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## **GATA6 expression distinguishes classical and basal-like subtypes in advanced pancreatic cancer.**

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GATA6 expression distinguishes classical and basal-like subtypes in advanced pancreatic cancer.

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1 **Abstract (250)**

2

3 Purpose:

4 To determine the impact of basal-like and classical subtypes in advanced PDAC and to  
5 explore GATA6 expression as a surrogate biomarker.

6

7 Experimental design

8 Within the COMPASS trial patients proceeding to chemotherapy for advanced PDAC  
9 undergo tumour biopsy for RNA sequencing. Overall response rate (ORR) and overall  
10 survival (OS) were stratified by subtypes and according to chemotherapy received.  
11 Correlation of *GATA6* with the subtypes using gene expression profiling, in situ  
12 hybridization (ISH) were explored.

13

14 Results:

15 Between December 2015-May 2019, 195 patients (95%) had enough tissue for RNA  
16 sequencing; 39 (20%) were classified as basal-like and 156 (80%) as classical. RECIST  
17 response data were available for 157 patients; 29 basal-like and 128 classical where the  
18 ORR was 10% vs. 33% respectively ( $p=0.02$ ). In patients with basal-like tumours treated  
19 with modified FOLFIRINOX (mFFX) ( $n=22$ ) the progression rate was 60% compared to  
20 15% in classical PDAC ( $p= 0.0002$ ). Median OS in the intention to treat population  
21 ( $n=195$ ) was 9.3 months for classical vs. 5.9 months for basal-like PDAC (HR 0.47 95% CI  
22 0.32-0.69,  $p=0.0001$ ). *GATA6* expression by RNAseq highly correlated with the classifier  
23 ( $p<0.001$ ) and ISH predicted the subtypes with sensitivity of 89% and specificity of 83%.  
24 In a multivariable analysis, *GATA6* expression was prognostic ( $p=0.02$ ). In exploratory  
25 analyses, basal-like tumours, could be identified by keratin 5, were more hypoxic and  
26 enriched for a T cell inflamed gene expression signature.

27

28 Conclusions

29 The basal-like subtype is chemoresistant and can be distinguished from classical PDAC  
30 by *GATA6* expression.

31

32

33

34 **Translational relevance**

35

36 The transcriptomic basal-like subtype is highly chemoresistant and patients have a  
37 shorter median overall survival compared to classical PDAC. In this study, survival was  
38 lowest in basal-like PDAC treated with modified FFX. *GATA6* expression by both RNAseq  
39 and in-situ hybridization (ISH) is highly associated with the classifier where low or  
40 absent *GATA6* is seen in the basal-like subtype.

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45

46 **Manuscript (3300)**

47

48 Introduction

49

50 By 2030, pancreatic ductal adenocarcinoma (PDAC) will become the second leading  
51 cause of cancer related mortality in North America(1). The majority of PDAC patients  
52 present with advanced disease where the mainstay of treatment remains combination  
53 chemotherapy. Modified FOLFIRINOX (FFX) and gemcitabine- nab-paclitaxel (GnP) are  
54 the most commonly used regimens resulting in median survival less than one year (2, 3).  
55 While the need to discover novel approaches is obvious, it is equally important to  
56 understand how to select the aforementioned regimens for current patients. There are  
57 no randomized data that shows superiority of either combination and patient inclusion  
58 differences are evident in the two pivotal phase III trials(2, 3). The only molecular  
59 predictor of response is prior knowledge of a pathogenic germline variant in a  
60 homologous recombination repair gene, which may influence the regimen of choice (4).  
61 Other currently targetable genomic variants are uncommon in PDAC.

62

63 Gene expression profiling, primarily in resected pancreatic tumours, describes a number  
64 of subtypes with considerable overlap, yet presently these do not inform clinical practice  
65 (5-7). Collisson et al. documented three subtypes (classical, quasimesenchymal, and  
66 exocrine-like)(6), Bailey et al. four subtypes (immunogenic, progenitor, ADEX and  
67 squamous)(5) and Moffitt et al. two subtypes (classical and basal-like)(7). The squamous  
68 (Bailey), quasimesenchymal (Collisson) and basal-like (Moffitt) cohorts align well across  
69 the classifiers and all three are associated with a poor prognosis in these studies. Despite  
70 this, varying tumour cellularity and heterogeneity in clustering methodologies leaves  
71 uncertainty as to the most appropriate classifier and furthermore, the clinical application  
72 of these subtypes to advanced stage disease is unclear.

73

74 In an effort to reconcile and apply existing knowledge, we established the COMPASS trial  
75 (Comprehensive Molecular Characterization of Advanced Pancreatic Ductal  
76 Adenocarcinomas (PDAC) for Better Treatment Selection: A Prospective Study, NCT

77 NCT02750657). Unique to this prospective study is the acquisition of tissue prior to  
78 chemotherapy in the advanced setting, which then undergoes laser capture  
79 microdissection (LCM) to ensure high tumour cellularity. The primary endpoint of  
80 feasibility in obtaining a high-quality genome report within 8 weeks in the first 50  
81 patients has been published (8). In this earlier analysis, we determined that a modified  
82 Moffitt RNA signature, optimized for use in advanced stage PDAC (classical vs. basal-like,  
83 Supplementary Figure 1) may have prognostic impact (8). Furthermore, we found that  
84 *GATA6*, a transcription factor required for normal pancreas development(9), which has  
85 been shown to align with the classical subtype could represent a surrogate marker for  
86 classical PDAC (8). Here, we evaluated the modified Moffitt basal-like and classical  
87 subtypes together with *GATA6* expression on outcomes in patients receiving mFFX or GnP  
88 regimens on the expanded COMPASS trial. We further explored specific clinical and  
89 pathologic characteristics of the subtypes and evaluated *GATA6* as a surrogate biomarker  
90 and clinical tool. Post-hoc exploratory analyses were performed to seek additional  
91 positive biomarkers for the basal-like subtype.

92

## 93 **Methods**

94

### 95 *Patient Population*

96 The COMPASS trial is a prospective multi-institutional Canadian cohort study. Patient  
97 eligibility for the study has been previously described(8). Briefly, patients require a  
98 radiologic or histologic diagnosis of locally advanced or metastatic PDAC, suitable for  
99 combination chemotherapy, and must consent to a fresh tumor biopsy prior to treatment  
100 start. Biopsies can be taken from the primary lesion or any metastatic sites. Patients must  
101 not have had prior treatment for advanced disease. Treatment decisions are at the  
102 discretion of their medical oncologist. Response to therapy is assessed using  
103 computerized tomography (CT) and measured using RECIST 1.1. Demographics and  
104 treatment details, including subsequent treatments are prospectively collected using an  
105 electronic MEDIDATA database. This report includes all patients enrolled from December  
106 2015 until May 2019 and follow-up censored on August 30<sup>th</sup>2019. Patients on this study  
107 were enrolled at the Princess Margaret Cancer Centre, McGill University Health Centre  
108 (MUHC) and Kingston General Hospital and the study was conducted in accordance with  
109 the Declaration of Helsinki. The COMPASS trial has been approved by participating site



110 Institutional Review Board (University Health Network, MUHC Centre for Applied Ethics,  
111 and Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics  
112 Board); each patient provided written informed consent prior to study entry.

113

#### 114 *RNA sequencing and GATA6 expression*

115 Frozen biospecimens underwent LCM for tumor enrichment. RNAseq analysis was  
116 performed at the Ontario Institute of Cancer Research as previously described (10).  
117 Briefly, reads were aligned to the human reference genome (hg38) and transcriptome  
118 (Ensembl v84) using STAR v.2.5.2a (11). Duplicated reads were marked using Picard v.  
119 1.121 (<https://github.com/broadinstitute/picard>). Gene expression was calculated in  
120 fragments per kilobase of exon per million reads mapped (FPKM) using the cufflinks  
121 package v. 2.2.1 (12). A modified Moffitt classification (classical vs. basal-like) was  
122 applied to each sample with sufficient RNA for analysis (**Supplementary Figure 1**). Cut-  
123 off threshold levels for *GATA6* expression were determined using the maximal chi-  
124 squared method on RECIST response and dichotomised *GATA6* expression.

125

#### 126 *GATA6 RNA in situ hybridization (ISH)*

127 Given our early results, the COMPASS trial was amended (01-Feb-2017) to include  
128 *GATA6* staining using an RNAscope® in situ hybridization (ISH) assay (Advanced Cell  
129 Diagnostics Inc., Hayward, CA). A semi-quantitative score was used by the study  
130 pathologist (SF) (**Supplementary Figure 2A**) as previously reported (8). Scoring was  
131 applied blinded to results of the modified Moffitt classifier.

132

#### 133 *Immunohistochemical analysis of GATA6 and keratins*

134 To provide more widely applicable diagnostic tests for PDAC subtypes, we optimized a  
135 protocol for *GATA6* immunohistochemistry (IHC) (**emethods**) using a polyclonal anti-  
136 *GATA6* antibody from R&D (Cat. Number AF1700), and secondary antibody from Vector  
137 (Cat. Number VECTABA5000). DAB+ (3,3-diaminobenzidine tetrahydrochloride plus,  
138 DAKO, Cat. Number K3468) was used as chromogen and nuclei were counterstained with  
139 Mayer's hematoxylin.) (**Supplementary Figure 2B**). To assess the pattern of *GATA6* staining  
140 across larger tumor regions, we used whole sections (n=30) from a previously described  
141 resection cohort with matched RNAseq data (10) together with biopsies (n=41) from the  
142 advanced cohort.

143

144 In an exploratory analysis, we sought additional clinical markers to aid subtype identification;  
145 cytokeratins associated with *GATA6* expression were identified from RNAseq data and further  
146 explored by IHC (**emethods**).

147

#### 148 *Image analysis*

149 To control for potential bias of manual scorings of both ISH or IHC, we performed image  
150 analysis on pre-annotated tumor sections using image analysis software QuPath v0.1.3  
151 (13). Detection parameters were established on unequivocal *GATA6*-high versus -low  
152 versus -absent tumors and confirmed by the study pathologist. Semi-quantitative (SQ)  
153 scores were also predicted from image analysis data using the maximal chi-squared  
154 method.

155

#### 156 *Statistical Analysis*

157 Qualitative variables were compared by Fisher's exact test, and quantitative variables by  
158 Wilcoxon rank sum test for pairwise comparison and the Kruskal-Wallis test for multiple  
159 group comparison. All patients receiving at least 1 cycle of chemotherapy were included  
160 in the analysis of overall response rate (ORR). Survival curves were plotted using the  
161 Kaplan–Meier method and hazard ratios were calculated using Cox proportional hazard  
162 regressions with *p*-values calculated using the Wald statistic. All tests were two-sided.  
163 Multiple tests *p*-values were adjusted using Benjamini and Hochberg method (14) for  
164 independent tests or Benjamini and Yekutieli method (15) for dependent tests,  
165 respectively. Statistical significance was set at  $p = 0.05$ . All analyses were conducted in R  
166 version 3.2 (16). Spearman correlation coefficients were ascertained for evaluating gene  
167 expression. Sensitivity, specificity and accuracy scores were computed to assess  
168 prediction quality.

169

170

## 171 **Results**

172

### 173 **Patient characteristics at baseline**

174

175 Between 30<sup>th</sup> December 2015 and May 30<sup>th</sup>2019, 250 patients were enrolled and 206  
176 were eligible (**Figure 1-Consort**). Of these, 195 patients (95%) had enough tissue for  
177 RNA analysis and are included in this report. **Table 1** shows baseline clinical and  
178 pathological characteristics of those patients. Using the modified Moffitt classifier, 39  
179 (20%) baseline tumor samples were basal-like, and 156 (80%) classical. Locally  
180 advanced disease at diagnosis was present in 24 (12%) and these cases, in this small  
181 subset, were all identified as classical (p=0.005). Liver metastases were present in 97%  
182 of basal-like tumors compared with 69% of classical tumors (which includes the locally  
183 advanced cases) (p<0.0001). Although basal-like tumors were more frequent in male  
184 patients (p=0.02) the overall sample size was small, Other characteristics were similar  
185 between the groups (**Table 1**).

186

#### 187 **Response to chemotherapy according to modified Moffitt classification.**

188

189 Of the 195 patients, 14 (7%) did not receive any chemotherapy and were considered non-  
190 evaluable (NE). A further 23 patients (12%) died as a result of rapid functional decline  
191 prior to their first scan, of which 19 received only 1 cycle of chemotherapy; five of these  
192 23 had basal-like PDAC. One patient receiving mFFX did not have measurable disease at  
193 enrolment. Accordingly, RECIST response data were available for 157 patients (81%)  
194 including 29 patients with basal-like tumours and 128 with classical tumours (**Figure**  
195 **2A**). The ORR in classical PDAC was 33% vs. 10% in basal-like PDAC (p=0.02). The rates  
196 of progression by RECIST criteria at first CT image were much higher in basal-like vs.  
197 classical PDAC (52% vs. 16% <0.0001). **Figure 2A** shows the percentage change in target  
198 lesions, demonstrating chemoresistance of the basal-like subtype. In patients treated  
199 with mFFX and evaluable for response (n=91), progression was evident in 60% of basal-  
200 like vs. 15% of classical PDAC (p=0.0002) (**Figure 2B**). The ORR was 29.6% vs. 10% in  
201 classical vs. basal-like PDAC (p=0.09). One patient in the latter group with a partial  
202 response (PR) had a germline *BRCA2* pathogenic variant and displayed genomic  
203 characteristics of homologous recombination deficiency. The numbers treated with GnP  
204 regimens and available for response were small, progression of disease was seen in 3/9  
205 (33%) patients with basal-like vs. 8/54 (15%) with classical tumours (p=0.18) (**Figure**  
206 **2C**). The ORR was 39% vs. 11% in classical vs. basal-like PDAC respectively (p=0.14). Of  
207 note, 20/63 (32%) received additional experimental agents in this group.

208

209 **Overall survival according to the modified Moffitt classification**

210

211 Overall survival in the intention to treat population (n=195) is shown in **Figure 3A**.  
212 Median follow-up is 7.17 months. Median overall survival according to receipt of  
213 chemotherapy is shown in **Figure 3B**. In patients receiving mFFX (n=103) where  
214 performance status was less likely to confound results, median overall survival was 6.5  
215 months in basal-like vs. 10.6 months in classical subgroups (HR 0.33 95% CI 0.19-0.60,  
216 p=0.0001). These observations suggest favourable impact of mFFX in classical PDAC but  
217 little impact of mFFX in the basal-like population (**Figure 3C**). In contrast, there was no  
218 difference between subgroups when treated with GnP regimens, where median overall  
219 survival, was 8.12 months in basal-like vs. 8.19 months in classical groups respectively  
220 (HR 0.80 95% CI 0.40-1.60, p=0.53) (**Figure 3D**). In a multivariable Cox proportional  
221 hazard regression analysis, the Moffitt subtype remained highly prognostic (p=0.018).  
222 Substituting GATA6 expression for the Moffitt subtype also demonstrated the prognostic  
223 impact of GATA6 in the model, again supporting its use as a biomarker of the subtypes.  
224 Of note, stage (locally advanced versus metastatic) or chemotherapy type had no impact  
225 in this observational cohort study (**Supplementary Figure 3**). To further explore if there  
226 was a significant interaction between FFX or GnP and the subtypes, we performed an  
227 interaction analysis. There was no statistically significant difference to suggest one  
228 chemotherapy regimen for one particular subtype, although basal-like tumours trended  
229 toward improved survival with GnP, p=0.08. Of note, the modified Moffitt classifier used  
230 in this study, outperforms the previously published Moffitt classifier in identifying the  
231 poor prognostic basal-like subtype (**Supplementary Figure 1B**).

232

233 **GATA6 expression by RNAseq and RNA ISH is associated with modified Moffitt**  
234 **subtypes.**

235

236 GATA6 expression remained strongly associated with the modified Moffitt  
237 transcriptomic classifier (p<0.001) in this expanded cohort (**Figure 4A, left**). In addition,  
238 the proposed RNA ISH Semiquantitative (SQ) score was highly associated with GATA6  
239 gene expression (RNASeq) (p<0.001) (**Figure 4A, right**). Matched RNAseq and ISH  
240 results were available in 106 patients (23 with basal-like, 83 with classical subtypes). SQ

241 scoring of *GATA6* ISH confirmed higher *GATA6* expression (74/83 score 2-4) in classical  
242 vs. basal-like PDAC (19/23 score 0-1). Furthermore, *GATA6* ISH correlated with modified  
243 Moffitt with a sensitivity of 89%, specificity of 83% and accuracy of 88%. Both manual  
244 scores and modified Moffitt calls could be predicted from image analysis data with  
245 concordance of 92% and 81%, respectively, confirming reproducibility of  
246 semiquantitative assessment (n= 106). The modified Moffitt signature remained  
247 prognostic in this staining sub-cohort (**Figure 4B**); both *GATA6* ISH SQ scoring (**Figure**  
248 **4C**) and subtyping inferred from image analysis of *GATA6* ISH (**Figure 4D**) predicted  
249 outcome in a similar manner.

250

251

### 252 ***GATA6* IHC may discriminate basal-like from classical PDAC**

253

254 Matched IHC and ISH results were available in only 78 advanced PDAC cases. *GATA6*  
255 levels by IHC and ISH were well correlated using quantitative assessment  
256 (**Supplementary Figure 4A**) and semi-quantitative scoring (concordance 88%),  
257 indicating that *GATA6* protein levels mirror RNA expression and could aid subtype  
258 identification when RNA detection is not feasible. Indeed, IHC-based semi-quantitative  
259 scoring identified most patients with classical subtype tumors by strong and moderate  
260 *GATA6* staining (52/63 with scores 2-4) while basal-like subtype patients mostly  
261 exhibited no or weak *GATA6* staining (9/15 with scores 0-1), so that *GATA6* protein  
262 detection by IHC was associated with modified Moffitt subtypes in advanced PDAC with  
263 a sensitivity of 83%, specificity of 60%, and accuracy of 78%. Once more, this was  
264 confirmed by quantitative assessment (**Supplementary Figure 4B**) and the concordance  
265 between prediction of *GATA6* scoring from image analysis to manual scoring of *GATA6*  
266 was 90%.

267

### 268 **Tissue distribution of *GATA6* by IHC in a subset of resectable and metastatic PDAC**

269

270 Recent data are emerging that basal and classical subtypes can co-exist in PDAC (17, 18).  
271 We therefore explored potential variation in *GATA6* expression patterns. We used whole  
272 sections from selected resected cases (n=30) in addition to needle biopsies (n=41).  
273 Although early stage tumours may not necessarily reflect the biology of advanced disease

274 adequate tumor content is available for more complete evaluation. GATA6 staining (IHC)  
275 in resected specimens that were basal-like (n=14) and classical (n=16) also associated  
276 with the Moffitt subtypes: extensive immunopositivity for GATA6 (>50% of tumour cells  
277 with score 2 or higher) was found in 9/16 (56%) classical tumours vs. 1/14 (7%) basal-  
278 like tumours. (**Supplementary Table 1**). Interestingly, variable GATA6  
279 immunopositivity (<50% of tumour cells with score 2 or higher) was present in 4/16  
280 (25%) and 4/14 (28%) of classical and basal-like tumours, respectively, documenting a  
281 group where these subtypes may co-exist. This was furthermore observed in a number  
282 of advanced PDAC biopsies, which also exhibited variable GATA6 expression by ISH and  
283 IHC (**Supplementary Figure 5**), demonstrating that regional GATA heterogeneity can  
284 exist in resectable and advanced stage tumors. These differences were also observed at  
285 the cellular level by image analysis (**Supplementary Figure 6**). In sum, GATA6 staining  
286 patterns were widely comparable across whole sections of 22/30 (73%) resection cases.  
287 Variable GATA6 immunopositivity was present in a subset of both, classical and basal-  
288 like subtypes, in resectable and advanced disease, which may point at the presence of  
289 classical and basal regions in the same tumor.

290

291

### 292 **Keratin 5 may positively identify the basal-like subtype.**

293 GATA6 positively identifies classical PDAC, but markers for the basal subtype are lacking.  
294 In an exploratory analysis, we evaluated keratin markers associated with GATA6  
295 expression. In line with their use as basal markers in other tumor types (19, 20), keratins  
296 15, 5/6, 23 and 14 were inversely correlated with GATA6 expression and thus the  
297 classical subtype (**Supplementary Figure 7A**). In this post-hoc analysis, none of the  
298 identified cytokeratins were superior to GATA6 in their association with modified Moffitt  
299 subgroups, including keratin 17, a prognostic marker in PDAC (21) (**Supplementary**  
300 **Figure 7B**). Among these, keratin 5 (CK5) demonstrated the strongest expression  
301 differences between basal-like and classical tumors and was found to be complementary  
302 to GATA6 expression in our cohort (**Supplementary 8**). Furthermore, GATA6 and  
303 keratin 5 often demonstrated complementary staining pattern by IHC in PDAC tissues,  
304 including in 41 COMPASS biopsies and 30 resected PDAC whole sections  
305 (**Supplementary Table 1, Figure 5**). From these specimens, we observed the presence  
306 of both GATA6 and CK5 staining in a subset of cases (**Figure 5**, bottom panels). Indeed,

307 the intratumoral staining pattern of the two markers was predominantly inversely  
308 correlated in 149 individual tumor regions from the 30 resected cases (**Figure 6A**). Of  
309 note, this analysis revealed a small number of regions that contained considerable  
310 number of both CK5+ and GATA6+ cells (**Figure 6A**). Double immuno-staining confirmed  
311 distinct GATA6+/CK5- and GATA6-/CK5+ regions within the same tumor (**Figure 6B**)  
312 and in individual ducts (**Figure 6C**), which further support the notion that basal-like and  
313 classical programs can co-exist in the same tumor. Overall, many basal-like cases of  
314 advanced PDAC showed CK5 positivity (10/19, 53%) whereas most classical tumors  
315 (22/23, 96%) exhibited scant (<10%) or negative CK5 staining. Keratin 5 was thus highly  
316 specific and also showed remarkable intratumoral complementarity to GATA6 staining  
317 suggesting a clinically relevant biomarker of the basal-like subtype.

318

### 319 **Additional molecular characteristics of the basal-like phenotype**

320

321 Among the 195 eligible COMPASS patients, all 8 (4%) with adenosquamous histology  
322 were basal-like and stained positive for keratin 5 by IHC, with negligible *GATA6*  
323 expression by RNA ISH. We have previously shown that the basal-like subgroup is  
324 enriched in a hypoxia-associated gene signature by gene set enrichment analysis (22)  
325 and this observation is retained in this expanded dataset ( $p=0.0003$ ). In addition, we  
326 found higher PD-L1 expression in the basal-like cohort ( $p<0.001$ ), higher PD-1 expression  
327 ( $p<0.001$ ) and enrichment of a T-cell inflamed signature previously reported (23, 24)  
328 ( $p=0.007$ ) (**Supplementary Figure 9**). Tumour mutational burden was not different  
329 between groups (2.02 mutations/Mb vs 1.96 mutations/Mb) and was consistent with  
330 that seen in an unselected PDAC cohort (25).

331

332

### 333 **Discussion**

334

335 Combination chemotherapy is used in the treatment of most patients with advanced  
336 PDAC, yet the field is lacking robust biomarkers of outcome to guide regimen selection.  
337 Here, we show that patients with tumors of a modified 'basal-like' phenotype, or those  
338 with low *GATA6* RNA expression, have inferior outcomes compared to those with the  
339 'classical' phenotype. The latter are accurately identified by high *GATA6* expression and

340 positive GATA6 staining by in situ hybridization (ISH). Our data also suggests that basal-  
341 like tumours are particularly resistant to mFFX, warranting further investigation.

342

343 Both the PRODIGE4/ACCORD 11 and the MPACT PDAC trials of metastatic disease  
344 demonstrated an improved in survival with FOLFIRINOX and GnP across all sub-cohorts  
345 compared with gemcitabine alone(2, 3), yet they provide little insight into which  
346 subgroups might benefit the most. Notably, our study shows no superiority in either  
347 regimen in an unselected population with regard to survival. In the aforementioned  
348 trials, histological groups were not documented in either study, which is not unusual  
349 since many patients have a diagnosis made from very small samples or brushings. In  
350 contrast, the histological classification in resected specimens can be more easily reported  
351 and the PRODIGE24/ CCTG PA6 trial of mFFX in the adjuvant setting documented the  
352 prognostic impact of tumour grade in multivariable analysis (26) In patients receiving  
353 mFFX, those with well-differentiated tumors benefited the most (HR 0.52, 95% CI 0.34-  
354 0.81) whereas the impact in poorly differentiated tumors was not significant. Although  
355 limitations to the three-tiered histological classification (poor, moderate and well-  
356 differentiated) in PDAC have been noted (27), well-differentiated tumors highly express  
357 the classical program and *GATA6* (28).

358

359 The resistance of the basal-like subtype to mFFX is supported by a recent collaborative  
360 study by Tiriac et al (29)demonstrating that patient- derived organoid (PDO)  
361 chemotherapy signatures may predict treatment response. The signatures indicative of  
362 individual cytotoxic agents were applied to our COMPASS cohort suggesting that the  
363 basal-like cohort subgroup was most likely to have a non-oxaliplatin sensitive  
364 signature(29). We furthermore hypothesize that basal-like tumours may have limited  
365 sensitivity to 5-Fluorouracil. Martinelli et al. demonstrated *GATA6* loss in resected PDAC  
366 with a basal-like phenotype in the ESPAC-3 trial, and shorter survival in these patients  
367 when treated with adjuvant 5-Fluorouracil . This study also showed that *GATA6* low cell  
368 lines derived from patient-derived xenografts were particularly resistant to 5-FU but not  
369 gemcitabine (30). Notably oxaliplatin was not evaluated. In search of treatment  
370 alternatives, we report here that basal-like tumors had higher hypoxia scores, and higher  
371 PD-1 and PD-L1 expression with enrichment of a T cell inflamed signature (24) which  
372 may be predictive of immunotherapy response(23), suggesting a therapeutic strategy for



373 clinical trial design in this chemoresistant group. Similarly, triple negative breast cancers,  
374 although associated with worse outcomes, have higher levels of tumour infiltrating  
375 lymphocytes compared to hormone receptor positive, HER2-ve tumours. The impact of  
376 immune populations within subtypes in PDAC will require further investigation (31).

377

378 Clinical applicability of RNA sequencing and tumor enrichment by LCM is currently  
379 limited given tissue acquisition, cost and time to reporting. GATA6 detection from FFPE  
380 needle biopsies at diagnosis is therefore an attractive surrogate for transcriptomic  
381 classifiers. We demonstrate concordance of GATA6 ISH with the subtypes with sensitivity  
382 and specificity of over 80% in our tumor-enriched samples. Of note, the *GATA6* gene is  
383 not part of the original Moffitt subtype signatures but rather the Bailey squamous  
384 classifier, which largely overlaps with Moffitt calls in high purity samples (5). The  
385 number of tissue specimens available for matched ISH, IHC and RNAseq was low in our  
386 study (n=78, 40%). Therefore, although specificity was much lower for IHC compared to  
387 ISH, a prospective study with adequate tissue for matched analysis is needed.  
388 Recognizing that the identification of the basal-like subtype is critical and that GATA6 is  
389 a negative marker we sought additional positive keratin biomarkers that may be more  
390 feasible for the practicing clinician. Of these, keratin 5 predicted outcomes best after  
391 *GATA6* expression and was found to exhibit high complementarity to GATA6 staining  
392 pattern and RNA expression levels. Moreover, combined keratin 5 and GATA6 stainings  
393 on serial sections and by double immune-staining have consistently suggested that basal-  
394 like and classical elements can co-exist in a subset of PDAC cases, which strongly  
395 reinforces the need for a positive basal-like biomarker and has major implications for  
396 rationalizing subtype-specific treatments. We are currently evaluating combined staining  
397 of GATA6 and keratin 5 on the COMPASS trial.

398

399 Notably microdissected tissue, although impractical in laboratory medicine practice,  
400 most accurately detects tumour gene expression, with comparatively less exocrine and  
401 immune compartments compared to TCGA datasets, as recently described(32). This  
402 therefore implies that more reliable biomarkers can be determined from highly cellular  
403 specimens. CA-19.9 is the only approved biomarker for monitoring disease in the  
404 advanced setting (33) and the POLO trial has now documented a benefit for maintenance  
405 PARPi in patients with germline *BRCA* mutations(4). Robust subtyping of pancreatic

406 cancer will be critical to advancing the field, GATA6 as a single biomarker and highly  
407 correlated with the Moffitt classifier will now be evaluated in a prospective trial.

408

409 This study is limited by few progression biopsies to understand the stability of the  
410 subtypes under selective pressure during chemotherapy. This is especially interesting in  
411 light of the co-existence of basal-like and classical elements, documented here and  
412 elsewhere (17, 18). In addition, the numbers of basal-like tumours treated with GnP  
413 regimens is low and the GnP group is potentially confounded by performance status. The  
414 interaction term for chemotherapy type and subtype was not significant in this study  
415 although numbers were low. We therefore cannot conclude whether GnP is a better  
416 strategy in the basal-like cohort, rather our data suggests alternative therapies are  
417 urgently needed and clinical trials to evaluate this particular group are warranted. With  
418 mFFX as current treatment of choice in the adjuvant setting, understanding  
419 chemotherapy response to subtypes has increasing importance. It should also be noted  
420 that the response rates and survival between those receiving mFFX and GnP were not  
421 statistically different in this analysis. This is supported by the recent HALO trial 109-321  
422 study where response rates and overall survival are comparable to historical outcomes  
423 with mFFX(2, 34). This furthermore supports the need to understand which populations  
424 can benefit most from these regimens and a prospective trial has now been planned.

425

426 In the major tumor types of lung and colorectal cancer, factors such as histological  
427 subtype, molecular profile and PD-L1 status can influence the choice of upfront systemic  
428 treatment in advanced disease and have resulted in survival gains (35-37). Since PDAC  
429 will soon become the second leading cause of cancer related mortality, it behooves the  
430 oncology community to invest in biomarkers helpful for selecting standard  
431 chemotherapy. In this study, we confirm the prognostic impact of the modified Moffitt  
432 subtypes and demonstrate that basal-like PDAC responds poorly to mFFX. The basal-like  
433 cohort can be accurately identified by GATA6 RNA expression, providing a putative single  
434 important biomarker in clinical trial design.

## **Figure Legends**

**Figure 1:** Consort Diagram of patients enrolled and included on the COMPASS trial. 250 patients were enrolled and 232 patients underwent biopsies. Biopsy sites included liver, pancreas and peritoneum/omentum. 195 patients were eligible with RNAseq data representing the study population.

**Table 1:** Baseline characteristics of cases enrolled according to modified Moffitt classification (classical vs. basal-like)

### **Figure 2: Waterfall plots demonstrating tumour size change according to modified Moffitt classifier**

- A) Tumour size change in all patients included (n=194\*): This includes any chemotherapy received. The Non Evaluable patients did not have imaging to determine response
- B) Tumour size change in patients receiving first-line modified FOLFIRINOX (mFFX) (n=102\*)
- C) Tumour size change in patients receiving gemcitabine/nab-paclitaxel (GnP) regimens (n=71)

\*1 patient with non-measurable disease is not included

NE: non evaluable

mFFX: modified FOLFIRINOX

GnP: gemcitabine/nab-paclitaxel

New lesions

### **Figure 3: Kaplan Meier overall survival curves according to modified Moffitt subtype and chemotherapy received**

- A) Overall survival in the intention to treat population (n=195) which includes patients who did not receive chemotherapy or who were non evaluable
- B) Overall survival in patients receiving first line mFFX or GnP regimens (at least 1 cycle) and is presented according to modified Moffitt subtype (n=174). This graph integrates curves in 3C and 3D.
- C) Overall survival in patients receiving  $\geq 1$  cycle mFFX (n=103) according to modified Moffitt subtype
- D) Overall survival in patients receiving  $\geq 1$  cycle GnP regimens (n=71) according to modified Moffitt subtype.

### **Figure 4: GATA6 expression is associated with modified Moffitt subtypes in advanced PDAC**

- A) Gata6 expression by RNAseq versus modified Moffitt subtypes (left), and GATA6 expression by RNAseq versus GATA6 ISH (right). Scores of 0-1 reflect the basal-like subtype and 2-5 the classical subtype.
- B) Kaplan Meier curve of overall survival by modified Moffitt in patients with matched tissue for RNAseq and GATA6 ISH analysis (n=106).
- C) Kaplan Meier curves of overall survival by GATA6 ISH semi-quantitative analysis in patients with matched tissue for RNAseq and GATA6 ISH analysis (n=106).

D) Kaplan Meier curve of GATA6 by QuPath image analysis in those patients with matched tissue for RNAseq and GATA6 ISH (n=106).

**Figure 5: Pathology images comparing GATA6 staining by ISH and IHC, together with CK5 IHC staining**

**A) Advanced PDAC cases:**

COMP-022: Classic with glandular architecture (HE), GATA6 ISH score 3, GATA6 IHC score 2, CK5 negative (rare positive cells), magn 100x

COMP-0234: Basal-like with squamous features (HE), GATA6 ISH score 1, GATA6 IHC score 1 (weak/focal), CK5 positive, magn 100x

COMP-0135: Basal-like with poor differentiation (HE), GATA6 ISH score 2 (variable distribution), GATA6 IHC score 2 (variable distribution), CK5 positive, magn 100x

**B) Resected PDAC cases:**

Expression pattern of GATA6 and CK5 in resected PDAC.

PCSI\_639: Classic with glandular architecture (HE), GATA6 ISH score 3, GATA6 IHC score 2, CK5 negative, magn 100x.

PCSI\_588: Basal-like with squamous features (HE), GATA6 IHC score 1 (weak/focal), CK5 positive, magn 100x.

PCSI\_645: Classic with dual phenotype (glandular and squamous) on HE, GATA6 IHC score 2 (variable distribution), CK5 positive, magn 25x.

**Figure 6: Tissue pattern of GATA6 and keratin 5 expression**

**A)** IHC staining of GATA6 and keratin 5 on serial sections from resected PDAC specimen (n = 30). Representative images, magn 25x (left). Quantification of the percentage of GATA6+ or CK5+ cells, respectively, in 149 matched regions on adjacent sections (right).

**B)** Dual immunostaining of GATA6 (brown) or CK5 (magenta) in resected PDAC revealing distinct regions of GATA6 or CK5 immunoreactivity, magn 25x.

**C)** Dual immunostaining of GATA6 (brown) or CK5 (magenta) revealing GATA6 and CK5 immunoreactivity in the same tumour ducts. Resected PDAC (left); advanced PDAC (right); magn 400x.

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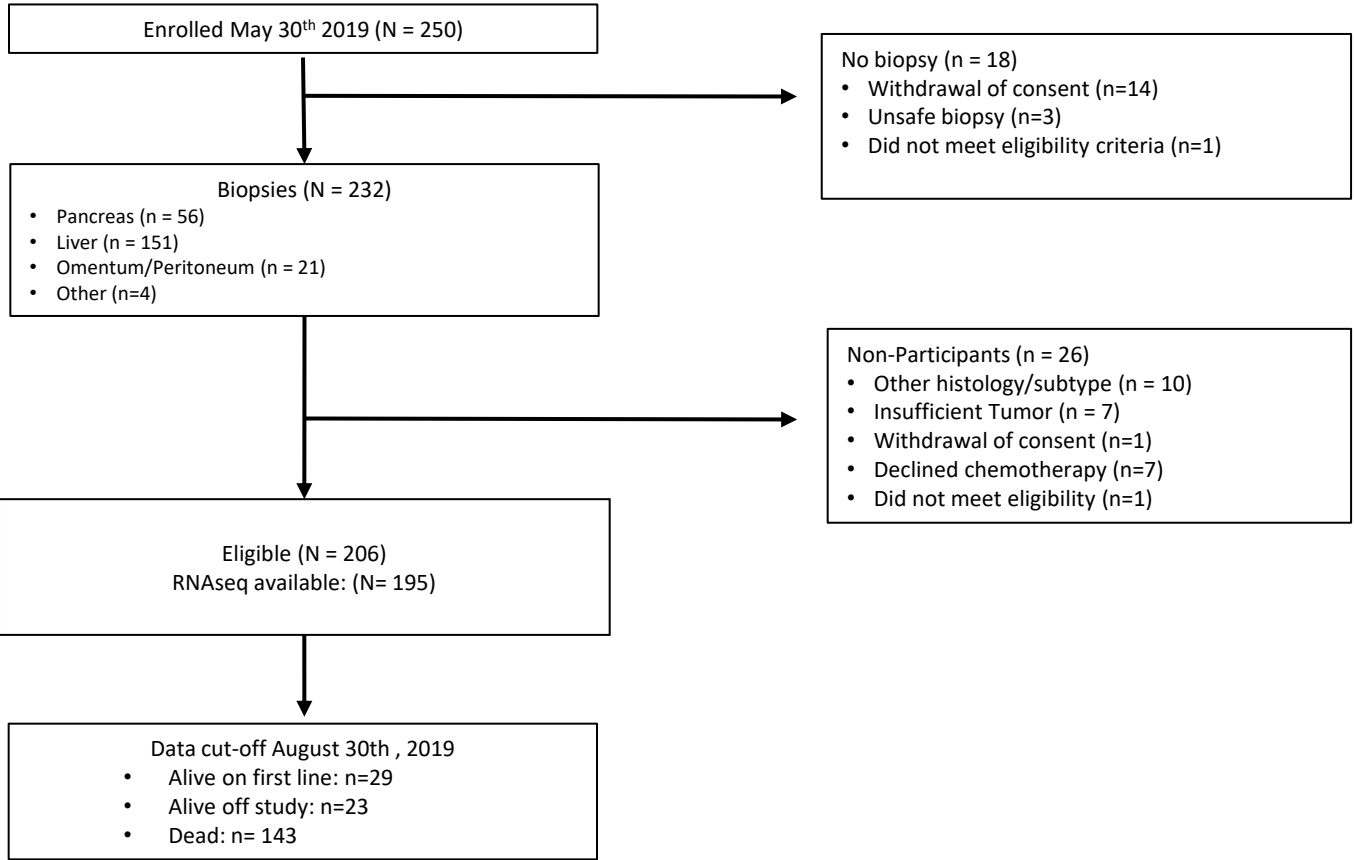
Table 1: Baseline characteristics of patients included (n=195)

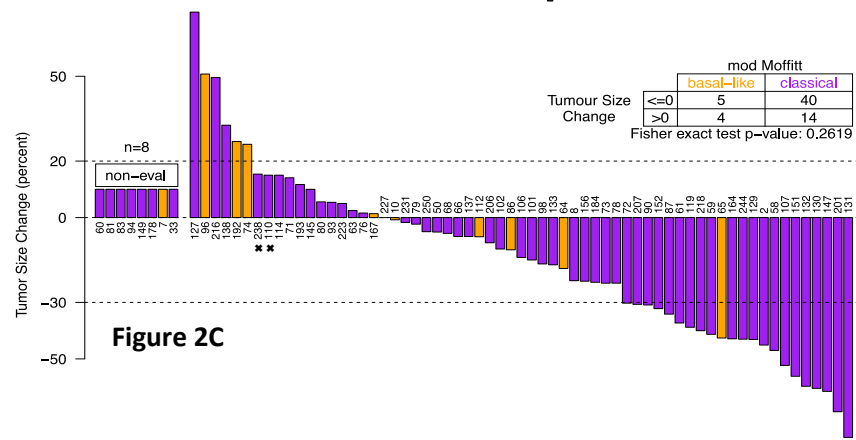
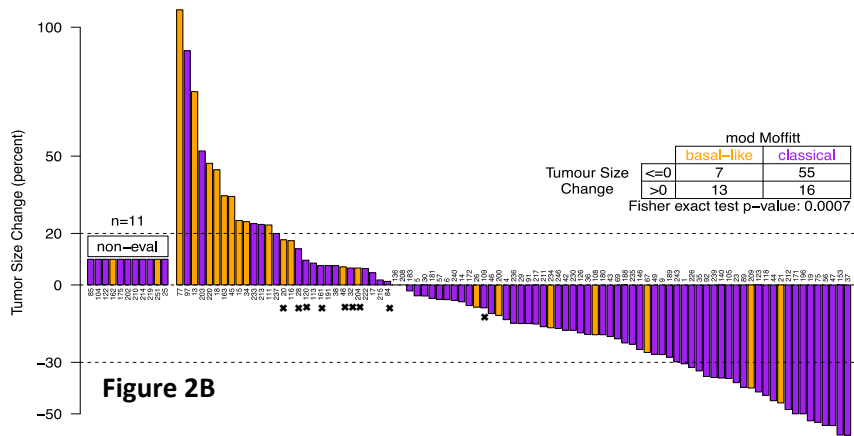
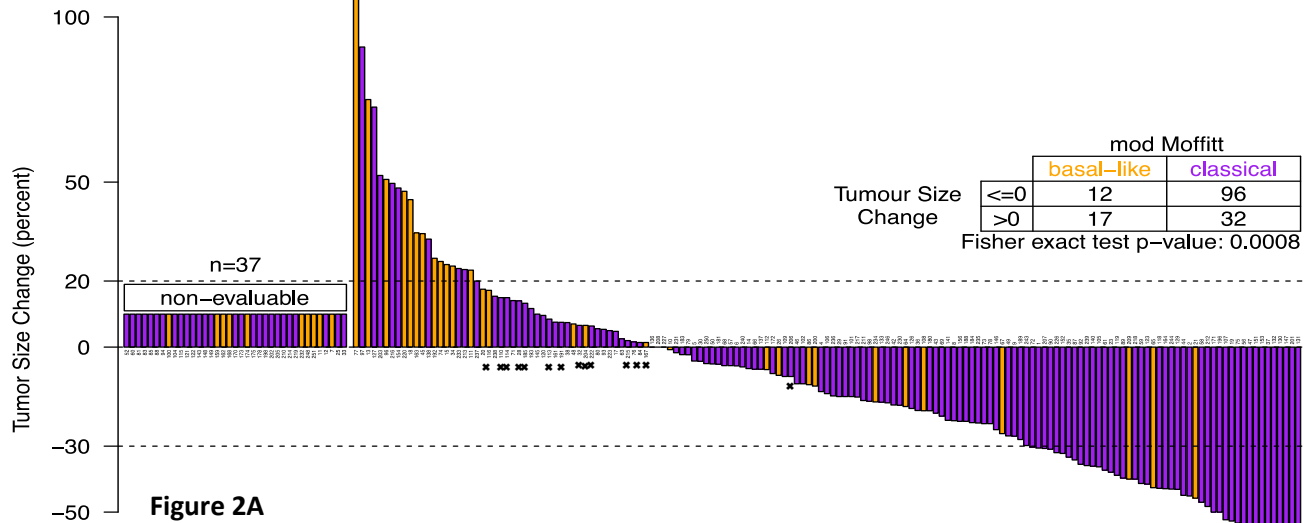
	Classical (N= 156) N, %	Basal-like (N=39) N, %	p value
<b>Median age (yrs)</b>	64.0 (29-84)	65.0 (44-83)	0.75
<b>Sex</b>			
<b>Male</b>	83 (53)	29 (74)	0.02
<b>Female</b>	73 (47)	10 (26)	
<b>Stage</b>			
<b>Metastatic</b>	132 (85)	39 (100)	0.005
<b>Locally advanced</b>	24 (15)	0 (0)	
<b>Race</b>			
<b>White</b>	119 (79)	27 (77)	0.23
<b>Asian</b>	28 (19)	6 (17)	
<b>African/other</b>	4 (3)	2 (6)	
<b>Unknown</b>	5	4	
<b>Prior resection</b>			
<b>Yes</b>	13 (8)	3 (8)	0.99
<b>No</b>	143 (92)	36 (92)	
<b>CA19.9 (median, range)</b>	1832 (1-371847)	1124 (1-71956)	0.24
<b>Ever Smoker</b>			
<b>Yes</b>	80 (51)	23 (59)	0.47
<b>No</b>	76 (49)	16 (41)	
<b>Type II DM ≥18mths</b>			
<b>Yes</b>	32 (21)	8 (21)	0.99
<b>No</b>	120 (79)	30 (79)	
<b>Unknown</b>	4	1	
<b>Liver metastases</b>			
<b>Yes</b>	108(69)	38 (97)	<0.0001
<b>No</b>	48(31)	1 (3)	
<b>HRD genotype*</b>			
<b>Yes</b>	14 (9)	2 (5)	0.74
<b>No</b>	142 (91)	37 (95)	
<b>First chemotherapy</b>			
<b>mFolfirinox</b>	81 (52)	22 (59)	0.25
<b>GnP-regimens</b>	61 (39)	10 (26)	
<i>Gem/nab-paclitaxel alone</i>	43	8	
<i>Gem/nab-paclitaxel+experimental</i>	18	2	
<b>Cisplatin/Gem or Gem alone</b>	5 (3)	2 (5)	
<b>None</b>	9 (6)	5 (13)	

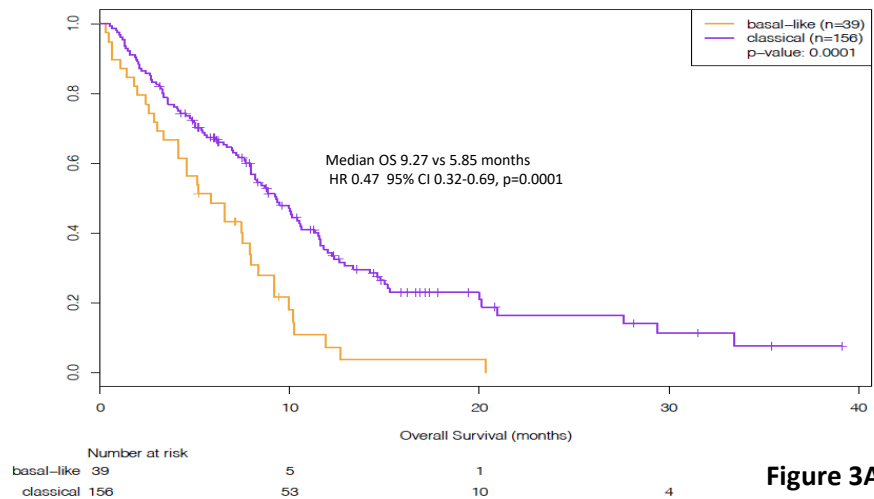


Figure 1

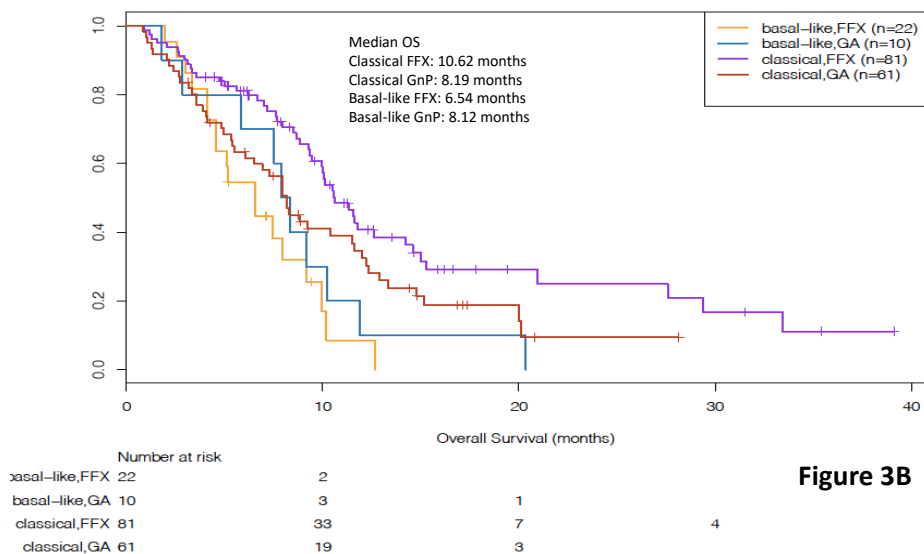
# Consort Diagram



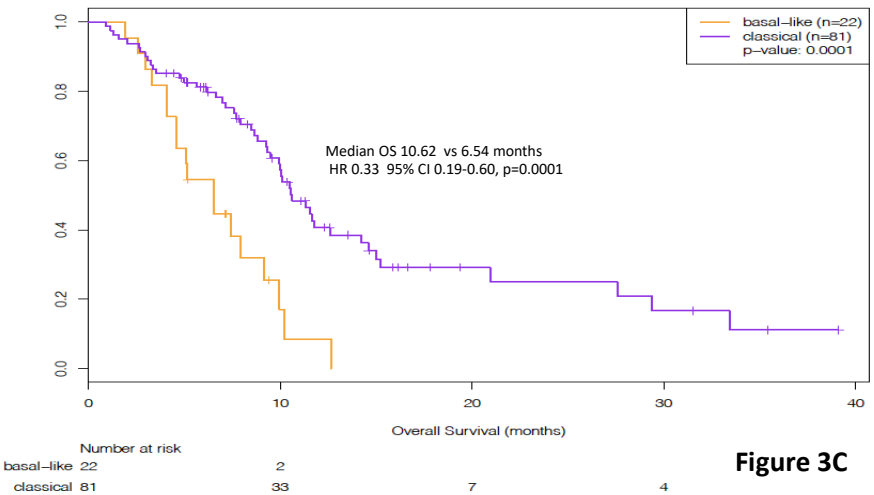




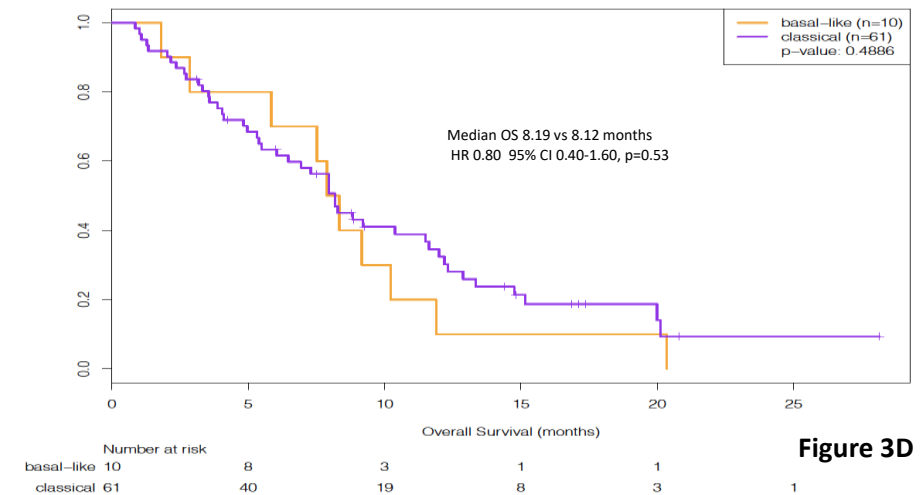
**Figure 3A**



**Figure 3B**

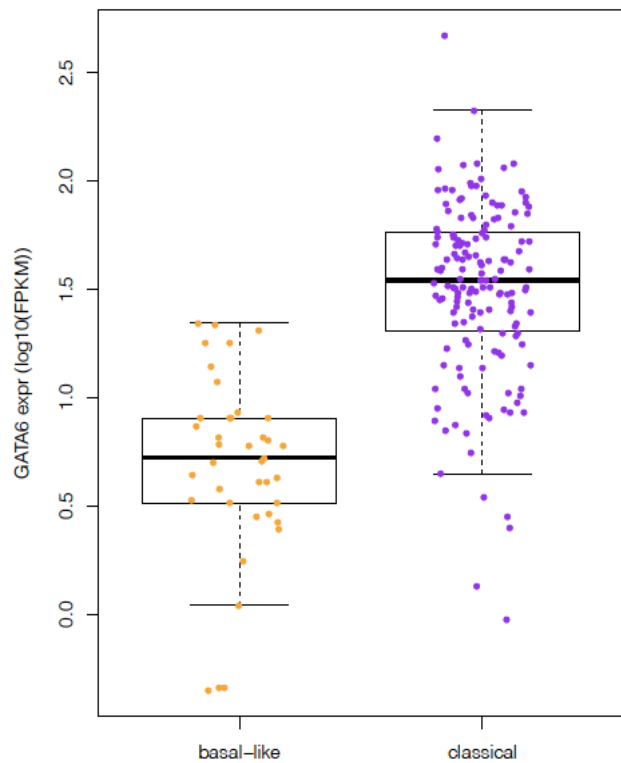


**Figure 3C**

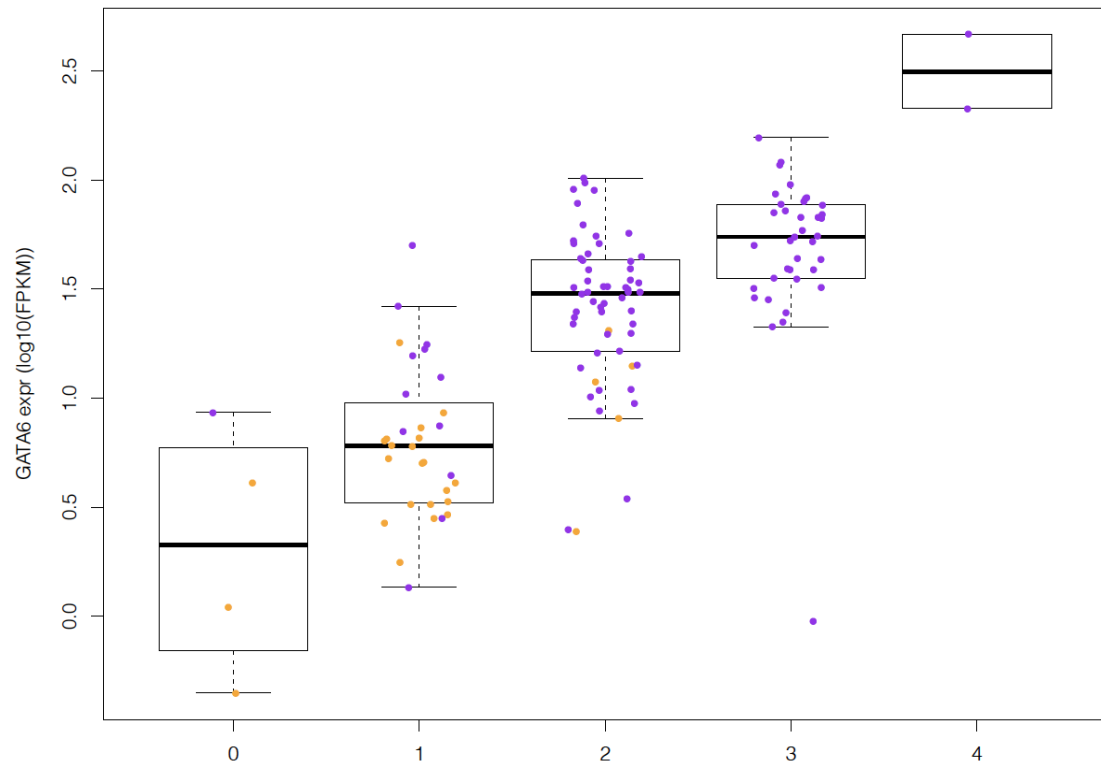


**Figure 3D**

Figure 4A



Wilcoxon Rank Sum test  $1.096 \times 10^{-16}$



Kruskal-Wallis test  $1.118 \times 10^{-14}$

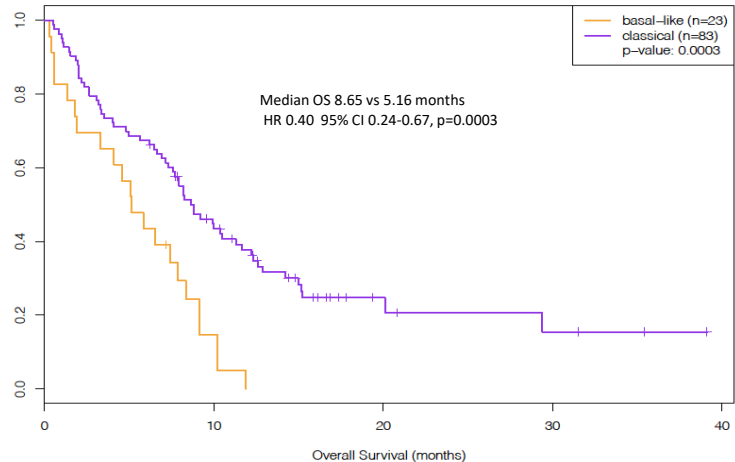


Figure 4B

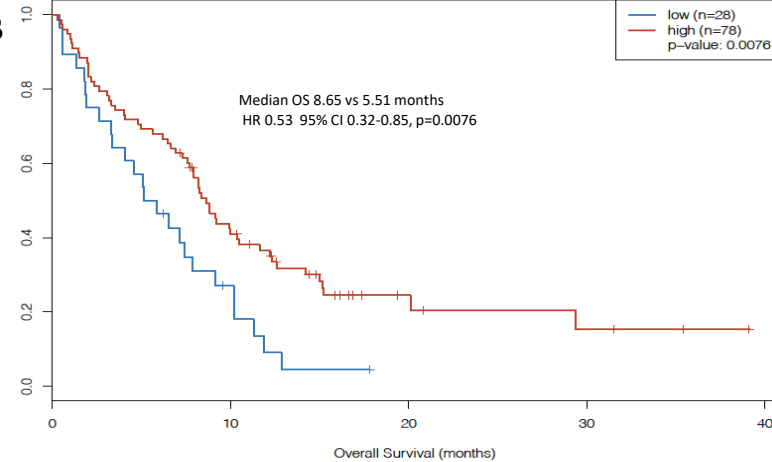


Figure 4C

Number at risk

basal-like	23	3		
classical	83	32	6	3

Number at risk

low	28	6		
high	78	29	6	3

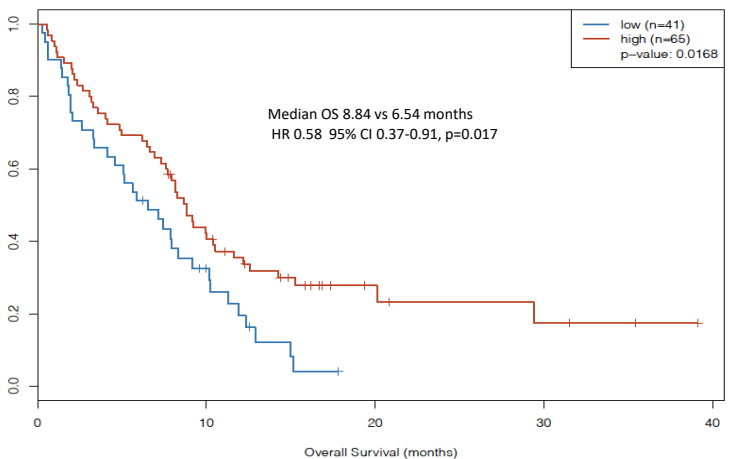


Figure 4D

Number at risk

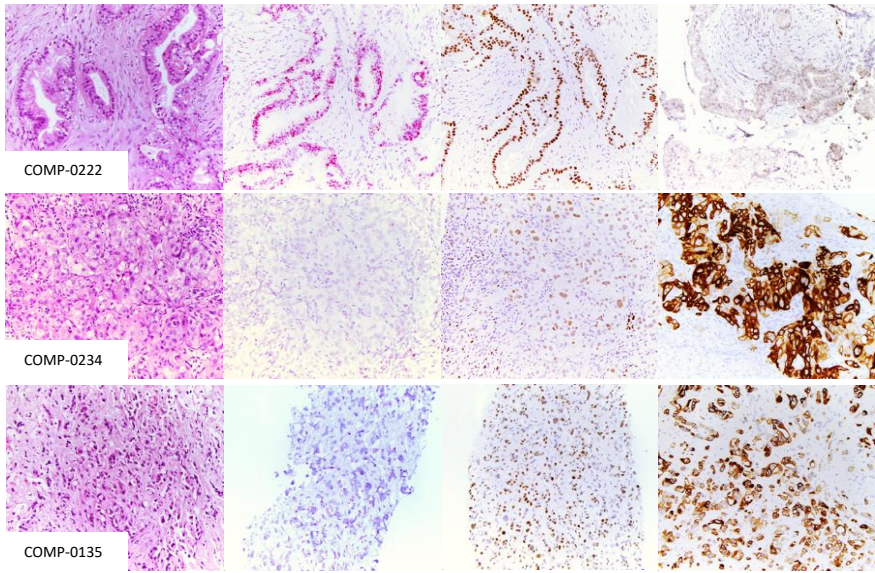
low	41	10		
high	65	25	6	3

**Figure 5A**

GATA6 RNA ISH

GATA6 IHC

Keratin 5 IHC



**Figure 5B**

GATA6 IHC

Keratin 5 IHC

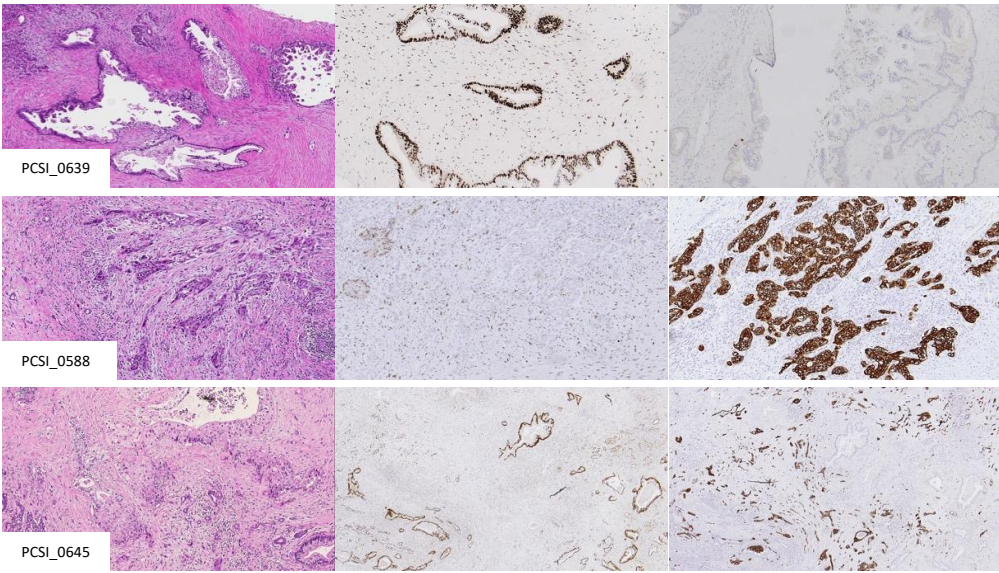




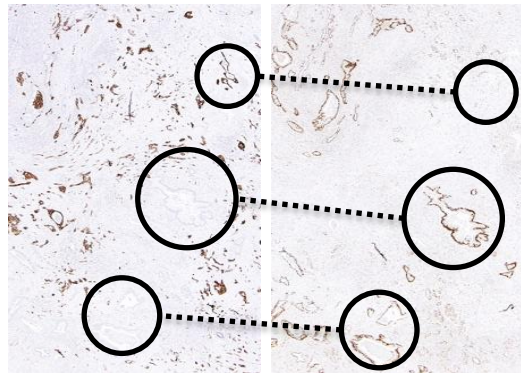
Figure 6

**A**

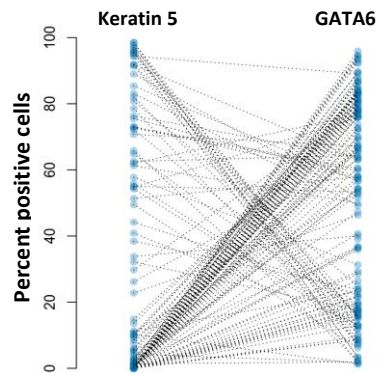
Matched regions on PDAC serial sections

Keratin 5 IHC

GATA6 IHC

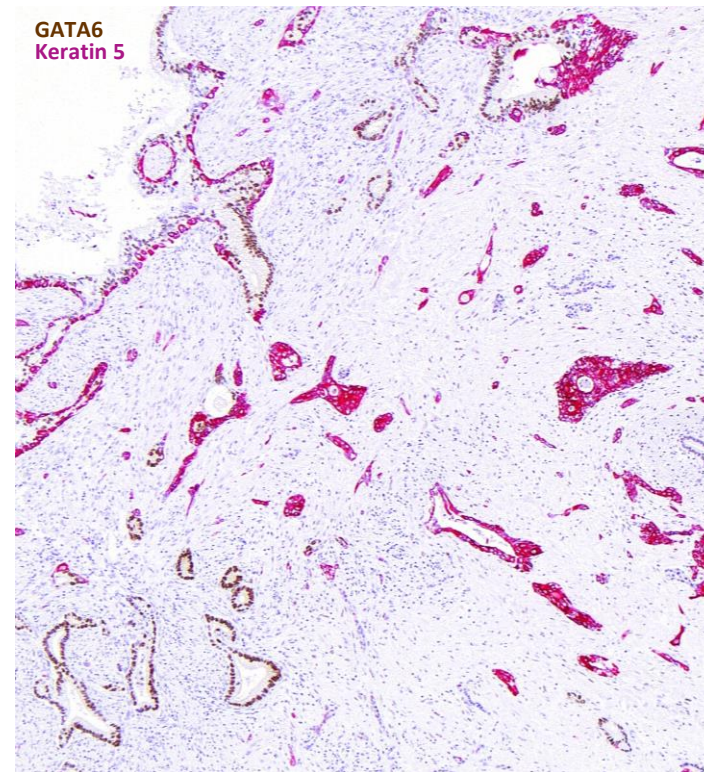


Quantification



**B**

Double Immunostaining



**C**

Resectable PDAC

Advanced PDAC

