological variables available post-operatively

(age, tumor size, T-stage, tumor location, vascular invasion, perineural invasion, margin status, presence of lymph node metastases, and differentiation), and the other containing only variables assessable pre-operatively (age, tumor size, tumor location, and S100A2 and S100A4 status). Patient sex was not included in models due to its known poor prognostic value in PDAC.<sup>10</sup> To improve the clinical utility of the nomogram, follow-up was truncated at 24 months to focus prognosis prediction in this most clinically critical period following surgery. To simplify generation and application of the predictive nomograms, violations of the proportional hazards assumption were addressed by stratifying the baseline hazard by predictive variables, rather than introducing interaction with a time-dependent stratum.

#### *Nomogram Testing*

Nomograms were tested for discrimination and calibration against validation cohorts (Glasgow and Germany) using established methods.<sup>36, 37</sup> Variability of the Brier score assessment of overall fit was estimated using 5,000 bootstrap rounds.<sup>36, 37</sup>

#### **EUS-FNA and Cell Block Construction**

**EUS-FNA samples were collected and processed as per the standard diagnostic pathway using endoscopic and cytohistological techniques according to local practice.** Formalin-fixed EUS-FNA tissue fragments or cell block preparations were embedded in paraffin, sectioned (4  $\mu$ m) and H&E stained as standard. Staining for S100A2 and A4 were performed as described above and compared with corresponding resection specimen from 17 consecutive patients undergoing both EUS-FNA and pancreatectomy.

## RESULTS

Patient characteristics are summarized in Table 1, with detailed descriptions provided in the Supplementary Material.

### **High S100A2 and S100A4 Expression is associated with Poor Survival after Pancreatectomy**

In all three cohorts, high expression of S100A2 calcium-binding protein was associated with poor survival after pancreatectomy. In the APGI cohort, expression of S100A2 was high in 115 of 507 patients (22.7%) (median survival 21.0 Vs 15.0 months;  $P = 0.023$ ) (Table 1, Figure 2). S100A2 expression was high in 63 out of 198 patients (31.8%) in the Glasgow cohort (median survival 24.7 vs 13 months;  $P < 0.001$ ) and in 118 out of 400 patients (29.5%) in the German cohort (median survival 18.2 vs 11.9 months;  $P < 0.001$ ) (Table 1, Figure 2).

High S100A2 expression remained a significant independent prognostic factor in a combined multivariate model of all 3 cohorts (Table 2; HR = 1.64, 95% CI 1.33 – 2.02  $P < 0.001$ ). This was also the case in the APGI (Table S3; HR 1.32, 95% CI 0.97 – 1.80,  $P < 0.001$ ) and Glasgow (Table S4; HR 2.00, 95% CI 1.36 – 2.90,  $P < 0.001$ ) cohorts, but not the German (Table S5; HR 1.48, 95% CI 0.95 – 2.29,  $P = 0.076$ ) cohort. It is likely that this reflects reduced power or cohort-specific variable collinearities, as the influence of S100A2 expression on survival was not significantly different between cohorts (Likelihood ratio test,  $\chi^2 = 4.86$ ,  $df = 2$ ,  $P = 0.09$ ). Furthermore, S100A2 was associated with poor survival in the German cohort (univariate cox regression (HR 1.69, 95% CI 1.34 – 2.14,  $P < 0.001$ ) and log rank survival analysis (Figure 2C)). When considered alongside a previous study with over

400 (200 non-redundant) patients this provides a total of over 1300 cases supporting the association of high S100A2 expression and poor survival.<sup>19</sup>

Positive expression of S100A4 was associated with poor survival after pancreatectomy in all three cohorts. In the APGI cohort, 345 out of 514 patients (67.1%) had positive expression of S100A4, **which was associated with a** significantly worse outcome (median survival 29.9 Vs 16.2 months;  $P < 0.001$ ) (Table 1, Figure 2). These findings were recapitulated in the Glasgow cohort with 137 out of 198 patients (69.2%) with analyzable tissue demonstrating positive expression of S100A4, that was again associated with poor outcome (median survival 26.4 Vs 16.2 months;  $P = 0.010$ ) (Table 1, Figure 2). In the German cohort, 288 out of 430 patients (72.0%) with positive S100A4 expression demonstrated significantly worse outcome (median survival 22.0 vs 14.6 months,  $P = 0.013$ ) (Table 1, Figure 2).

In a combined multivariate model, S100A4 remained strongly prognostic in a time dependent manner with its effect on prognosis decreasing after 24 months in both the combined (HR 2.06, 95% CI 1.30 – 3.28,  $P < 0.001$  at 12 months), and individual models (Tables 2, S3-S5). This data suggests that S100A4 is a strong predictor of disease recurrence in the first 24 months after surgery, with its effect decreasing after this period. S100A4 was an independent prognostic factor in multivariate models in the APGI (Table S3, HR 2.13, 95% CI 1.08 – 4.17,  $P = 0.018$  at 12 months) and Glasgow (Table S5, HR 2.37, 95% CI 0.97 – 5.79,  $P = 0.048$  at 12 months) cohorts. Similar to S100A2, S100A4 was not significant in a multivariate model in the German cohort (Table S5), however, was predictive in univariate cox regression (HR 1.33, 95% CI 1.06 – 1.67,  $P = 0.013$ ) and log rank survival analysis (Figure 2).

### **Expression of S100A2 and S100A4 Stratifies Patients into 3 Prognostic Groups**

The combined prognostic effects of the two biomarkers were assessed independently in all three cohorts (Figure 1). **In all three cohorts, tumors with high S100A2 expression were more likely to be associated with positive S100A4 expression (Table S6 in Supplementary Material,  $P \leq 0.001$ ).** In the APGI cohort, patients with low or no expression of either S100A2 or S100A4 had the best prognosis, followed by patients with either high S100A2 or positive S100A4 expression, and patients with both biomarkers positive had the worst prognosis (median survival 29.8 vs 17.0 vs 13.2 months respectively,  $P < 0.001$ ) (Figure 1). These findings were validated in the Glasgow (median survival 26.5 vs 20.1 vs 9.3 months;  $P < 0.001$ ) and the German (median survival 22.9 vs 14.3 vs 12.9 months;  $P < 0.001$ ) cohorts (Figure 1). When combining all 3 cohorts, patients with both biomarkers positive had a 12-month survival rate of only 54% after pancreatectomy, compared to 79% and 66% in the biomarker negative or single biomarker positive groups respectively ( $P < 0.001$ ) (Figure 1).

**There was no difference in the use of adjuvant chemotherapy between patients who were biomarker negative and those with a single biomarker positive in all three cohorts (Table S7 in Supplementary Material).** In the APGI cohort, patients with both biomarkers positive had lower adjuvant therapy use rate (Table S7,  $P = 0.025$ ). There were no significant differences in adjuvant therapy use amongst biomarker groups in the Glasgow and German cohorts (Table S7 in Supplementary Material).

**Expression of S100A2 and S100A4 co-segregates with the Squamous subtype of PDAC**

In 96 patients who underwent whole transcriptome sequencing analysis as part of the APGI (ICGC) cohort, S100A2 and S100A4 mRNA expression was significantly associated with the recently described squamous subtype<sup>20</sup> ( $P < 0.01$ ; Figure 3, Table **S8** in Supplementary Material). This was recapitulated in the 235 patients who underwent micro-array mRNA expression analysis, as described by Bailey *et al.*<sup>20</sup> Using both mRNA and protein expression, patients with high S100A2 ( $P = 0.002$ ) and positive S100A4 ( $P < 0.001$ ) expression were associated with the squamous subtype, with the strongest correlation in those with both biomarkers positive (Figure 3, Table **S9** in Supplementary Material). Squamous subtype tumors<sup>20</sup> were associated with a significantly higher mean nomogram score than other subtypes (140 vs 103;  $P = 0.004$ ).

### **MSKCC Prognostic Nomogram Validation for Resectable PDAC**

After demonstrating the association of S100A2 and S100A4 expression with the pro-metastatic squamous subtype and poor prognosis in PDAC, the potential clinical utility of a prognostic nomogram incorporating molecular biomarkers was explored. The overall fit of the published MSKCC nomogram to all three cohorts was assessed by stratifying patients using predicted survival score and comparing observed and MSKCC predicted survival (Figure 1D(i)). Relative to the true outcome, MSKCC nomogram predictions were optimistic, particularly at later time points, this was especially true for the German cohort (Figure S1, Supplementary Material). The MSKCC nomogram risk score was prognostic in the APGI and Glasgow cohorts (Cox regression coefficients 0.79 and 1.35, likelihood ratio test for coefficient not zero,  $P = 5.0 \times 10^{-5}$  and 0.025, respectively), but not the German cohort (coefficient 0.15,  $P = 0.31$ ). The MSKCC risk score was well-calibrated against the APGI and Glasgow

cohorts (Likelihood-ratio test for coefficient not unity,  $P = 0.28$  and  $0.56$ , respectively), but less well against the German cohort ( $P = 2.6 \times 10^{-9}$ ) (Figures 1D(ii) and S1, Supplementary Material).

### **Pre-Operative Molecular Nomogram Predicts Survival after Pancreatectomy as accurately as Post-Operative Clinicopathological Nomogram**

A molecular nomogram incorporating S100A2 and S100A4 expression was then constructed. The APGI cohort was used to construct two prognostic nomograms based on the Cox proportional hazards model: one employing traditional post-operatively available variables (“Post-operative Prognostic Nomogram”) (Figures 1E(iii) and S2, Supplementary Material), and one employing only variables that can be measured pre-operatively (“Pre-operative Prognostic Nomogram”) (Figures 1E(iv) and 4). To improve the clinical utility of predicting early recurrence and to incorporate the prognostic value of S100A4 over the initial 24 months following surgery, follow-up was truncated at 24 months. Both models included tumor location (pancreatic head vs body / tail), after exploratory analysis in the APGI cohort indicated differences in baseline hazard between these patient groups (Table **S10**, Supplementary Material).<sup>38</sup>

Risk stratification accuracy of the APGI pre-operative and APGI post-operative nomograms were assessed in the Glasgow and German validation cohorts. The pre-operative prognostic nomogram displayed good discrimination on both validation cohorts (risk score Cox coefficients  $0.59$  and  $0.66$ ,  $P = 1.7 \times 10^{-3}$  and  $1.2 \times 10^{-5}$  for Glasgow and German respectively) (Figure 1E(v)). Notably, the APGI pre-operative nomogram was superior to the MSKCC post-operative prognostic nomogram in both

its spread of risk scores, and the accuracy of its absolute survival estimates (Figures S1 and S3, Supplementary Material).

The overall accuracy of the APGI pre-operative nomogram in predicting patient survival was assessed and compared to the APGI post-operative nomogram. Although the pre-operative nomogram was slightly optimistic by predicting marginally better outcome probabilities than those observed in the Glasgow and German cohorts, it was more accurate than the MSKCC post-operative nomogram (Figures S1 and S3, Supplementary Material). Brier scores was used to formally evaluate the relative performance of the APGI pre-operative and post-operative nomograms with over 5,000 bootstrap draws of each validation cohort. This demonstrated the APGI pre-operative nomogram was more accurate than the MSKCC post-operative nomogram, and as accurate as the APGI post-operative nomogram in outcome predictions (Figures S4 and S5, Supplementary Material).

### **Pre-operative Assessment of Biomarker Expression in EUS-FNA Samples**

A pilot study was performed on 17 consecutive patients to compare biomarker expression status between pre-operative EUS-FNA cell blocks and the corresponding surgical resection specimen (Table **S11**, Supplementary Material). S100A2 and S100A4 expression correlated in 15 (88%) and 14 (82%) out of 17 patients respectively, based on the EUS-FNA cell block and the surgical specimen for both biomarkers examined (representative images are shown in Figure 5). This demonstrates that biomarker status can be measured pre-operatively using immunohistochemistry, and it is likely that EUS biopsy assessment will improve with



the current development of more effective biopsy needles and standardization of processing techniques.

## DISCUSSION

Despite our increasing understanding of the molecular heterogeneity in morphologically identical cancers, and the advances in molecularly guided targeted therapy selection, the impact of these findings in surgical decision-making has not been addressed. Perioperative mortality for pancreatectomy has improved dramatically over the last 30 years and the definition of “resectability” has been expanded over the last decade with increasingly aggressive surgery being performed.<sup>39, 40</sup> However, early recurrence remains the Achilles’ heel of surgical resection, making better patient selection for surgery a priority area of research.

In this study, the expression of two molecules, S100A2 and S100A4, which functionally promote carcinogenesis and metastasis, were validated as prognostic biomarkers in multiple independent cohorts of patients with resectable PDAC ( $n = 1184$ ), in keeping with earlier studies ( $n = \sim 400$ ).<sup>19</sup> These two biomarkers were used to stratify patients with resectable PDAC into distinct prognostic phenotypes after pancreatectomy. Patients with both biomarkers positive are at significant risk of early recurrence, with almost half of these patients succumbing within 12 months after pancreatectomy (12-month survival rate = 54%). Suggesting that disease recurrence occur at around 6 months or earlier for the majority of this group. **The risk and pattern of disease recurrence following pancreatectomy is not proportional and early recurrence has recently been defined as within 12 months following surgery.**<sup>35, 41</sup> We focused **on outcome** in the first 24 months post-operatively for nomogram construction since

the majority of patients that develop early recurrence will succumb to the disease by this point, and thus improve its clinical utility.<sup>41</sup> A pre-operative prognostic nomogram incorporating these two biomarkers, and pre-operatively determined variables including age, tumor size and location was developed and independently validated. This pre-operative prognostic nomogram performed as well as the published MSKCC post-operative prognostic nomogram, which is the most widely used, and currently considered the gold standard. In our study, a number of variables used in the MSKCC nomogram was missing and thus comparing the performance of both nomograms in these cohorts is not optimal. The APGI pre-operative nomogram, however, utilizes less variables and all are obtainable prior to surgical resection to aid decision making. Finally, as a proof-of-concept, biomarker expression status was assessed using immunohistochemical staining of the EUS-FNA cell blocks.

**However, due to the retrospective nature of the study, and the cohorts acquired were mature, with long term follow up, there are a few limitations with the study. First, a proportion of the recurrence pattern data were not available, therefore, the association between recurrence patterns and biomarker expression was not assessed. Secondly, pre-operative CA19-9 measurements were not available for a significant proportion of the patients. Increased serum levels of CA19-9 has been shown to be associated with early recurrence, and may improve the performance of a pre-operative nomogram.<sup>41</sup> Thirdly, only a small number of pre-operative EUS samples had sufficient material available for comparison with post-operative S100A2 and S100A4 immunostaining. Therefore, in order to further validate the clinical utility of the pre-operative nomogram, its use should be tested in parallel with trials in PDAC comparing upfront resection and**

neoadjuvant therapy. Finally, this study was not powered nor designed to assess S100A2 and S100A4 expression and response to adjuvant chemotherapy. Chemotherapy response in the adjuvant setting is difficult to reliably assess as survival difference is dependent on many factors including residual occult metastatic disease and performance status, requiring multi-center personalized medicine trials (such as *PRECISION-Panc* in the UK and *Precision Promise* in the USA) to further delineate this relationship. Interestingly, in the APGI cohort, patients with both biomarkers positive were less likely to be administered adjuvant therapy, possibly due to an aggressive disease phenotype leading to more early recurrence and declining performance status and subsequently reduced adjuvant chemotherapy use.

Aberrant S100A2 and S100A4 expression correlated to the recently described poor prognosis 'squamous' (also termed QM or Basal)<sup>21, 22</sup> subtype of PDAC, which is enriched for transcriptional programs associated with proliferation, inflammation and metastasis.<sup>20</sup> Mechanistically, S100A2 hypomethylation and associated increased expression is a feature of the squamous subtype. The regulation of S100A4 expression is more complex, potentially involving both tumour and microenvironment factors, and will require further investigation.<sup>43</sup> Interestingly, the squamous subtype was associated with a higher mean nomogram score and demonstrates the potential clinical utility of the currently presented molecular prognostic nomogram in identifying patients with aggressive tumor biology and a pro-metastatic phenotype. These patients are at high risk of early recurrence and are unlikely to benefit from pancreatectomy and are perhaps better treated with neoadjuvant therapy (an increasingly popular approach to PDAC in many centers), as

occult metastatic disease that is not detected by current staging modalities will likely manifest itself during this period. **However, the use of neoadjuvant therapy is not universal and a significant proportion of patients may not respond to this approach.**<sup>44</sup> Thus, patients predicted to have a favorable prognosis, may be better served with upfront surgery and adjuvant therapy with median survival up to 54 months reported using adjuvant modified FOLFIRINOX in patient cohorts with favorable post-operative prognostic features.<sup>45</sup> Furthermore, the growing interest in more aggressive and extensive surgery in the setting of borderline resectable or locally advanced disease could be justified **by prognostic indicators prior to initiating therapy.**<sup>39,40</sup> Accurate prognostication can assist multidisciplinary and shared decision-making, especially in patients with borderline fitness for surgery, which is a significant proportion of patients with PDAC, delivering a more personalized treatment plan. This approach has the potential to improve the overall outcomes and quality of life for patients with PDAC.

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## Figure Legends

**Figure 1. Methodology for biomarker validation and Nomogram construction and validation.** **A.** The expression of biomarkers S100A2 and A4 was determined using immunohistochemistry in 3 independent cohorts of PDAC and correlated with survival after pancreatectomy. Biomarker expression prevalence is presented as individual pie charts. **B** and **C.** Survival following pancreatectomy for all 3 cohorts (**b**) individually and (**c**) combined, stratified by biomarker expression (both negative, one positive, both positive), is represented by Kaplan-Meier survival curves. Patients with both biomarkers positive had a survival rate of only 54%, 26% and 6% at 1, 2 and 5 years respectively. This was found to be 79%, 54% and 18% in the biomarker negative and 66%, 38% and 14% in the single biomarker positive groups respectively. **D.** Clinicopathological variables for all 3 cohorts were independently entered into the



MSKCC post-operative nomogram to validate its performance in the patient cohorts. The MSKCC nomogram predicted survival in the APGI ( $P = 5.0 \times 10^{-5}$ ) and Glasgow cohorts ( $P = 0.025$ ) (green), but not the German cohort ( $P = 0.31$ ) (red). **E.** The APGI training cohort was used to construct two Cox proportional hazard models, one was termed the APGI post-operative prognostic nomogram, and one the APGI pre-operative prognostic nomogram. These were assessed and validated against the Glasgow ( $P = 1.7 \times 10^{-3}$ ) and German ( $P = 1.2 \times 10^{-5}$ ) validation cohorts with excellent fit in both cohorts.

**Figure 2:** Kaplan-Meier survival curves for S100A2 expression in the (A) APGI, (B) Glasgow, (C) German cohorts; S100A4 expression in (D) APGI, (E) Glasgow, (F) German Cohorts.

**Figure 3:** High S100A2 and positive S100A4 expression correlates with the squamous sub-type of PDAC. Patients are ranked according to S100A2 mRNA expression and the relative expression Z-score is represented by a waterfall plot, IHC staining and Bailey subtype is shown below. High S100A2 and positive S100A4 expression associated strongly with the squamous subtype ( $P < 0.001$ ).

**Figure 4:** A pre-operative molecular prognostic nomogram for resectable pancreatic cancer.

**Figure 5:** Immunohistochemistry of EUS-FNA versus resection specimen in 2 patients