Molecular understanding of Hedgehog-dependent cancers

From pathways to patients

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Citation for published version (APA):
Veenstra, V. L. (2018). Molecular understanding of Hedgehog-dependent cancers: From pathways to patients.

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“Why does this magnificent applied science which saves work and makes life easier bring us so little happiness? … The simple answer runs: Because we have not yet learned to make sensible use of it.”

- Albert Einstein -
ADAM12 is a circulating marker for stromal activation in pancreatic cancer and predicts response to chemotherapy

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Oncogenesis, 2018
ABSTRACT
Pancreatic ductal adenocarcinoma (PDAC) is characterized by abundant stroma that harbors tumor-promoting properties. No good biomarkers exist to monitor the effect of stromal targeting therapies or to predict response. We set out to identify such non-invasive markers for PDAC stroma and predict response to therapy. Gene expression datasets, co-culture experiments, xenografts and patient samples were analyzed. Serum samples were measured from a cohort of 58 resected patients, and 87 metastatic or locally advanced PDAC patients. Baseline and follow-up levels were assessed in 372 additional metastatic PDAC patients who received nab-paclitaxel with gemcitabine (n=184) or gemcitabine monotherapy (n=188) in the phase III MPACT trial. Increased levels of ADAM12 were found in PDAC patients compared to healthy controls (p<0.0001, n=157 and n=38). High levels of ADAM12 significantly associated with poor outcome in resected PDAC (HR 2.07, p=0.04). In the MPACT trial survival was significantly longer for patients who received nab-paclitaxel and had undetectable ADAM12 levels before treatment (OS 12.3m vs 7.9m p=0.0046). Consistently undetectable or decreased ADAM12 levels during treatment significantly associated with longer survival as well (OS 14.4m and 11.2m respectively vs 8.3, p=0.0054). We conclude that ADAM12 is a blood-borne proxy for stromal activation, the levels of which have prognostic significance and correlate with treatment benefit.

Novelty and Impact
The pancreatic cancer stroma is known to hamper treatment. Novel chemotherapeutics against the stroma have been developed, but predictive markers are urgently needed. We aimed to identify such markers and found that the metalloprotease ADAM12 associates with activated stroma in pancreatic cancer. ADAM12 can be detected in the circulation of patients, and predicts response to treatment. If confirmed in other studies, ADAM12 could be used to stratify patients that are to receive stroma-targeting agents.
INTRODUCTION
Survival of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) has remained poor for many decades.\(^1\) Median survival after diagnosis is around 6 months, and even patients treated with a combination of surgery and chemotherapy typically succumb to the disease within 5 years.\(^2\) The vast majority of patients are diagnosed with advanced disease, precluding resection of the tumor. Treatment options for these patients are limited and clinical trials typically demonstrate only partial benefit, in a limited number of patients. Biomarkers that allow patient stratification and early response monitoring are expected to improve outcome.

In recent years, research on PDAC has shifted focus from tumor cells to the abundant non-malignant compartment known as the stroma. This stroma consists of extracellular matrix proteins, cancer associated fibroblasts (CAFs), and immune cells.\(^3\) The stroma typically forms the vast majority of tumor bulk and contributes to chemoresistance by acting as a barrier to the delivery of chemotherapeutics. Non-mechanical tumor promoting properties have been attributed to the stroma as well.\(^4\)

Nab-paclitaxel is an albumin-bound nanoparticle formulation of paclitaxel that enhances distribution and penetration into tumor tissues.\(^5,\) \(^6\) While the initially suspected role for SPARC (which is expressed on stromal fibroblasts and binds albumin) in conferring enhanced sensitivity to nab-paclitaxel in PDAC was not confirmed,\(^7,\) \(^8\) the combination of nab-paclitaxel with gemcitabine is effective compared to gemcitabine monotherapy and median survival increases from 6.6 to 8.7 months.\(^9\) Other stroma targeting agents have also been tested in trials, but these have failed to demonstrate favorable outcomes.\(^10\) Given the practical hurdles to obtaining tissue from the pancreas for histopathological assessment, good non-invasive markers are urgently needed that inform on the PDAC stroma to allow selection of patients for stroma-targeting therapies, and/or to monitor the response to such regimens.

ADAM12 is a member of the A Disintegrin And Metalloproteases (ADAM) protein family and harbors extracellular metalloprotease, and intracellular signaling properties. The protein is involved in cell adhesion by binding to integrins and syndecans, as well as the proteolytic cleavage of substrates from producing cells, a process known as ectodomain shedding.\(^11\) Cancer-related substrates of ADAM12 include epidermal growth factor (EGF), and Sonic Hedgehog (SHH).\(^12-14\) Expression of ADAM12 is elevated in glioblastoma, breast, bladder, lung, prostate, and liver cancer.\(^15-20\) ADAM12 expression correlates with tumor stage in breast and bladder cancer, and is prognostic in small cell lung cancer.\(^15,\) \(^20,\) \(^21\) A previous study showed the upregulation of ADAM12 in pancreatic cancer associated fibroblasts (CAFs) compared to fibroblasts from non-neoplastic tissue, but the clinical or functional relevance was not explored.\(^22\) Here, we show that ADAM12 is associated with the activated stroma, and poor-prognosis mesenchymal molecular subclasses, in PDAC. It is elevated in the serum of PDAC patients and associates with poor outcome. In an exploratory analysis of patients treated with nab-paclitaxel and gemcitabine, circulating ADAM12 levels predicted outcome.
MATERIALS AND METHODS

Collection of blood samples
In the AMC cohort, serum samples were obtained perioperatively from 60 patients undergoing resection, or before the start of treatment in case of unresectable patients (n=89). Clinicopathological data were obtained from medical records and included age, gender, tumor diameter, differentiation grade, lymph node ratio (positive lymph nodes/total number of lymph nodes examined), therapies received, and tumor-node-metastasis (TNM) staging. Collection of material was approved by the institute’s ethics committee, and written informed consent was received from all participants (AMC 2014_181). Blood samples of 38 non-age matched healthy individuals without any indication of malignancy were collected as a control group. In the MPACT trial cohort, collection of plasma samples for biomarker development was optional and separate written consent was obtained for sample collection and biomarker analysis. The patients and methods of the MPACT trial have been described previously. In brief, patients were ≥18 years of age, had confirmed measurable metastatic adenocarcinoma of the pancreas, a Karnofsky performance status (KPS) of ≥70, and did not receive prior chemotherapy for metastatic disease. Patients were randomized 1:1 (stratified by performance status, presence of liver metastases, and region) to receive nab-paclitaxel 125 mg/m² plus gemcitabine 1,000 mg/m² on days 1, 8, and 15 every 28 days or gemcitabine alone 1,000 mg/m² on days 1, 8, 15, 22, 29, 36, and 43 in 56 days (cycle 1) and then on days 1, 8, and 15 every 28 days (cycle ≥2). Treatment continued until disease progression or unacceptable toxicity.

Establishment of patient-derived xenografts and primary cell lines
Details on methods pertaining to the establishment of patient-derived xenografts and primary cell lines, are found in the Supporting Information Materials and Methods. STR profiling was performed to confirm donor-cell line matching.

Transcript and gene expression analysis
Methods related to transcript and gene expression analysis are in the Supporting Information.

ELISA analysis of serum samples (AMC cohort)
For the reporting on association of ADAM12 levels with prognosis, the pertinent guidelines were considered. Serum was obtained by centrifugation of blood for 10 min at room temperature at 1300g, and storage at -80°C until analysis. ADAM12 levels were determined using the human ADAM12 DuoSet ELISA kit (R&D Systems, Minneapolis, MN), according to manufacturer’s recommendations. Briefly, after coating the 96-well plates (Greiner Nunc MaxiSorp, Kremsmünster, Austria) with capture antibody overnight at room temperature, blocking the plate with 1% BSA the following day, 80µl of serum samples were added. After incubation for two hours at room temperature and mild washing steps, samples were incubated with biotinylated detection antibody for additional two hours followed by a 20 min incubation step with horse-radish peroxidase (HRP)-labeled streptavidin. Substrate was tetramethylbenzidine substrate solution (TMB), added for
an additional 20 min. Absorbance was measured at 450nm and 570nm with a microplate reader (BioTek Synergy BioTek, Winooski, VT) after addition of 1M H$_2$SO$_4$ stop solution. For wavelength correction, 570nm readings were subtracted from the 450nm values before further analysis.

**ELISA analysis of plasma samples (MPACT cohort)**

All MPACT trial blood samples were collected in EDTA tubes to chelate calcium and prevent blood clotting, and storage at -80°C until analysis. To allow the analysis of these plasma samples using the ADAM12 DuoSet ELISA, recalcification was required. 100µl of EDTA plasma was complemented with 12mM CaCl$_2$ (final concentration) and incubated overnight at 4°C. The following day, clots were removed manually and 50µl sample was used for the assay. The ELISA was performed as for the serum samples. Sensitivity of the assay was reduced compared to measurement in serum by a factor of 0.44, explaining why in the MPACT cohort, the lowest quartile was composed of samples that had undetectable plasma ADAM12. Measurement of ADAM12 in matched serum and plasma samples revealed high correlation ($R^2=0.9971$, $p$-value: $2.0\times10^{-6}$, $n=6$).

**Statistics**

Student’s t-tests were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). R was used for linear regression analysis of gene expression. All tests were two-sided and $p<0.05$ was considered statistically significant. For the AMC cohort patient data, SPSS package 24 (IBM Analytics, Armonk, NY) was used for Chi-square testing, Mann-Whitney U test, Spearman’s rank correlation, Cox proportional-hazard regression modeling, Kaplan-Meier survival analysis and log-rank test. Patients who died within 30 days after operation were excluded from survival analysis as these likely did not succumb to cancer ($n=5$). Cox proportional hazard regression was used for univariate and multivariate analyses to investigate the correlation of OS with ADAM12 and potential prognostic factors.

For the MPACT cohort, baseline ADAM12 values were categorized into undetectable (0) and detectable (>0). The fold change of ADAM12 at cycle 2, day 1 (C2D1) from baseline (cycle 1, day 1; C1D1) was calculated by the results at C2D1 divided by the value at C1D1. These were then assigned to three groups (0: both values below detection limit; <1 if levels decreased, >1 if values increased). Descriptive statistics summary of demographics and baseline characteristics were performed. Correlation of ADAM12 groups with categorical variables was tested using CMH statistics or Cochran-Armitage Trend Test statistics, or one-way ANOVA for continuous variables. Overall survival was analyzed using Kaplan-Meier method and log-rank test. SAS 9.2 (SAS Institute Inc., Cary, NC) was used for all statistical analyses in the MPACT study.
RESULTS
ADAM12 associates with activated pancreatic cancer stroma and poor-prognosis molecular subclasses

We identified ADAM12 in a previous screen for stromal targets of tumor-derived SHH. To confirm that ADAM12 is expressed in human pancreatic cancers, we queried publically available gene expression datasets that contain normal pancreas and pancreatic cancer tissue. ADAM12 was significantly higher expressed in tumor tissue (Fig. 1a and Supporting Information Fig. S1a), and high expression of ADAM12 was associated with worse survival following resection (Fig. 1b and Supporting Information Fig. S1b). Microdissected tumor tissue expression data confirmed a predominantly stromal expression of ADAM12 (Fig. 1c). To further delineate the source of ADAM12 expression, we measured its expression by species-specific qPCR in patient-derived xenografts (PDXs). Mouse Adam12 expression in stromal host cells was found to be high compared to other well-characterized paralogs (Adam10 and -17; Fig. 1d).

To determine if ADAM12 expression is a hallmark of tumors with highly activated stromal stellate cells and (myo)fibroblasts, its correlation with known markers for such cells was determined by qPCR in bulk tumor tissue (Fig. 1e, and Supporting Information Fig. S2a). A strong correlation of ADAM12 was found with secreted protein acidic and cysteine rich (SPARC), α- muscle actin (ACTA2), and fibroblast activation protein (FAP). No strong inverse correlations with tumor cellularity were found. Gene set enrichment analysis revealed a significant enrichment of extracellular matrix and stromal pathway signatures in patients with high ADAM12 expression (Supporting Information Fig. S2b).

Subclasses of PDAC have been defined at the gene expression level. All current classifications identify a subtype that is characterized by mesenchymal features and increased stromal infiltration. We found that ADAM12 expression associated with both the Collisson et al. quasi-mesenchymal and the Bailey et al. squamous subtype tumors (Fig. 1f). Patients clustered with the activated stroma signature from Moffit et al. also showed high expression of ADAM12 (Fig. 1g). These analyses show that the expression of ADAM12 associates with poor-prognosis mesenchymal subgroups of PDAC.

ADAM12 expression is driven by tumor cell-derived TGF-β

Several tumor-derived signals have been identified that shape the stroma by activating the cells that reside in it. For instance, transforming growth factor beta (TGF-β) is a strong activator of cancer-associated fibroblasts (CAFs) and pancreatic stellate cells (PSCs) during cancer progression. To functionally confirm this activation mechanism to drive ADAM12 expression, we treated human stellate cells with TGF-β and other ligands known to be involved in tumor-stroma crosstalk. An upregulation of ADAM12 was only apparent in stellate cells treated with TGF-β (Fig. 2a).

ADAM12 exists as soluble proteins. These forms can be generated by shedding of the cell-bound protein, but in humans a soluble isoform (ADAM12-S) also exists. To determine if soluble ADAM12
is produced by activated stellate cells, PS-1 cells were stimulated using TGF-β or by coculturing with primary PDAC tumor cells, and ADAM12 was measured by ELISA in the supernatant of these cultures (Fig. 2b). As for the transcript analysis, a strong upregulation of ADAM12 was observed following TGF-β-dependent activation of stellate cells. Coculture of human stellate cells with primary tumor cells led to a significant upregulation of soluble ADAM12 that could be blocked by the TGF-β pathway inhibitor A83-01, confirming that active TGF-β ligand is present in these cocultures and able to drive ADAM12 secretion in stromal cells.36

**Figure 1.** ADAM12 associates with activated pancreatic cancer stroma and poor-prognosis molecular subclasses. (a) Boxes indicate median with first and third quartiles of log2 transformed gene expression values from two U133 Plus 2.0 microarray datasets of pancreatic cancer patients comparing normal and tumor tissue. Badea et al. set (GSE15471), n=36 paired biopsies; Pei et al. set (GSE16515); n=16 (normal), n=36 (tumor). ***p<0.001, statistical testing was by two-tailed Student’s t test. (b) Kaplan-Meier analysis of patients from the ICGC cohort, dichotomized for median ADAM12 expression.27 (c) Log2 transformed ADAM12 expression values from the Pilarksy et al. (E-MEXP-1121) gene expression set obtained from microdissected pancreatic cancer tissue. *p<0.05, testing by two-tailed Student’s t test. (d) Transcript levels for indicated Adam/ADAM paralogs relative to Gapdh/GAPDH were measured in xenografts by qPCR using mouse- or human-specific primers. Boxplots show data from 10 individual patient grafts. Difference between groups was tested by ANOVA for both panels p<0.0001. (e) Association of ADAM12 expression with stromal activation markers in the AMC patient cohort was measured by qRT-PCR, n=15. Size of dots indicates tumor cellularity scored by a pathologist. (f) Patients from GSE17891 dataset were grouped by published PDAssigner subtype labels and log2 expression of ADAM12 and paralog ADAM10 is shown (n=27). Patients from GSE17891 were grouped by published PC class labels (n=96). (g) Patients were k-means clustered using the activated stroma gene set and ADAM12 and –10 expression is shown (n=132).
Figure 2. ADAM12 expression is induced by tumor cell-derived TGF-β.
(a) Immortalized stellate cells (PS-1) were starved with 0.5% FCS for 24h and subsequently treated with the indicated ligands for 48h. Concentrations used: TGF-β 5 ng/ml; TGF-β receptor I inhibitor A83-01, 1µM; ShhN supernatant from 293T cells, 1:4; EGF, 50 ng/ml; bFGF, 10 ng/ml; HGF, 10 ng/ml; IL-1α, 10 ng/ml; IL-1β, 10 ng/ml. In addition to ADAM12, expression analysis of stromal activation marker genes ACTA and SPARC in response to TGF-β is shown. Two-tailed Student’s t test comparing control vs. TGF-β, ***p<0.001, **p<0.001. (b) To ascertain that tumor cell-derived TGF-β drives ADAM12 expression, stellate cells were treated as for panel a or cocultured with tumour cells of the 84 primary culture. Supernatant was harvested and cleared by centrifugation. Soluble ADAM12 levels were measured by ELISA. At least three replicates are shown, statistical testing was by two-tailed Student’s t test comparing control vs. TGF-β, and control vs. coculture, ***p<0.001.

ADAM12 is elevated in the serum of PDAC patients and predicts outcome after resection
Having established the association of ADAM12 with stromal activation and poor-prognosis molecular subclasses, we proceeded to evaluate ADAM12 as a non-invasive biomarker in PDAC. Patients diagnosed with PDAC before therapeutic intervention showed a significant elevation of serum ADAM12 compared to healthy individuals (Fig. 3a). The association of serum ADAM12 levels with clinical parameters was analyzed (Supporting Information Table S1). Patients were dichotomized using serum ADAM12 levels determined by receiver-operator-characteristics (ROC, for live-dead resected patients at time of analysis; 316 pg/ml). No correlations of serum ADAM12 with age, primary tumor size, and disease stage were found (Supporting Information Table S2). High ADAM12 levels in the resected cohort associated with poor survival (HR=2.07, p=0.04), as did high CA19-9 levels and high LNR (Table 1). In a multivariate analysis no significant associations were found (Supporting Information Table S3). The impact of serum ADAM12 on overall survival was analyzed by Kaplan–Meier analysis and log-rank test. We found that whereas serum ADAM12 did not significantly associate with survival in unresectable patients, in resected patients higher ADAM12 levels were strongly associated with shorter survival (Fig. 3b,c). It thus appears that activated stroma, as revealed by high serum ADAM12 levels, contributes to poor disease outcome when the tumor is at a resectable stage.
Table 1. Cox proportional hazard regression model. Univariate analysis for overall survival in PDAC patients (resected N=58; unresectable N=86). Dichotomization of resected patients by CA19-9 was at 41.5 kU/L, by ADAM12 at 316 pg/mL. Dichotomization of unresectable patients by CA19-9 was at 354 kU/L. LNR, lymph node ratio.

|                       | resected HR 95% CI P-value | unresectable HR 95% CI P-value |
|-----------------------|-----------------------------|--------------------------------|
| age                   | 1.01 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| gender                |                             |                                |
| male                  | 1.00 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| female                | 0.64 0.31-1.32 0.225        | 1.32 0.84-2.07 0.236           |
| stage                 |                             |                                |
| I                     | 1.00 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| II                    | 2.37 0.56-10.01 0.242       | 1.00 0.02-20.36 0.242          |
| III                   | 2.14 0.41-11.12 0.368       | 1.44 0.59-3.54 0.424           |
| IV                    | 3.86 0.35-42.93 0.273       | 2.51 1.06-5.99 0.038           |
| tumor size            |                             |                                |
| ≤20mm                 | 1.00 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| >20mm                 | 2.01 0.63-4.92 0.124        | 25.36 0.37-174 0.134           |
| CA19-9                |                             |                                |
| low                   | 1.00 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| high                  | 3.28 1.05-10.19 0.04        | 2.11 1.21-3.69 0.008           |
| LNR                   | 7.49 1.61-34.89 0.01        | n/a n/a n/a                    |
| ADAM12                |                             |                                |
| low                   | 1.00 0.97-1.04 0.73         | 1.02 0.99-1.05 0.203           |
| high                  | 2.07 1.03-4.16 0.041        | 0.67 0.41-1.10 0.116           |

Figure 3. ADAM12 is elevated in the serum of PDAC patients and predicts poor outcome in patients undergoing resection. (a) ADAM12 levels were measured by ELISA in serum of healthy individuals (n=38), and patients diagnosed with PDAC (n=157). Boxplots show median with interquartile range. ***p<0.0001; tested by Mann-Whitney U-test against healthy controls. (b) Kaplan-Meier survival analysis of AMC PDAC patients who underwent resection of the tumor, dichotomized by baseline serum levels determined by receiver-operator-characteristics (ROC; 316 pg/mL). (c) As for panel b, for patients who did not undergo resection.
ADAM12 levels predict favorable outcome in patients treated with nab-paclitaxel

The phase III MPACT trial showed survival benefit of nab-paclitaxel with gemcitabine compared to gemcitabine in metastasized PDAC patients, and is relatively well tolerated. To determine if ADAM12 levels associate with response to nab-paclitaxel, we measured its levels in plasma samples from the MPACT cohort.\textsuperscript{9, 24, 37}

Baseline samples were measured and it was observed that the decreased sensitivity of detection in plasma (rather than serum) resulted in a considerable number of samples that had undetectable levels of ADAM12 as defined by 2 \times \text{standard deviation of the optical density of blanks}. Dichotomization of the MPACT cohort by this cutoff resulted in groups with similar size across treatment arms and baseline characteristics (Supporting Information Table S4 and S5) but significantly worse survival for patients with detectable ADAM12 (Supporting Information Fig. S3a). Univariate Cox regression revealed a HR of 1.41 (1.10–1.81 95\%CI; \( p = 0.0062 \)) for detectable ADAM12 (Supporting Information Table S6) in this cohort.

When the trial arms were analyzed separately, ADAM12 levels did not significantly associate with survival in patients that received gemcitabine monotherapy (Fig. 4a, dashed lines, \( P = 0.2543 \)). Conversely, in patients that received nab-paclitaxel with gemcitabine, undetectable plasma ADAM12 strongly associated with favorable outcome (solid lines, \( P = 0.0046 \)). Patients with undetectable ADM12 showed a median survival benefit of over 4.0 months from the addition of nab-paclitaxel to gemcitabine, as compared to a benefit of 1.9 months for patients with detectable ADAM12. Baseline ADAM12 levels were significantly associated with outcome in a multivariate model including KPS, and treatment as factors (Supporting Information Table S7). Inclusion of CA19.9 in the model yielded a non-significant association of ADAM12 with survival.

Next, we determined the predictive power of the change in ADAM12 levels during treatment from cycle 1, day 1 (baseline) to cycle 2, day 1 (follow-up) samples (Supporting Information Table S4b, baseline characteristics in Supporting Information Table S5B). A reduction in ADAM12 levels is likely to be caused by a diminished stromal activation, or a reduced tumor load. Indeed, a reduction in plasma ADAM12 associated with improved survival (Supporting Information Fig. S3b). Importantly, these associations were all driven by the favorable outcome of patients with reduced (OS 11.2m) or repeatedly undetectable (OS 14.4m) ADAM12 in the nab-paclitaxel with gemcitabine-treated trial arm, as compared to the increased levels (OS 8.3) of ADAM12 (Fig. 4b,c). Although statistical significance was not reached in the gemcitabine arm, the rank ordering of the groups defined by change in ADAM12 levels on treatment was the same in both arms of the trial. In the poorest-outcome group (in which ADAM12 became detectable or increased at cycle 2), the overall survival was numerically superior at 8.3 months in the nab-paclitaxel with gemcitabine arm as compared to the monotherapy arm at 6.9 months. Thus, our results do not indicate an absence of treatment benefit from nab-paclitaxel in the patients with high or increased ADAM12.
Figure 4. Plasma ADAM12 predicts favorable outcome in patients treated with nab-paclitaxel and gemcitabine. (a) Baseline recalcified plasma samples from the MPACT cohort were analyzed for ADAM12 and patients were dichotomized for ADAM12 levels above or below detection (2x SD of blanks). Patients in the arm receiving gemcitabine monotherapy are indicated by dashed lines. Numbers in parentheses indicate number of patients in the group (n), and median survival for that group (OS). Analysis of patients in the arm receiving gemcitabine and nab-paclitaxel is shown with solid lines. (b) Baseline (cycle 1, day 1) and follow-up (cycle 2, day 1) samples were measured and fold change was calculated. Samples with undetectable ADAM12 levels at both baseline and follow-up did not show a fold change and are indicated by the red line. Patients in the arm receiving gemcitabine monotherapy are shown. (c) As for panel b, analysis of patients in the arm receiving gemcitabine and nab-paclitaxel.
In conclusion, we established that ADAM12 is a serum-borne proxy for the stromal activation of pancreatic cancers, and that its levels associate with poor disease outcome. A low level of or decrease in ADAM12 is associated with improved survival in patients treated with nab-paclitaxel and gemcitabine, and could possibly be used to stratify patients in future trials using this or other treatment combinations.

**DISCUSSION**

PDAC is characterized by an abundance of activated stroma that harbors both tumor-promoting, as well as suppressive properties. Here, analysis of gene expression data, patient-derived xenografts and co-culture experiments identified ADAM12 as a circulating marker for activated stroma and tumors of poor-prognosis molecular subclasses. In patients with resectable disease, high ADAM12 levels predicted inferior survival outcomes. This could imply that ADAM12 is not a general stromal activation marker, but that there are very specific biological programs affected by this protein. Known roles for stromal ADAM12 are manifold: The well characterized function of ADAM12 as a sheddase of growth factor receptor ligands such as EGF can provide a nurturing environment for tumor cells. Furthermore, ADAM12 has been shown to participate in matrix remodeling by cleavage of extracellular matrix proteins, suggesting involvement in cancer invasion and metastasis formation. Further studies, similar to ones performed for breast and prostate cancer, are needed to elucidate the functional contributions of ADAM12 to pancreatic cancer.

In metastatic PDAC patients treated with gemcitabine, ADAM12 was not associated with survival. However, low ADAM12 levels did predict response to treatment in the cohort of patients treated with nab-paclitaxel and gemcitabine. This suggests that the supportive role of the stroma in the metastases predominates, to induce resistance to cytotoxic treatment. In this case, low ADAM12 levels identify lesions that are more sensitive to cytotoxic treatment. Clearly, such an effect becomes more apparent in effective treatment regimens, such as nab-paclitaxel with gemcitabine. Future studies are needed to show that ADAM12 retains its predictive power for other effective cytotoxic treatment regimens, such as FOLFIRINOX.

Alternatively, it could be argued that the stroma-targeting properties of nab-paclitaxel explain why low ADAM12 predicts favorable outcome of nab-paclitaxel and gemcitabine. Nab-paclitaxel is accepted to have at least some degree of specificity for the stroma: The conjugation to albumin has been suspected to increase taxane concentration at sites of high SPARC (an albumin receptor) expression such as stromal (myo)fibroblasts. In tumors with relatively few activated stromal cells, nab-paclitaxel will therefore be able to effectively eradicate the vast majority of the stroma, whereas in tumors with highly abundant stroma, the ablation is only partial and not effective in the long term.

One limitation of the current study is the exploratory, retrospective nature of this analysis. The MPACT trial was not statistically powered to test the interaction between serum markers such as ADAM12 and treatment groups. Furthermore, it is possible that the predictive value of ADAM12 as
inferred from the differences in response to treatment is a result of the longer survival of patients receiving nab-paclitaxel with gemcitabine. Moreover, it should be noted that other biomarker discovery studies have not yet yielded applicable markers. For instance the initially suspected role for SPARC in conferring enhanced sensitivity to nab-paclitaxel in PDAC was not confirmed, and its expression did not correlate with response to nab-paclitaxel with gemcitabine. Decreased CA19-9 levels were initially described to associate with response rate in the experimental arm of the MPACT trial, and high baseline CA19.9 was associated with reduction in risk of death from the combination treatment (gemcitabine with nab-paclitaxel). However, in a follow-up analysis CA19-9 levels did not predict survival in the combination treatment arm.

Although the significant association of serum ADAM12 with outcome was not sustained in multivariate analysis of the MPACT samples when CA19-9 was included in our model, levels of ADAM12 were very strongly associated with favorable responses to nab-paclitaxel with gemcitabine suggesting that the analysis of a sufficiently large cohort could confirm predictive power. This will offer important possibilities for patient stratification. ADAM12, or similar blood-borne proxies for stromal activation could possibly be used to stratify patients in future trials with experimental stroma targeting agents, as well as for currently used (chemo)therapeutics.
ACKNOWLEDGEMENTS

We thank Tom van Leusden for technical assistance, and all patients for participating.

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Keywords

pancreatic cancer, stroma, serum marker, ADAM12, nab-paclitaxel

Abbreviations

ACTA2: α-smooth muscle actin gene; ADAM: a disintegrin and metalloprotease; CAF: cancer associated fibroblast; EGF: epidermal growth factor; FAP: fibroblast activation protein; FOLFIRINOX: folinic acid, fluorouracil, irinotecan, oxaliplatin; KPS: Karnofsky performance status; LNR: lymph node ratio; PDAC: pancreatic ductal adenocarcinoma; PSC: pancreatic stellate cell; SHH: Sonic Hedgehog; SPARC: secreted protein acidic and cysteine rich; TGF-β: transforming growth factor-β; TNM: tumor/lymph node/metastasis

Grant sponsor

KWF Dutch Cancer Society

Grant number

UVA 2012-5607

Grant sponsor

KWF Dutch Cancer Society

Grant number

UVA 2013-5932
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ADAM12 is a circulating marker for stromal activation in pancreatic cancer and predicts response to chemotherapy.
SUPPLEMENTARY METHODS

Establishment of patient-derived xenografts and primary cell lines

Collection of tumor material was approved by the institute’s ethics committee in accordance with the Declaration of Helsinki (AMC METC 2014_181). Written informed consent was received from all participants. All PDAC patients treated in the Academic Medical Center were diagnosed by pathology or cytology. Specimens were processed and inspected according to national and international guidelines. An experienced pathologist performed microscopic assessment. Final diagnosis was set in accordance with the WHO classification, and the pTNM classification of malignant tumors. Freshly excised tumor pieces (approximately 3x3x3 mm) originating from the primary tumor or liver metastasis were washed several times in PBS containing 10 µg/ml gentamycin (Lonza, Basel, Switzerland) and 1% penicillin-streptamycin. Pieces were grafted subcutaneously into the flank of immunocompromised NOD.Cg-PrkdcscaIl2rg-scid/Il2rg<sup>scid</sup>/SzJ (NSG) mice (JAX 005557) with Matrigel (BD, Franklin Lakes, NJ). Animals were bred and maintained at the local animal facility according to the pertinent legislation, and ethical approval was obtained for all procedures (DTB/LEX102348). After outgrowth to a size of approximately 500 mm<sup>3</sup>, tumors were harvested and passaged, and/or used to establish in vitro cultures. For this, harvested xenografts were minced with a scalpel blade, placed in IMDM with 8% FBS and collagenase IV (0.5 mg/ml, Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 60 min with vortexing every 15 min. The dissociated suspension was passed through a 70 µm cell strainer, washed with culture medium and grown in IMDM containing 8% FBS and 50µM β-mercaptoethanol. During the first 5-10 passages, cultures contained colonies of human epithelial cells and a layer of murine fibroblasts. A culture without epithelial component, as confirmed by flow cytometry using an anti-EPCAM antibody (DAKO, F0860 at 1:100), was used for stimulation experiments.

Treatment of cultures

Stellate cells were seeded in 12 well culture plates and upon reaching confluence, pre-starved overnight in 0.5% FBS containing medium and treated for 24h with 5ng/ml recombinant human TGF-β1 (Peprotech, London, United Kingdom) in the presence or absence of 1µM ALK4/5/7 inhibitor A83-01 (Tocris Bioscience, Bristol, United Kingdom). ShhN supernatant from 293T cells was added 1:4, EGF was used at 50 ng/ml, bFGF, 10 ng/ml; HGF 10 ng/ml; IL-1α, 10 ng/ml; IL-1β, 10 ng/ml.

RNA isolation and quantitative real-time PCR

Small pieces of PDX tumor were homogenized using an Ultra-Turrax tissue homogenizer T8 (IKA-Werke, Staufen im Breisgau, Germany) in 1ml of Trizol (ThermoFisher). Primary cells were lysed in Trizol and RNA isolation was performed according to the manufacturer’s protocol. Snap frozen patient tumor samples were embedded in Tissue-Tek OCT (Sakura FineTek, Japan) and 30 slices of 20 µm each were cut on a cryotome. Cut tissue was immersed in 1 ml of RNA Bee (Amsbio, Abingdon, United Kingdom), homogenized, and RNA isolation was performed according
to manufacturer’s protocol (Qiagen, Hilden, Germany). For tumor percentage scoring, a 10 µm slice was kept before the tissue was cut in 20 µm slices, and processed for H&E staining. Scoring of tumor percentage was performed by an experienced pathologist. cDNA was synthesized using Superscript III (ThermoFisher) and random primers. Real-time quantitative RT-PCR was performed with SYBR green (Roche, Basel, Switzerland) on a Lightcycler LC480 II (Roche). Relative expression of genes was calculated using the comparative threshold cycle (Cp) method and values were normalized to reference gene GAPDH/Gapdh. Primer sequences are: hGAPDH Fw 5’ gaaggtgaaggtcggagtct 3’; hGAPDH Rv 5’ tggaagatgtgatggatt 3’; hADAM10 Fw 5’ ttcatgcaaatacagca 3’; hADAM10 Rv 5’ ttctctcctcgacagtct 3’; hADAM12 Fw 5’ ttccacacccctctcagac 3’; hADAM12 Rv 5’ gctctgaaactctcggtt 3’; hADAM17 Fw 5’ gggaaacatgagcgtctc 3’; hADAM17 Rv 5’ accgaatgtcgtggtatt 3’; hACTA2 Fw 5’ caaagccgccctcaagag 3’; hACTA2 Rv 5’ agcccagccaaactc 3’; hFAP Fw 5’ tcagggtgagttctcattg 3’; hFAP Rv 5’ gctgtgcttgcctatttg 3’; hSPARC Fw 5’ gaaagatgacccgcaagct 3’; hSPARC Rv 5’ ctcagactgccggaga 3’; hKRT19 Fw 5’ ttagagggtgaagatccgca 3’; mGapdh Fw 5’ ctcatgccacccatggcg 3’; mGapdh Rv 5’ cgtgctgcttgggtgacg 3’; mAdam10 Fw 5’ aagatgtggttgcggccag 3’; mAdam10 Rv 5’ cgcctcctgactgagct 3’; mAdam12 Fw 5’ gcctgaggacagcagacag 3’; mAdam12 Rv 5’ cgcctcctgtttctttct 3’; mAdam17 Fw 5’ gcctcctgtttctttct 3’.

Gene Set Enrichment Analysis (GSEA) and expression analysis
Gene set enrichment analysis (GSEA, v2.0.14) software was downloaded from the Broad Institute website (http://www.broadinstitute.org/gsea) and used according to the author’s guidelines. Median ADAM12 expression was used to dichotomize samples. Gene set for the GO term ‘extracellular matrix’ was downloaded from the Molecular Signature Database (MSigDB; V4.0); the pancreatic stroma signature was published by Binkley et al. 2000 phenotype permutations were used to determine significance of the enrichment score. Gene expression data were collected and processed for use in the AMC in-house R2 Genomics Analysis and Visualization Platform: (http://r2.amc.nl). For visualization of gene expression, data were imported in R and plotted using ggplot2, or plotted in Graphpad Prism.
Supplementary Figure S1. ADAM12 expression correlates with stromal activation in pancreatic cancer. (a) Boxes indicate median with first and third quartiles of log2 transformed gene expression values from two U133 Plus 2.0 microarray datasets of pancreatic cancer patients comparing normal and tumor tissue, using the Zhang et al. Human Gene Chip 1.0 platform expression data (GSE28735; 5). n=45 paired biopsies, ***p<0.001. (b) Kaplan-Meier plot of patients from the GSE15471 set, dichotomized for median ADAM12 expression.

Supplementary Figure S2. (a) Scatter plot of ADAM12 gene expression and indicated stromal activation markers in bulk patient tumors (GSE16515 6, GSE15471 7, GSE28735 5). Line indicates linear regression fit, shaded area indicates standard error confidence bounds, determined using the R linear model function. Size of dots indicates expression of epithelial marker gene KRT19. No strong inverse correlations with tumor cell content as inferred from cytokeratin 19 (KRT19) and epithelial cell adhesion molecule (EPCAM) were found. R-squared (R2) linear regression coefficients and statistical significance of regression are shown in tabular overview. (b) Gene set enrichment analysis (GSEA) on the GSE16515 dataset using gene ontology set ’extracellular matrix’ as a gene signature, pancreatic stromal signature by Binkley et al. 4. Samples were dichotomized by median ADAM12 expression. NES, normalized enrichment score, FWER p, family-wise error rate multiple testing-corrected significance.
Supplementary Figure S3. Plasma ADAM12 levels correlate with favorable outcome in metastatic PDAC patients. (a) Baseline recalcified plasma samples from the entire MPACT cohort were analyzed for ADAM12 and patients were dichotomized for ADAM12 levels above or below detection (2 x SD of the OD of blanks). Kaplan-Meier survival analysis was performed, with log-rank testing. (b) Baseline (cycle 1, day 1) and follow-up (cycle 2, day 1) samples were measured and fold change was calculated. Samples with undetectable ADAM12 levels at both baseline and follow-up did not show a fold change and are indicated by the red line.

Table S1. Baseline characteristics of AMC PDAC patients (2012-2015). Asterisks indicate parameters with incomplete coverage. The stage IV patient in the resected cohort had distant positive lymph nodes and was staged as M1. IQR: interquartile range, LNR: lymph node ratio.
Table S2. Correlations of ADAM12 serum levels and baseline clinicopathological parameters of the AMC cohort. Patients were divided into high and low ADAM12 serum level by ROC (316 pg/mL, determined on the resected cohort). CA19-9 and LNR were tested as continuous variables.

|                | resected |        |        |        |        |        |        |        |
|----------------|----------|--------|--------|--------|--------|--------|--------|--------|
|                | low      | high   | Total  | P value| Low    | High   | Total  | P value|
| age            | 34       | 26     | 60     | 0.303  | 32     | 57     | 89     | 0.416  |
| gender         |          |        |        |        |        |        |        |        |
| male           | 18       | 17     | 35     | 0.333  | 13     | 31     | 44     | 0.213  |
| female         | 16       | 9      | 25     | 0.276  | 19     | 26     | 45     | 0.51   |
| stage          |          |        |        |        |        |        |        |        |
| I              | 3        | 4      | 7      | 0.475  |        |        |        |        |
| II             | 26       | 16     | 42     |        | 4      | 6      | 10     | 0.948  |
| III            | 5        | 5      | 10     |        | 11     | 21     | 32     |        |
| IV             | 0        | 1      | 1      |        | 17     | 30     | 47     |        |
| tumor size     |          |        |        |        |        |        |        |        |
| ≤20mm          | 8        | 4      | 12     | 0.443  |        |        |        |        |
| >20mm          | 25       | 21     | 46     |        | 28     | 53     | 81     |        |
| CA19-9         |          |        |        |        |        |        |        |        |
| low            | 15       | 12     | 27     | 0.197  |        |        |        |        |
| high           | 20       | 49     | 69     |        |        |        |        |        |
| LNR            | 34       | 25     | 59     | 0.944  |        |        |        |        |

Table S2. Correlations of ADAM12 serum levels and baseline clinicopathological parameters of the AMC cohort. Patients were divided into high and low ADAM12 serum level by ROC (316 pg/mL, determined on the resected cohort). CA19-9 and LNR were tested as continuous variables.

Table S3. Cox proportional hazard regression model on overall survival in the AMC cohorts. Multivariate analysis for overall survival in PDAC patients (resected n=58; unresectable n=86). ADAM12 cut-off as for Supplementary Table 2. Dichotomization of resected patients by CA19-9 was at 41.5 kU/L, of unresectable patients at 354 kU/L.
### Table S4. Treatment group sizes by ADAM12 plasma level dichotomization of the MPACT cohort. (A) Baseline levels, and (B) fold change in ADAM12 levels at follow-up relative to baseline (C1D1 to C2D1).

|                | ADAM12 at C1D1                  |
|----------------|--------------------------------|
|                | undetectable       | detectable       |
| **Treatment**  | Nab-paclitaxel +Gemcitabine | 45/95 (47%)   | 139/277 (50%)  |
|                | Gemcitabine         | 50/95 (53%)   | 138/277 (50%)  |

|                | ADAM12 fold change |
|----------------|--------------------|
|                | 0                  | <1                | >1                |
| **Treatment**  | Nab-paclitaxel +Gemcitabine | 22/38 (58%) | 59/112 (53%) | 27/58 (47%) |
|                | Gemcitabine        | 16/38 (42%) | 53/112 (47%) | 31/58 (53%) |

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Table S5. Correlation of ADAM12 plasma levels to clinicopathological parameters of the MPACT cohort.

(A) Baseline characteristics of cohort divided by baseline ADAM12 levels are shown. NLR; neutrophil-to-lymphocyte ratio. KPS; Karnofsky performance status; #, F-test applied. (B) As panel A, for fold change in ADAM12 from C1D1 to C2D1. Asterisk indicated CMH tested p-value.

### Table S5

#### A

| Parameter                      | ADAM12 at C1D1 |                |                | CMH P-value |
|-------------------------------|----------------|----------------|----------------|-------------|
|                               | undetectable (N=95) | detectable (N=277) | total (N=372) |              |
| NLR                           |                |                |                |              |
| ≤5                            | 71             | 161            | 232 (62%)      | 0.004       |
| >5                            | 24             | 116            | 140 (38%)      |             |
| CA19-9                        |                |                |                |              |
| ≥59xuln                       | 48             | 132            | 180 (48%)      | 0.7447      |
| >uln - <59xuln                | 28             | 81             | 109 (29%)      |             |
| normal                        | 13             | 40             | 53 (14%)       |             |
| KPS at baseline               |                |                |                |              |
| 90-100                        | 65             | 161            | 226 (60%)      | 0.0892      |
| 70-80                         | 30             | 114            | 144 (39%)      |             |
| liver metastasis              |                |                |                |              |
| yes                           | 78             | 233            | 311 (84%)      | 0.6484      |
| no                            | 17             | 44             | 61 (16%)       |             |
| age (years)                   |                |                |                |              |
| <65                           | 52             | 166            | 218 (59%)      | 0.3760      |
| ≥65                           | 43             | 111            | 154 (41%)      |             |
| sum of longest diam at BL (cm)| least squares mean | 10.4 | 12.1 | 0.0526* |

#### B

| Parameter                      | ADAM12 fold change |                |                | Trend P-value |
|-------------------------------|--------------------|----------------|----------------|---------------|
|                               | 0 (N=38) | <1 (N=112) | >1 (N=58) | total (N=208) |              |
| NLR                           |          |            |          |                |              |
| ≤5                            | 31       | 83         | 34       | 148 (71%)      | 0.0106       |
| >5                            | 7        | 29         | 24       | 60 (29%)       |             |
| CA19-9                        |          |            |          |                |              |
| ≥59xuln                       | 18       | 54         | 34       | 106 (51%)      | 0.1852*      |
| >uln - <59xuln                | 12       | 36         | 17       | 65 (31%)       |             |
| normal                        | 6        | 16         | 5        | 27 (13%)       |             |
| KPS at baseline               |          |            |          |                |              |
| 90-100                        | 32       | 74         | 36       | 142 (68%)      | 0.0325       |
| 70-80                         | 6        | 38         | 22       | 66 (32%)       |             |
| liver metastasis              |          |            |          |                |              |
| yes                           | 28       | 93         | 50       | 171 (82%)      | 0.1340       |
| no                            | 10       | 19         | 8        | 37 (18%)       |             |
| age (years)                   |          |            |          |                |              |
| <65                           | 21       | 69         | 40       | 130 (63%)      | 0.1663       |
| ≥65                           | 17       | 43         | 18       | 78 (37%)       |             |
| sum of longest diam at BL (cm)| least squares mean | 8.5 | 10.4 | 13.9 | 0.0003* |

Table (A) Baseline characteristics of cohort divided by baseline ADAM12 levels are shown. NLR; neutrophil-to-lymphocyte ratio. KPS; Karnofsky performance status; #, F-test applied. (B) As panel A, for fold change in ADAM12 from C1D1 to C2D1. Asterisk indicated CMH tested p-value.
### Table S6. Univariate Cox Regression Model for the MPACT cohort. Including the individual potentially prognostic factors.

| Factor                        | Comparison                          | HR   | 95% CI      | P value |
|-------------------------------|-------------------------------------|------|-------------|---------|
| age                           | <65 vs ≥65                          | 0.86 | 0.69 - 1.07 | 0.1654  |
| liver metastasis              | no vs yes                           | 0.55 | 0.41 - 0.75 | 0.0001  |
| CA19-9 at baseline            | ≥59xULN vs normal                   | 1.36 | 0.98 - 1.88 | 0.0667  |
|                               | >ULN-<59xULN vs normal              | 1.12 | 0.79 - 1.58 | 0.5262  |
| NLR                           | ≤5 vs >5                            | 0.43 | 0.35 - 0.54 | <.0001  |
| ADAM12 baseline               | 0 vs >0                             | 0.71 | 0.55 - 0.91 | 0.0062  |
| ADAM12 Fold change            | <1 vs 0                             | 1.52 | 1.00 - 2.29 | 0.0478  |
|                               | >1 vs 0                             | 2.26 | 1.43 - 3.56 | 0.0005  |
| treatment                     | A+GEM vs GEM                        | 0.81 | 0.66 - 1.01 | 0.0582  |
| KPS                           | 90-100 vs 70-80                     | 0.57 | 0.46 - 0.71 | <.0001  |
| sum of longest diameter at baseline | every 5 cm increase                | 1.18 | 1.11 - 1.26 | <.0001  |

### Table S7. Multivariate Cox Regression Model. Including ADAM12 at baseline, treatment arm, and KPS as major factors.

| Factor                        | Comparison                          | HR   | 95% CI      | P-value  |
|-------------------------------|-------------------------------------|------|-------------|----------|
| ADAM12 baseline               | 0 vs >0                             | 0.75 | 0.59 - 0.96 | 0.0241   |
| treatment                     | A+GEM vs GEM                        | 0.77 | 0.62 - 0.96 | 0.0194   |
| KPS                           | 90-100 vs 70-80                     | 0.58 | 0.47 - 0.73 | <.0001   |
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