

## **POINT-TO-POINT REPLY TO REVIEWERS COMMENT**

On behalf of all the Authors, I would like to thank the Editor for the opportunity to consider this paper and the Reviewers for their comments and suggestions that allowed improving the quality of the manuscript.

The manuscript has been modified; changes have been highlighted in the updated version submitted for the review.

Please, find below a point-to-point reply to the comments of the Reviewers.

### **REVIEWER 1**

General comment: The review by Caputo et al focuses on an interesting topic that has potential to advance the field. However, several items need to be addressed to strengthen the work.

#### 1) English language needs polishing

**Reply:** We thank the Reviewer for Her/His comment. The English language of the manuscript has been revised by a native-English speaker (James McNamara jmcnamara@pnac.org). According to the BPG recommendation, we attached the certification provided by James McNamara.

#### 2) What databases were used for the literature search? Any limitations such as date? Size of study? Type of study? Of 32 total references, 13 are the authors' own publications (first, co- or senior author).

**Reply:** We thank the Reviewer for Her/His comment. We apologize for not being clear in the methods section. We reported that the database consulted was Pubmed but detailed information are of course needed. According to the Reviewer suggestion, the selection process has been clarified adding in the methods the following sentences:

- "...was queried for articles published up to December 2020. Terms pancreatic cancer, pancreatic adenocarcinoma, nanotechnology, nanomedicine, nanoparticles, diagnosis, detection, therapy and management have been used in different combinations. References reported in the selected articles have been also considered as other bibliographic sources..."

- "...without limitation concerning article types (original articles, review, etc.), subjects involved in the research (e.g., humans, mice), experimental condition (in vitro or in vivo) and population size have been considered..."

Regarding the number of self-citations, it was not the intention of the authors to be self-referential; the number of self-citations was due to the lack of abundance in the literature of contributions published on the subject of nanomedicine and early diagnosis of pancreatic cancer. However,

apologizing to the Reviewers and the Publisher, and in accordance with Their suggestions, we limited self-citations where not strictly necessary. The bibliography has therefore been modified.

- 3) More details should be provided for statements/results throughout the review. As an example, the statement “Nevertheless, the studies performed analyzing the PC of PDACs demonstrated that also the stage of the disease determines changes in PC composition [12]” is too general. Authors should describe specifically how stage changes the composition (1 or 2 sentences).

**Reply:** We thank the Reviewer for Her/His comment. In the revised manuscript, more details have been reported for each study in both the section regarding the early diagnosis and therapy.

Sentences we added and highlighted in the “NANOTECHNOLOGY AND PDAC EARLY DIAGNOSIS” section are:

- “...analysing plasma samples collected from 11 patients affected by untreated PDAC and 13 patients undergoing surgery for benign disease (“healthy controls”). The samples were let to interact with anionic liposomes (ALs) made of 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG); the obtained PCs general characteristics (i.e., dimension and surface charge and protein composition) were assessed by Dynamic Light Scattering and Laser Doppler Electrophoresis techniques. The Authors demonstrated significant differences in the surface charge ...”
- “The lack of proteomics-based mass spectrometry investigations did not allow to fully characterize the proteins forming the PCs in the two group.”
- “...More in details, liposomal formulations composed of HSPC (hydrogenated soy phosphatidylcholine), cholesterol (Chol) and DSPG (1,2-distearoyl-sn-glycero-3- [phospho-rac- (1-glycerol)]) interacted with plasma samples collected from 20 PDAC patients and 5 non-oncological controls. Protein patterns of PCs were characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Semi-quantitative comparison of electrophoretic gel results was performed by densitometry analysis and significant differences in protein band intensity profiles of controls vs. PDACs were found. These differences were confirmed and amplified by Principal Component Analysis (PCA) of the identified intensities...”
- “...Differently from other NPs, GO nanoflakes bind low levels of the most abundant proteins present in human blood (e.g.; albumin) and have the ability to adsorb other poorly-concentrated plasma proteins. As shown by Papi et al., when PCs formed around GO nanoflakes of 25 pancreatic ductal adenocarcinoma patients were compared with those of 25 controls, PDAC patients PCs were significantly less enriched of proteins with molecular weight ranging between 20-30 kDa. This difference allowed to distinguish between cancer and non-cancer subjects ...”

- "...Particularly, SDS-PAGE analysis of PCs formed around DOPG liposomes allowed to distinguish patients affected by T1-T2 PDACs from T3s and metastatic ones. More in detail, PC intensity profiles of T1-T2 pancreatic ductal adenocarcinomas significantly differed from those of the T3s because of the dissimilar abundance of a subset of proteins with molecular weight ranging between 25 and 50 kDa (p 0.04). Comparing T1-T2 to metastatic PDACs, significant differences in intensity profiles were also found in the subgroups of proteins with molecular weights of 25-50 kDa (p 0.01) and 50-120 kDa (p 0.04). The differences highlighted in the protein intensities would reflect the stage-related different expression of proteins (e.g., VEGF, CDK2, adhesion molecules, etc.) already reported as result of more sophisticated and expensive technologies..."

- "...In this multiplexed strategy that combines Hb levels with the characterization of PC formed around GO nanosheets, the Authors developed a NET able to distinguish PDACs from controls with 82.4% sensitivity and 97.1% specificity. Notably, the prediction ability of the combined parameters (Hb and PC technology) overcame the ability of each single one..."

Sentences we added and highlighted in the "NANOTECHNOLOGY AND PDAC THERAPY" are:

- "...In 2008, Patra et al. demonstrated, in vitro and in vivo, a significant inhibition of pancreatic cancer cells proliferation and growth using a formulation of gold NPs transporting cetuximab (C225) and gemcitabine..."

- "... The efficacy of Onivyde's® was demonstrated in a randomized trial performed on a series of 417 patients affected by metastatic PDACs. Patients who received the combination of Onivyde® and 5-fluorouracil (5-FU)/leucovorin gained on average 2 months of survival and showed an average delay in the time to tumor growth of 3.1 months when compared to those who received only 5-FU/leucovorin..."

- "... As shown by Mirshafiee and colleagues, after exposure to plasma NPs present significant inhibition (up to 94%) of their targeting ability. This inhibition is due to the formation of the PC that impairs the biodistribution and the cellular uptake of the NPs, and to the effect of the organic filters (liver and the spleen).

~~On the other hand, it has been reported how a minority of blood proteins bound to liposome surface can act like fingerprints and are able to boost the uptake in cancer cells.~~

Moreover, since it has been proved that only a minority of proteins forming the PC are internalized within tumour cells, acting as PC-fingerprints, in 2019 Palchetti et al. synthesized a collection of liposomes with different levels of fingerprints for pancreatic cancer cells. Notably, fluorescence

flow-assisted cell sorting (FACS) technology allowed Palchetti and coworkers to demonstrate that the most effective liposomal formulation identified in her panel was significantly more internalized in pancreatic cancer cells than the liposomal formulation used in the Onivyde® (95% and 10% respectively).

Nonetheless, Liu demonstrated in PDAC animal models that, when compared with liposomes, mesoporous silica nanoparticles (MSNs) improve irinotecan loading and efficacy. More in detail, the Author developed a supported lipid bilayer coated MSN carrier with increased loading capacity and cancer cells killing ability when compared to the conventional liposomal formulation. Furthermore, these nanocarriers showed improved stability decreasing the irinotecan toxicity by the reduction of the systemic leakage of the drug. The MSN carrier developed by Liu and coworkers was also more efficient in the treatment of pancreatic cancer metastatic cells and, according to the Author opinion, could be used in FOLFIRINOX regimen treatment for PDAC...”

- “...As demonstrated in mice by Meng et al in 2015, coated bilayer MSNs can...”

- “...In this paper, Meng also focused on the role that MSN nanocarriers have in overcoming the challenges of PDAC stromal barrier and abnormal perfusion that have been widely recognized among the main factors contributing to the failure of oncological treatments...”

- “...The Authors also reported the innovation of using a nanocarrier to produce a synergistic immune response by the concomitant delivery of an immunogenic cell death stimulus and interfering in immune suppression...”

- “...As reported in the review of Sadoughi et al., chitosan proved its efficacy in PDAC therapy as nanocarrier of both gemcitabine and 5-FU...”

4) A table or figure would be helpful to summarize findings/current state of the art.

**Reply:** We thank the Reviewer for Her/His comment and totally agree with Her/Him. According to this suggestion, two tables were added in the revised manuscript resuming the studies considered. Particularly, Table 1 resumes the application of the nanotechnologies in the early detection of pancreatic ductal adenocarcinoma, Table 2 resumes the application of the nanotechnologies in the treatment of PDAC.

The following sentence and Table have been added in highlighted in the “NANOTECHNOLOGY AND PDAC EARLY DIAGNOSIS” section:

“All the studies focused on PDAC early diagnosis and considered in this paper have been resumed in Table 1.”

Table 1 Nanotechnology and PDAC early diagnosis.

Year	Reference	NP type	Main findings
2012	[8]	Gold, Silica, Polystyrene	Characteristics of NPs (material, size, electric charge, hydrophobicity) influence the composition of the PC.
2012	[10]	Silica, Carbon, Iron oxide, Polystyrene	Different experimental sources (e.g. plasma, urine), affect the composition of the PC
2013	[9]	Silica, Polystyrene	Environmental factors (e.g. incubation time) influence the composition of PC
2014	[14]	Liposomes	After interaction of NPs with plasma of PDACs and non-oncological subjects, differences in the electric charge (zeta potential) and size of the PCs allow to distinguish between the two groups
2015	[12]	Gold	Gold-NPs successfully used for early detection of prostate cancer.
2017	[11]	Silica, Polystyrene, Gold, Liposomes	Different patients affected by different pathological conditions have “personalized” PC.
2017	[15]	Liposomes	Development of NP enabled plasma test able to discriminate PDACs from controls (sensitivity 85%, specificity 100%).
2018	[17]	Liposomes	Analysis of PCs formed around DOPG liposomes allowed to distinguish patients affected by T1-T2 PDACs from T3s and metastatic ones.
2019	[13]	Silica	Successful application of silica NPs in early diagnosis of the head and neck solid squamous carcinoma.
2019	[16]	GO	Analysis of BC formed around GO nanoflakes distinguished PDAC from healthy subjects (sensitivity 92%).
2020	[18]	GO	Development of a NET based on the combination of Hb levels with the characterization of the PC formed around GO nanosheets distinguished PDAC from controls (sensitivity 86.7%, specificity 95.8%).

Nanoparticle (NP); Protein Corona (PC); Pancreatic ductal adenocarcinoma (PDAC); 1, 2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG); Biomolecular Corona (BC); Graphene Oxide (GO); NP-enabled tool (NET).

The following sentence and Table have been added in highlighted in the “NANOTECHNOLOGY AND PDAC THERAPY” section:

“All the studies focused on the application of nanotechnology in PDAC therapy considered in this paper have been resumed in Table 2.”

Year	Reference	NP type	Main Findings
2008	[21]	Gold	Gold NPs used as nanocarrier improve the effect of cetuximab and gemcitabine in reducing the growth and the proliferation of pancreatic cancer cells in murine model.
2013	[23]	Silica	The exposure to human plasma significantly reduces the targeting ability of NPs.
2015	[27]	MSN	Coated bilayer MSNs improve the synergic delivery of gemcitabine and paclitaxel in PANC-1 injected in murine models.
2015	[22]	Liposomal irinotecan (Onivyde®)	Approval of Onivyde® for clinical use in metastatic PDAC, who already have received gemcitabine.
2016	[25]	MSN	In KPC pancreatic cancer cells injected into murine models, biocompatibility and therapeutic efficacy of MSNs-irinotecan formulation are superior to liposomal-irinotecan formulation.
2017	[28]	MSN	MSNs improve oxaliplatin and indoximod bioavailability in KPC pancreatic cancer cells injected into murine models.
2017	[26]	NP albumin	FDA approved the clinical use of NP albumin-bound paclitaxel (Abraxane) for pancreatic cancer.
2019	[24]	Liposomes	Targeting ability of liposomal formulation with specific fingerprints for pancreatic cancer cells is superior to Onivyde.
2020	[30]	Chitosan	The use of chitosan as carrier of gemcitabine and 5-FU improves therapeutic effect decreasing toxicity in pancreatic cancer cells.

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Table 2 Nanotechnology and PDAC therapy.

Nanoparticle (NP); Mesoporous silica NP (MSN); Kras<sup>LSL-G12D/+</sup>/Trp53<sup>LSL-R172H/+</sup>/Pdx-1-Cre (KPC); Pancreatic Cancer Cell (PANC-1); Mesoporous silica nanoparticle (MSN); Pancreatic ductal adenocarcinoma (PDAC); 5-Fluorouracil (5-FU);

### **REVIEWER 3**

The authors did a thorough literature search on nanoparticles used for pancreatic cancer diagnosis and treatments. The topic is very interesting. However, the manuscript only listed these approaches without providing sufficient detailed information, especially for the treatment part. After reading this paper, readers only know what nanotechnologies have been applied. More details, such as targeting and releasing mechanisms, should be provided.

**Reply:** We thank the Reviewer for Her/His comment and agree with Her/Him. More detailed information of all the studies included in this review have been given. Sentences have been added and highlighted in the manuscript (see reply to Reviewer n<sup>o</sup>1).

## **POINT TO POINT REPLY TO EDITOR COMMENTS OF RE-REVIEW**

On behalf of all the Authors, I would like to thank the reviewer for the comments. The manuscript has been modified according to these comments. We took the opportunity to change the word “resumed” with “summarized” as previously suggest by one of the Reviewer. All changes have been highlighted in the updated version. Please, find below a point-to-point reply to your comment.

Kindest regards,

The corresponding Author"