Appendix Detailed Response to Reviewer's Comments

Reviewer #1: The manuscript by Di Franco ad colleagues analyses zebrafish embryos as a novel xenotransplantation model for pancreatic cancer patients. This is an interesting and relevant study. There are, however, several concerns that should be addressed:

1. Xenotransplantation models are of interest, but the need has not increased 'exponentially'. There are other models such as for example patient-derived organoids. Further whole tumor genome sequencing has been another promising approach for personalized medicine.

RESPONSE: Thanks for your suggestion. We clarify that the use of zebrafish embryos is one of the research fields for the personalized medicine. To do this we changed the sentence "After the first experiment reported in 2005[4], the use of zebrafish in vivo model of xenotransplantation has increased exponentially" with the sentence "After the first experiment reported in 2005[9], nowadays the use of the zebrafish model of xenotransplantation is one option for implementing strategies of personalized medicine, together with other models such as mouse patients-derived xenografts, patient-derived organoids[10,11] or the whole tumor genome sequencing[12,13]."

2. Lederfolin is not a common English term.

RESPONSE: we changed Lederfolin with "Folinic acid".

3. What is meant by "exclusive treatment"?

RESPONSE: we changed "exclusive treatment" with "unique treatment in non resectable PDAC".

4. How variable was the volume of the tumor and the number of tumor cells implanted? Could the authors provide estimates for this?

RESPONSE: We would like to thank the reviewer for the important questions. We carried out a volumetric analysis of the volume occupied by the nuclei of the patient's tumor fragment inoculated in the zebrafish embryos. The volume was 6411±10.96 μm^3 (mean ± SEM) derived from two embryos xenotransplanted with different patient's tumors. We used for this analysis the 3D Objects Counter function of the software ImageJ. For example, for a typical mammalian nucleus of 6 µm, the value corresponds to about 60 cells per xenotransplant. We added in the Materials and Method section the following sentence: "*Xenografts imaging and quantification*

All xenografts were imaged by fluorescence Nikon Eclipse E600 microscope to monitor the RTA and analyzed using ImageJ software. For the volumetric analysis of the nuclei the images were acquired by using a Nikon A1 confocal microscope with a 5 µm z-stacks. The quantifications were performed using the 3D Objects Counter function of ImageJ software." Moreover, in the Results section we added the following sentences: "The volumetric analysis of the volume occupied by the nuclei of the patient's tumor fragment inoculated in the zebrafish embryos revealed a volume of 6411±10.96 μm^3 (mean ± SEM) derived from two embryos xenotransplanted with different patient's tumors. Considering a diameter of 6 µm for a typical mammalian nucleus, this value corresponds to about 60 cells per xenotransplant. We calculated the percentage of epithelial cells (mean PDAC counterpart) out of the total surface area (31.8 ± 4.9 %, n=3, Figure 2D)."

5. What was the reason for the 50% mortality rate at dpi2 in the model?

RESPONSE: The survival rate at 2 dpi was 55%, 51%, 47%, 52% and 55% respectively for control, Gemcitabine, GEMOX, GEM/nab-P and FOLFOXIRI. We suppose that the reason for the high mortality was the high invasiveness of the tumor xenografts and the everyday anesthetization and manipulation to take image of the tumor. We added a comment on this

aspect in the discussion section modifying the following sentence: "Indeed, in all cases the tumor cells successfully engrafted in the xenotransplanted zebrafish embryos and the mean xenotransplanted zebrafish embryos survival rate was 71.5% at 1 dpi but decreased at 52.4% at 2 dpi, probably due to the high invasiveness of the tumor xenografts and the everyday embryo anesthetization and manipulation to image the tumor."

6. The effect of chemotherapy was assessed after 2 days. How valid is such a short duration time period?

RESPONSE: We commented this advantage of the use of zebrafish embryos for patient-derived avatar adding the following sentences in the discussion section:

"The patients-derived zebrafish avatars do not require tumor cell expansion and the results of the chemosensitivity assay can be obtained in just few days. Previously study with different tumor type (patient derived gastric xenografts) demonstrate sensitivity to chemotherapy after 2 days post treatment[33]. The difference in time scale of the assay is not due to the fast zebrafish's biology, but rather because zebrafish larvae are 10,000 times smaller than adult mice therefore they require reduced cells number to perform the injection (ranging from 500 to 5000 cells in each zebrafish embryo instead of 1×10^6 in the mouse). A lower quantity of PDAC cells let to perform a higher number of xenograft that provide powerful statistical analysis[23,34]. All these aspects make possible to evaluate an evident effect of chemotherapy in just few days."

7. The statistical analysis is difficult to follow, as multiple parameters are analysed.

RESPONSE: The need to compare the avatar results with current data from the literature makes it necessary to use a complex statistical analysis. We are sorry if we analyzed multiple parameters but we had to deal with the need to transfer the data reported on humans in

literature with the data obtained from avatar tests. We tried to make easer the comprehension of the analysis performed specifying the formulas used and with the use of tables and diagrams.

8. It would have been important to compare this model to other established models, e.g. organoids.

RESPONSE: we critically discussed the zebrafish model in comparison with organoids model by adding the following sentences in the discussion section: "Different patients-derived tumor models, both in vitro and in vivo, have been developed. Organoid cultures were a major breakthrough in the in vitro culture of tumor cells from patients, becoming the most attractive tool to be used as an in vitro screening platform. Recently, this technique was further developed to generate organoids from patient-derived cancer tissues[23]. However, only few retrospective studies correlated patient clinical outcomes with organoid drug response[24–26]. Moreover, the use of organoids has some limitations in comparison to the zebrafish model. First of all, the initial organoid generation requires 4-5 weeks[23,27], which is border line for the time frame needed to guide first clinical decisions. Moreover, for the lack of stromal components, these models lack many complex interactions observed in the TME or in a living organism and do not allow, for instance, the evaluation of the metastatic or angiogenic potential[23]. Patient-derived xenografts (PDX) into immunodeficient mice has been considered the gold standard model for assessing pre-clinical efficacy of cancer drugs. The methodology of initiation and propagation consists in sectioning the fresh surgical tissue into ~ 3 mm^3 pieces, followed by subcutaneous or orthotopic implantation into the flank of an immunodeficient mouse. During the engraftment phase, tumors are allowed to establish and grow and then are harvested upon reaching a size of 1500 mm^3. Similar protocols are employed for subsequent expansion cohort and treatment cohort[28]. Despite this preclinical model more closely recapitulate the

heterogeneity of human tumors, there are inherent limitations. For example, mice are expensive and require large vivarium space, limiting the scale of experimentation. Moreover, the tumor propagation required a long amount of time, resulting in several genetic, pathological, histological and micro-environmental niche changes, that may not mirror the patient's tumor accurately. Mice are also furred, and is not possible to image disseminated cancer cells throughout the whole animal[29]. To address these issues, have been developed zebrafish xenograft transplantation approaches to engraft human cancers into two days post fertilization zebrafish embryos at a stage prior to the development of the adaptive immune system[15]."

9. There are several grammatical errors that should be corrected.

RESPONSE: a native English speaker performed the revision of the manuscript.

Reviewer #2: The authors are presenting a study for the use of zebrafish embryos as avatars for the analysis of potential therapeutic interventions in human pancreatic cancer patients. The proposal is well presented and discussed. The experimental protocol is described in detail and authors are transferring samples from patients to the embryos, followed by the test of several chemotherapeutic drugs. In addition to this, authors are comparing the avatar results with current data from the literature, after administration of several therapeutic drugs. This approach might accelerate the transfer to a 'personalized' medicine for the treatment of pancreatic cancer.

RESPONSE: We would like to thank the reviewer looking at the appreciation of our work.

Reviewer #3: This is an interesting study. The authors developed a model to use zebrafish embryos as avatar of patients with PDAC, standardizing the protocol for the xenotransplantation of pancreatic tumor tissue, for the exposition of the xenotransplanted zebrafish embryos to the chemotherapy drugs, and for the evaluation of the effects of chemotherapy on the xenotransplanted tumor tissue. This model has some clinical significance and could service as a useful experimental tool. But the paper needs very significant improvement before acceptance for publication. My detailed comments are as follows:

1. PDX and organoid are two of efficient model in vitro for chemotherapy screening. PDX and organiod both have restricted capability to passage and freeze the tumor tissue samples. The zebrafish model seems to lack of this function. It is more likely to be an one-off tool. Would the results on zebrafish model stand for the chemosensitivity of matched PDAC patients? Are there any supporting data? How do you prove it?

RESPONSE: Thank you for your comment. We added in discussion section the following sentences: "Moreover, both in organoids and in mPDX, to amplify tumor tissue to obtain enough material for chemoresponse analysis, the cells are subjected to a strong selection pressure, diverging from the original tumor[30]. For our purpose, we do not need cell expansion, instead we directly observe the drug response of human tumor material xenotransplanted in zebrafish embryos. We believe that this is an appeal of zebrafish xenotransplantation and not a limitation." and "Very few studies evaluate the use of zebrafish embryos for personalized medicine and passaging to the best of our knowledge there are no results in literature of treatment correlation between zebrafish xenografts and matched PDAC patients. However a preclinical human cancer xenotransplantation platform has been recently developed in zebrafish to inform therapeutic decisions in patients with T-cell acute lymphoblastic leukemia[37]. Moreover, a retrospective study with zPDXs from multiple myeloma cells, demonstrate that zPDXs showed a response equivalent to patient's clinical outcome[39]. Similarly, Wu J.Q. et al. showed a retrospective correlation

with one gastric tumor patient clinical outcome[33]. In 2017 Fior et al. performed a retrospective study, showing that colorectal patient's avatars are predictive of patient clinical outcome in 4 out 5 patients (80%)[34]. These results bode well for our co-clinical trial (NCT03668418)."

2. The authors performed 4 kinds of chemotherapy on zebrafish models. And quantify the curative effect of chemotherapy by the reduced area of Dil on ImageJ. Would you provide the evidence of apoptosis of cancer tissue after treatment?

RESPONSE: We would like to thank the reviewer to highlight this aspect. A panel to highlight the evidence of apoptosis in zPDX after treatment was provided. Moreover, we investigate the presence of human PDACs and their nuclear alteration, performing FFPE tissues and H&E staining in zebrafish embryos. The nuclear alteration on human PDACs, could be associated with a chemotherapy damage. We compared three different groups of embryos including: GEM/nab-P, FOLFOXIRI and Controls. The images of embryos and the nuclear damages (present in GEM/nab-P and FOLFOXIRI cases) associated with treatment were reported in the added figure 3. In the Results section, we modified the following sentence to cite the histological evaluation that we performed to evaluate the effect of the chemotherapy on the xenotransplanted cells: "In absence of chemotherapy (control group), the Dil-stained areas showed a statistically significant increase over time in all cases, while we observed a tendency to a reduction of the mean RTA in treated subgroups. In Figure 3 it is possible to observe the alteration due to the chemotherapy of xenotransplanted cells of some treated xenotransplanted zebrafish embryos." Moreover, in the Materials and Methods section we added the following sentences:

"*Formalin-Fixed Paraffin Embedded samples preparation of Xenografts*

Histological analysis

2 days post treatment zPDX were sampled for histopathological examination. Briefly, the embryos were fixed in 10% neutral buffered Formalin and dehydrated through increasing ethanol scale. Then the zPDX were embedded after immersion in liquid paraffin at 60°C for 2 h. The Formalin-Fixed Paraffin Embedded (FFPE) were sectioned at 3 µm-thick, then rehydrated using xylene and ethanol solutions. Finally, zPDX sections were stained with Hematoxylin and Eosin (H&E) for morphological analysis."

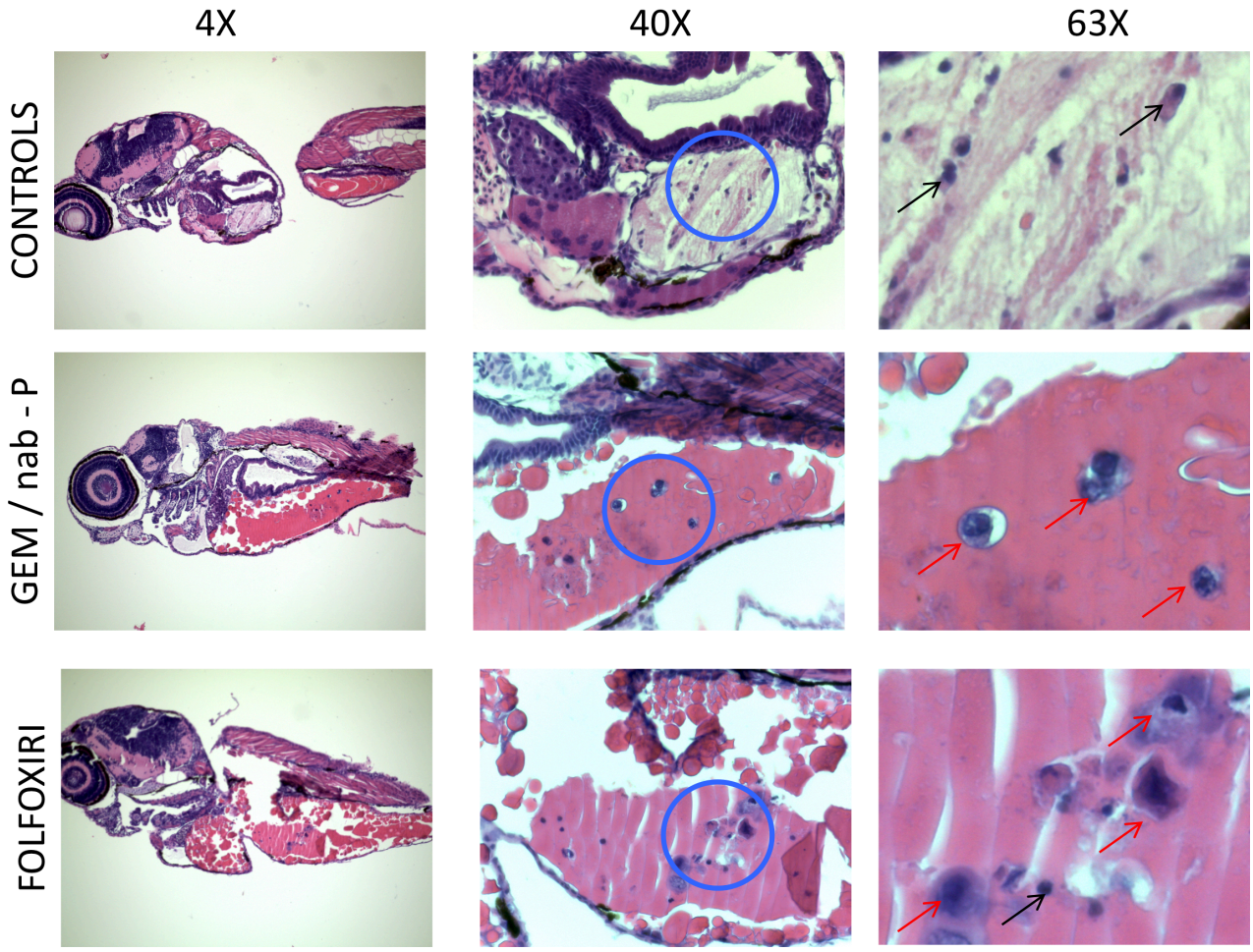

Figure 3. Representative H&E stained sections of zPDX. Visible morphologic alteration of the human PDAC nuclei (red arrows) in zPDX exposed to GEM/nab-P and FOLFOXIRI could be associated with a chemotherapy damage. Control group shows normal nuclei (black arrows).

3. In fig 2a, the right H-E staining image seemed that it was not from the left view field. And the images were not clear enough. In fig2, tumor tissue fragments were stained with CM-Dil, and fig2 seemed to indicate the success of transplanting PDAC tissue into zebrafish. I think H-E staining and Dil existence were unconvincing. Specific cancer cell markers should be stained with IHC.

RESPONSE: we would like to thank the reviewer for the suggestions. We changed the figure 2 with a new figure in which, in addition to the image of xenotransplanted zebrafish embryos stained with H&E, we added an image of xenotransplanted zebrafish embryos stained with anti-Human Pan Cytokeratin Antibody (PanCK) (Figure 2E). This staining revealed a positivity for PDAC cells xenotransplanted into the yolk of zebrafish embryos. H&E and IHC images were obtained from the same FFPE block using consecutive histological sections, in order to overlap the H&E and IHC images associated with the same hPDAC cells.

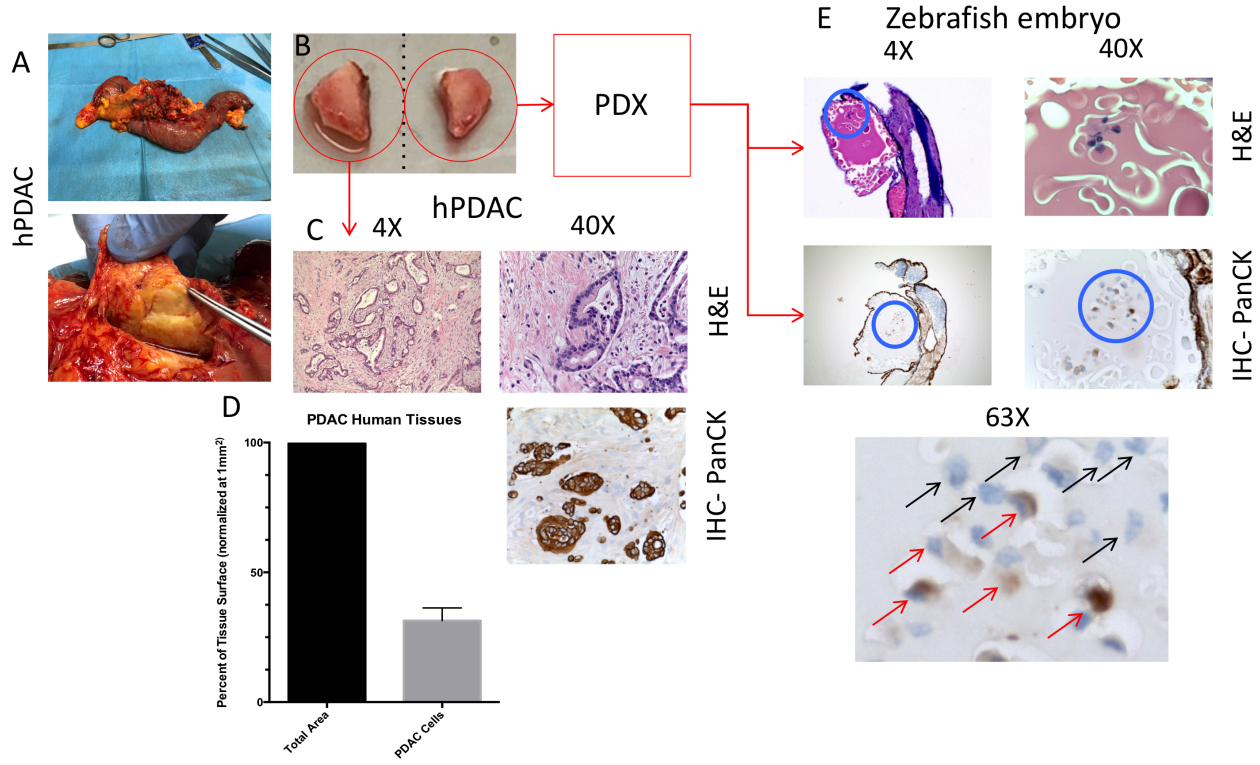

Figure 2. Surgical specimen was obtained from patient with diagnosed PDAC (A). Two fragments of the tumor (B) were taken. One of them was used for histological evaluation (C)

revealing that the percentage of epithelial cells (mean PDAC counterpart) out of the total surface area is 31.8 ± 4.9 % (n=3) (D). The second fragment of the tumor was used for xenotransplantation into the yolk of zebrafish embryos 2 dpf. After 2 days post xenotransplantation, the zPDX are Formalin-Fixed Paraffin Embedded. We performed the H&E staining and IHC using anti-Human Pan Cytokeratin Antibody (PanCK) on zPDX sections, highlighting the presence of epithelial PDAC cells (red arrows) and the immune-negative counterpart (black arrows) that might be associated with the microenvironment side of PDAC human tissue (E).

4. What's more, would the microenvirnment of tumor remain? How does it convert after transplanted on zebrafish?

RESPONSE: Thanks to the reviewer for this additional question.

This is really important to demonstrate the presence of PDAC tissue inside the ZF models. Looking figure 2D, we calculated the percentage of epithelial cells (means PDAC counterpart) out of the total surface area. We labeled the remaining part as "tumor microenvironment" comprising the all other types of cells. PanCk positivity and negativity, could be associated with the epithelial and stromal counterpart, respectively. Of course, using only one IHC marker, is possible to highlight the presence and their percentage of epithelial PDAC cells. So far, we can assume that the immune-negative counterpart inside the yolk, might be associated with the microenvironment side of PDAC human tissue (figure 2E).

5. In fig3, seeing the relative area (a.u.) for POO2, although the mean value is higher in FOLFOXIRI group, the deviation of data in FOLFOXIRI group is smaller that the deviation in Gem+nab-P group.

There was significant difference in Gem+nab-P group but no in FOLFOXIRI group, compared with control group. Would you provide the detailed SPSS statistical analysis file?

RESPONSE: The detailed statistical analysis was reported in the table below.

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Va	A-	?	
Control vs. FOLFOXIRI	1.209	-0.01553 to 2.434	No	ns	0.0537	B		FOLFOXIRI
Control vs. GEMOX	0.776	-0.5215 to 2.073	No	ns	0.3612	C		GEMOX
Control vs. GEM/nab-P	1.435	0.09908 to 2.771	Yes	*	0.0324	D		GEM/nab-P
Control vs. GEM	0.29	-0.9776 to 1.558	No	ns	0.9367	E		GEM

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control vs. FOLFOXIRI	1.964	0.7548	1.209	0.475	4	10	2.546	30
Control vs. GEMOX	1.964	1.188	0.776	0.5032	4	7	1.542	30
Control vs. GEM/nab-P	1.964	0.5287	1.435	0.5182	4	6	2.769	30
Control vs. GEM	1.964	1.674	0.29	0.4917	4	8	0.5899	30

6. As for table 6, i don't think those comparisons were logical. You can't compare your zebrafish survival data with those literature data representing the real clinical effective rate of chemotherapy on human being. This part of result should be deleted.

RESPONSE: Thanks for your comments. Our model is the first one described using zebrafish embryos as avatar for patients with PDAC. Our data are very preliminary data and a co-clinical trial to compare results reported in the test with xenotransplanted zebrafish embryos and enrolled patients are underway. However, the follow-up of the enrolled patients is too short to compare the zebrafish model with the clinical data reported during the follow-up. Then, to evaluate if our initial results could be considered positively to continue with further tests using our developed model, we tried to evaluate if the results of chemotherapy efficacy reported with the different chemotherapy schemes administered to the xenotransplanted zebrafish embryos are coherent with data reported in literature. In other words, we evaluate the efficacy of the different chemotherapy schemes reported in literature using an objective parameter, the percentages of partial response reported in the

clinical practice for each tested chemotherapy scheme, and we tried to compare them with the results of our test, to evaluate if our data are coherent with the theoretically expected data obtainable during the follow-up of our enrolled patients. We know that this comparison has important limitations and could not replace the comparison with data obtainable with the follow-up of the enrolled patients. However, we performed this comparison only for orientation of our research project without any intention to draw conclusive statements. We tried to better explain all these aspects in the Materials and Method section modifying the following sentence: "Moreover, we evaluated the possibility to use in the zebrafish model the "Response evaluation criteria in solid tumors (RECIST)" used in the common clinical practice to evaluate the response to the chemotherapy in oncological patients[21] as an objective parameter for evaluation of data obtained with zebrafish embryos tests." Moreover, in the discussion section we modified the following sentences: "Moreover, due to the too short follow-up of the enrolled patients and the consequent absence of clinical data of response to chemotherapy treatments, we tried to use the RECIST criteria in the experiment with the xenotransplanted zebrafish embryos, with the intent to use an objective parameter in the evaluation of the robustness our model. In this way, we evaluate if the results of the tests with the different chemotherapy scheme could be in accordance with data reported in large number of series published in literature." and "We know that this comparison has important limitations and could not replace the comparison with data obtainable with the follow-up of the enrolled patients. We performed this comparison only to give an initial assessment of the reliability of our model. However, taking in consideration the previous premises, these preliminary results are encouraging because first of all they confirmed the possibility to successfully xenotransplant directly the tumor tissue in the zebrafish embryos." Moreover, we deleted the sentence "*So, we can speculate that those cases in which the xenotransplanted zebrafish embryos exposed to a chemotherapy*

scheme reported a reduction of the mean RTA higher than 60% in comparison to the control group could correspond to the patients with a possible partial response to the chemotherapy treatment" because it draws a conclusion beyond the scope of our article.

Minor problems...

1. page 8, "introduction" paragraph 1 line 3. The data about the median survival time and 5-year overall survival rate of PDAC should be update.

RESPONSE: we changed the sentence with the following update data: "with a median survival time of less than 18 months and with a 5-years survival rate for pancreatic cancer increased from 6% to 9% from 2014 to 2018[1,2]."

2 page 8, "introduction" paragraph 1 line 3. The authors stated "The annual death rate for PDAC is almost equal to the incidence rate". Is there any references supporting this statement?

RESPONSE: we added the following reference supporting the statement: "Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;**68**:394–424 [PMID: 30207593 DOI: 10.3322/caac.21492]".

3. page 8, line 13-14. The data about the rate of severe side effects and the specific chemotherapy regimens should be clearly stated.

RESPONSE: We specified with more details the incidence of the side effects for the different chemotherapy regimens modifying the sentence "However, severe side effects (Grade 3–4) quite frequently occur and raise questions of patient selection, asking for dose-reduced or dose-modified schedules" with the following sentences: "Different regimens have been developed such as gemcitabine plus nab-paclitaxel and FOLFIRINOX and direct

comparisons of these regimens are not available[6,7]. Moreover, the incidence of adverse events increases with the use of combination regimens compared to gemcitabine: the proportion of patients who experienced at least one grade 3 or higher treatment-related, treatment-emergent adverse event was 77% with nab-paclitaxel plus gemcitabine and 51% with gemcitabine-alone in the MPACT study[6]. FOLFIRINOX produced higher rate of both hematological and non-hematological grade 3 or higher toxicities such as diarrhea and sensory neuropathy[7]."

4 According to reference 6, the zebrafish model had been used to transplant PDAC tissue. And zebrafish model also was used on other cancer types. Is any similar studies performed on other cancer types towards personalized medicine, like breast cancer?

RESPONSE: Very few studies evaluate the use of zebrafish embryos for a personalized medicine. We cited in the discussion section the study that evaluated the possible use of zebrafish embryos towards personalize medicine. We added the following sentences: "Very few studies evaluate the use of zebrafish embryos for personalized medicine and passaging to the best of our knowledge there are no results in literature of treatment correlation between zebrafish xenografts and matched PDAC patients. However a preclinical human cancer xenotransplantation platform has been recently developed in zebrafish to inform therapeutic decisions in patients with T-cell acute lymphoblastic leukemia[37]. Moreover, a retrospective study with zPDXs from multiple myeloma cells, demonstrate that zPDXs showed a response equivalent to patient's clinical outcome[39]. Similarly, Wu J.Q. et al. showed a retrospective correlation with one gastric tumor patient clinical outcome[33]. In 2017 Fior et al. performed a retrospective study, showing that colorectal patient's avatars are predictive of patient clinical outcome in 4 out 5 patients (80%)[34]."

Reviewer #4 Recension of manuscript No. 03714071: „ The use of zebrafish embryos as Avatar of patients with pancreatic cancer: A new xenotransplantation model towards personalized medicine, written by Gregorio Di Franco, Alice Usai, Matteo Palmeri, Matteo Bianchini, Desirée Gianardi, Niccolò Furbetta, Simone Guadagni, Luca Emanuele Pollina, Niccola Funel, Enrico Vasile, Alfredo Falcone, Vittoria Raffa, Luca Morelli", which will be published in World Journal of Gastroenterology. The structure of manuscript is in keeping with the common required criteria. The topic of the work is very actual, because pancreatic cancer is a very aggressive malignancy with a poor prognosis. The response to chemotherapy treatment of patients with pancreatic ductal adenocarcinoma (PDAC) is difficult to predict and the identification of patients who most likely will benefit from aggressive chemotherapy approaches is crucial. Authors developed a simple, not expensive, diffusible zebrafish embryo model as avatar for patients affected by PDAC. Authors investigated the response of zebrafish xenografts to the chemotherapy options and authors analyzed the results by monitoring the fluorescent stained area. The described model appears to be an effective, usable and not expensive model for the xenotransplantation of pancreatic tumor tissue and for the evaluation of the efficacy of the different chemotherapy schemes available for the treatment of patients with PDAC. Work is clearly legible, brings summarizes new knowledge. The citations are actual and their format respect usual standards. This study could open a new frontier to personalized medicine because the results of the tests obtained in the present model in the xenotransplanted zebrafish embryos could reflect the clinical course of the patients' medical history, and such an approach might improve the evaluation of the patient's prognosis and the identification of the most appropriate individualized therapy. I recommend the manuscript to be published. Kosice, 21. February 2020 MUDr. Jana Katuchova, PhD. Professor of Department of Surgery University Hospital Košice Slovakia

RESPONSE: We would like to thank the reviewer looking at the appreciation of our work.