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# Transitional type Circulating Tumor Cells Predict Systemic Recurrence and Support Risk Stratification for Chemotherapy After Resection of Pancreatic Ductal Adenocarcinoma: Long-term Outcomes of the CLUSTER Trial

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**Running title:** Circulating tumor cells for pancreatic cancer treatment

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**Keywords**: Pancreatic neoplasms, circulating tumor cells, epithelial mesenchymal transition, recurrence

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**STRUCTURED ABSTRACT:**

**Aim:** To evaluate whether transitional circulating tumor cells (trCTCs) predict systemic recurrence of pancreatic ductal adenocarcinoma (PDAC) and assess their potential role in risk stratification for systemic treatment.

**Background:** The high metastatic potential of PDAC is believed to be associated with early dissemination after cancer cell reprogramming via an epithelial-to-mesenchymal transition. These cells are detectable in circulation as trCTCs and could serve as valuable biomarker capturing systemic disease involvement.

**Methods:** The prospective CLUSTER trial enrolled patients scheduled for PDAC resection (2016–2018). Pre- and postoperative CTCs were isolated with the Isolation-by-SizE-of-Tumor-Cells device and characterized by immunofluorescence. Cox regression with spline terms assessed associations between preoperative biomarkers and systemic recurrence, while multivariable subgroup analyses with interaction tests evaluated overall survival (OS) stratified by adjuvant chemotherapy.

**Results:** In preoperative samples, trCTCs were detected in 82 (67%) of 123 patients with a median number of two cells per ml (IQR 1-3). A linear association between preoperative trCTC

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counts and systemic recurrence ($\chi^2$=13.2, p=0.004) was observed, but no relevant correlation with CA19-9 levels was found (Pearson correlation=0.05, 95% CI:–0.13-0.23). Furthermore, trCTC-positivity after resection predicts recurrence and is associated with prolonged OS associated with adjuvant therapy (HR 0.21, 95%CI: 0.09-0.49) after adjustment for tumor stage and neoadjuvant chemotherapy.

**Conclusions:** Preoperatively, higher trCTC counts are associated with increased risk of systemic recurrence, while postoperative presence reflects minimal residual disease. Integrating trCTC assessment alongside currently used biomarkers into the clinical pathway for patients with PDAC could enhance risk stratification and support more personalized treatment decisions.

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## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive epithelial cancers marked by metastatic disease at the time of diagnosis or by systemic progression in patients undergoing oncologic resection in the majority of patients.[1] For those with clinically localized disease, surgical resection in combination with systemic therapy provides the best chance for long-term survival.[2] However, despite curative intent surgery, approximately three out of four patients with resected PDAC will develop recurrence, predominantly at a systemic site and succumb to their disease.[3,4]

Currently, CA19-9 is the only circulating biomarker in routine clinical use, but lacks specificity due to biliary obstruction and sensitivity due to the approximately 20% of individuals who are non-secretors.[5] Furthermore, CA19-9 has shown limited value for predicting systemic recurrence, underscoring the need for novel biomarkers.[5] In the past has shown that CTCs can be isolated from the peripheral blood of patients with PDAC, thereafter showing promising results for predicting early recurrence.[6] Epithelial-mesenchymal transition (EMT) has been proposed as one of the key drivers of early dissemination of pancreatic cancer cells.[7,8] During this process, epithelial tumor cells gain mesenchymal characteristics that allow them to lose apico-basal polarity and translocate from the primary tumor site.[9] After detachment from the primary tumor and extravasation into circulation these cells are referred to as circulating tumor cells (CTCs).[6] While a majority of extravasated cancer cells die in circulation through shear forces, immune cell mediated elimination (mainly NK cells), or oxidative stress, cancer cell reprogramming through EMT permits a subset of CTCs to survive the harsh circulatory environment and evade the immune system.[9] Those CTCs that manage to survive can attach to the endothelium, migrate into

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the metastatic site, and undergo mesenchymal-endothelial transition (MET) to form metastases.[9] Therefore, CTCs which have transitional (mixed epithelial and mesenchymal, trCTCs), or mesenchymal properties are thought have metastatic potential driving the poor outcomes of patients with PDAC.[10]

Our previous work from the CLUSTER trial has demonstrated a) significantly lower CTC counts are observed in those receiving neoadjuvant chemotherapy b) high burden of preoperative CTCs predict early disease recurrence and c) CTC increase after resection predicts disease recurrence. Therefore, CTCs have shown to reflect disease progression and response to treatment. However, the ability of trCTC to predict long-term benefits of systemic treatment and risk assessment of systemic outgrowth of minimal residual disease remains unknown. Therefore, the goal of this study was to predict systemic disease involvement prior to resection and assess the benefit of adjuvant treatment stratified by trCTC using the CLUSTER cohort.

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## METHODS

The current study enrolled patients from the previously published prospective CLUSTER Trial (CircuLating tUmor cellS in pancreaTic cancer, NCT02974764) which was conducted at Johns Hopkins Hospital from March 2016 to March 2018. In that study, patients were prospectively enrolled based on a clinical suspicion of non-metastatic (Stage I-III) surgically resectable PDAC. Exclusion criteria included a personal history of malignancy other than PDAC, prior pancreatectomy for PDAC, or age < 18 years. For the current study additional patients were excluded with failed preoperative and postoperative CTC assessment, pathological diagnoses other than PDAC, and loss to follow-up or cancer unrelated postoperative death within one year

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were excluded. This trial was designed as an observational trial to test the clinical applicability of CTCs where no treatment decisions were made on basis of CTC assessment. Treating physicians were blinded to the CTC data and vice versa, the researcher performing CTC enumeration was blinded to the clinical data at time point of assessment. All enrolled patients signed a detailed informed consent and were followed from the time of diagnosis or presentation to the clinic until patient death. This study was carried out in compliance with the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki and was approved by the Institutional Review Board for Human Research and complied with all Health Insurance Portability and Accountability Act regulations. The study abided by the strengthening the reporting of observational studies in epidemiology (STROBE) guidelines.[11]

*CTC assessment*

For CTC assessment, peripheral blood (10 ml in ethylenediaminetetraacetic acid, EDTA) was drawn prior to incision and at 4-6 days postoperatively. Blood samples were then processed with the Isolation by Size of Epithelial Tumor Cells assay (ISET; Rarecells®) within 4 hours according to the manufacturers protocol.[12] Hereby, the ISET assay entraps cells by filtering cells with size >8μm. Thereafter, all isolated cells were fixed on the membrane and preserved in -20°C for further assessment. CTCs enumeration and phenotype characterization were performed with an optimized immunofluorescent staining protocol. For this, a combination of pan-cytokeratin and vimentin antibodies were used to assess epithelial and mesenchymal cell traits, respectively. To separate white cell populations, additional staining with anti-CD45, CD11b, CD14, CD34 was performed to ensure specificity of CTC assessment. CTCs were then stratified

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as epithelial-type (pan-cytokeratin+, vimentin-, CD-), mesenchymal type (vimentin+, pan-cytokeratin-, CD-), and mixed epithelial/mesenchymal-type further referred to as transitional CTCs (trCTCs, pan-cytokeratin+, vimentin+, CD-). Detected cells were additionally assessed for morphology characteristics (nuclei and cytoplasmic structures). No restrictions in cell size were applied. Due to the aims of this study at assessing systemic disease, only CTCs with mesenchymal properties were analyzed. As a control, healthy volunteer samples of five participants were obtained in which no CTCs were observed. The full protocol with supplementary methods, Supplemental Digital Content 1, http://links.lww.com/SLA/F690 can be found in the reporting of early postoperative results of the CLUSTER trial as described by Gemenetzis et al.[13]

*Treatment and Follow-up*

In the postoperative setting, all patients who underwent resection were followed at the surgical outpatient clinic at three-monthly intervals for the first six months after surgery, and at six-monthly intervals thereafter. The decision for treatment with adjuvant chemotherapy was made by the blinded multidisciplinary team and the patient's own preference. Imaging for recurrence was conducted using abdominal/pelvic and chest computed tomography (CT) at three- to six-month intervals for the first two years and then annually, or earlier when indicated. The presence of metastatic or local disease progression was identified through imaging. Pathological confirmation of recurrence was not obligate if radiological suspicion was strong. The date of death was obtained from institutional medical records, online obituaries, or the

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Social Security Death Index. All data were collected, de-identified, and analyzed by an independent reviewer.

*Outcome definitions*

trCTC counts are provided as cells per ml. The pathological assessment of the pancreatic specimen was performed in accordance with the College of American Pathologists (CAP) pancreatic protocol and staging performed according to the 8$^{th}$ edition of the AJCC-TNM classification.[14] The resection margin was considered positive (R1) if a surgical margin (uncinate, pancreatic transection, biliary, or enteric margin) was involved within less than 1mm. Overall survival (OS) was defined as the time between the date of surgery and the date of death whereas time to progression/recurrence (TTP) was defined as the interval between surgery and recurrence. For all patients found to be alive at the time of last follow-up, the survival was censored at the date of last contact. Survival data for this study were updated till June 1, 2024 ensuring a follow-up of five years after resection for all patients.

*Statistical analysis*

Statistical analysis was conducted using the R statistical software (version 4.2.3). Categorical data were summarized as frequencies and percentages, while continuous data were reported as mean with standard deviation (SD) or median and inter-quartile ranges (IQR). Comparisons of differences in the distribution of categorical data was done with $\chi^2$ test, while the Mann-Whitney U test was used to assess continuous data. Any missing data were

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acknowledged in the baseline tables and excluded from group comparisons as a complete case analysis. The timepoint for analysis of long-term outcomes was set at five years postoperatively. Cox-regression models with spline terms were used to estimate non-linear associations between serologic biomarkers and systemic recurrence at five years. Log-Rank tests were used to assess survival in the tested cohorts with data visualization by Kaplan-Meier curves by using "Ggplot2". The predictive accuracy of multivariable risk models was evaluated by the concordance index (c-index). To assess the treatment effect of adjuvant therapy on OS, mantel-Cox regression and Cox proportional hazard regression with interaction analysis were performed using the "Survival", "Survminer", and "Publish" packages. Hazard ratios (HR) and 95% confidence intervals (95%CI) were calculated for each assessed variable. A two-sided p-value of <0.05 was considered statistically significant.

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## RESULT

*Patient Characteristics*

Of the 200 patients enrolled in the CLUSTER trial, 123 patients were available for systematic assessment of recurrence in the current study. The exclusion flowchart with criteria is provided in Supplementary **Figure 1**, Supplemental Digital Content 1, http://links.lww.com/SLA/F690. Neoadjuvant therapy was administered in 50 (41%) patients. CA19-9 was elevated >37 U/ml in 73 (66%) of patients. In preoperative samples, trCTCs were detected in 82 (67%) patients with a median number of two cells per ml (IQR 1-3/ml) (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/SLA/F690). Forty-nine (60%) patients with pre-operative detectable trCTCs had no detectable trCTCs after resection whereas 28 remained positive. After resection, trCTCs were detected in 31 (27%)

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patients with a median number of one cell per ml (IQR 1-1.5). Median OS of the whole cohort was 24.9 months (95%CI:21.0-27.6) with 22 patients (18%) being alive after a complete 5-year follow-up. Other clinicopathological characteristics are presented in **Table 1**.

*Systemic recurrence is frequently observed and associated with worse survival*

During a median follow-up of 73.9 months (IQR 65.0-78.6) for surviving patients, 75 (61%) were diagnosed with systemic recurrence, whereas 26 (21%) and 22 (18%) had local-only or no recurrence, respectively. Time to recurrence was significantly shorter in patients with systemic recurrence (median TTR: 8.9 months [95%CI:7.2-11.5]) as compared to patients with local-only recurrence (median TTR 15.9 months [95%CI:10.6-24.7], Log-Rank p=0.001, **Supplemental Figure 2a**, Supplemental Digital Content 1, http://links.lww.com/SLA/F690). Consequently, OS was shorter for patients having systemic recurrence (19.4 months (95%CI:16.8-24.6) compared to local-only recurrence (mOS: 29.1 months, 95%CI:20.6-49.8, Log-Rank p=0.003) and compared to patients without recurrence (median not reached, estimated five-year survival 80% (95%CI:64-100%), both comparisons Log-Rank p<0.001, **Supplemental Figure 2b**, Supplemental Digital Content 1, http://links.lww.com/SLA/F690).

*Preoperative blood biomarkers predict systemic recurrence*

The predicted five-year probability of systemic recurrence by preoperative measurement of CA19-9 and trCTC is presented in **Figure 1**. There was no significant correlation between CA19-9 and trCTC-levels (Pearson correlation 0.05, 95%CI: -0.13-0.23, p=0.605). Due to non-linearity of the CA19-9 association to systemic recurrence (ANOVA-p=0.012), an optimum of five spline terms were used, whereas there was no evidence for non-linearity for trCTCs with an

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optimal prediction at three spline terms (ANOVA-p=0.183). There was a significant association between systemic recurrence and both tested serological biomarkers; preoperative trCTC-levels ($\chi^2$=13.2, p=0.004) and preoperative CA19-9 levels ($\chi^2$=15.63, p=0.004).

*Postoperative biomarkers for prediction of systemic recurrence*

Of the 31 patients with postoperative trCTCs, 30 (97%) were diagnosed with recurrence with one patient lost to follow-up after 13.9 months. Of those, 25 (83%) were diagnosed with systemic recurrence. In patients without detectable postoperative trCTCs, recurrence was observed in 62 (76%) patients of which 44 (71%) had systemic recurrences. Therewith, both recurrence at any site (p=0.010) and systemic recurrence (p=0.009) were less often observed in postoperative trCTC negative patients. Additionally, time to recurrence was shorter in trCTC positive versus trCTC negative patients (mTTR 7.2 months [95%CI:3.8-12.3] vs. 15.6 months [95%CI:13.4-18.0], Log-Rank p<0.001, **Figure 2a**). Consequently, trCTC positivity was also associated with a shorter OS (mOS trCTC-positive: 16.8 months [95%CI:12.6-24.8] vs. 27.2 months [95%CI:24.2-46.1], Log-Rank p<0.001, **Figure 2b**). The association of trCTC positivity with worse oncological outcomes were confirmed after stage adjustment (pT and pN) for time to recurrence (HR 2.62, 95%CI: 1.63-4.22) and OS (HR 2.28, 95%CI: 1.40-3.70). In patients who were trCTC negative after surgery, their preoperative trCTC status was not associated with the rate of recurrence (negative: 24/33 [73%] vs. positive: 38/49 [78%], p=0.618). Also, time to recurrence was similar between patients who had trCTCs preoperatively but were negative postoperatively (mTTR 15.5 months, 95%CI:10.3-18.1) compared to patients who had no detectable trCTCs pre- and postoperatively (mTTR 16.0 months, 95%CI: 13.4-46.7, Log-Rank

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p=0.530, Figure 3a) whereas those who remained positive had significantly shorter TTR (mTTR 7.3 months, 95%CI:4.0-12.3, both comparisons Log Rank p<0.001, Figure 3b).

*Postoperative detection of trCTC was associated with benefit from adjuvant chemotherapy* Sixty-seven trCTC negative patients (91%) and 18 trCTC positive patients (60%) after resection received adjuvant chemotherapy. On multivariable subgroup analysis adjusting for neoadjuvant treatment, CA19-9 levels, tumor stage, and lymph node status, an association with longer survival in the subgroup treated with adjuvant therapy versus no adjuvant therapy (HR 0.21, 95%CI: 0.09-0.49) was observed in the subgroup of patients with postoperative detectable trCTCs, whereas there was no evidence for a treatment effect in trCTC negative patients (HR 1.62, 95%CI: 0.51-5.16, **Figure 4**). Furthermore, the interaction analysis showed that there was a significant difference in adjuvant treatment effect on OS ($P_{interaction}$ = 0.004). Other subgroups associated with an improved survival in the adjuvant treated subgroup versus the group not receiving adjuvant treatment were patients undergoing upfront resection (HR 0.26, 95%CI: 0.10-0.67), patients with a CA19-9 above 100 U/ml (HR 0.26, 95%CI: 0.10-0.71), patients with pT3-4 tumors (HR: 0.35, 95%CI: 0.16-0.74), and patients with pathologically confirmed lymph node involvement (HR 0.29, 95%CI: 0.13-0.65). However, no significant differences were observed between subgroups of CA19-9 ($P_{interaction}$ = 0.504), tumor stage ($P_{interaction}$ = 0.136), lymph node status ($P_{interaction}$ = 0.069) or receipt of neoadjuvant treatment ($P_{interaction}$ = 0.099). The associated benefit of adjuvant treatment for patients with postoperative detectable trCTC was corroborated in a delay of recurrence (HR: 0.07, 95%CI: 0.03-0.19, median time to recurrence adjuvant: 10.0 months [95%CI: 7.5-17.7] vs. no adjuvant: 2.7 months [95%CI: 2.2-not reached], p<0.001) but not for recurrence rates (estimated 5-year recurrence

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rates both 100%). Nine out of 18 patients who received adjuvant chemotherapy and had detectable postoperative trCTCs had samples drawn after completion of adjuvant treatment of which three normalized and six remained positive.

## DISCUSSION

In the CLUSTER trial, it was demonstrated that CTCs dynamics reflect progression of disease and response to treatment through the key findings of 1) lower CTC counts after neoadjuvant treatment as compared to those undergoing upfront surgery, 2) lower numbers of CTCs observed after treatment and 3) the value of longitudinal assessment of CTCs for predicting early disease recurrence, In this analysis of the long-term follow-up of the prospective CLUSTER, we demonstrate that trCTCs reflect the systemic burden of disease and therefore predict systemic recurrence after resection for PDAC for risk assessment prior to surgical resection.We further demonstrate that all patients who had positive trCTCs after resection were diagnosed with recurrence after a five year follow-up time, whereas recurrence was observed less frequently in trCTC negative patients reflecting minimal residual disease. Consequently, those with trCTC positivity derived an OS-benefit associated with adjuvant chemotherapy whereas those without may have limited treatment effect.

Despite sequential genomic alterations from KRAS followed by CDKN2A in early and loss of TP53 and SMAD4 in late carcinogenesis being described,[15] recent studies suggest that carcinogenesis in PDAC can develop rapidly and mutations can occur in parallel instead of sequentially leading to aggressive tumor characteristics early on.[16] Overall this results in systemic progression of PDAC in many patients while still remaining radiologically undetectable. The ability of a cell to undergo EMT is a reminiscent of cell migration during

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embryologic processes such as gastrulation, neural crest cell migration, and organogenesis being misused by cancer cells to detach from the primary tumor en route to metastatic seeding.[9] This is a complex process and cancer cells face multiple challenges including the harsh circulatory environment and the immune system. Therefore, it is estimated that only <0.01% of cells successfully seed systemic site and can therefore be the origin of cancer metastasis which is eventually the major cause of death in patients with PDAC.[17,18] Therefore, if trCTCs are detected preoperatively, while there is disease in circulation it does not necessarily mean that involvement of systemic organs has already occurred, i.e. the trCTCs could origin from the primary tumor.[19] However, the higher the preoperative trCTC count, the higher also the chance for systemic seeding of tumor cells and therefore of systemic recurrence as shown in a linear association of trCTCs and systemic recurrence in this study.

Contrastingly, postoperatively trCTC positivity may represent local or systemic minimal residual disease as measurements were performed four to six days after surgery, thus well beyond the two hours half-life of CTCs.[20] As trCTC positivity was associated with a 100% estimated recurrence rate with the majority being diagnosed with systemic recurrence after a sufficient follow-up time, we hypothesize that tumor cell shedding most likely can originate from a systemic site in those patients without local recurrence.[21] This illustrates systemic minimal residual disease despite assumed complete local tumor resection and that metastatic cells can undergo EMT and secondary tumor cell shedding.[21] These results are in line with a study from Park et al. where CTC positivity emerged as an independent predictor of systemic recurrence in a population of 36 patients after resection for PDAC.[22] In that study, 70% of patients with positive CTCs (1/3$^{rd}$ of the study population) were diagnosed with systemic recurrence whereas the other

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two thirds of patients who were CTC negative had a 30% rate of systemic recurrence.[22] In contrast to this study, CTCs were isolated by centrifugation and staining for EpCAM/CK and DAPI (if negative for CD45), therefore not allowing for differentiation between epithelial and transitional/mesenchymal subtypes. This may explain the difference in predictive ability as in this study (data not shown) and in early analyses of the CLUSTER trial, epithelial CTCs had limited predictability of worse outcomes including early recurrence.[13,23,24]

As this analysis shows, although trCTCs are highly predictive of systemic tumor recurrence, specificity of CTC detection remains a drawback of this approach. Of the trCTC negative patients after resection of PDAC, three out of four patients were diagnosed with recurrence. This may be explained by two central reasons; 1) Experimental studies show, that EMT is not an absolute requirement for tumor metastasis and therefore in these patients trCTC may not be detectable despite systemic tumor involvement.[25,26] To address this in the future, further subtyping and investigation of the mechanisms driving the metastatic cascade are required. 2) trCTCs may not have been detected because of the limits of detection considering the isolation technique we used (ISET). This is reflected by the low yield of trCTCs observed in the study population, especially after resection (median 1 cell per ml). Theoretically, patients who had recurrence of disease despite a trCTC negative status postoperatively may have had trCTCs which were not detected by our measuring. In this study trCTCs were detected in 67% preoperatively and 27% after resection which is comparable to other publications in radiologically localized PDAC where CTC positivity varies between 33% and 56% assessed with different methods and at different time points.[22,27,28] Recent advances such as the VAR2-based approach, a plasmodium falciparum derived protein that binds to oncofetal chondroitin sulfate

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glycosaminoglycans combined with isolation by immunodensity cell separation may improve detection of CTCs with mesenchymal properties as shown by the results of Tang et al.[29] However, owing the lowered sensitivity of CTCs with current isolation techniques, a multiomic approach that integrates multiple biomarkers into a multi-analyte test is needed to help overcome the limitations.[30,31] In the current study, this was investigated using the clinically available biomarker CA19-9. Preoperatively, CA19-9 is often used as a surrogate for tumor activity and systemic tumor involvement. However, drawbacks of CA19-9 are that some 10 to 20% are non-producers.[31] When excluding them, CA19-9 can predict oncological outcomes with a discriminative ability, however, a c-index of 0.6 is not acceptable for the precision medicine we aim to perform nowadays.[5] Therefore, introduction of novel biomarker such as trCTCs are warranted. In this study, both CA19-9 and trCTCs were associated with systemic recurrence. Interestingly, there was no statistical correlation shown for the two serological biomarkers. As shown in Figure 2, the additive value of preoperative trCTC assessment to the routinely used CA19-9 measurements is visualized. Although due to the study design no statement can be on the value of resection of the pancreatic tumor itself, this information could aid in treatment decisions of borderline cases with disputable indications e.g. in patients questionable condition for major surgery.[32-34]

Overall, several randomized clinical trials have concluded a benefit of adjuvant chemotherapy after upfront surgery of PDAC.[35,36] After neoadjuvant treatment, the benefit of adjuvant treatment is disputed due to insecurity of additional treatment effect and potential side effects due to dose accumulation including peripheral neuropathy especially in patients treated with mFOLFIRINOX.[37] As the aforementioned prognostic factors are surrogates for risk of

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systemic tumor involvement, adjuvant chemotherapy may be warranted in patients having these risk factors.[38] In this study, patient subgroups with present surrogates of systemic tumor involvement, in particular trCTC positivity, highly elevated CA19-9, and pathologically confirmed lymph node involvement, have shown to be significantly associated with an OS improvement associated with the receipt of adjuvant chemotherapy. Although no other analysis on trCTCs with long-term results are yet published, the other clinically available factors are in line with a recent international multicenter study of 767 neoadjuvantly treated patients, which showed an overall benefit of adjuvant treatment but no clinically relevant treatment effect for the subgroup of patients with ypN0, partial or complete radiological response, or if patients were treated with ≥8 neoadjuvant cycles.[39] Given that trCTC positivity, advanced tumor stage and CA19-9 >100 U/ml had similar HR associated with an adjuvant treatment effect trCTC assessment but are not necessarily correlated, there may be an additive value for capturing systemic disease. To date, however, CTC assessment remains exploratory as platforms are not yet broadly available clinic and costs remain relatively high (approximately 250’000$ for acquisition of the isolation device plus 1’000 per patient analyzed). Further improvement of technologies and streamlining of procedures may facilitate applicability. Therefore, in the future, trCTC assessment along with other prognostic factors may aid in optimizing individual treatment decisions making.

This study is the first prospective study with long-term outcomes of the value of trCTCs in PDAC with systematic follow-up for recurrence. It may be possible that regardless, some recurrences may have been missed due to death without prior imaging or cancer unrelated death with yet undetectable recurrence. To minimize this bias, patients with cancer unrelated death

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within 12 months after surgery were excluded.[40] Furthermore, the treatment effect of adjuvant chemotherapy may be different depending on prior preoperative induction or neoadjuvant treatment.[41] In the included analysis, this was accounted for but due to limited power, not every variable could be tested in the subgroup analysis stratified by receipt of preoperative chemotherapy. Also, conditional reasons for not undergoing adjuvant treatment which are also associated with poor outcomes may influence this analysis. Therefore, the findings of this study along with other studies reporting on determinants of a proposed benefit of adjuvant chemotherapy must be interpreted with caution and validated with higher level evidence through well-designed randomized trials investigating a biomarker based individualized precision approach.[38,39,41]

## CONCLUSIONS

Preoperative trCTC and CA19-9 are not correlated, but both are independent predictors of systemic recurrence in patients with resected PDAC. The combination of the two serologic biomarkers could aid in risk stratification of systemic tumor involvement in those patients with PDAC prior to pancreatectomy. Furthermore, results of this study confirm the role of postoperative trCTC for the detection of minimal residual disease after resection. While in trCTC positive patients, eventually progression of this minimal residual disease results in recurrence, the time to clinical apparent metastases can be prolonged by adjuvant chemotherapy. Therefore, although not curative, there is an associated relevant OS benefit derived from adjuvant chemotherapy in these patients. The integration of trCTC into the clinical pathway for patients with PDAC could enhance risk stratification and enable more personalized treatment decisions.

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**Figure 1: Preoperative serologic biomarker assessment for prediction of systemic recurrence**

Figure 3 shows the joint association of pre-operative trCTC count and CA19-9. There was no correlation between both preoperative assessed serologic biomarkers however both were associated with systemic recurrence. For example, a CA19-9 within normal range (5-37) and two detectable trCTCs has similar predictivity as negative trCTCs with a CA19-9 of 200, whereas a patient with two trCTCs and a CA19-9 of 200 was predicted to have an 85-90% chance of systemic recurrence. White dots represent actual patient values.

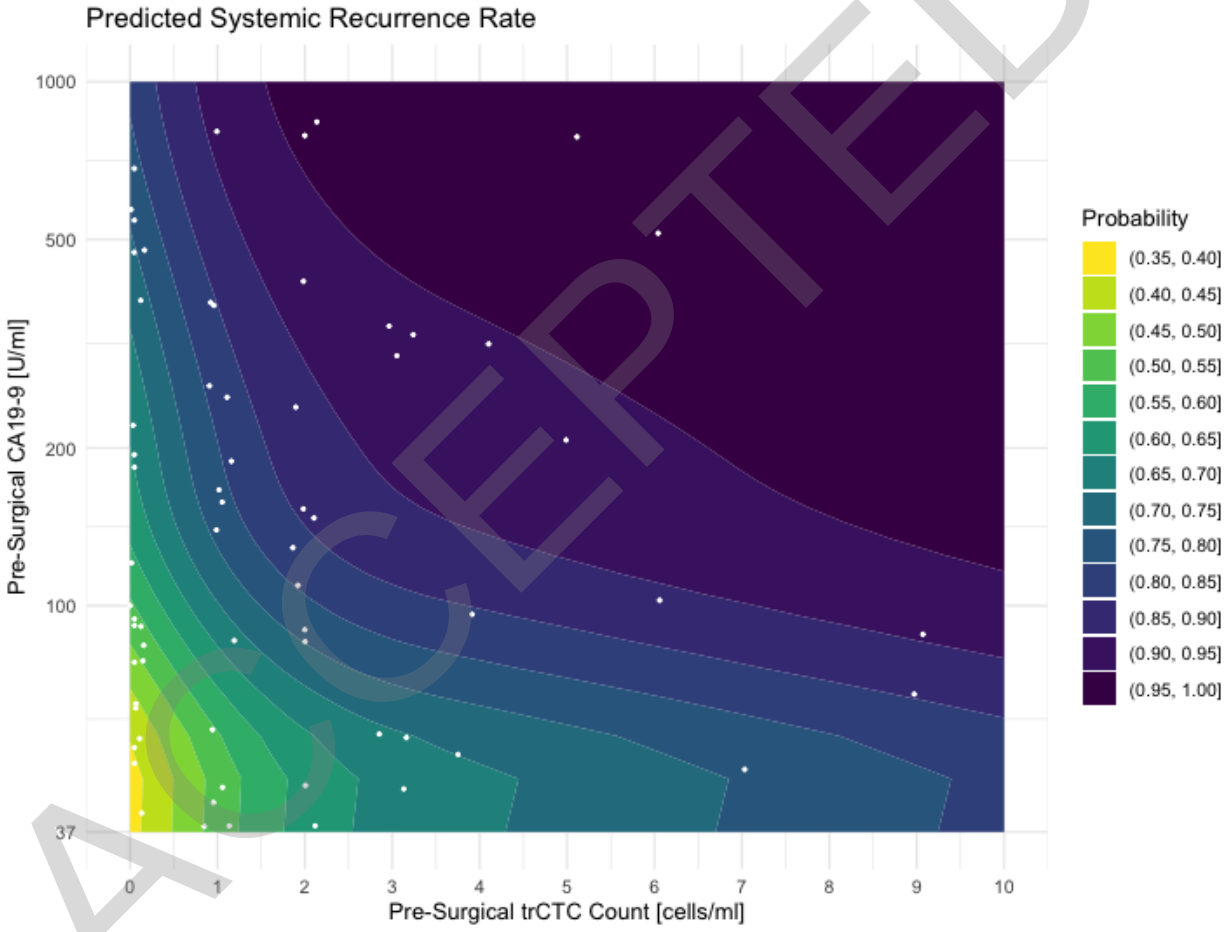


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**Figure 2: Kaplan-Meier curves for time to recurrence (a) and overall survival (b) stratified by postoperative trCTC positivity**

Figure legend: Kaplan-Meier Curves showing unadjusted survival for subgroups. 95%-Intervals are shown in hatched color, crosses represent time of censoring.

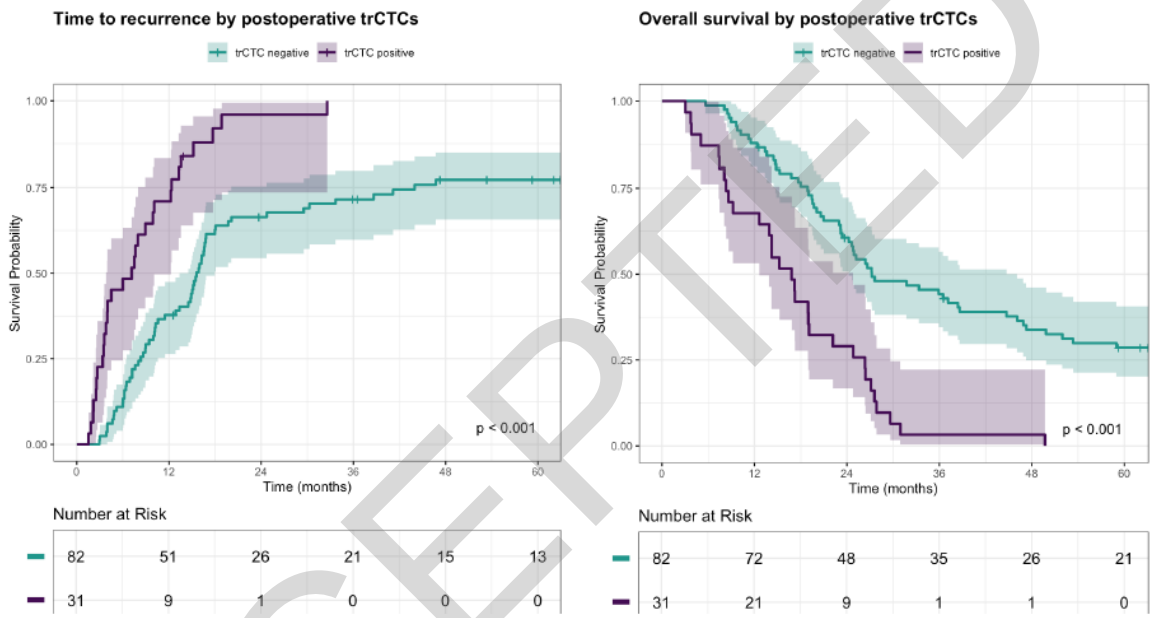


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**Figure 3: Kaplan-Meier curves for time to recurrence (a) and overall survival (b) stratified by pre- to postoperative trCTC dynamics**

Figure legend: Kaplan-Meier Curves showing unadjusted survival for subgroups. 95%-Intervals are shown in hatched color, crosses represent time of censoring.

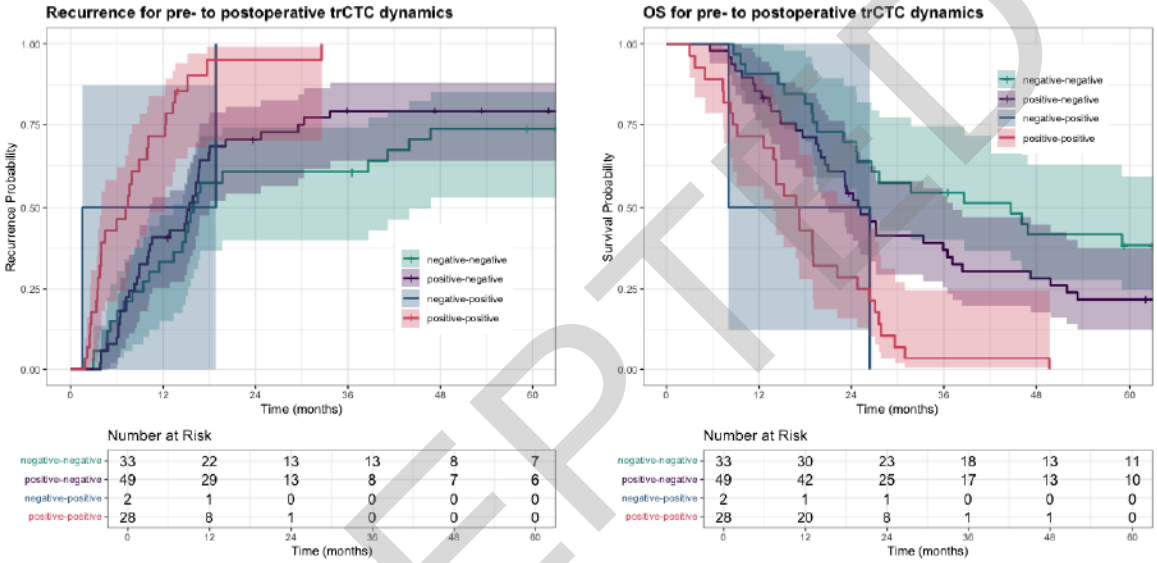


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**Figure 4: Multivariable subgroup and interaction analysis on the overall survival benefit derived from adjuvant chemotherapy**

Figure legend: Subgroup analyses show different OS-improvement associated with the receipt of adjuvant treatment. P-Interaction for differing treatment effects between subgroups: postoperative trCTC (0.004). CA19-9 (0.504), tumor stage (0.136), lymph node status (0.069), receipt of neoadjuvant treatment (0.099). dots represent estimates, whereas lines represent 95%CI, * represent out of bounds 95*CI.

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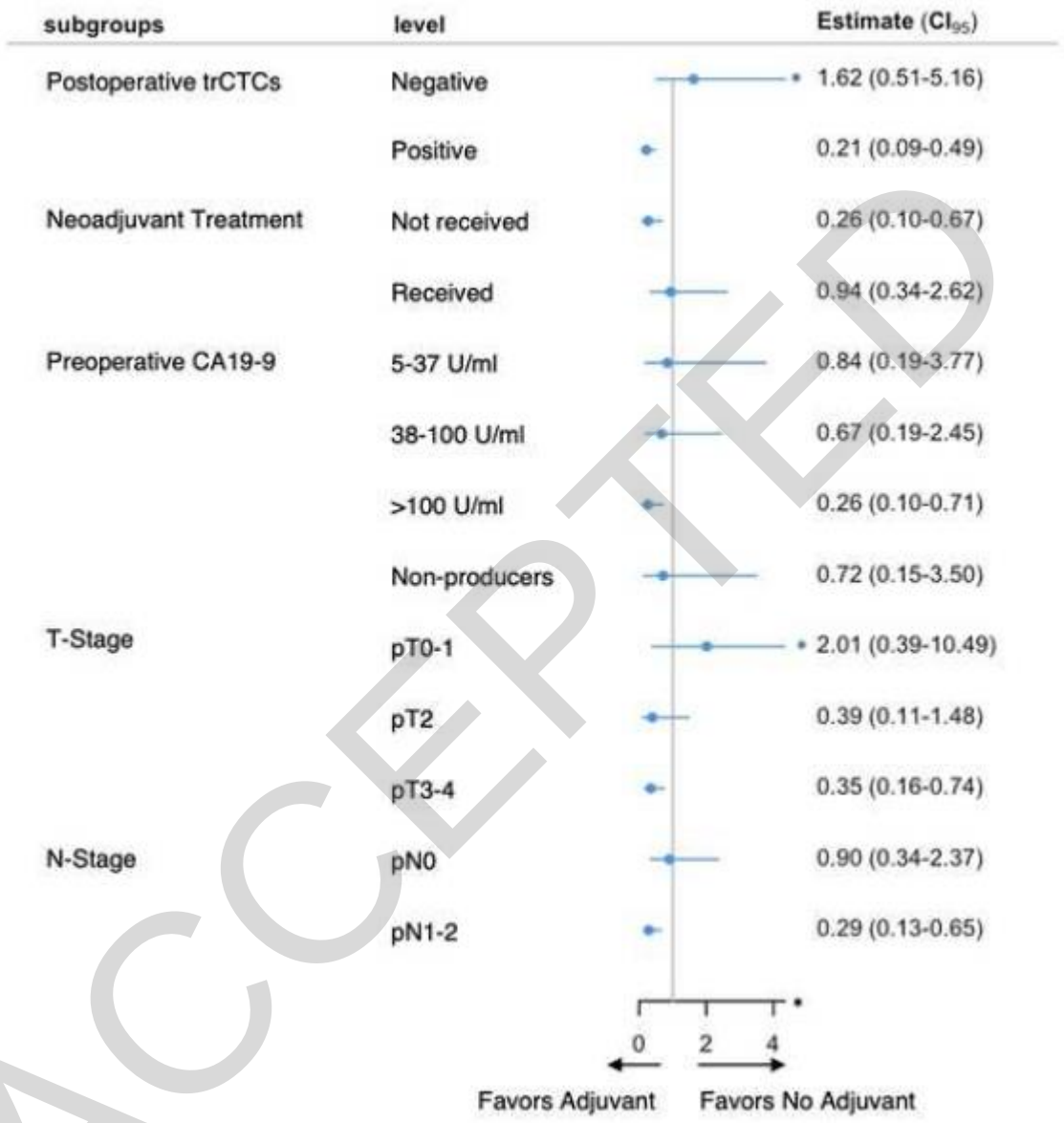


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**Table 1: Clinicopathologic characteristics of all patients and comparisons of post-resection trCTC cohorts**

| | all patients, N = 123[1] | trCTC negative, N = 82[1] | trCTC positive, N = 31[1] | p-value[2] |
|---|---|---|---|---|
| Age | 67.9 (58.6- 74.6) | 68.4 (59.2-74.7) | 69.2 (59.9-74.6) | 0.731 |
| Sex [female] | 56 (46%) | 38 (46%) | 13 (42%) | 0.675 |
| Presurgical trCTCs [median] | 1 (0-2.75) | 1 (0-2) | 3 (2-4.75) | <0.001 |
| Unknown | 1 | 0 | 1 | |
| Presurgical trCTC positivity | | | | <0.001 |
| positive | 82 (67%) | 49 (60%) | 28 (93%) | |
| negative | 40 (33%) | 33 (40%) | 2 (6.7%) | |
| Unknown | 1 | 0 | 1 | |
| CA19-9 | | | | 0.025 |
| 5-37 U/ml | 28 (25%) | 23 (31%) | 4 (14%) | |
| 38-100 U/ml | 31 (28%) | 21 (28%) | 6 (21%) | |
| >100 U/ml | 42 (38%) | 27 (36%) | 13 (45%) | |
| non-secreter | 9 (8.2%) | 3 (4.1%) | 6 (21%) | |
| Unknown | 13 | 8 | 2 | |
| Neoadjuvant chemotherapy | 50 (41%) | 34 (41%) | 11 (35%) | 0.562 |
| Surgical procedure | | | | 0.825 |
| PancreatoduFodenectomy | 88 (72%) | 58 (71%) | 21 (68%) | |
| Distal pancreatectomy | 27 (22%) | 19 (23%) | 7 (23%) | |
| Total pancreatectomy | 8 (6.5%) | 5 (6.1%) | 3 (9.7%) | |
| Tumor stage | | | | 0.010 |
| pT0-1 | 19 (15%) | 17 (21%) | 1 (3.2%) | |
| pT2 | 46 (37%) | 33 (40%) | 9 (29%) | |
| pT3-4 | 58 (47%) | 32 (39%) | 21 (68%) | |
| Lymph node status | | | | 0.061 |
| pN0 | 48 (39%) | 37 (45%) | 8 (26%) | |
| pN1-2 | 75 (61%) | 45 (55%) | 23 (74%) | |
| Grade of differentiation | | | | 0.131 |
| Well-moderate | 74 (69%) | 51 (73%) | 16 (57%) | |
| Poor | 34 (31%) | 19 (27%) | 12 (43%) | |
| Treatment effect/Unknown | 15 | 12 | 3 | |
| Complete pathological response | 5 (4.1%) | 5 (6.1%) | 0 (0%) | 0.320 |
| Resection margin status [R1] | 19 (15%) | 9 (11%) | 8 (26%) | 0.074 |
| Perineural invasion [present] | 98 (80%) | 62 (76%) | 28 (90%) | 0.083 |
| Lymphovascular invasion [present] | 64 (52%) | 40 (49%) | 19 (61%) | 0.235 |
| Receipt of adjuvant treatment | 95 (83%) | 67 (91%) | 18 (60%) | <0.001 |

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| | | | |
|---|---|---|---|
| Unknown | 9 | 8 | 1 |

Table legend: n (%) or median (IQR), p-value by Pearson's $\chi^2$, Wilcoxon rank sum test; Fisher's exact test, 10 patients with missing data on postoperative trCTCs.

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