

## **SUPPLEMENTARY METHODS**

### **CTCs Isolation and Enrichment with the ISET assay**

The acquired blood sample was placed on a rotator in room temperature until the isolation process ( $t < 4$  hours). For the initial step, the 10 mL sample was diluted in the ISET provided buffer (three different powder buffers mixed in sterile water with 1M of NaOH for a final pH of 7.2-7.4) on a 1:10 volume-to-volume (v/v) ratio, and was split into two 50 mL Eppendorf tubes, each of them also containing 900 $\mu$ L of 37% formaldehyde solution for cell fixation purposes. Preparation during this step promotes extensive red cell lysis, which will allow filtration of the sample and avoid membrane clogging. The ISET cartridge was preconditioned with 10 mL of 1X Phosphate-buffered saline (PBS) solution prior to sample processing. The diluted sample was filtered through the 8 $\mu$ m-pore membrane with a suction pressure of -8 to -11kPa. A second 1X PBS washing step was performed and the membrane was separated from the cartridge and left to dry in room temperature for 30 minutes. Storage of the membrane was done in the dark at -20°C, until further analysis.

### **Immunofluorescent staining**

For immunofluorescent (IF) staining, commercially available conjugated primary antibodies or a combination of unconjugated primary and a secondary antibody were utilized. More specifically, a list of IF monoclonal antibodies used in this experiment is: pan-cytokeratin conjugated antibody (FITC, ThermoFisher), vimentin conjugated antibody (Alexa Fluor 594, ThermoFisher), CD45 conjugated antibody (Alexa Fluor 647, ThermoFisher), CD11b (Alexa Fluor 647, ThermoFisher), CD34 (Alexa Fluor 647, ThermoFisher), and CD14 unconjugated primary with a secondary antibody (Alexa Fluor 647, ThermoFisher). Antibodies were stored in 4°C per manufacturer's instructions and were all used in a 1% concentration for sample staining. The staining protocol was performed as follows: one core of each sample membrane was cut

and re-hydrated in 1 mL of 1X tris-buffered saline (TBS) for 10 minutes. It was then sunk in 500µl of 1X TBS with 0.2% Triton-X for 5-6 minutes to achieve adequate cell membrane permeabilization for staining of targeted cytoplasmic elements. After two 5-minute 1X TBS washing steps, the membrane core was incubated in 1 mL of 5% milk-based blocking buffer (lab-scientific non-fat dry milk diluted in 5% w/v of 1X TBS with 2% Tween) for 1 hour. This preconditioning step promotes down-regulation of cell surface Fc receptors and blocking of nonspecific monoclonal antibody binding, whereas the addition of Tween prevents non-specific protein interactions. The membrane core is then incubated in the prepared antibody cocktail (all antibodies in 1% concentration diluted in the milk-based blocking buffer) for 2 hours and washed twice before placed on a glass microscope slide (Fisherbrand™ Superfrost™). A glycerol-based antifade mount ant (ThermoFisher) containing DAPI or NucStain for nuclear staining was applied directly and the membrane core was covered with a coverslip and stored at 4°C overnight prior to review. After review, all slides were stored in the dark at -20°C.

### **Hematoxylin-Eosin (H&E) staining protocol**

A separate membrane core was initially re-hydrated in 1X PBS for 5 minutes. It was then submerged sequentially in hematoxylin for 3 minutes, deionized water, eosin for 1 minute and washed again in deionized water. The membrane core was then affixed on a glass microscope slide with a 10% glycerol solution.

### **CTCs immunofluorescent review and characterization**

Review of the stained membrane cores was performed using the Nikon T/E inverted microscope system (Nikon, Japan). The membrane core has an approximate surface of 28mm<sup>2</sup> and combined scanning with all IF filters (FITC, DAPI, Alexa Fluor 594, and Alexa Fluor 647) was performed, together with a phase-contrast microscopy of the detected cells. Cell identification was done with 20X and/or 40X magnification and cells were photographed with the

Nikon NIS Elements imaging software (version 4.20.02, build 988-64 bit). Exposure times for each filter were 100ms for DAPI, 500ms for Alexa Fluor 594 (Vimentin), 1 second for FITC (pan-cytokeratin), and 2 seconds for Alexa Fluor 647 (CD antibodies). Captured images for each wavelength were combined and saved for future reference and re-evaluation.

### **Pancreatic cancer cell line spiking experiments**

A series of different spiking experiments was performed prior to patient sample processing to assess two major experimental parameters: 1. the sensitivity and specificity of IF antibodies to the targeted cell populations, and 2. the capacity of ISET to capture cells of interest. For both control experiments, the AsPC-1 human pancreatic cancer cell line (CRL-1682<sup>TM</sup>, American Type Culture Collection ATCC) was used. Briefly, culture conditions were followed based on the manufacturer's recommendations: RPMI-1640 (with L-Glutamine, Gibco, Life Technologies) with 10% v/v Fetal Bovine Serum (FBS) and 0.5% v/v Penicillin-Streptomycin (Sigma Aldrich). Cells were harvested by adding 0.25% v/v trypsin/EDTA solution and incubating at 37°C for 5 minutes. Finally, the cells were stained with Trypan-Blue for viability assessment and counted with the TC20 Automated Cell Counter (Bio-Rad).

Initially, AsPC-1 and CAF cell lines were stained for pan-cytokeratin and vimentin antibodies, respectively, using the immunofluorescent staining protocol and were reviewed under the IF microscope. A consistent expression of epithelial and mesenchymal markers was observed, whereas none of the cell lines were positive for CD staining.

For validation of ISET capacity for cell capture, two different experiments were performed in which 50, and 100 viable AsPC-1 cells per mL of blood were spiked in 5mL of peripheral blood from a healthy volunteer. The recovery rates after processing the spiked samples with the standardized ISET protocol were 81% (202 cells) and 79% (397 cells), respectively (Supplementary Figure 11).

### **Flow-assisted cell sorting (FACS)**

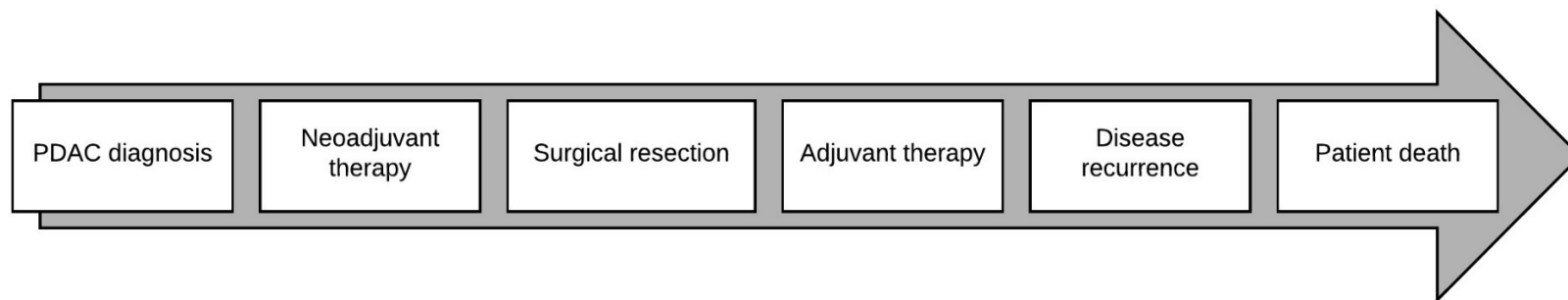
For FACS, an additional sample of 5 mL of peripheral blood was separately drawn. An initial negative-selection step was performed using the RosetteSep™ Human CD45 Depletion Cocktail (Stemcell Technologies, Cambridge MA, USA). Red blood cells and white blood cells expressing CD66b and CD45 proteins were marked with antibody complexes and depleted with gradient centrifugation over a buoyant density medium (1200g for 20 minutes). After centrifugation, the targeted cell population is highly-enriched between the plasma and the medium (“buffy coat”) and is transferred to a separate Eppendorf tube. Two washing steps follow with HBSS buffer (Hank’s Balanced Salt Solution with 2% FBS) and centrifugation at 300g for 10 minutes. After removal of the supernatant, the pellet (approximately 300µl) was stained for epithelial cell surface markers (EpCAM, MUC-1, CD61 - integrin alpha V/beta 3; all antibodies conjugated with phycoerythrin – PE, Abcam) and CD45 for exclusion of leftover white blood cells, conjugated with allophycocyanin (APC, Biolegend) at a 1% v/v concentration. The stained pellet is washed once more with HBSS buffer after incubation in the dark for 30 minutes and kept in ice until flow cytometry. Prior to sorting, cells are stained with Propidium Iodide (PI) for viability assessment. FACS was performed using with the MoFlo XDP Cell Sorter (Beckman Coulter Life Sciences, Indianapolis, USA). Prior to FACS, instrument tuning is performed with negative and positive control samples from peripheral blood of a healthy volunteer (CD45+, EpCAM-) and the AsPC-1 cell line (CD45-, EpCAM+), respectively. Targeted cells were sorted in a 500µl Eppendorf tube and stored in -80°C. In a predetermined number of enrolled patients (n=30), additional blood was drawn for separate flow-assisted cell sorting (FACS) analysis. After FACS, the sorted cells were immediately stored in -80oC.

### **CTCs DNA extraction and quantification**

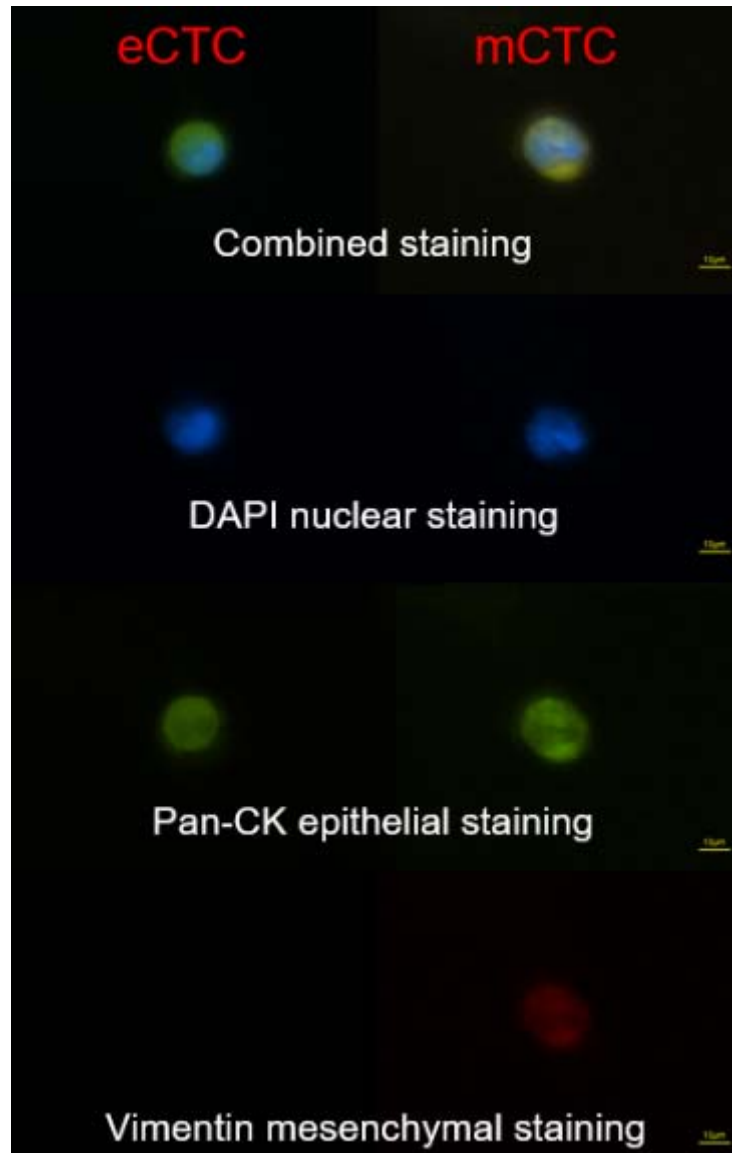
For downstream genetic analysis of isolated CTCs, three cores of each membrane were used. The cores were incubated in a Proteinase-K buffer in 65°C for 16 hours and then placed

in a heating block at 95°C for 10 minutes, according to the Arcturus® PicoPure® DNA Extraction Kit protocol (ThermoFisher Scientific). A next step of DNA purification followed using the Agencourt AMPure XP beads (Beckman Coulter, Life Sciences) and the purified DNA sample was quantified with the Qubit® 3.0 Fluorometer and the Quantifiler® Human DNA Quantification Kit (ThermoFisher Scientific). An Ampliseq® custom panel was designed targeting three driver genes in PDAC (KRAS, TP53, and DPC4) with a minimum sequencing library depth of 500X. Digital next-generation sequencing was performed with the Ion Torrent system (Life Technologies).

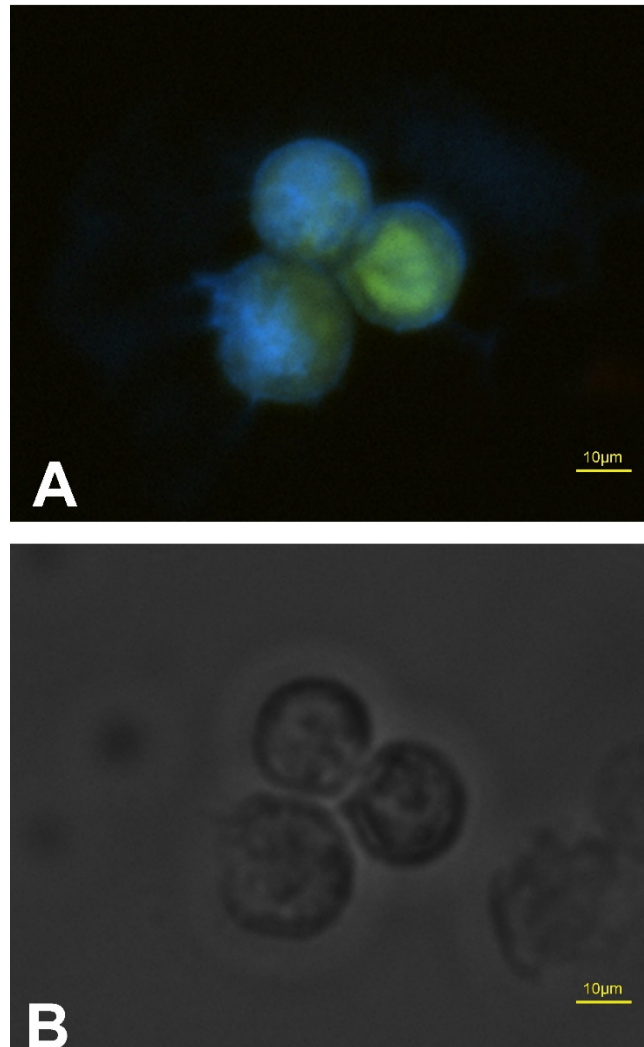
**Supplementary Figure 1:** Study design illustration



**Supplementary Figure 2:** Immunofluorescent staining of circulating tumor cells in patients with pancreatic adenocarcinoma. The left column illustrates a cell that expresses an epithelial phenotype (eCTC) and is positive for pan-cytokeratin only. The right column illustrates a cell that expresses a combined epithelial/mesenchymal phenotype (mCTC) and is positive for pan-cytokeratin and vimentin.

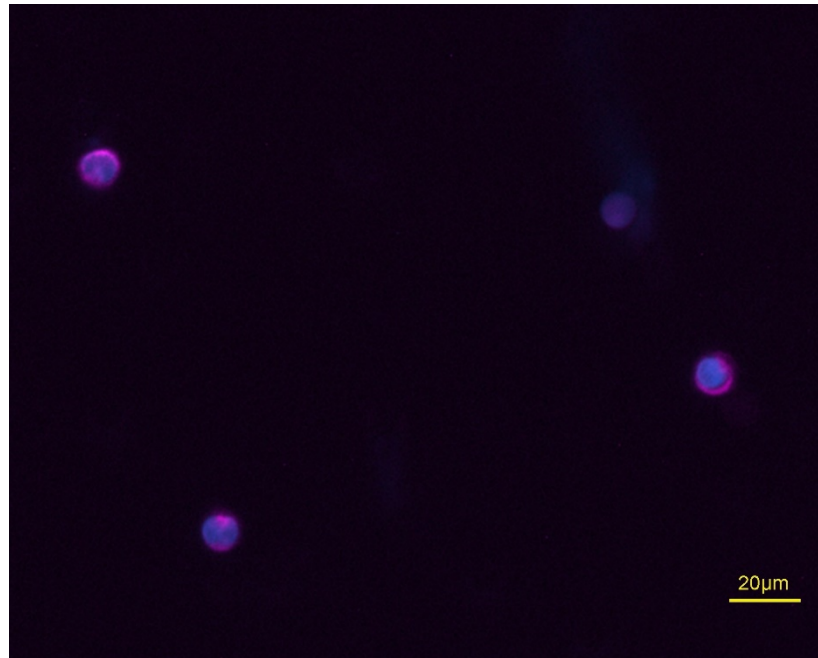


**Supplementary Figure 3:** Isolated cluster of 3 CTCs. **A.** Immunofluorescent staining expressing only pan-cytokeratin (eCTCs phenotype), **B.** Photographic image of the identified CTC cluster.

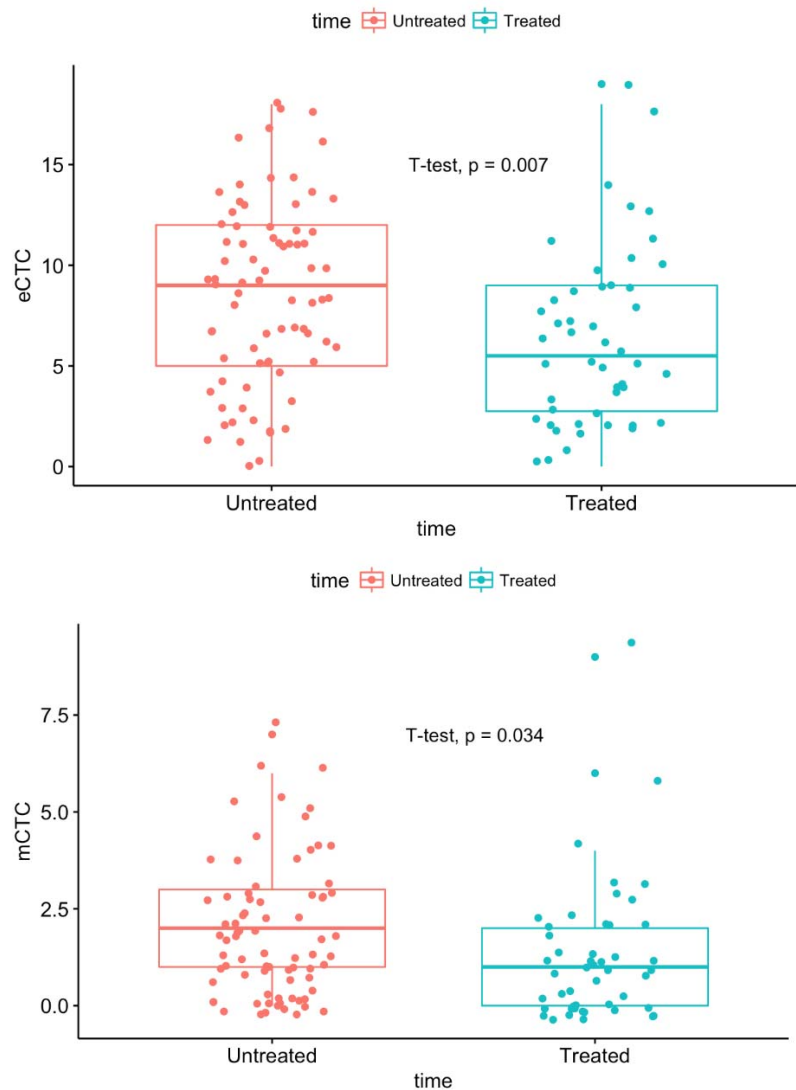




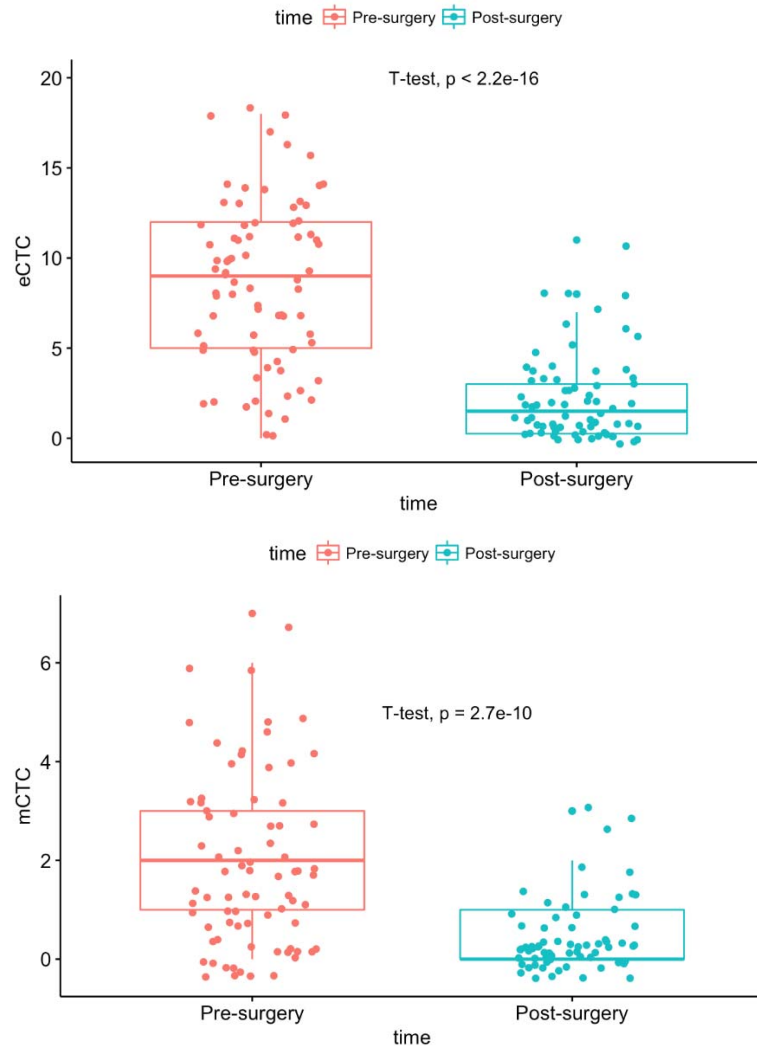
**Supplementary Figure 4:** Immunofluorescent staining of white blood cells from healthy control specimen. The cells are expressing a CD phenotype (purple color). No cells expressing an epithelial or mesenchymal phenotype were identified in the peripheral blood of healthy controls.



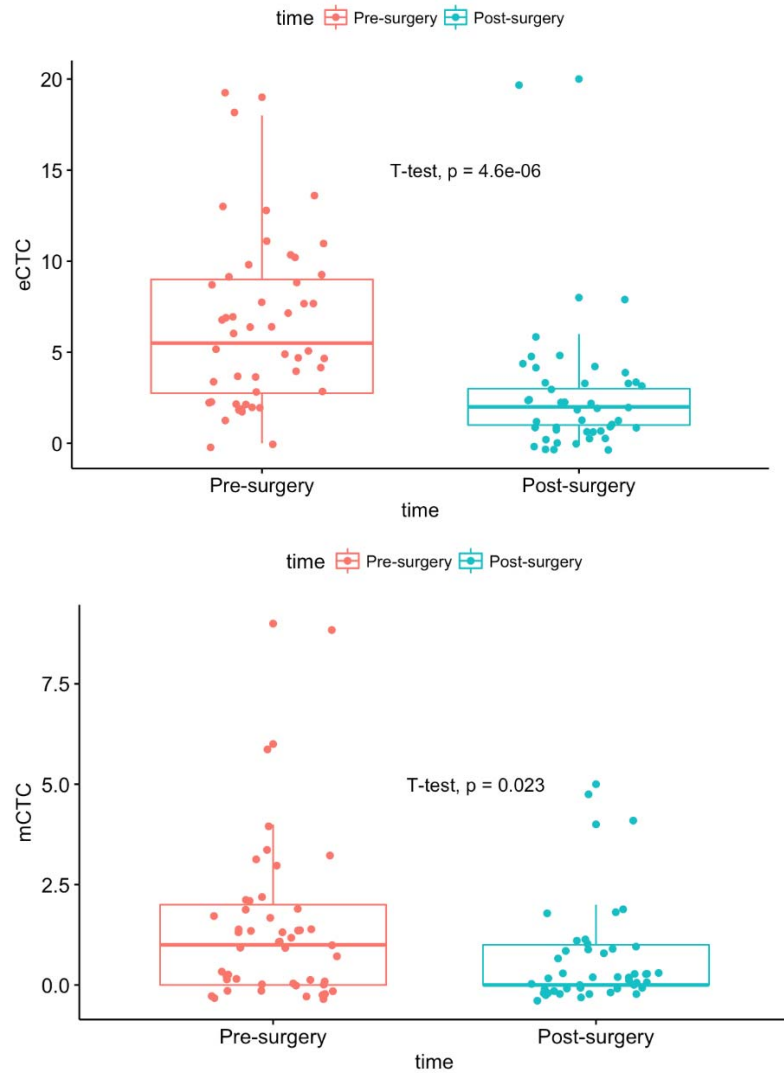
**Supplementary figure 5:** Boxplots of comparison between eCTCs (A) and mCTCs (B) between preoperative blood samples in chemo-naïve patients and patients who underwent neoadjuvant therapy. eCTCs: epithelial phenotype CTCs/mL of blood, mCTCs epithelial/mesenchymal phenotype CTCs/mL of blood.



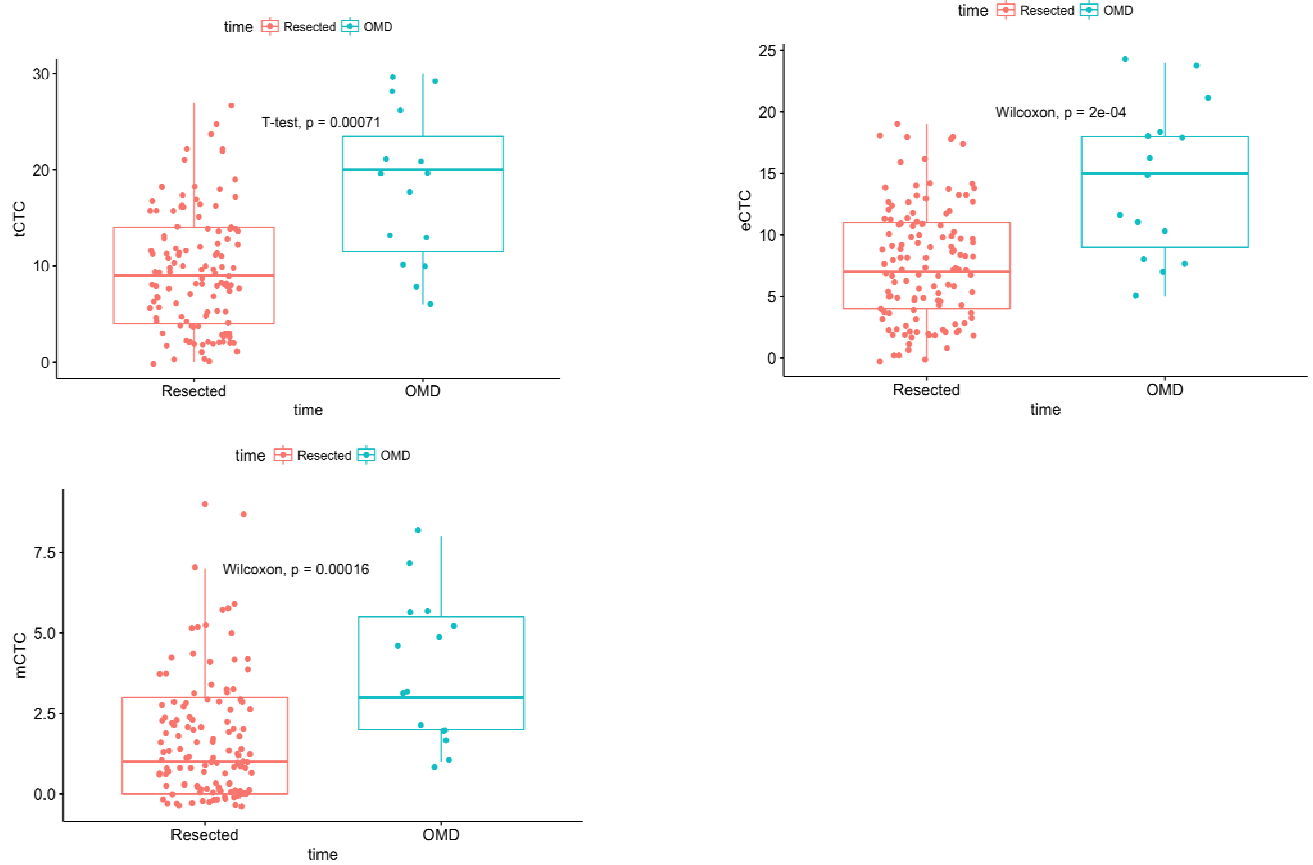
**Supplementary Figure 6:** Boxplots of comparison between eCTCs (**A**) and mCTCs (**B**) of preoperative and postoperative (POD 4-7) blood samples in chemo-naïve patients. eCTCs: epithelial phenotype CTCs/mL of blood, mCTCs epithelial/mesenchymal phenotype CTCs/mL of blood, POD: postoperative day.



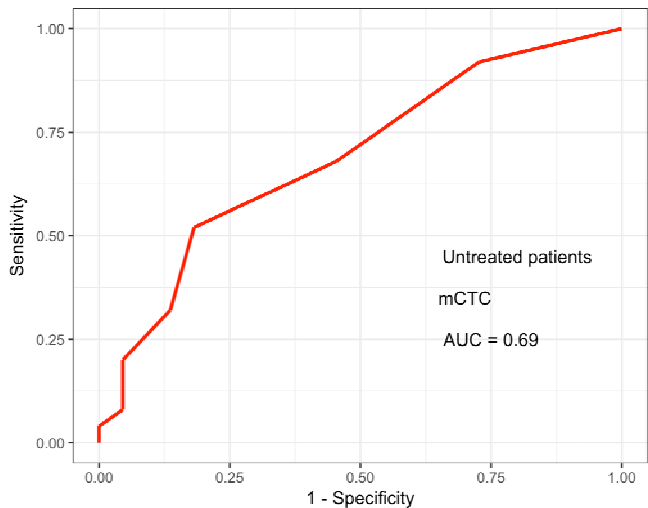
**Supplementary Figure 7:** Boxplots of comparison between eCTCs (**A**) and mCTCs (**B**) of preoperative and postoperative (POD 4-7) blood samples in patients who underwent neoadjuvant treatment. eCTCs: epithelial phenotype CTCs/mL of blood, mCTCs epithelial/mesenchymal phenotype CTCs/mL of blood, POD: postoperative day.



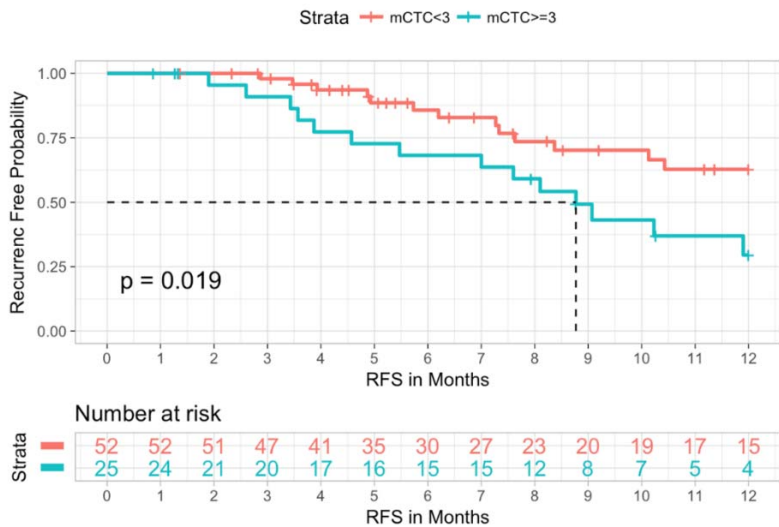
**Supplementary Figure 8:** Boxplots of comparison between tCTCs (A), eCTCs (B) and mCTCs (C) of OMD and resected non-OMD patients. *eCTCs*: epithelial phenotype CTCs/mL of blood, *mCTCs* epithelial/mesenchymal phenotype CTCs/mL of blood, *OMD*: intraoperatively-discovered occult metastatic disease.



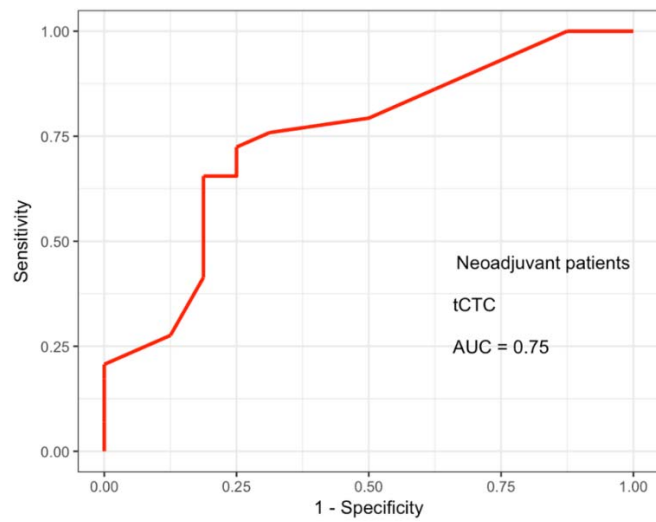
**Supplementary Figure 9: A.** ROC curve (area under the curve 0.690) for preoperative mCTCs with an optimal cut-off of 3 cells/mL of blood for predicting early recurrence in chemo-naïve patients (<12 months, 52% sensitivity, 82% specificity) and **B.** associated Kaplan-Meier curve for post-operative 1-year recurrence. **C:** ROC curve (area under the curve 0.750) for pre-operative tCTCs with an optimal cut-off of 6 cells/mL of blood for predicting early recurrence in patients who underwent neoadjuvant therapy (<12 months, 72% sensitivity, 75% specificity) and **D.** associated Kaplan-Meier curve for post-operative 1-year recurrence. *tCTCs: total CTCs/mL of blood, mCTCs: epithelial/mesenchymal phenotype CTCs/mL of blood, ROC: receiver operating characteristic.*



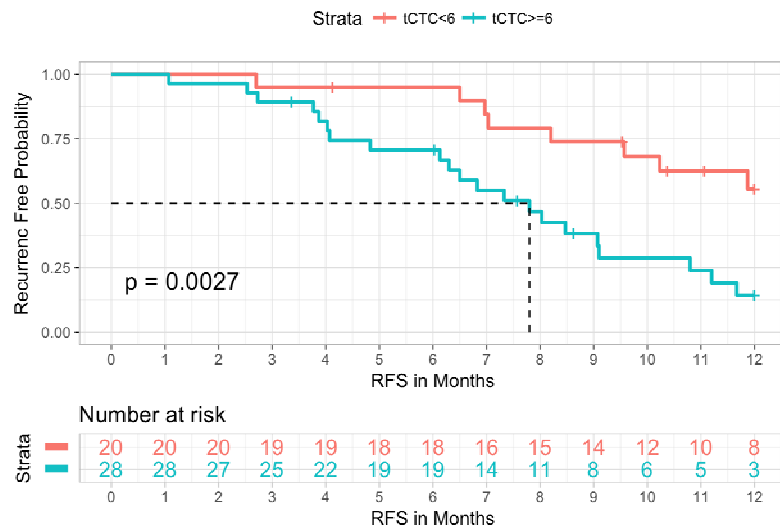
A.



B.

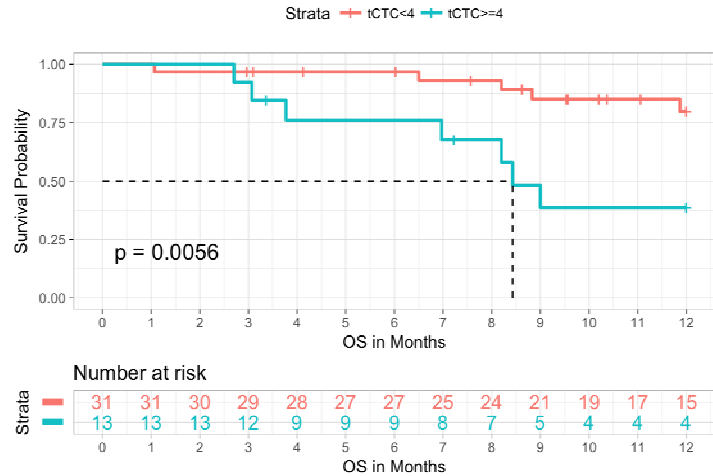


C.

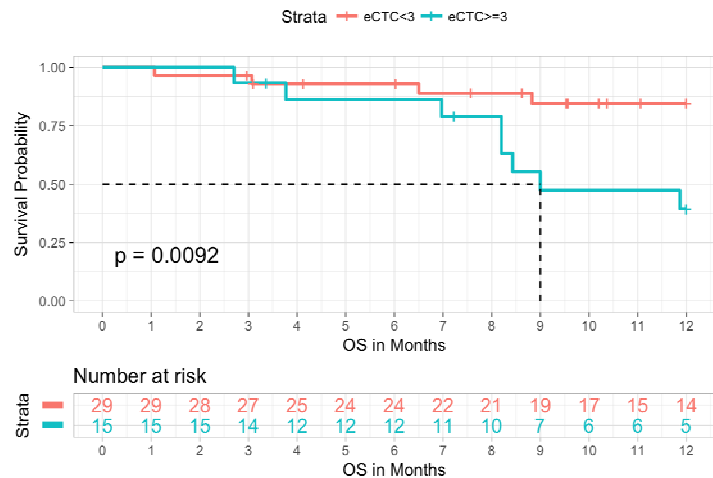


D.

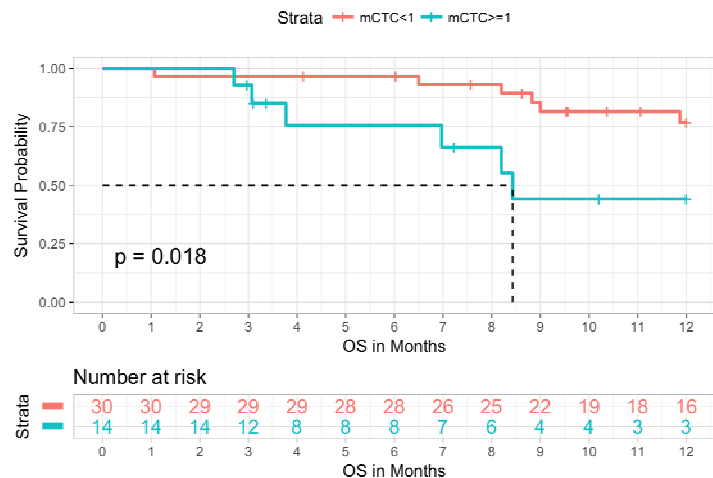
**Supplementary Figure 10:** Kaplan-Meier curves for 1-year disease-specific mortality associated with postoperative (POD 4-7) **A.** tCTCs ( $\geq 4/\text{mL}$ ), **B.** eCTCs ( $\geq 3/\text{mL}$ ), and **C.** mCTCs ( $\geq 1/\text{mL}$ ) in patients who underwent neoadjuvant therapy. *tCTCs*: total CTCs/mL of blood, *eCTCs*: epithelial phenotype CTCs/mL of blood, *mCTCs*: epithelial/mesenchymal phenotype CTCs/mL of blood, *POD*: postoperative day.



A.



B.



C.



**Supplementary Figure 11:** Combined immunofluorescent staining (DAPI, pan-cytokeratin, vimentin) of AsPC-1 cancer cells spiked in human blood and isolated using the ISET assay.

