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ORIGINAL ARTICLE

# A transcriptomic signature to predict adjuvant gemcitabine sensitivity in pancreatic adenocarcinoma

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**Background:** Chemotherapy is the only systemic treatment approved for pancreatic ductal adenocarcinoma (PDAC), with a selection of regimens based on patients' performance status and expected efficacy. The establishment of a potent stratification associated with chemotherapeutic efficacy could potentially improve prognosis by tailoring treatments.

**Patients and methods:** Concomitant chemosensitivity and genome-wide RNA profiles were carried out on preclinical models (primary cell cultures and patient-derived xenografts) derived from patients with PDAC included in the PaCaOmics program (NCT01692873). The RNA-based stratification was tested in a monocentric cohort and validated in a multicentric cohort, both retrospectively collected from resected PDAC samples (67 and 368 patients, respectively). Forty-three (65%) and 203 (55%) patients received adjuvant gemcitabine in the monocentric and the multicentric cohorts, respectively. The relationships between predicted gemcitabine sensitivity and patients' overall survival (OS) and disease-free survival were investigated.

**Results:** The *GemPred* RNA signature was derived from preclinical models, defining gemcitabine sensitive PDAC as *GemPred*+. Among the patients who received gemcitabine in the test and validation cohorts, the *GemPred*+ patients had a higher OS than *GemPred*− ($P = 0.046$ and $P = 0.00216$). In both cohorts, the *GemPred* stratification was not associated with OS among patients who did not receive gemcitabine. Among gemcitabine-treated patients, *GemPred*+ patients had significantly higher OS than the *GemPred*−: 91.3 months [95% confidence interval (CI): 61.2-not reached] versus 33 months (95% CI: 24-35.2); hazard ratio 0.403 (95% CI: 0.221-0.735, $P = 0.00216$). The interaction test for gemcitabine and *GemPred*+ stratification was significant ($P = 0.0245$). Multivariate analysis in the gemcitabine-treated population retained an independent predictive value.

**Conclusion:** The RNA-based *GemPred* stratification predicts the benefit of adjuvant gemcitabine in PDAC patients.

**Key words:** pancreatic cancer, gemcitabine, chemosensitivity prediction, transcriptomic signature, precision medicine

## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related mortality in Western countries and bears one of the poorest prognosis with a 5-year survival rate of 9%.[1] In addition to frequent late-stage diagnosis, this dismal outcome can also be explained by the lack of effective therapies.[2] Targeted therapies and immunotherapies have failed to improve unselected patient outcomes, making chemotherapy the only effective systemic treatment.[3] Despite being a generally refractory cancer, it has been shown that the molecularly-guided selection of small patient subgroups increased the efficacy for some therapies such as olaparib for patients with germline *BRCA* mutations[4] (∼5% of patients) or immunotherapy[5] for mismatch repair-deficient tumors (∼1% of patients). These studies demonstrate the advantage of molecular stratification for therapeutic decisions and introduce an incremental model for PDAC, resolving one subgroup at a time.

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For patients with excellent performance status, the polychemotherapy regimen 5 fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) was shown to be more effective than gemcitabine alone in both the adjuvant[6] and metastatic settings[7] at the expense of high toxicities. The selection of systemic therapy is, therefore, based as much on the patient's fitness as on the potential efficacy of a particular chemotherapeutic regimen. Companion diagnostics are used to guide the choice of therapy by selecting patients who have a higher chance of responding to a given therapeutic agent. With an ever-growing number of regimens composed of multiple chemotherapies, this has not only the potential to improve survival by matching drugs to likely responders, but also to reduce adverse effects by avoiding unnecessary highly toxic multiagent regimens.[8]

Gemcitabine is the preferred companion of nab-paclitaxel, another systemic therapy that can be used in some countries as the first line in the advanced setting.[9] However, there is, to date, no upfront comparison of modified FOLFIRINOX and gemcitabine-nab paclitaxel. It could be proposed that patients with homologous recombination deficiency should be treated with platinum salts while for the others a gemcitabine efficacy predictive biomarker could help select patients for the gemcitabine-nab paclitaxel regimen.

Gemcitabine remains to date the most effective monotherapy in PDAC with an estimated response rate of 10%-23% in advanced patients[7,10] and is often used in patients unfit for more aggressive therapies.[11,12] Mostly based on gemcitabine metabolism, the association of single gene biomarkers with the response to gemcitabine has been demonstrated both at the level of protein expression[13] and genetic polymorphisms.[14,15] In particular, the nucleoside transporter hENT1 has been extensively studied and its protein expression was shown in multiple studies to be associated with gemcitabine sensitivity in PDAC[13,16-18] as well as in other malignancies.[19] The stratification of patients by hENT1 expression, however, is hindered by the difficulty to robustly assess the protein's expression given the high discrepancies between available antibodies.[20]

Multigene signatures based on RNA expression measurements have been shown to provide robust predictive tools in breast[21,22] and prostate[23] cancer. In PDAC, RNA signatures provide an in-depth description of tumor phenotypes, summarized by the basal-like and classical epithelial subtypes, with robust prognostic and suggested predictive values.[24,25] GATA6, a surrogate of the molecular subtypes,[26] was associated with response to chemotherapy, specifically to the 5-fluorouracil/leucovorin regimen, in the ESPAC3 trial.[27] Overall, RNA signatures, whether defining general molecular phenotypes or drug-sensitive phenotypes,[28] have only shown limited predictive value compared with the unfortunately unavailable biomarkers derived from gemcitabine metabolism such as hENT1.

The purpose of this study was to establish an RNA-based signature predictive of gemcitabine sensitivity in PDAC relying on preclinical models, patient-derived primary cell cultures and xenografts, with concomitant genome-wide RNA profiles and gemcitabine sensitivity analyses. A robust statistical approach was then used to derive from these preclinical models a large-scale multigene signature predictive of gemcitabine sensitivity. This signature was finally tested in a monocentric cohort and validated in a large multicentric cohort of patients with resected PDAC.

## MATERIAL AND METHODS

The Reporting recommendations for tumor MARKer prognostic studies (REMARK) were followed.[29]

### *In vitro and in vivo models*

*In vitro* and *in vivo* models were derived from patients included under the PaCaOmics clinical trial (ClinicalTrials.gov: NCT01692873). Fresh tumor samples were first used to generate patient-derived xenografts (PDX) which were then used to derive primary cell cultures. This study was approved by the Paoli-Calmettes hospital ethics committee following patient informed consent. Animal experiments were approved by the local ethics committee and carried out following the guidelines of our center (Cancer Research Center of Marseille).

Thirty-eight primary cell cultures (patients' clinical data in supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.10.601) were used for *in vitro* chemosensitivity tests, carried out in triplicates and repeated three times by measuring cell viability at different concentrations of gemcitabine. RNA sequencing (RNAseq) was applied to untreated cells to obtain transcriptomic profiles. Twelve PDX (patients' clinical data in supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2020.10.601) were tested for gemcitabine sensitivity. Eight of these patients were also included in the cell lines series. The first 16 PDX for each patient that reached 200 $mm^3$ were randomized to gemcitabine treatment or vehicle and tumor growth was monitored twice a week. RNAseq was applied to untreated PDX and only human transcripts were analyzed. Details in supplementary information, available at https://doi.org/10.1016/j.annonc.2020.10.601.

### *De novo gemcitabine sensitivity signature*

The strategy used to derive the *GemPred* signature is outlined in supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2020.10.601 and detailed in the supplementary information, available at https://doi.org/10.1016/j.annonc.2020.10.601. In essence, a dimensionality reduction method is used to derive RNA signatures defining candidate latent space from primary cell culture RNA profiles. The latent space that best distinguishes cell proliferation and *in vivo* response to gemcitabine is selected and compared with PDX. Finally, a linear combination of both, proliferation and sensitivity RNA signatures, is used to select a threshold that best discriminates the primary cell cultures that are the most sensitive to gemcitabine (i.e. in which gemcitabine induces the highest cytotoxicity). All decision thresholds providing at least three cell lines in

the smallest group were tested. A 20% cut-off showed the highest statistical difference as measured by Student's *t*-test (supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2020.10.601). The identified RNA signatures (proliferation and response) can be used on any genome-wide RNA profiling assay to project any new sample on each of these spaces. This approach was shown to give highly robust results and to score samples independently of technological considerations.[30] A web application is provided to apply the *GemPred* signature on whole transcription profiles, preferentially using an identical RNAseq methodology: http://cit-apps.ligue-cancer.net/pancreatic_cancer/GemPred. The genes with the highest contribution to the sensitivity signature, both positively and negatively, are reported in supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2020.10.601.

### *Patient cohorts of resected pancreatic adenocarcinoma*

This study was approved by the institutional review board (2010/01NICB IRB:00003835) and included two patient cohorts of consecutive and unselected patients subject to curative surgery for PDAC between September 1996 and August 2009.[13,25] Exclusion criteria were identical in both cohorts: preoperative chemotherapy or chemoradiotherapy, macroscopically incomplete resection, histology other than PDAC and death due to postoperative complications within 30 days following surgery.

The first monocentric cohort, referred to as the test cohort, included 86 patients from a university center with expertise in the management of PDAC (Erasme in Bruxelles, Belgium). Archived tissue for 19 patients had failed RNA profiles in a previous study and could not be retrieved to be reassessed for this study, making 67 patients assessable.

The second cohort, referred to as the validation multicentric cohort, included 385 patients from four French university centers with expertise in the management of PDAC. Archived tissue could not be retrieved for 7 patients and adjuvant treatment was not known for 10 patients, leaving 368 assessable patients.

Each participating center maintains a prospective PDAC database, including patient demographics, clinical and pathological variables. An aggregated clinical database was created with standardized clinicopathological variables, including sex, age at diagnosis, preoperative assessment of clinical disease stage, tumor stage according to the Union for International Cancer Control, TNM (tumor—node—metastasis) classification, histologic grade, adjuvant therapy and relevant outcome parameters including overall survival (OS) and disease-free survival (DFS). DFS was not evaluated for all patients. Details of RNA profiling are available in supplementary information, available at https://doi.org/10.1016/j.annonc.2020.10.601.

## RESULTS

### *Patient-derived model populations*

Thirty-eight PDAC samples were used in this work to produce PDAC primary cell cultures, 16 from resected tumors and 22 from endoscopic ultrasound (EUS)-guided biopsy (clinical characteristics in supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.10.601). Gemcitabine *in vitro* response assays were carried out on the primary cell cultures to determine their sensitivity using a dose-response approach. Gemcitabine showed a wide range of *in vitro* responses, measured by the plateau effect on cell viability [Einf, median = 0.329; range (2.12e-8, 0.628)], as well as a wide range of potency [EC50, median = 0.0169 μM; range (1.0e-5, 0.755)], on the collection of primary cell cultures. Twelve PDX were also generated and the *in vivo* tumor sensitivity to gemcitabine was assessed. Eleven tumors were obtained from surgical biopsies and one from an EUS-guided biopsy (clinical characteristics in supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2020.10.601). Genome-wide RNA expression profiles were obtained on the 38 primary cultures and 12PDX using RNAseq.

To both evaluate our study design and to test the possibility of using predictive biomarkers at the RNA level, the association of the expression of genes previously linked with the *in vitro* gemcitabine response was tested. *hENT1* and *CDA* were the only genes associated with an *in vitro* sensitivity (supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2020.10.601).

### *De novo RNA signatures of gemcitabine sensitivity with in vivo and in vitro models*

Single-gene based biomarkers alone are insufficient to propose an effective stratification strategy to select highly gemcitabine-responsive patients.[20] To define a robust multigene signature predicting gemcitabine sensitivity, we applied a procedure using the combination of *in vitro* gemcitabine response, estimated with dose-response curves of cell viability (Figure 1A), and whole-transcriptome profiling by RNAseq. Briefly, the strategy aims at the decomposition of the primary cell culture transcriptomic dataset into a set of independent RNA signatures (i.e. components derived from an independent component analysis) to extract distinct signatures associated with *in vitro* gemcitabine response and *in vitro* replication time as a measure of proliferation. This approach applied to the 38 primary cell cultures uncovered two distinct and independent (i.e. uncorrelated) RNA signatures, one associated with *in vitro* response to gemcitabine and one with *in vitro* proliferation (Figure 1B). The first was estimated by the area under the dose-response curve, representing the overall *in vitro* response, as well as the plateau effect measuring the efficacy at high dosage. Figure 1C and D illustrates the association between the gemcitabine *in vitro* response RNA signature and the dose-response curves. In 12 PDX with both transcriptomic profiles and the effect of gemcitabine treatment on tumor growth (Figure 1E), the gemcitabine *in vitro* response signature was significantly correlated to the gemcitabine versus control difference on tumor growth (Pearson's $r = 0.618$, $P = 0.0319$). Eight of the 12 PDX produced cellular lines that were used in this study. Only weak concordance was found between *in vitro* and *in vivo* models at the level of gemcitabine sensitivity and

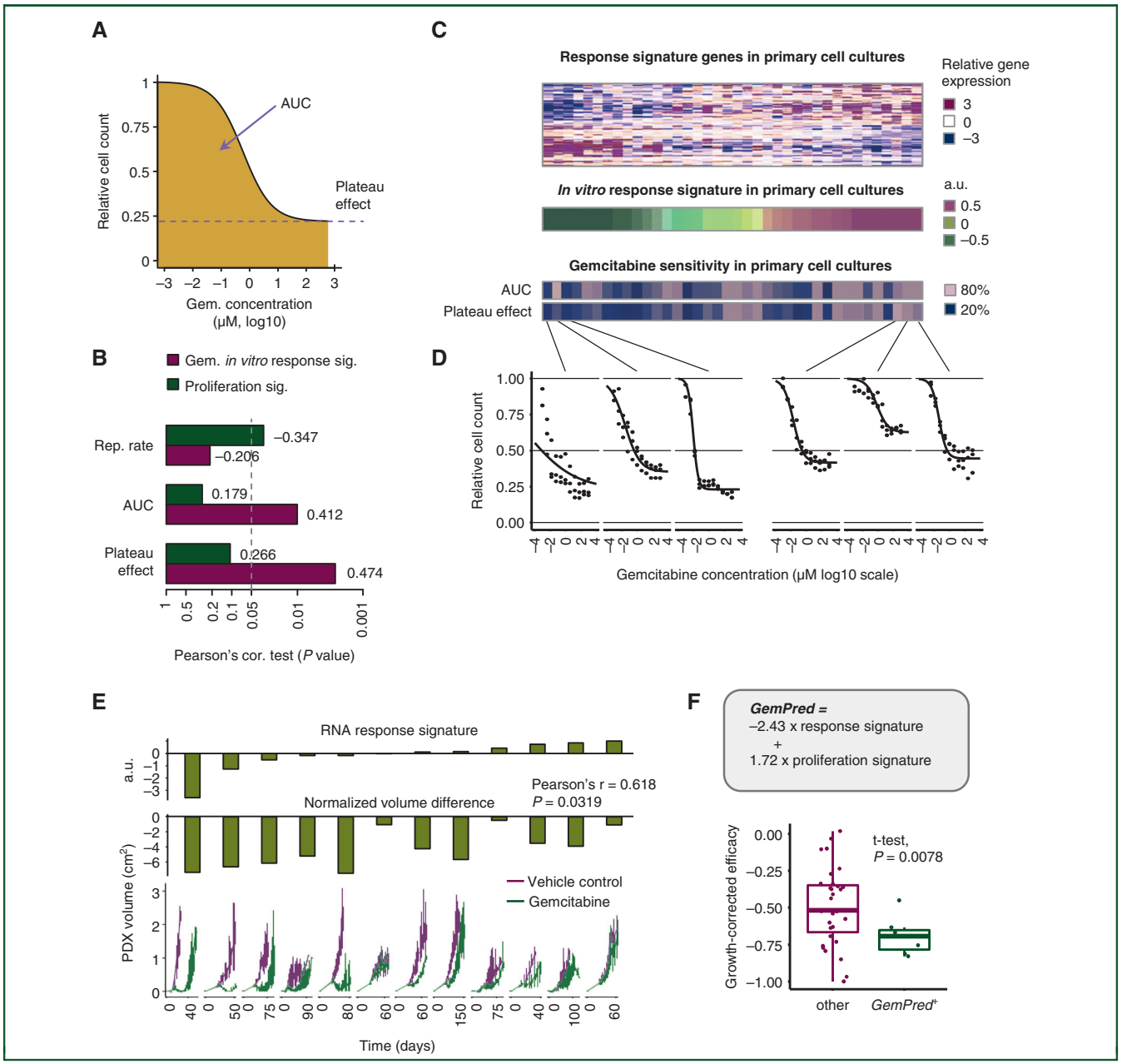


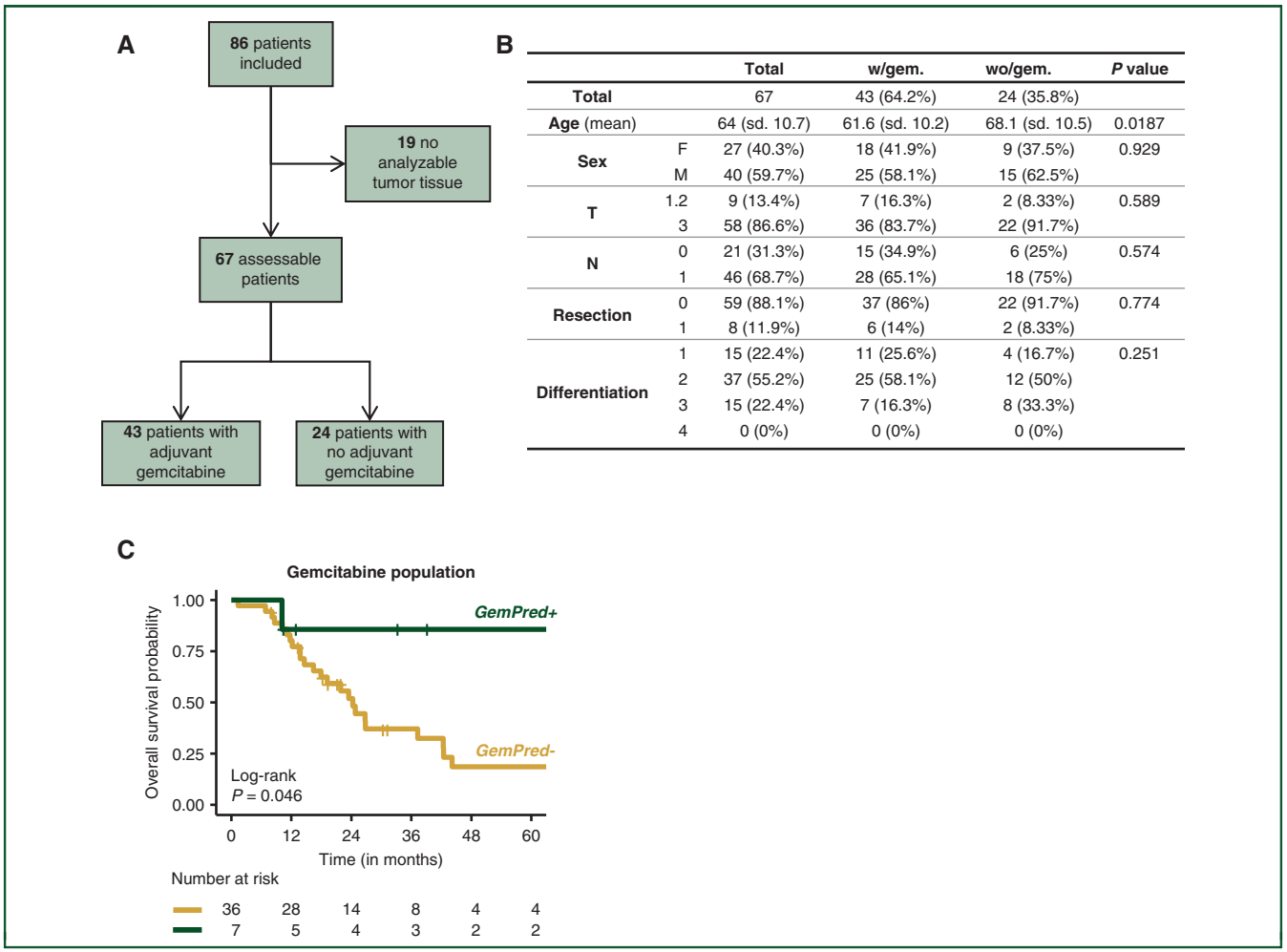

**Figure 1.** ***De novo*** **gemcitabine sensitivity RNA signature.**
(A) Exemplary dose-response curve and the two measures, area under the dose-response curve (AUC) and plateau effect, used to estimate *in vitro* response to gemcitabine. (B) Correlation *P* value of the two identified signatures with *in vitro* response to gemcitabine and proliferation. Pearson's correlation coefficient and *P* value values are shown between the two signatures and three primary cell culture features: proliferation measured by the replication rate (rep. rate), dose-response AUC and plateau effect. (C) Gemcitabine sensitivity signature in primary cell culture. Heatmap of single-gene expression with the highest contribution to the signature is shown along with the sensitivity signature (linear combination of gene expression values, arbitrary unit). AUC and plateau effect for each primary cell culture are shown. Primary cell cultures are ordered by their gemcitabine sensitivity signature value. (D) Dose-response curves for the three primary cell cultures with the highest and lowest values on the response to gemcitabine signature are shown. Cell counts at each concentration relative to the vehicle-treated primary cell cultures are shown at each concentration. (E) Association of patient-derived xenograft (PDX) response to gemcitabine with the sensitivity signature applied to PDX RNA profiles. The 12 PDX are ordered by the value of the RNA gemcitabine sensitivity signature and a normalized value of the volume difference between gemcitabine-treated and control PDX. For each PDX, tumor volume is shown in the bottom panel for eight replicates in each of the gemcitabine and the control arms, with curves following the median in green and purple, respectively. Vertical segments range from min to max values. (F) The *GemPred* signature is shown as a linear combination of the proliferation and gemcitabine sensitivity signatures. The boxplot presents the distribution of the growth-corrected efficacy of gemcitabine in the *GemPred*+ subgroup of primary cell culture ($n = 7$) and the remainder ($n = 31$).
Cor., correlation; Gem., gemcitabine; sig., signature.

gene expression. Therefore, these two series of models were considered independently. Finally, to obtain a more generalizable signature, we derived a linear combination of the proliferation and gemcitabine *in vitro* response signatures to be able to differentiate the cytotoxic and cytostatic *in vitro* responses. The combined signatures were associated with the growth-corrected efficacy *in vitro*,[30] i.e. an estimate of cytotoxicity at high dosage. To define a decision rule, every possible threshold of the combined signature was tested against the growth-corrected dose-response of gemcitabine

efficacy. A 20% cut-off of the combined signatures was identified as the threshold which best differentiated a group of primary cell cultures in which gemcitabine had the highest cytotoxic effect, thereby defining a subtype entitled *GemPred+* (Figure 1F and supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2020.10.601). To assess the specificity of the *GemPred* signature to gemcitabine, we tested the sensitivity of the *GemPred+* primary cell cultures to 5-FU, taxotere and SN-38, none of which were found to have a differential cytotoxic effect associated with the *GemPred* stratification (supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2020.10.601).

### *Evaluation of the GemPred signature in a test cohort*

The *GemPred* signature was first assessed in a monocentric test cohort of patients from the Erasme Hospital in Brussels (Belgium). A total of 67 assessable patients were included; all had had curative intent PDAC resection. Some 43 (64.2%) then received a gemcitabine-based adjuvant treatment and 24 (35.8) did not (Figure 2A). There were no significant clinical differences between the whole cohort of patients ($n = 86$) and the subset of assessable patients ($n = 67$) for which RNA profiles were obtained (supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2020.10.601). Patients who did not receive adjuvant gemcitabine were older, but overall had similar characteristics (Figure 2B). The median follow-up was 72.1 months [95% confidence interval (CI): 33.2-not reached] and the median OS 21.0 months (95% CI: 16.4-26.8). Of the 43 patients who received gemcitabine, 7 (16%) were identified as *GemPred+*. The median OS was not reached for this group and the 5-year survival rate was 85.7% (95% CI: 63.3-100). The *GemPred−* patients treated by gemcitabine had a median OS rate of 24.3 months (95% CI: 17.9-42.5) and a 5-year survival rate of 18.5% (95% CI: 8.02-42.8) (Figure 2C). There was no significant difference in OS between the *GemPred+* ($n = 6$) and *GemPred−* ($n = 18$) subgroups



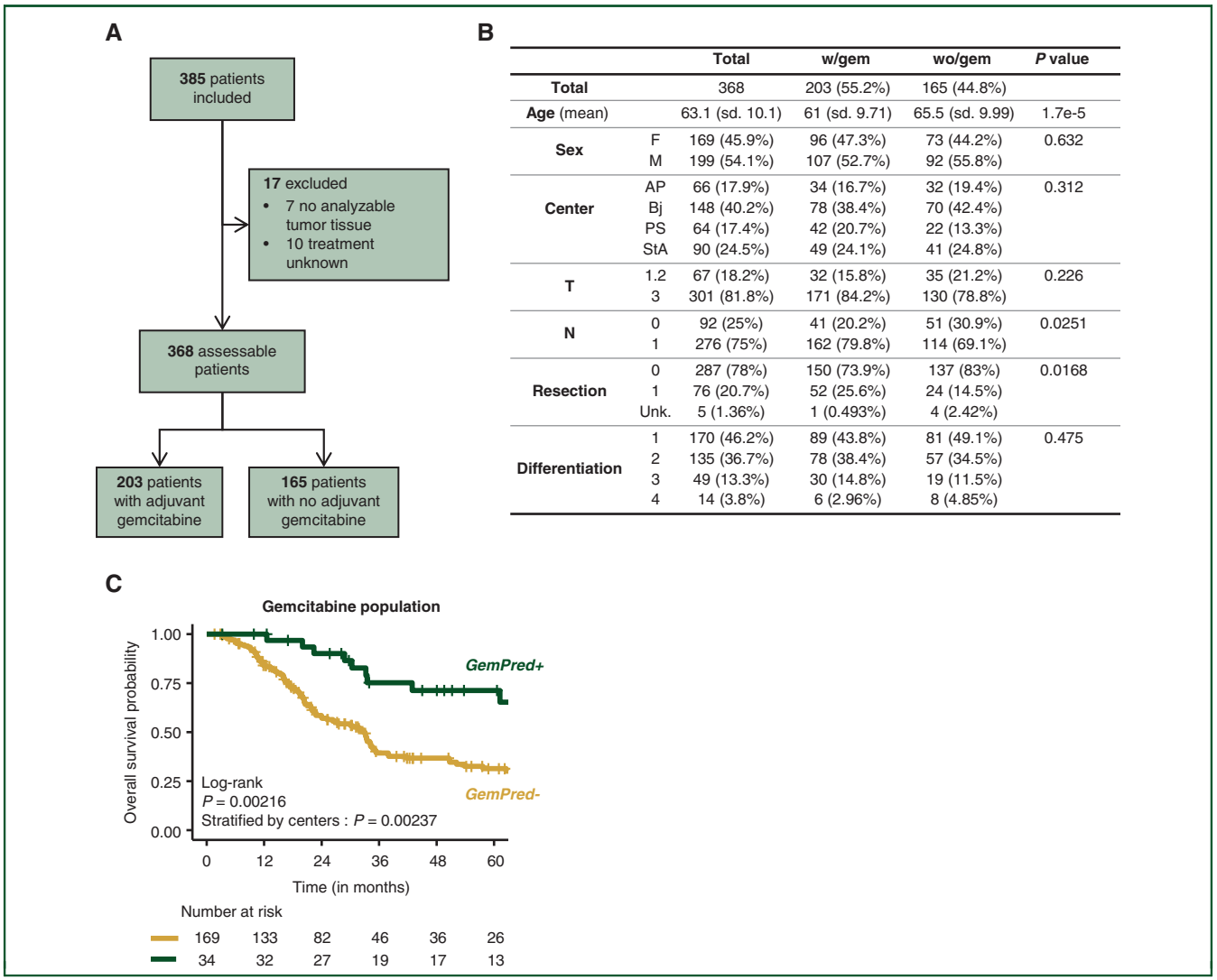

| | | Total | w/gem. | wo/gem. | *P* value |
|---|---|---|---|---|---|
| **Total** | | 67 | 43 (64.2%) | 24 (35.8%) | |
| **Age** (mean) | | 64 (sd. 10.7) | 61.6 (sd. 10.2) | 68.1 (sd. 10.5) | 0.0187 |
| **Sex** | F | 27 (40.3%) | 18 (41.9%) | 9 (37.5%) | 0.929 |
| | M | 40 (59.7%) | 25 (58.1%) | 15 (62.5%) | |
| **T** | 1.2 | 9 (13.4%) | 7 (16.3%) | 2 (8.33%) | 0.589 |
| | 3 | 58 (86.6%) | 36 (83.7%) | 22 (91.7%) | |
| **N** | 0 | 21 (31.3%) | 15 (34.9%) | 6 (25%) | 0.574 |
| | 1 | 46 (68.7%) | 28 (65.1%) | 18 (75%) | |
| **Resection** | 0 | 59 (88.1%) | 37 (86%) | 22 (91.7%) | 0.774 |
| | 1 | 8 (11.9%) | 6 (14%) | 2 (8.33%) | |
| **Differentiation** | 1 | 15 (22.4%) | 11 (25.6%) | 4 (16.7%) | 0.251 |
| | 2 | 37 (55.2%) | 25 (58.1%) | 12 (50%) | |
| | 3 | 15 (22.4%) | 7 (16.3%) | 8 (33.3%) | |
| | 4 | 0 (0%) | 0 (0%) | 0 (0%) | |

**Figure 2. Gemcitabine sensitivity signature in the test cohort.**
(A) Test cohort flowchart. (B) Clinico-pathological description of the test cohort including the total number of assessable patients ($n = 67$), the subgroup of patients who received adjuvant gemcitabine ($n = 43$), the subgroup of patients who did not receive adjuvant gemcitabine ($n = 24$) and a statistical comparison between patients with or without adjuvant gemcitabine (respectively, w/gem and wo/gem). *P* values of Student's *t*-test or chi-square test are shown. (C) Kaplan–Meier curves for overall survival in the adjuvant gemcitabine subgroup ($n = 43$), stratified by gemcitabine sensitivity prediction, *GemPred+* in dark green ($n = 7$) versus *GemPred−* dark brown ($n = 36$).
sd., standard deviation.

among the patients who did not receive adjuvant gemcitabine (supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2020.10.601).

### *Evaluation of the GemPred signature in a validation cohort*

The *GemPred* signature was then assessed in a multicentric validation cohort of patients from four clinical centers in the Paris region (France). A total of 368 assessable patients were included; all of them had curative intent PDAC resection. Some 203 (55.2%) then received a gemcitabine-based adjuvant treatment and 165 (44.8%) did not (Figure 3A). There were no significant clinical differences between the whole cohort of patients ($n = 385$) and the subset of assessable patients ($n = 368$) for which RNA profiles were obtained (supplementary Table S5, available at https://doi.org/10.1016/j.annonc.2020.10.601). Patients who did not receive adjuvant gemcitabine were older, had less often positive lymph nodes and less frequent positive resection margins (Figure 3B). The median follow-up was 72 months (95% CI: 62.2-99.5) and the median OS was 33.2 months (95% CI: 30-36.1). Of the 203 patients who received gemcitabine, 34 (17%) were identified as *GemPred*+. These patients had a median OS of 91.3 months (95% CI: 61.2-not reached) and a 71.3% 5-year survival rate (95% CI: 56.2-90.4), while the 169 *GemPred*− patients had a median OS rate of 33 months (95% CI: 24-35.2) and a 5-year survival rate of 31.3% (95% CI: 24.1-40.8) (Figure 3C). Among patients who received adjuvant gemcitabine, *GemPred*+ patients had significantly higher OS than *GemPred*− (hazard ratio 0.403, 95% CI: 0.221-0.735, $P = 0.0022$). There was no difference between the *GemPred*+ and *GemPred*− subgroups among the patients who did not receive adjuvant gemcitabine or between the *GemPred*− patients who did




| | | Total | w/gem | wo/gem | *P* value |
|---|---|---|---|---|---|
| **Total** | | 368 | 203 (55.2%) | 165 (44.8%) | |
| **Age** (mean) | | 63.1 (sd. 10.1) | 61 (sd. 9.71) | 65.5 (sd. 9.99) | 1.7e-5 |
| **Sex** | F | 169 (45.9%) | 96 (47.3%) | 73 (44.2%) | 0.632 |
| | M | 199 (54.1%) | 107 (52.7%) | 92 (55.8%) | |
| **Center** | AP | 66 (17.9%) | 34 (16.7%) | 32 (19.4%) | 0.312 |
| | Bj | 148 (40.2%) | 78 (38.4%) | 70 (42.4%) | |
| | PS | 64 (17.4%) | 42 (20.7%) | 22 (13.3%) | |
| | StA | 90 (24.5%) | 49 (24.1%) | 41 (24.8%) | |
| **T** | 1.2 | 67 (18.2%) | 32 (15.8%) | 35 (21.2%) | 0.226 |
| | 3 | 301 (81.8%) | 171 (84.2%) | 130 (78.8%) | |
| **N** | 0 | 92 (25%) | 41 (20.2%) | 51 (30.9%) | 0.0251 |
| | 1 | 276 (75%) | 162 (79.8%) | 114 (69.1%) | |
| **Resection** | 0 | 287 (78%) | 150 (73.9%) | 137 (83%) | 0.0168 |
| | 1 | 76 (20.7%) | 52 (25.6%) | 24 (14.5%) | |
| | Unk. | 5 (1.36%) | 1 (0.493%) | 4 (2.42%) | |
| **Differentiation** | 1 | 170 (46.2%) | 89 (43.8%) | 81 (49.1%) | 0.475 |
| | 2 | 135 (36.7%) | 78 (38.4%) | 57 (34.5%) | |
| | 3 | 49 (13.3%) | 30 (14.8%) | 19 (11.5%) | |
| | 4 | 14 (3.8%) | 6 (2.96%) | 8 (4.85%) | |

**Figure 3. Gemcitabine sensitivity signature in the validation cohort.**
(A) Validation cohort flowchart. (B) Clinico-pathological description of the test cohort including the total number of assessable patients ($n = 368$), the subgroup of patients who received adjuvant gemcitabine ($n = 203$), the subgroup of patients who did not receive adjuvant gemcitabine ($n = 165$) and a statistical comparison between patients with or without adjuvant gemcitabine (respectively, w/gem and wo/gem). *P* values of Student's *t*-test or chi-square test are shown. (C) Kaplan–Meier curves for overall survival in the adjuvant gemcitabine subgroup ($n = 203$), stratified by gemcitabine sensitivity prediction, *GemPred*+ ($n = 34$) versus *GemPred*− ($n = 169$).
sd., standard deviation.

and did not receive adjuvant gemcitabine (supplementary Figure S4, available at https://doi.org/10.1016/j.annonc.2020.10.601).

### *Evaluation of the interaction between the GemPred signature and adjuvant gemcitabine*

To increase statistical power and improve the relevance of multivariate analyses, the two cohorts were pooled resulting in a cohort of 435 patients, among which 246 (57%) received adjuvant gemcitabine. Simultaneously stratifying by adjuvant gemcitabine and by the gemcitabine sensitivity signature *GemPred* resulted in four groups (Figure 4A): *GemPred*− with ($n = 205$) or without ($n = 145$) adjuvant gemcitabine had, respectively, a median OS of 31.7 and 23.7 months (95% CI: 24-34.2 and 18.4-34.9) as well as a 29.1% and 27.1% 5-year survival rate (95% CI: 22.6-37.5 and 20.5-35.9), *GemPred*+ without adjuvant gemcitabine ($n = 44$) had a median OS of 31.4 months (95% CI: 21-43.1) and a 26.7% 5-year survival rate (95% CI: 15.4-46.3), while the *GemPred*+ subgroup of patients who received gemcitabine ($n = 41$) had a median OS of 91.3 months (95% CI: 63.1-not

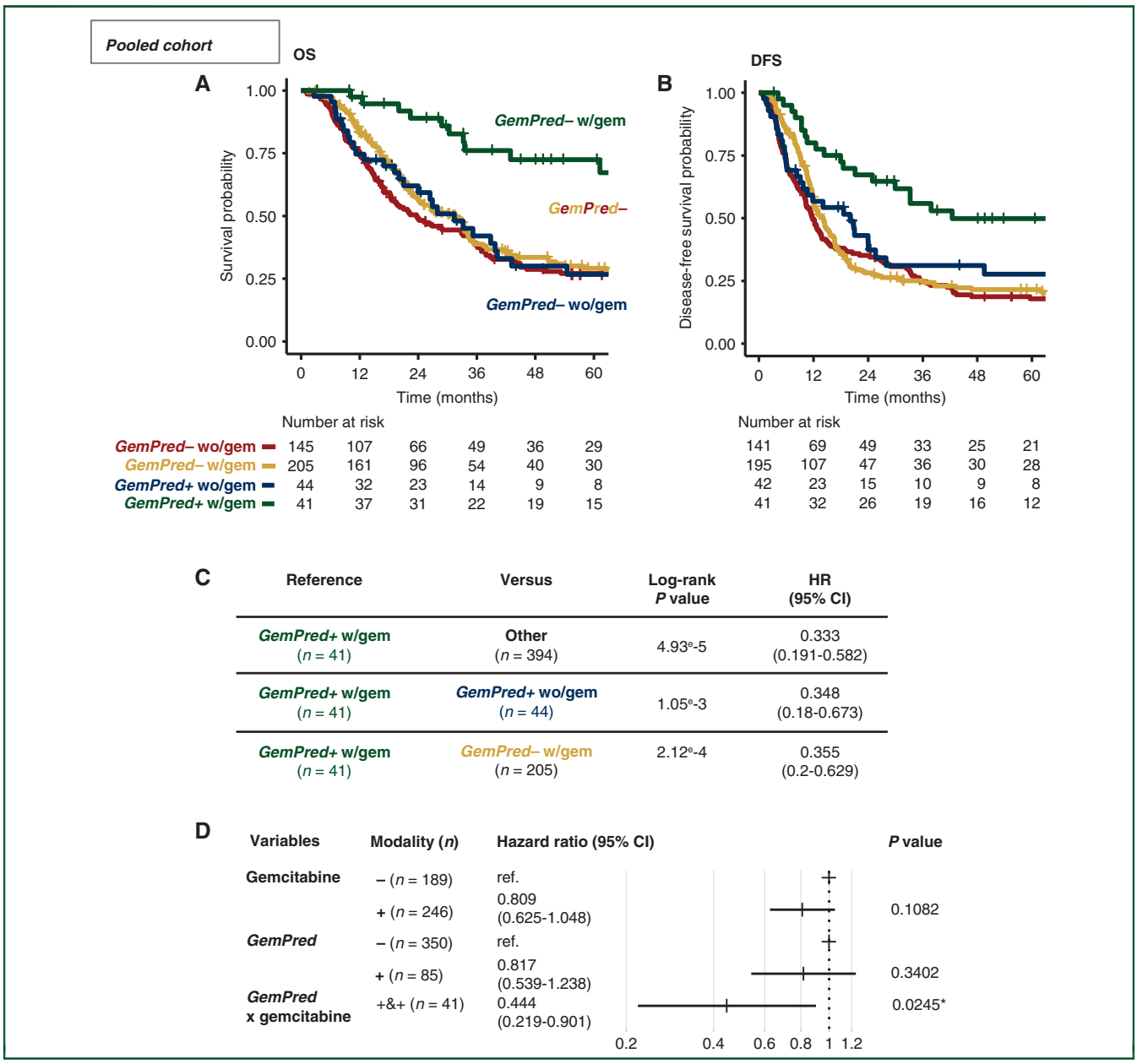



**Figure 4. Pooled cohort analysis.**

(A) Kaplan–Meier curves for OS in the pooled cohort ($n = 435$), stratified by gemcitabine sensitivity prediction (*GemPred*+ and *GemPred*−) and by adjuvant gemcitabine (with or without adjuvant gemcitabine). (B) Kaplan–Meier curves for DFS in the pooled cohort ($n = 419$), stratified by gemcitabine sensitivity prediction (*GemPred*+ and *GemPred*−) and by adjuvant gemcitabine (with or without adjuvant gemcitabine). (C) Summarizing comparison between the overall survival of the subgroup of *GemPred*+ patients predicted as sensitive to gemcitabine and who received adjuvant gemcitabine and other subgroups of patients. (D) Forest plot of a Cox proportional hazards regression model including the *GemPred* stratification, adjuvant gemcitabine and their interaction.

CI, confidence interval; DFS, disease-free survival; HR, hazard ratio; OS, overall survival; w/gem, with adjuvant gemcitabine; wo/gem, without adjuvant gemcitabine.

* <5%.

reached) and a 72.5% 5-year survival rate (95% CI: 58.5-89.8). *GemPred*+ patients having received gemcitabine had a significantly longer DFS with a median 42.5 months (95% CI: 29.9- not reached) against 13.4 months (95% CI: 10.3-15.5) for the pool of all other groups of patients (Figure 4B). Overall, the *GemPred*+ patients who received adjuvant gemcitabine had significantly longer OS than any other subgroups (Figure 4C). In a Cox proportional hazards regression model including an interaction term, a significant interaction was found between adjuvant gemcitabine and the *GemPred*+ stratification (hazard ratio 0.444, 95% CI: 0.219-0.901, $P = 0.0245$), indicating an effective predictor of response to adjuvant gemcitabine (Figure 4D). The inclusion of patients' age—the most different characteristic in both cohorts between patients having received gemcitabine and others—in the multivariate interaction model showed that the predictive value of the *GemPred*+ stratification remained significant ($P = 0.0265$) independent of age (supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2020.10.601).

### *Comparison and multivariate analysis of GemPred*

Previous studies have proposed biomarkers and signatures predictive of response to gemcitabine in PDAC. RNA expression of genes involved in gemcitabine metabolism, specific expression ratios or an organoid-derived signature[28] had either no predictive value or an unspecific prognostic value on either OS (Figure 5A) or DFS (supplementary Figure S6, available at https://doi.org/10.1016/j.annonc.2020.10.601). As previously reported,[13] the non-commercially available hENT1 antibody immunohistochemistry (IHC) quantification had a high predictive value of gemcitabine response. *GemPred*+ and hENT1 IHC selected independent groups of patients and had independent predictive values on both OS and DFS (supplementary Figure S6, available at https://doi.org/10.1016/j.annonc.2020.10.601), suggesting these potentially select complementary sets of gemcitabine-sensitive patients. The *GemPred*+ patients were all found to be a subset of the classical subtype[24] (Figure 5B, 92 of 389 classical, none of 72 basal-like), supported by their distribution in the upper half of the pancreatic cancer molecular gradient[31] (Figure 5C). A multivariate analysis among patients who received adjuvant gemcitabine in the pooled cohort showed that *GemPred*+ was a predictor of OS independent of clinicopathological features, the RNA levels of genes previously described to be associated with response to gemcitabine (i.e. *DCK*, *hENT1*, *CDA*) and molecular subtype (Figure 5D). *GemPred*+ is also an independent predictor of DFS in a multivariate analysis of the patients who received gemcitabine (Figure 5E).

## DISCUSSION

The practice of oncology continually faces the challenge of matching the right therapeutic regimen to the right individual, balancing risks with expected benefits to achieve the most favorable outcome. Relying on tumor molecular profiles to personalize treatments is a major challenge in oncology that until now mostly focused on the identification of—often rare—actionable mutations. The transcriptome, i.e. the genome-wide quantification of RNA, has a frequently overlooked potential to assess efficient phenotypic signatures as demonstrated by clinically implemented assays of risk stratifications such as PAM50,[32] MammaPrint,[33] Oncotype Dx® Breast[21] or Oncotype Dx® Colon.[34] More importantly than predicting recurrence risks, RNA may be expected to predict the sensitivity to therapies with no specifically known targets, such as chemotherapy.

In this work, we present *GemPred*, an RNA-based whole transcriptome signature predicting sensitivity to gemcitabine for patients with PDAC. The predictive value of the *GemPred* signature for gemcitabine response was first tested in a monocentric cohort then validated in a multicentric cohort. The *GemPred* signature had a significant predictive value of both OS and DFS in the subgroup of patients who received adjuvant gemcitabine and had no prognostic value in the subgroup of patients who did not. The relationship between the *GemPred*+ stratification and the more general molecular subtypes of PDAC suggests a subdivision of the larger classical subtype into a gemcitabine-sensitive subgroup.

In PDAC, actionable mutations can be identified in up to 30% of patients[35,36] and result in increased survival when matched therapies can be administered.[37] These studies, which include RNA profiling for gene-fusion detection, demonstrate the feasibility of successfully profiling PDAC biopsies to adopt or modify a course of treatment in a reasonable timeframe. The *GemPred* signature takes advantage of genome-wide RNA profiles to identify gemcitabine-sensitive tumors. An important point is that the genome-wide RNA profiles in this study were obtained from routine formalin-fixed, paraffin-embedded samples with an overall low cost, supporting a wide application of the *GemPred* signature. Previous work has also shown that sequencing RNA from fine needle aspirates, including for metastatic patients, is feasible.[31] It should also be noted that, while the signature was developed on tumor cells, it performed well on more complex samples such as surgical specimens that have abundant stroma, suggesting its relevance on diagnostic biopsies.

Despite the use of more effective, yet more toxic, polychemotherapies, gemcitabine is still one of the recommended monotherapies for PDAC,[38] one of the standard metastatic first-line regimens combined with nab-paclitaxel[9] and the backbone of most therapeutic combinations in development.[39-41] The selection of the best option between a gemcitabine-based or non-gemcitabine-based regimen is still often undetermined. For instance, in an adjuvant setting, it was shown that bolus fluorouracil plus folinic acid had similar efficacy to gemcitabine, and one could suggest that it is also true in advanced patients unfit for aggressive combinations.[42] Similarly, nab-paclitaxel in metastatic patients was shown to be as effective with either 5-fluoruracil or gemcitabine.[43] The availability of a potent predictor of gemcitabine efficacy would allow selecting the most relevant regimen. The *GemPred* stratification is an RNA-based assay able to identify a subgroup

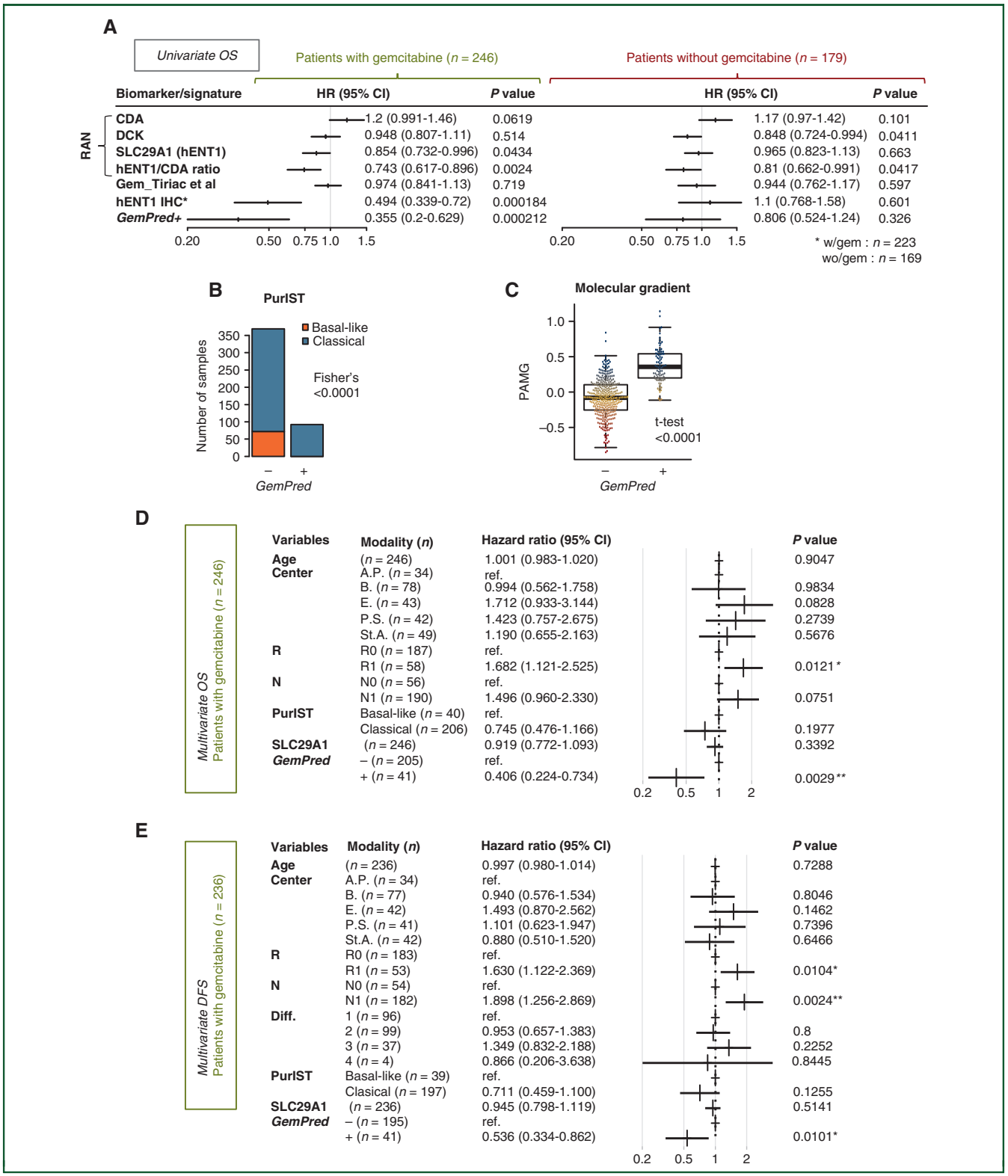



**Figure 5. Comparison and multivariate analyses.**

(A) Univariate analysis of gemcitabine-related biomarkers on overall survival in both the gemcitabine ($n = 246$ for RNA signatures, $n = 223$ for hENT1 immunohistochemistry) and non-gemcitabine populations ($n = 179$ for RNA signatures, $n = 169$ for hENT1 immunohistochemistry). Log-rank *P* values are shown. (B) Association between the *GemPred* stratification and the molecular subtypes of pancreatic ductal adenocarcinoma (PDAC). (C) Association between the *GemPred* stratification and the molecular gradient of PDAC. Forest plot of the multivariate analysis of OS (D, $n = 246$) and DFS (E, $n = 236$) among patients who received adjuvant gemcitabine. Variables in the model included: any generally available clinico-pathological variables with significant univariate association ($\alpha = 5\%$, supplementary Figure S7, available at https://doi.org/10.1016/j.annonc.2020.10.601), age, center and PurIST subtype to control for potential confounding factors and the gene expression of SLC29A1 (hENT1).

CI, confidence interval; DFS, disease-free survival; Diff., differentiation; HR, hazard ratio; N, N status; OS, overall survival; PAMG, pancreatic adenocarcinoma molecular gradient; R, resection margins; w/gem, with adjuvant gemcitabine; wo/gem, without adjuvant gemcitabine.

* <5%; ** <1%.

of *GemPred*+ patients who benefit from the use of adjuvant gemcitabine with 76.1% 3-year OS (CI: 95% 62.8-92.2, $n = 41$). In comparison to the 63.4% 3-year OS observed with modified FOLFIRINOX (mFOLFIRINOX),[6] *GemPred*+ patients may get a similar outcome with gemcitabine-based regimens. It may be expected that with such a favorable benefit, *GemPred*+ patients are better suited for less toxic gemcitabine-based regimens, and potentially even from gemcitabine alone, compared with polychemotherapies such as mFOLFIRINOX, especially if they have no deficiency in the homologous recombination DNA repair system. In these particular patients with *GemPred*+ and homologous recombination, gemcitabine-oxaliplatin could be an effective combination.[44]

Despite a large number of patients and the absence of patient selection bias due to the selection of all consecutive patients of all participating clinical centers, the main limitation of this work is the retrospective nature of the cohorts. For instance, patients having received gemcitabine tended to be younger, although age does not affect the predictive value of *GemPred* (supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2020.10.601). It would be of high interest to assess the predictive value of the *GemPred* signature in patients included in the PRODIGE24 phase III randomized trial that compared mFOLFIRINOX with gemcitabine in the adjuvant setting.[6]

This work entails further investigations to establish its clinical applicability. While we demonstrated the *GemPred* predictive value in an adjuvant setting, it is yet to be demonstrated that it may be useful in a neoadjuvant/induction context or for locally advanced/metastatic patients. Simultaneous molecular profiling of PDAC primary tumors and metastasis of the same patients have shown their broad molecular similarity,[45,46] suggesting the *GemPred* stratification could preserve its relevance in metastatic samples. Another limitation of this work is that it does not evaluate the *GemPred* signature on gemcitabine used in combination with other drugs, in particular with nab-paclitaxel. It was demonstrated that most combination therapies in oncology lack drug synergy and benefit on a populational level by benefiting different patient subgroups,[47] suggesting that the *GemPred* signature is likely to be associated with the response to gemcitabine-based therapeutic combinations. Finally, the validation of the *GemPred* signature in a clinical trial in which *GemPred*+ patients are randomly assigned to receive either adjuvant gemcitabine or FOLFIRINOX is necessary to fully validate the clinical value of the signature.

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## DISCLOSURE

RN, YB, MG, JI and NJD have a pending patent entitled 'Evaluation of the efficiency of an anticancer compound for a PDAC patient' filed 23 January 2020 (European patent application number EP20305052.1). All other authors have declared no conflicts of interest.

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