Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo 35

Settore Concorsuale: 06/D3 - MALATTIE DEL SANGUE, ONCOLOGIA E REUMATOLOGIA

Settore Scientifico Disciplinare: MED/06 - ONCOLOGIA MEDICA

ANALYSIS OF EXTRACELLULAR VESICLES FROM PATIENTS WITH ADVANCED PANCREATIC CANCER IDENTIFIES MIRNAS WITH PREDICTIVE VALUE FOR TREATMENT WITH GEMCITABINE + NAB-PACLITAXEL

Presentata da: Ilario Giovanni Rapposelli

Coordinatore Dottorato

Manuela Ferracin

Supervisore

Giovanni Luca Paolo Frassineti

Co-supervisore

Lorenzo Montanaro

Esame finale anno 2023

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Pancreatic cancer

Pancreatic cancer (PC) is the fourth leading cause of cancer death in Italy and seventh in the world [1,2](Figure 1). Its incidence is increasing worldwide [3](Figure 2); in the United States, where it is currently the fourth cancer for number of deaths in both sexes [4], PC is expected to become the second leading cause of cancer death by 2030 [5]. The 5-year survival rate of PC is about 11% [4]. Indeed, only about 20% of patients can undergo treatment with curative intent, whereas about 80% of patients receive diagnosis at an advanced stage [6].

Rank increased | No change | Rank decreased

Rank	Cancer 2007	Cancer 2017	Rank	Change in Absolute YLLs, % (UI)	Change in Age-Standardized YLL Rate, % (UI)
1	Tracheal, bronchus, and lung cancer	Tracheal, bronchus, and lung cancer	1	24.8 (21.7 to 27.6)	-4.1 (-6.5 to -2.0)
2	Stomach cancer	Liver cancer	2	21.2 (17.0 to 27.4)	-4.6 (-8.0 to 0.1)
3	Liver cancer	Stomach cancer	3	4.8 (2.4 to 7.4)	-18.6 (-20.5 to -16.6)
4	Colon and rectum cancer	Colon and rectum cancer	4	23.8 (19.2 to 27.6)	-4.5 (-8.0 to -1.7)
5	Breast cancer	Breast cancer	5	23.9 (17.3 to 28.7)	-1.7 (-6.8 to 2.1)
6	Esophageal cancer	Esophageal cancer	6	8.9 (5.8 to 12.2)	-16.2 (-18.6 to -13.7)
7	Brain and nervous system cancer	Pancreatic cancer	7	35.8 (32.5 to 38.6)	4.0 (1.5 to 6.1)
8	Cervical cancer	Brain and nervous system cancer	8	18.4 (11.9 to 24.6)	0 (-5.6 to 5.3)
9	Pancreatic cancer	Cervical cancer	9	15.1 (9.4 to 19.1)	-7.2 (-11.8 to -3.9)
10	Non-Hodgkin lymphoma	Non-Hodgkin lymphoma	10	22.1 (15.6 to 26.9)	0.2 (-5.2 to 4.3)

Figure 1. Cancers ranked by absolute years of life lost (YLLs) between 2007 and 2017. Data from the Global Burden of Disease study, that involved 195 countries. Pancreatic cancer moved from ninth place in 2007 to seventh in 2017. From [2].

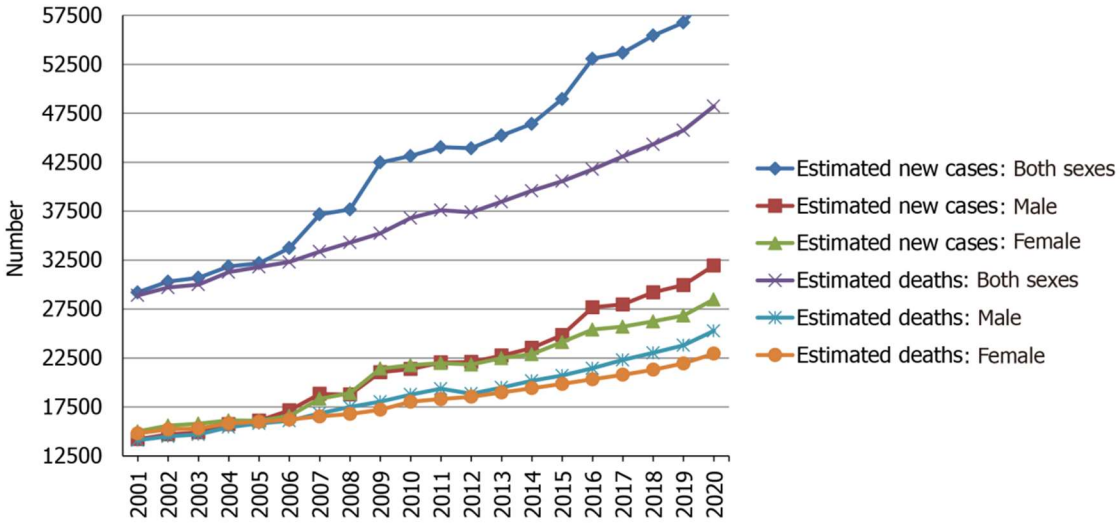


Figure 2. The incidence and the estimated deaths of pancreatic cancer are increasing worldwide. Depicted data refer to the United States. Pancreatic cancer is expected to become the second leading cause of cancer death in the United States by 2030. From [3].

Since about 90% of cases of PC are composed of pancreatic cancer adenocarcinoma [7], hereafter PC will be used for pancreatic cancer adenocarcinoma.

In advanced disease, chemotherapy is the only treatment. Gemcitabine used to be the standard of care until a few years ago [8]. In recent years, the introduction of combination regimens such as FOLFIRINOX (5-fluorouracil/leucovorin + irinotecan + oxaliplatin), gemcitabine + nab-paclitaxel and PAXG (cisplatin + nab-paclitaxel + capecitabine + gemcitabine) led to an improvement in patients' survival [9-11]. FOLFIRINOX and gemcitabine + nab-paclitaxel showed an improved survival compared to gemcitabine alone in two phase 3 trials: for FOLFIRINOX, median overall survival (OS) 11.1 months vs 6.8 with gemcitabine, for gemcitabine + nab-paclitaxel, median OS 8.5 months vs 6.7 with gemcitabine alone [9,10]. On the other hand, a randomized phase 2 trial with PAXG showed its superior activity compared with gemcitabine + nab-paclitaxel: patients free from progression at 6 months (primary endpoint of the trial) were 74% with PAXG and 46% in the control arm [11].

The abovementioned regimens are current treatment options for first-line therapy in patients with good performance status (PS). Response rates of these schedules are 31.6% for FOLFIRINOX (with 38.6% of patients reporting stable disease [SD]), 23% for gemcitabine + nab-paclitaxel (SD 27%), 50% for PAXG (SD 33%)[9-11]. These numbers highlight a significant amount of resistance to therapy. Since progressive disease often comes along with patient's deterioration in PS, only about 49% of patients receive second-line therapy (and about 19% receive third-line treatment)[12].

Therefore, choice of first-line therapy is crucial. In this context, the identification of predictive biomarkers, i.e. able to predict response (or resistance) to treatment, is a crucial point in order to improve management of PC, not only in advanced disease, but also in the preoperative setting, and would result in a great clinical benefit.

Currently, choice of treatment is mainly based on patient's clinical evaluation and on a sequential strategy of therapies. The only know predictive markers are alterations in genes involved in DNA damage repair such as *BRCA1/2* and *PALB2*, which confer sensitivity to platinum-containing regimens [13,14]. Furthermore, patients carrying germline *BRCA1/2* mutations, can receive a maintenance treatment with PARP inhibitor

olaparib after first-line platinum-based chemotherapy [15]. However, germline *BRCA* mutations are present in about 8% of patients [16], and *PALB2* mutations account for another 1% [17]. Thus, for the great majority of patients no predictive factors can guide choice of treatment, and the identification of predictive markers is still an unmet clinical need in PC.

1.2 Extracellular vesicles

Extracellular vesicles (EVs) are small particles, coated by a lipid bilayer, released by cells [18]. They contain several biomolecules such as DNA, RNA, proteins, glycans, lipids, and are able to transfer their content into target cells, facilitating intercellular communication and regulating several cell functions, e.g. proliferation, survival, apoptosis and migration [19].

More specifically, EVs are involved in several key processes of cancer biology, also in PC: proliferation, survival, apoptosis, invasion, migration, epithelial-mesenchymal transition (EMT), tumor microenvironment (TME) remodeling, immune evasion, pre-metastatic niche formation, chemoresistance [19-22](Figure 3).

Interestingly, in the complex scenario of cancer biology, EVs are released not only by tumor cells, but also from cells of the TME, that can influence tumor behaviour [24]. Moreover, cancer-derived EVs can induce stromal recipient cells to support pre-metastatic niche formation and may induce malignant transformation in resident mesenchymal stem cells [19,25]. This is of special interest in PC: indeed, a hallmark of this disease is the peculiar structure of TME, with a desmoplastic stroma and several cell types, including pancreatic stellate cells and cancer-associated fibroblasts, responsible for the production of the fibrous tissue [26,27].

EVs can be easily isolated from blood. Tumor cells secrete more EVs than non-malignant cells, and serum of cancer patients is enriched in EVs [28]. Moreover, a prognostic role in cancer patients has been shown for specific subsets of EVs [29]. The easy isolation of EVs prompted a large amount of studies about their role as potential biomarkers for PC [30,31]. Their easy isolation, along with the number of biomolecules contained and the number of functions they regulate (including chemoresistance), makes EVs an attractive

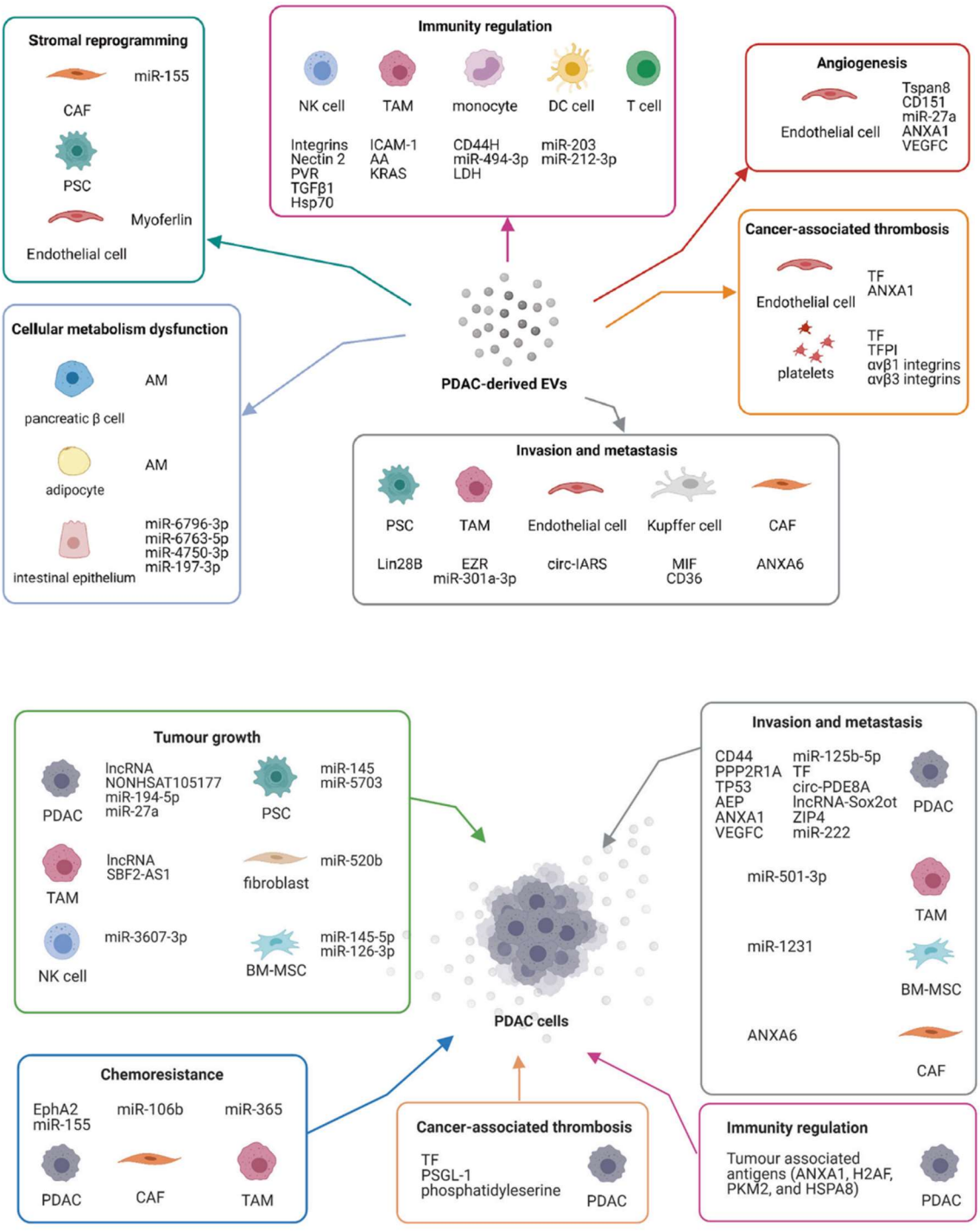


Figure 3. The communication mediated by extracellular vesicles between pancreatic cancer cells and tumour microenvironment. Extracellular vesicles from tumor cells and from cells of the microenvironment act in a complex interplay that modulates several key processes of cancer biology. PDAC pancreatic ductal adenocarcinoma, PSC pancreatic stellate cell, TAM tumour-associated macrophage, CAF cancer-associated fibroblast, BM-MSC bone marrow-derived mesenchymal stem cell, NK natural killer cell, DC dendritic cell. From [23].

tool for identification of prognostic or predictive markers in PC, by exploiting the minimally invasive approach of liquid biopsy, i.e. a blood sample in order to obtain information about one patient's tumor features [32,33]. More specifically, the analysis of EVs from PC patients may enlighten molecular patterns and mechanisms that underlie drug response or resistance, with the aim to identify features that can be exploited as predictive factors in order to select the right therapy for every patient.

1.3 MicroRNAs

Among the processes regulated by the EVs, chemoresistance is of special interest if investigating possible predictive biomarkers, i.e. able to predict response (or resistance) to therapy.

In EV-mediated chemoresistance, a key role is played by microRNAs (miRNAs). These are small, non protein-coding RNA fragments (about 20-25 nucleotides), that regulate the expression of specific target proteins through degradation of mRNA or interference with the translational process [34](Figure 4). Based on this mechanism, miRNAs are

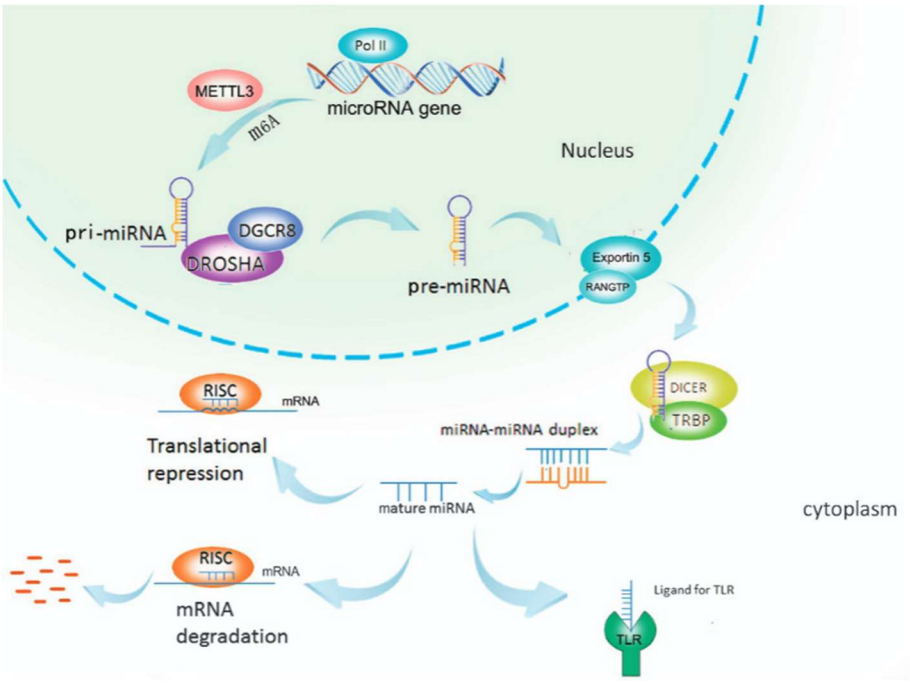


Figure 4. Biogenesis and mechanism of action of miRNAs. After transcription of miRNA gene by RNA polymerase II and processing, that involves several steps, mature miRNA is produced. Mature miRNA is incorporated into the protein complex RISC. This mediates gene silencing by cleavage and degradation of mRNA, or by translational repression. MiRNAs may also bind to Toll-like receptors (TLR) and activate downstream signaling pathways. From [35].

involved in several processes such as cell proliferation and differentiation, metabolism, apoptosis, signaling and hematopoiesis [34].

EVs from cancer cells are enriched in miRNAs and are able to perform cell-independent miRNA biogenesis [36]. The aberrant expression of miRNAs in cancer has been involved in several key mechanisms such as uncontrolled cell proliferation, cell death modulation, tumor invasion, angiogenesis, EMT, metastases, immune evasion, chemoresistance [22,34](Figure 5). Chemoresistance is induced by miRNAs by targeting drug-resistance-related genes or influencing genes related to cell proliferation, cell cycle, and apoptosis [37]. Indeed, miRNAs can promote anti-apoptotic activity, down-regulate key drug-metabolizing enzymes or activate survival pathways.

Specifically in PC, a role of certain miRNAs in chemoresistance has been already showed. For example, PC cells incubated with gemcitabine upregulate miR-155, which is transferred to other PC cells via EVs, and is able to promote gemcitabine resistance through facilitation of anti-apoptotic activity and suppression of deoxycytidine kinase, a key gemcitabine-metabolizing enzyme [38,39]. EVs released by cancer-associated fibroblasts may contribute to gemcitabine resistance, through the upregulation of chemoresistance-inducing factor Snail and its target miR-146a in recipient PC cells [40]. Given the above premises, miRNAs are a promising tool in biomarker-directed studies, and specifically in studies aiming to identify circulating predictive factors [41,42].

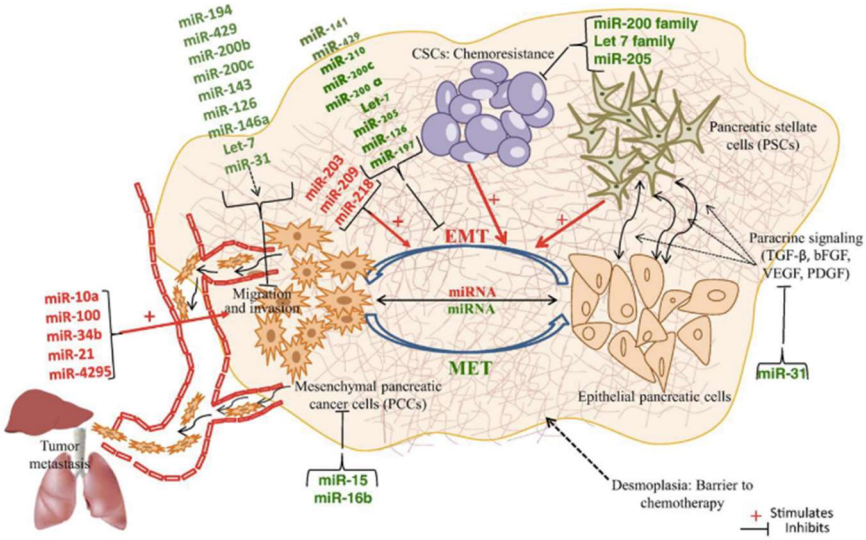


Figure 5. An example of the action of miRNAs in the milieu of pancreatic cancer. This figure depicts the role of some miRNAs in pancreatic cancer, in a complex scenario that involves not only tumor cells, but also pancreatic stellate cells and cancer stem cells (CSCs). In addition to oncogenic miRNAs (red), tumor suppressor miRNAs (green) are also shown: e.g., members of the miR-200 family can revert epithelial-mesenchymal transition (EMT) to mesenchymal-to-epithelial transition (MET). From [34].

2. AIM OF THE WORK

The identification of predictive biomarkers is an unmet clinical need in PC. Based on the easy isolation of EVs and the role of EV-borne miRNAs in chemoresistance, the present work aims to investigate a possible predictive molecular signature in PC, by analyzing EVs and their miRNA content in samples collected from patients before first-line treatment.

The first part of the present work was an exploratory analysis aimed to establish methods for isolation and enrichment of EVs, flow cytometry analysis of EV surface biomarkers and analysis of miRNA content, by comparing EVs from PC patients with EVs from healthy donors.

The second part of the work analyzed samples from advanced PC patients, collected before first-line treatment with gemcitabine + nab-paclitaxel. By analyzing EVs and their content in miRNA, and by matching miRNA expression in baseline samples with clinical outcome (response assessment), we aimed to identify a possible predictive molecular signature, thus investigating a possible role of liquid biopsy in order to predict response (or resistance) to therapy. This would translate in an improvement of patients' outcome through a personalized medicine approach.

3. METHODS

3.1 Patient population

The study population includes patients with metastatic or locally advanced pancreatic cancer, treated at IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori" - Meldola (FC), Italy. All patients had histologically or cytologically confirmed diagnosis of pancreatic adenocarcinoma and did not receive previous therapy for advanced disease (adjuvant therapy was allowed).

Patient received first-line treatment in routine clinical practice, according to standard schedules: for patients treated with gemcitabine + nab-paclitaxel, gemcitabine 1000 mg/m^2 + nab-paclitaxel 125 mg/m^2, d1,8,15 q28 (treatment schedule as in [10]); if necessary, dose reductions were applied as per standard clinical practice. All patients signed informed consent for collection of samples for translational research. Samples were collected before first-line treatment and stored in the local biobank facility. All baseline samples were collected between May 2015 and April 2021.

Collection of clinical data included demographic data, medical history, ECOG PS, treatment (schedule, administration, toxicity), biochemical assessments (including CA19.9), baseline tumor assessment and tumor response assessments, survival status.

Tumor assessment was performed with thoracic-abdominal contrast-enhanced computed tomography scan every 3 months, and tumor response was evaluated according to Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 [43].

The present study has been approved by local ethics committee (CEROM IRSTB118). The study complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki and local laws and fulfilled regulation about the protection of personal data.

3.2 Sample collection

Blood samples (5 ml) have been collected in EDTA-containing tubes before starting of treatment (0 to 14 days before d1 of first cycle). Plasma has been isolated, by

centrifugation at 2000 g for 15 min at room temperature, within two hours from blood withdrawal. Plasma has been divided in cryogenic vials (0.5 ml each) and stored at -80 °C until use in IRST biobank facility.

3.3 EV isolation

EVs have been isolated from 1 ml of plasma by size exclusion chromatography (SEC) columns of polysaccharide resin (qEV 70 nm columns, IZON, Lyon, France) following manufacturer's instructions. The EV-enriched fractions have been collected and used for subsequent analyses.

3.4 Nanoparticle tracking analysis

The concentration (number/ml) and size (nm) of EVs have been evaluated through Nanoparticle tracking analysis (NTA). This has been performed with NanoSight NS300 (Malvern Instruments, Malvern, UK), equipped with NTA 2.3 analytical software laser. Before analysis, all samples have been diluted in 0.1 µm filtered PBS and subsequently three videos per sample (30 seconds each) have been recorded at a camera level of 12 and in light scattering mode following manufacturer's instructions. The NTA software version 2.3 has been used for data analysis.

3.5 Flow cytometry

Multiplex bead-based flow cytometry has been used to analyze EV surface proteins (MACSPlex Exosome Kit, human, Miltenyi Biotec, Bergisch Gladbach, Germany). This method allows to analyze 37 different epitopes on EV surface, including specific markers for the identification of exosomes (CD9, CD81, CD63). Briefly, 70 µl of EV SEC eluate have been diluted with MACSPlex buffer to obtain a final volume of 120 µl. Each diluted sample has been incubated for 1 hour at room temperature on an orbital shaker at 450 rpm with different antibody-coated bead subsets and APC-conjugated anti-CD9, anti-CD63, and anti-CD81 detection antibodies. After washes with MACSPlex buffer according to manufacturer's instructions, the samples have been analyzed with the flow

cytometer (BD FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA) obtaining the raw value of the median fluorescence intensity (MFI) for each epitope. The MFI value of the negative control has been subtracted from the raw MFI value of each epitope.

3.6 RNA extraction

RNA has been extracted from EVs by using the Plasma/Serum RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) as indicated in the manufacturer's protocol. The extracted RNAs have been evaluated qualitatively with the Bioanalyzer 2100 instrument (Agilent Technologies, Cernusco sul Naviglio, Italy) using RNA 6000 Pico chips (Agilent Technologies).

3.7 Analysis of EV miRNA content

Starting from 5 µl of total RNA, microRNA libraries have been prepared with QIAseq miRNA Library Kit (Qiagen, Milan, Italy), following manufacturer's instructions for low input samples. Libraries have been quantified with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and quality checked on DNA high sensitivity chips (Agilent Technologies). Normalized libraries have been sequenced on NextSeq 550 System (Illumina, San Diego, CA, USA), approximately at a sequencing depth of 20 million reads per sample.

3.8 Bioinformatic analysis

Local Run Manager of NextSeq 550 System has been used for demultiplexing. Reads have been then trimmed, corrected for UMI reduction and aligned to miRBase v22 using the ready-to-use workflow for miRNA quantification of CLC Genomics Workbench, Biomedical Genomics Analysis plugin (Qiagen). Data normalization (using the Trimmed Mean of M-values method) and differential expression analysis have been performed using the CLC Genomic Workbench as well. Differentially expressed miRNAs have been identified by setting the threshold $|\log_2 FC| > 1{,}5$ and $p < 0.05$ using multi-factorial statistics based on a negative binomial generalized linear model. Graphical

representations have been elaborated with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3.9 Statistical analysis

The comparison of the EV mean diameters, mode diameters and expression of EV surface epitopes between patient groups has been performed with the Mann-Whitney U test. A two-sided testing has been used to obtain all *p* values which have been considered significant with a value less then 0.05.

For survival analysis, overall survival (OS) was defined as the time interval from the first day of treatment to the day of death or last follow-up visit. Progression-free survival (PFS) was defined as the time interval from the first day of treatment to the day of tumor progression or death, whichever occurred first. OS and PFS have been estimated by the Kaplan–Meier method.

SAS software version 9.4 (SAS Institute, Cary, NC, USA), R statistical package version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) and MedCalc® Statistical Software version 20 (MedCalc Software Ltd, Ostend, Belgium) have been used for data analysis.

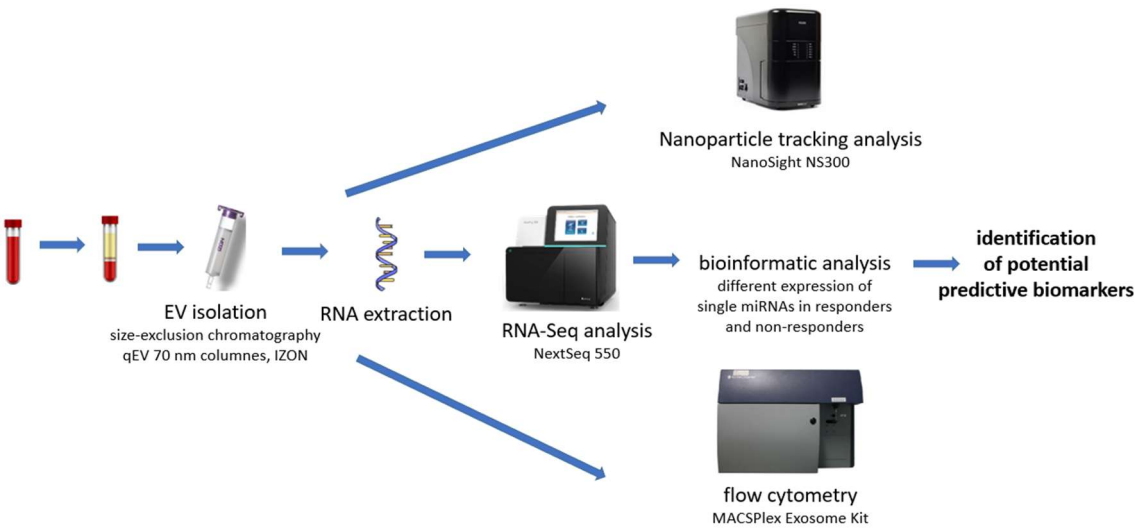


Figure 6. Workflow of the study. After blood sampling, plasma has been separated by centrifugation, then extracellular vesicles have been isolated by size-exclusion chromatography. Then, three different analytical pathways have been followed: 1) Nanoparticle tracking analysis for concentration and size of extracellular vesicles; 2) flow cytometry for surface marker analysis); 3) RNA extraction and RNA-Seq analysis for miRNA content; then, different expression of single miRNAs in responder and non-responder patients has been evaluated in order to identify potential predictive biomarkers.

4. RESULTS

4.1 Comparison of EVs from PC patients and healthy subjects

4.1.1 Baseline characteristics

For the first part of the work, i.e. an exploratory analysis of blood-derived EVs aiming to establish methodology for subsequent analyses, we used samples from advanced PC patients (n = 28), collected before first-line treatment, and healthy subjects (n = 7). Baseline characteristics of study population are reported in Table 1.

Patients with advanced PC	**n**	**%**
age (years)		
median 66 (range 43-82)		
sex		
female	16	57.1
male	12	42.9
stage		
metastatic	15	53.6
locally advanced	13	46.4
chemotherapy regimen		
gemcitabine + nab-paclitaxel	23	82.2
FOLFIRINOX	3	10.7
PAXG	2	7.1
baseline CA19.9		
≤ULN	5	17.8
>ULN and <10 ULN	7	25.0
≥10 ULN	15	53.6
NA	1	3.6
median	435.3	
Healthy subjects	**n**	**%**
age (years)		
median 47 (range 37-58)		
sex		
female	3	42.9
male	4	57.1

Table 1. Baseline characteristics of study population for the first part of the study (comparison of extracellular vesicles from pancreatic cancer patients and healthy subjects). ULN, upper limit of normal; NA, not available.

4.1.2 Nanoparticle tracking analysis

The analysis of EV concentration did not show any significant difference between PC patients and healthy subjects: in both cohorts, a concentration of 10×10^{10} EVs/ml has been found (Figure 7).

On the other hand, different mean size and distribution of EVs have been found: size (mean ± standard deviation) was 145.0 ± 9.89 nm in PC patients and 113.4 ± 12.78 nm in healthy subjects (p = 0.036), thus suggesting the presence of different EV subpopulations in patients (Figure 7 and Table 2).

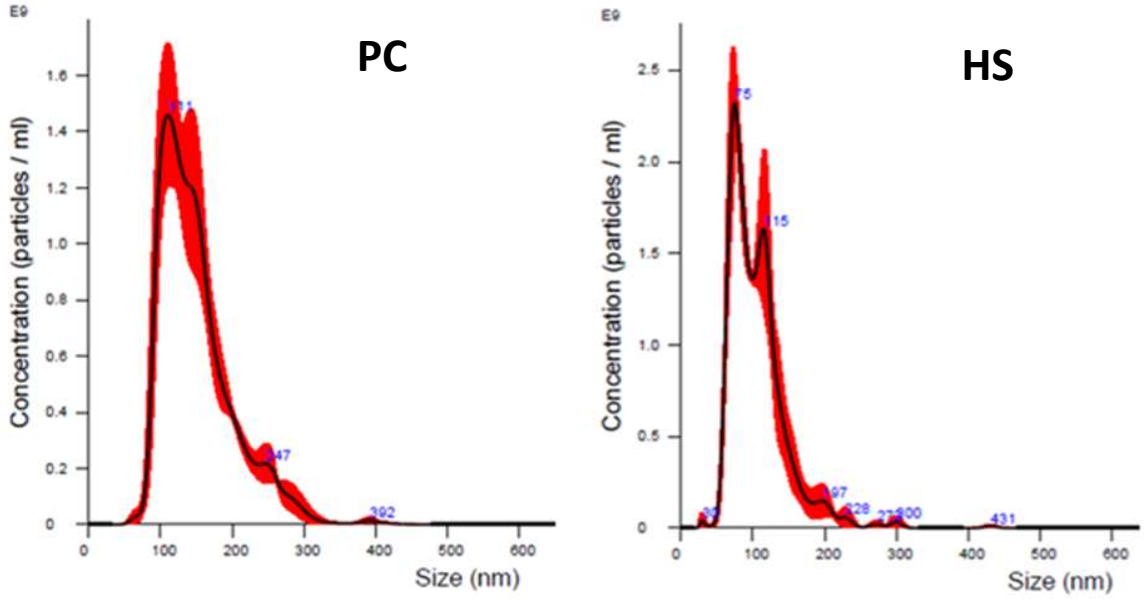


Figure 7. Nanoparticle tracking analysis. A representative graph is showed for each group: patients with pancreatic cancer (PC) and healthy subjects (HS).

	HS	PC	
count (EVs/ml)	$10x10^{10}$	$10x10^{10}$	
size (nm; mean ± SD)	113.4 nm ± 12.78	145.0 ± 9.89	*
	median (95% CI)		
50-150 nm	0.784 (0.599 - 0.917)	0.462 (0.378 - 0.547)	*
150-300 nm	0.213 (0.076 - 0.389)	0.524 (0.441 - 0.608)	*

Table 2. Nanoparticle tracking analysis. Data for extracellular vesicle count and size analysis are reported. HS, healthy subjects. PC, patients with pancreatic cancer. * $p < 0.05$.

4.1.3 Analysis of EV surface markers

Flow cytometry with MACSPlex Exosome Kit showed a different surface antigen expression in samples from PC patients and healthy subjects. More specifically, samples from PC patients showed an increased expression of several surface markers such as CD31, CD29, CD42a, CD41b, CD9, CD62P, CD40, HLA-BC, and HLA-DR (Figure 8).

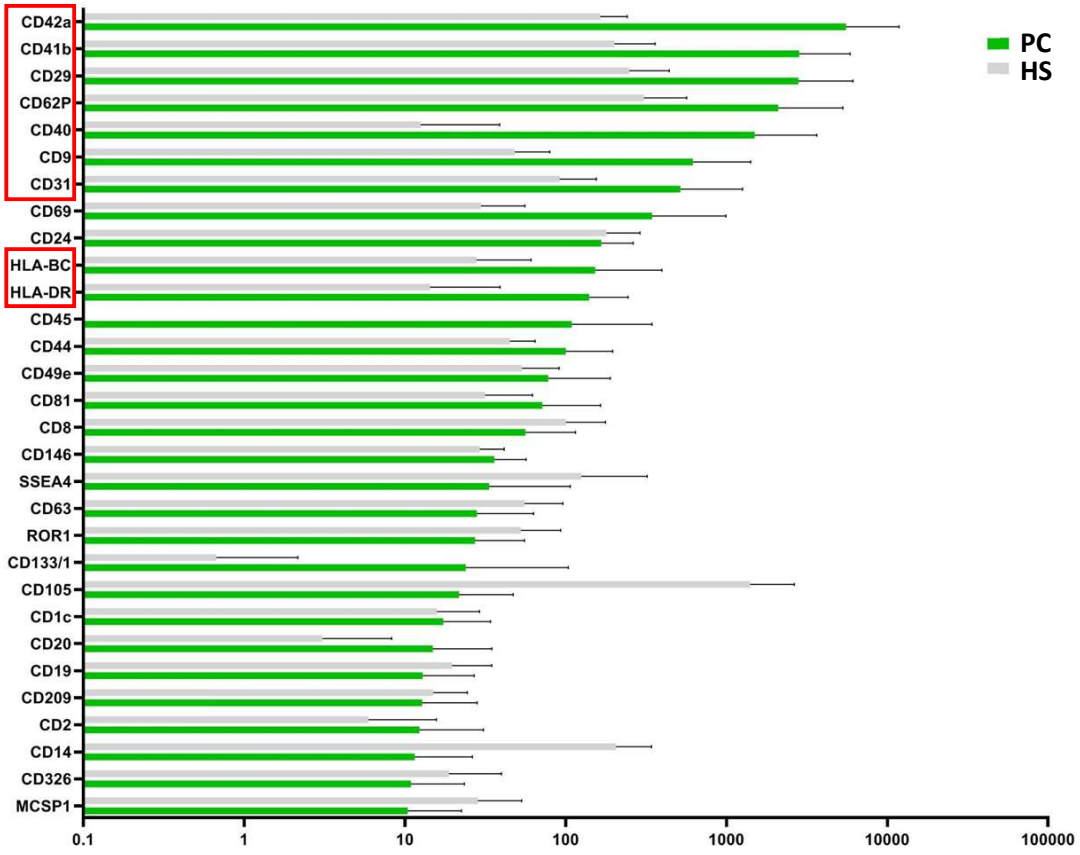


Figure 8. Flow cytometry. The mean MFI of each surface marker is showed, bars are standard deviation. Gray, healthy subjects (HS); green, patients with pancreatic cancer (PC).

4.1.4 Analysis of EV miRNA content

By analyzing the miRNA content of EVs, we have detected the expression of more than 600 miRNAs. When comparing samples from PC patients and healthy subjects, we have observed a significantly different expression ($p < 0.05$) of 28 miRNAs, including miRNAs with previously reported oncogenic or tumor suppressor activity (e.g., miR-431-5p and miR-188-3p, respectively). The most significant findings ($p < 0.01$) are showed in Table 3.

up-regulated	*p*	down-regulated	*p*
miR-485-3p	0.0056	miR-6802-3p	0.0086
miR-431-5p	0.0099	miR-4652-5p	0.0014
miR-5186	0.0062	miR-188-3p	0.0033
miR-6796-3p	0.0042	miR-7850-5p	0.0093
miR-4487	0.0085	miR-105-3p	0.0022
miR-4695-5p	0.0019	miR-216b-3p	0.0020
miR-4446-5p	0.0041	miR-138-1-3p	0.0019
miR-1909-5p	0.0004	miR-4485-3p	0.0084
miR-758-3p	0.0096	miR-6851-5p	0.0080
miR-7157-3p	0.0096	miR-3147	0.0045

Table 3. Analysis of miRNA content of extracellular vesicles. Up- or down-regulated miRNAs in patients with pancreatic cancer (compared to healthy subjects) are shown.

4.2 Analysis of samples from advanced PC patients collected before first-line treatment with gemcitabine + nab-paclitaxel

After confirming the ability of our approach to detect cancer-related EV biomarkers, we have analyzed samples from advanced PC patients, collected before first-line treatment with gemcitabine + nab-paclitaxel, to investigate a possible predictive molecular signature.

4.2.1 Patient baseline characteristics and outcome

Patient characteristics are reported in Table 4 and summarized in Table 5. Of 21 patients analyzed, at baseline 12 had metastatic disease, 9 locally advanced disease. As for best response assessment during treatment with gemcitabine + nab-paclitaxel, evaluated according to RECIST 1.1, 13 patients reported an objective response (partial response, none had a complete response), whereas 8 were classified as non-responders (4 with stable disease, 4 with progressive disease). Median PFS was 9.2 months for responders

Patient	Sex	Age	Stage	Site of metastases	Basal CA19.9 (U/ml)	Best response	Reason for discontinuation	Time to progression (months)	Survival (months)
1	m	70	M	liver, lymph nodes, bone	651.0	PR	PD	7.6	13.8
2	f	74	M	peritoneum	1434.0	PD	PD	2.5	6.2
3	f	54	M	liver, bone	417.0	PR	maintenance	NR	> 18.4 *
4	m	62	M	peritoneum, bone	2903.0	PR	maintenance	9.2	13.7
5	f	75	LA	-	292.8	SD	PD	8.1	15.0
6	m	65	LA	-	132.0	PR	surgery	15.9	> 31.0 *
7	f	61	M	liver, lymph nodes	30.4	PR	PD	6.6	7.3
8	m	65	M	liver	586.7	PR	maintenance	8.0	13.3
9	f	74	LA	-	926.0	PR	PD	10.5	13.6
10	f	65	M	liver	17.0	PR	maintenance	6.5	8.9
11	f	60	M	liver	11.1	PD	PD	2.0	6.6
12	f	74	M	liver	310.3	PR	maintenance	9.2	13.0
13	f	64	M	liver	32.8	PR	maintenance	12.9	13.9
14	f	72	LA	-	435.3	SD	toxicity	7.1	8.6
15	f	80	LA	-	289.0	PR	PD	9.1	11.1
16	m	73	LA	-	>10000.0	PD	PD	2.0	2.1
17	f	63	M	liver, lung, peritoneum, lymph nodes	816.0	PR	PD	9.6	14.2
18	m	71	LA	-	12.9	SD	surgery	18.2	30.4
19	f	71	LA	-	242.7	SD	PD	4.1	19.5
20	m	68	M	bone	3419.0	PR	PD	4.6	5.8
21	m	82	LA	-	585.0	PD	PD	2.9	6.2

Table 4. Baseline characteristics and treatment outcomes of patient population for the second part of the study (analysis of samples from advanced pancreatic patients collected before first-line treatment with gemcitabine + nab-paclitaxel). m, male; f, female; M, metastatic; LA, locally advanced; PR, partial response; SD, stable disease; PD, progressive disease; NR, progression not reached; * partial data (patient alive).

	n	%
age (years)		
median 70 (range 54-82)		
sex		
female	13	61.9
male	8	38.1
stage		
metastatic	12	57.1
locally advanced	9	42.9
best response		
complete response	0	0.0
partial response	13	62.0
stable disease	4	19.0
progressive disease	4	19.0
baseline CA19.9		
≤ULN	5	23.8
>ULN and <10 ULN	5	23.8
≥10 ULN	11	52.4
median	417.0	

Table 5. Summary of baseline characteristics and treatment outcomes of patient population. ULN, upper limit of normal.

and 2.9 months for non-responders (hazard ratio [HR] 0.39, 95% confidence interval [CI] 0.13-1.12; p = 0.08; Figure 9). Median OS was 13.6 months for responders and 6.6 months for non-responders (HR 0.77, 95% CI 0.29-2.04; p = 0.60; Figure 10). In the whole patient cohort, median was PFS 8.0 months and median OS 13.3 months.

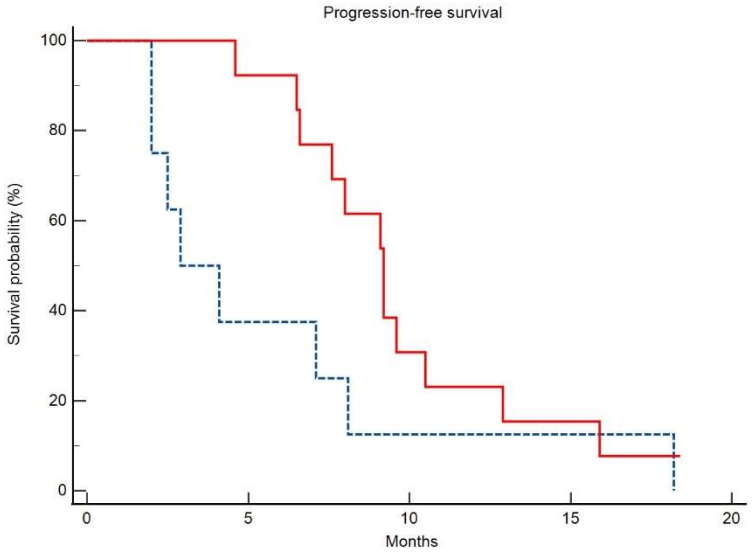


Figure 9. Kaplan-Meier analysis of progression-free survival. Median progression-free survival was 9.2 months for responders and 2.9 months for non-responders (HR 0.39, 95% CI 0.13-1.12; *p* = 0.08). Red line, responders; blue line, non-responders.

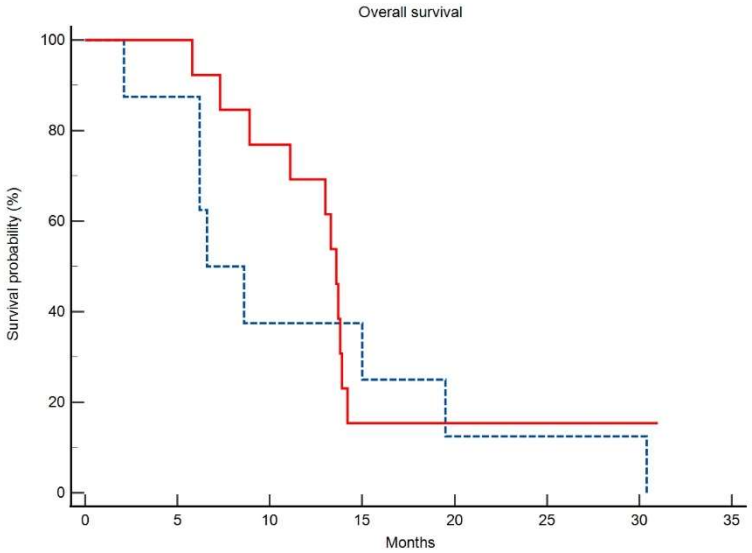


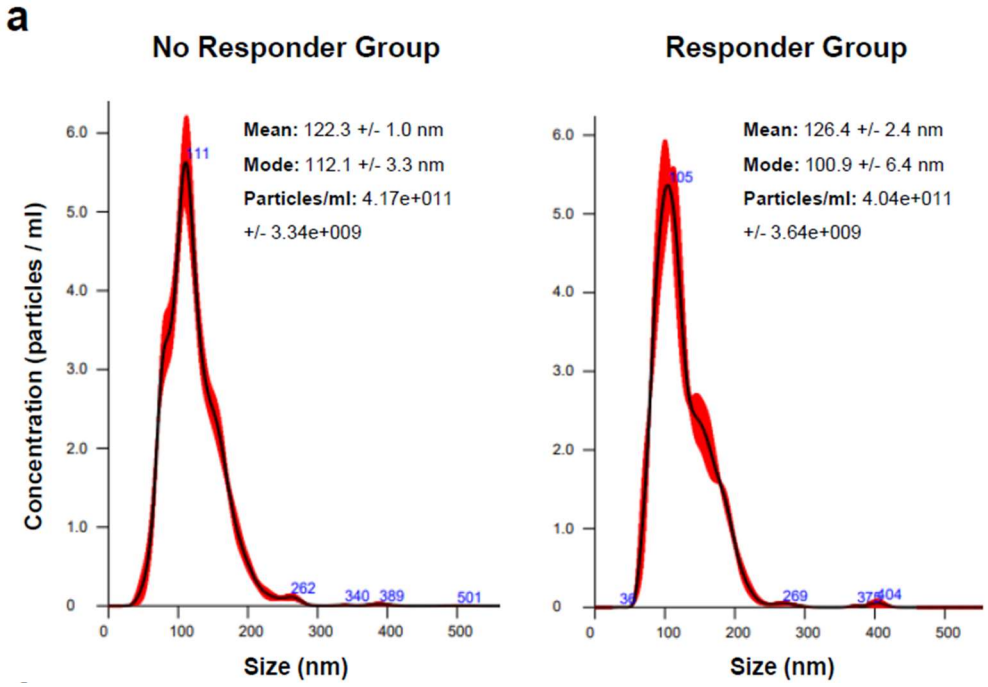
Figure 10. Kaplan-Meier analysis of overall survival. Median overall survival was 13.6 months for responders and 6.6 months for non-responders (HR 0.77, 95% CI 0.29-2.04; *p* = 0.60). Red line, responders; blue line, non-responders.

4.2.2 Nanoparticle tracking analysis

After isolation of EVs from plasma with SEC, nanoparticle tracking analysis has been performed for EV concentration and size. The analysis did not show any significant difference between responder and non-responder patients. A representative analysis for each group is shown in Figure 11a.

We have not observed any significant difference between the two patient groups in relative concentration of EVs between 50 nm and 150 nm or between 150 nm and 300 nm. For EVs with a diameter between 50 nm and 150 nm, we have observed a median relative concentration of 0.68 (range 0.52-0.86) in responders and 0.73 (range 0.52-0.82) in non-responders (p = 0.59; Figure 11b). For EVs with a diameter between 150 nm and 300 nm, we have observed a median relative concentration of 0.30 (range 0.13-0.45) in responders and 0.26 (range 0.18-0.45) in non-responders (p = 0.5; Figure 11c).

With regard to EV size, in responder patients we have reported a median of mean EV diameter of 134.3 nm (range 108.2-150.4 nm) and a median of mode diameter of 108.5 nm (range 78.5-126.5 nm). In non-responder patients, we have reported a median of mean EV diameter of 132.5 nm (range 119.3-147.1 nm) and a median of mode diameter of 105 nm (range 81.5-134.5 nm). We have not observed any significant difference, between the two groups, in the median of mean EV diameters (p = 0.5) or in the median of mode EV diameters (p = 0.98; Figure 11d-e).



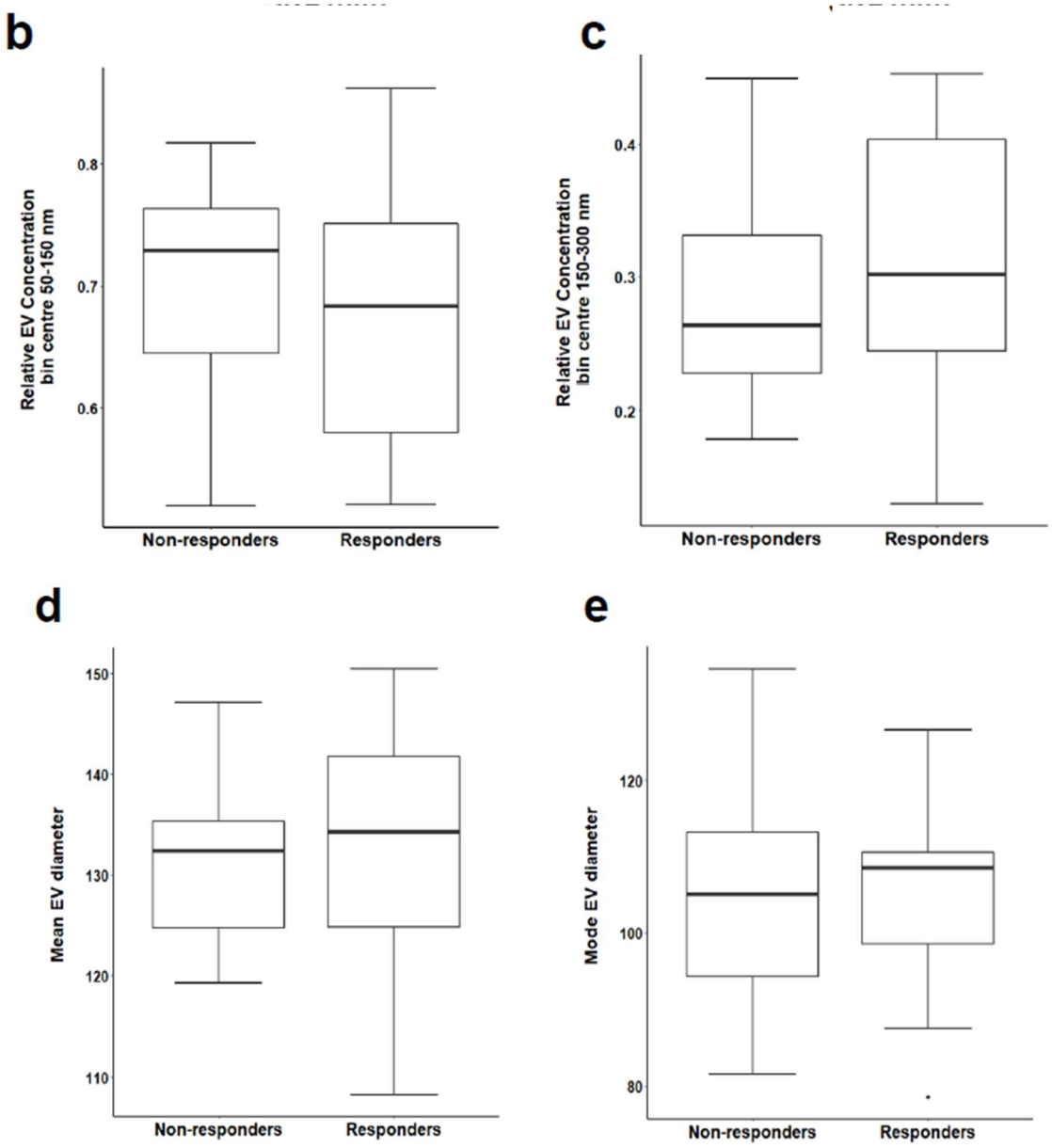


Figure 11. Nanoparticle tracking analysis. (a) A representative graph is showed for each group: non-responder and responder patients. **(b)** Relative concentration of EVs with 50-150 diameter (50-150 nm EV concentration / total concentration) in responder and non-responders. **(c)** Relative concentration of EVs with 150-300 diamter (150-300 nm EV concentration / total concentration) in responders and non-responders. **(d)** Median of mean EV diameter in responders and non-responders. **(e)** Median of mode EV diameter in responders and non-responders. In box plots black line is the median; lower and upper line of box are first and third quartile, respectively.

4.2.3 Analysis of EV surface markers

Flow cytometry with MACSPlex Exosome Kit has been performed to analyze the expression of EV surface proteins. The MFI of each surface marker is depicted in Figure 12a. The expression of typical exosomal markers (CD9, CD63, CD81) has been observed both in responder and non-responder patients (Figure 12a). Notably, we have observed a significantly higher expression of CD81 and SSEA4 in non-responder compared to responder patients ($p < 0.03$; Figure 12b).

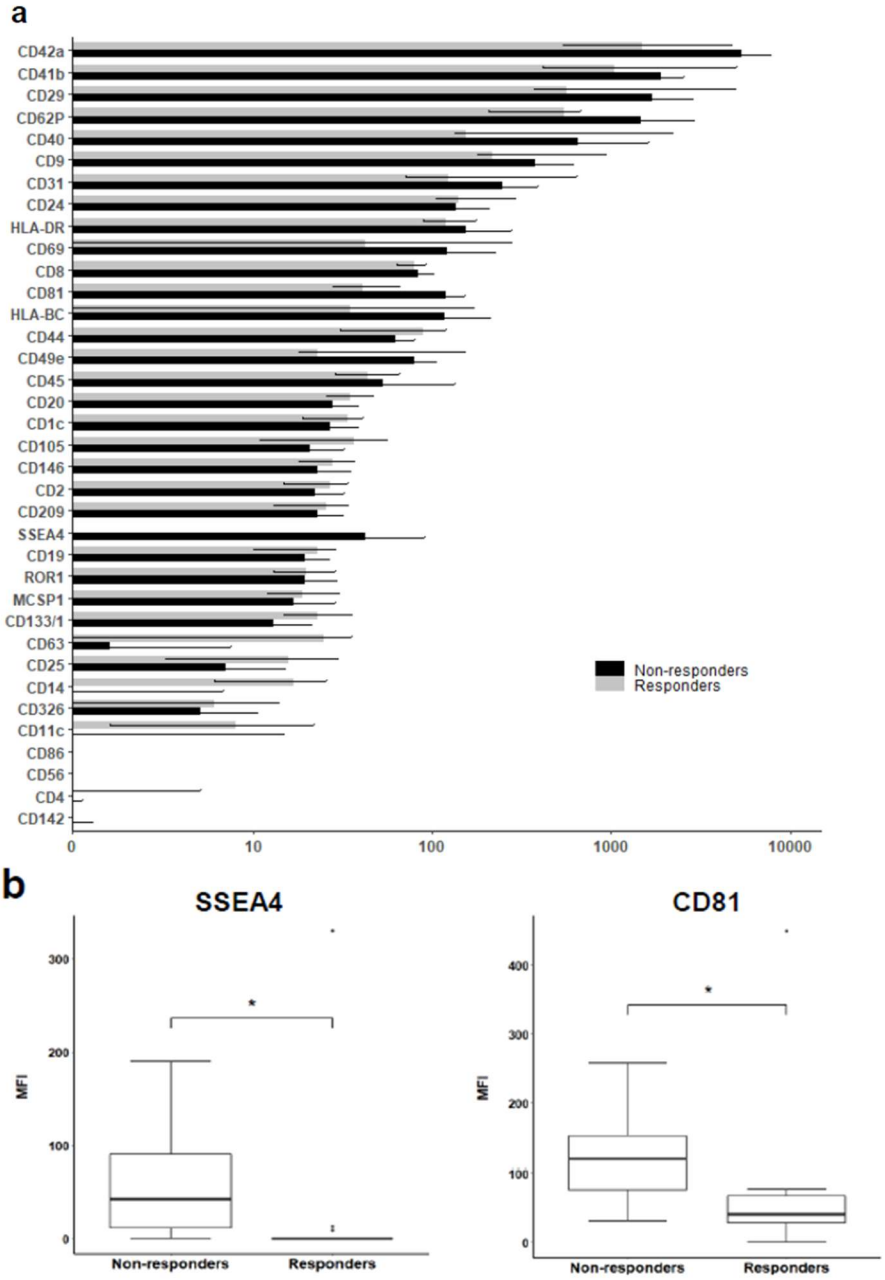


Figure 12. Flow cytometry. (a) The mean MFI of each surface marker is showed, bars are standard deviation. Gray, responders; black, non responders. **(b)** Surface expression of SSEA4 and CD81. In box plots black line is the median; lower and upper line of box are first and third quartile, respectively. * $p < 0.05$.

4.2.4 Analysis of EV miRNA content

Finally, after extracting RNA from EVs, we have analyzed the miRNA content of EVs with NextSeq 550 System and compared the expression of single miRNAs in responder and non-responder patients. We have observed a different expression of 44 miRNAs in EVs from responder and non-responder patients ($p < 0.05$; Figure 13). Of 44 miRNAs with different expression, 25 miRNAs were upregulated in responder patients (the most upregulated ones with a $\log_2$ fold change [FC] between 4.58 and 3.24), whereas the other 19 miRNAs were downregulated in responder patients (the most downregulated with a $\log_2$FC ranging from -2.86 to -1.83).

In particular, as for the most dysregulated molecules, we have observed an upregulation, in responder patients, of miR-141-3p, miR-141-5p, miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-375-3p, miR-429, and miR-545-5p (Figure 14). The possible tumor suppressor role of these miRNAs in PC has been searched in the literature; target proteins and their function are reported in Table 6.

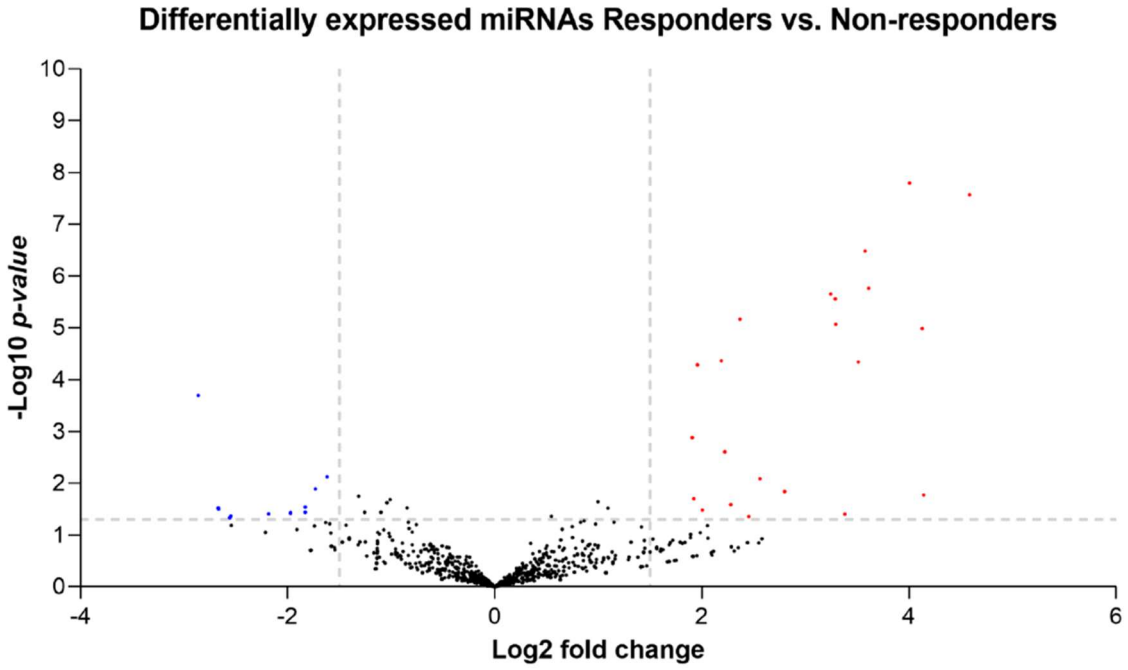


Figure 13. Analysis of miRNA expression in extracellular vesicles. Differentially expressed miRNAs between responder and non-responder patients are showed in the volcano plot. Red dots: miRNAs with $\log_2$FC > 1.5 and $p < 0.05$. Blue dots: miRNAs with $\log_2$FC < -1.5 and $p < 0.05$.

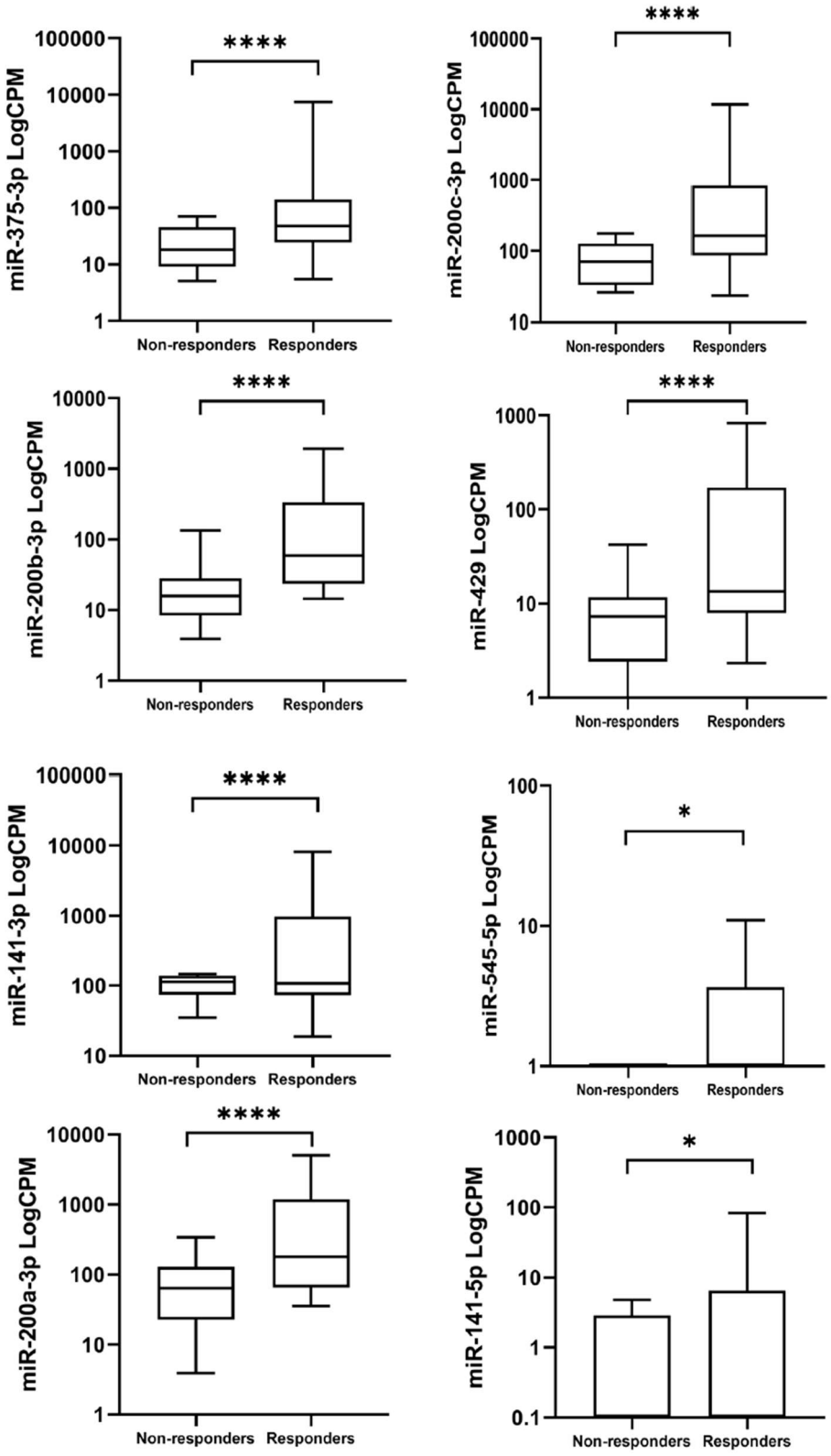


Figure 14. Expression of dysregulated miRNAs with possible role in pancreatic cancer. The most dysregulated miRNAs between responder and non-responder patients, with a possible role in pancreatic cancer, are shown. The expression of each miRNA is indicated with logarithmic scale of copy number per million value (LogCPM). In box plots black line is the median; lower and upper line of box are first and third quartile, respectively. * $p < 0.05$, **** $p < 0.0001$.

miRNA	Target	Function of target	Refs	log_2FC	*p*
miR-141-3p	**TM4SF1**	cell invasion and migration	77	3.576	3.298×10^{-7}
miR-141-5p	**TM4SF1**	cell invasion and migration	77	2.800	0.014
miR-200a-3p	**β-catenin**	cell proliferation and survival	72	3.287	2.736×10^{-6}
miR-200b-3p	**ZEB1**	epithelial-mesenchimal transition, gemcitabine resistance	73-76	3.242	2.220×10^{-6}
miR-200c-3p	**ZEB1**	epithelial-mesenchimal transition, gemcitabine resistance	71, 73-75	4.006	1.593×10^{-8}
miR-375-3p	**PDK1**	cell proliferation and survival, metabolism	78-79	4.584	2.685×10^{-8}
miR-429	**TBK1**	cell proliferation	81	3.611	1.728×10^{-6}
miR-545-5p	**RIG-I**	poor prognosis in PC	80	3.379	0.039

Table 6. Target and function of the most dysregulated miRNAs. For each of the most dysregulated miRNAs between responder and non-responder patients, here are reported target, function of target (with reference to previos reported role in pancreatic cancer), log_2 fold change (log_2FC) and *p* value.

5. DISCUSSION

In the present work, we show the feasibility of an EV-based liquid biopsy in order to obtain, in patients with advanced PC, predictive information for treatment with gemcitabine + nab-paclitaxel.

In the first part, we have compared samples from PC patients, treated with different chemotherapy regimens (gemcitabine + nab-paclitaxel, FOLFIRINOX, PAXG) and samples from healthy subjects, and we report a difference in EV size, expression of surface markers and miRNA content.

On the other hand, we did not observe a difference in EV concentration between advanced PC patients and healthy controls, whereas several studies previously reported an enhanced EV release by tumor cells compared to non-malignant ones, and accordingly a higher EV concentration in blood of patients with cancer [28,44,45]. This inconsistency is likely dependent on the difference in age between patients (median age 66, range 43-82) and healthy subjects (median 47, age 37-58). Indeed, it is known that circulating EV concentration decreases with aging [46]: this may compensate the expected difference between cancer patients and healthy donors, and give similar figures in our series. Moreover, the small size of cohorts, especially the healthy subjects (n = 7), has to be considered.

On the other hand, the differences in size and size distribution may suggest the present of different subpopulations in cancer patients: one may speculate that, as already reported, different EV subpopulations, with different miRNA content and function [47], may be present in PC patients compared to healthy subjects, consistently with a cancer scenario that adds even more complexity to the heterogeneity of EV populations [47].

As for the overexpressed surface markers in PC patients, it is not surprising to find several adhesion molecules: indeed, cancer hallmarks such as loss of cell-to-cell adhesion and anchorage-independent growth are dependent on cell adhesion molecules [48], and an altered expression of these may increase the malignant potential of cancer cells [48].

For example, CD31 is considered a marker of vascular density in PC. Interestingly, it has been associated with better OS in PC, and it has been proposed that this is related to the presence of stable vessels that supply anti-cancer immune cells [49]. CD31 plays a role in cell proliferation and migration, and, although usually related to endothelial cells, it is also expressed by tumor cells in several conditions (e.g., breast cancer and non-Hodgkin lymphomas) and contributes to tumor cell invasion [50]. In hepatocellular carcinoma, it has been found to promote metastases by inducing EMT and upregulating integrin β1 (also known as CD29) via the FAK/Akt signaling pathway [50].

Speaking of CD29, its upregulation in PC stem cells has been associated with increased invasion ability [51], and its expression has been associated with a critical role for metastatic process in PC [52]. The expression of CD29 in cancer stem cells has been associated with enhanced metastatic potential along with features of EMT in other tumor types such as breast cancer and squamous cell carcinoma [53,54].

As for miRNA content of EVs, some of the miRNAs we have found had been already described in relation to key features of tumor cells such as proliferation, survival, migration, and chemoresistance.

For example, miR-431-5p, increased in EVs from patients, has been reported to promote metastases of pancreatic neuroendocrine tumors by targeting DAB2 interacting protein (DAB2IP), a Ras GPTase activating protein, thus activating Ras/ERK pathway, and promoting EMT and cell migration and invasion [55].

On the other hand, miR-188-3p, decreased in PC samples, has been related to a tumor suppressor activity: its target BRD4 is an epigenetic regulator upregulated in PC cells and involved in cell proliferation growth and gemcitabine resistance of PC cells [56,57].

Other interesting findings are miR-216b-3p and miR-138-1-3p, both downregulated in samples from PC patients. Interestingly, miR-216b-3p is downregulated in PC tissue also, and inhibits pancreatic cancer cell progression and promotes apoptosis by down-regulating KRAS [58]. The target of miR-138 family is FOXC1, a member of the forkhead box family of transcription factors that has a crucial role: it increases the metastatic potential of PC cells by enhancing proliferation, migration, invasion, EMT, and angiogenesis [59].

Thus, we can conclude that the results discussed above, highlighting differences in EVs from PC patients and healthy subjects, especially in miRNA content, confirm the ability of our approach to detect cancer-related EV biomarkers that may provide information about tumor biology and potentially help to identify a predictive molecular signature.

Based on these premises, we have analyzed EVs from samples of advanced PC patients, collected before starting of first-line chemotherapy with gemcitabine + nab-paclitaxel, and investigated differences between responders and non-responders.

In our case series, we report a response rate of 62% with gemcitabine + nab-paclitaxel, that is higher than the figure reported in the phase 3 trial (23%)[10]. It must underlined that our cohort, differently from the population in the trial, included not only patients with metastatic disease, but also with locally advanced disease. Moreover, median baseline CA19.9 in our cohort was 417.0 U/ml, whereas in the whole trial population its value was 2469.7 U/ml (2293.7 U/ml in the arm treated with gemcitabine + nab-paclitaxel)[10], thus likely reflecting populations with different disease biology or tumor burden. Moreover, the small sample size of our cohort has to be taken into account (n = 21).

As for the survival analysis, in the whole patient cohort we report a median PFS of 8.0 months and a median OS of 13.3 months. In the abovementioned trial, PFS and OS were 5.5 and 8.5 months, respectively [10]. For these differences, the same caveats expressed above, including our small sample size, must be considered. As for the survival analysis comparing responder and non-responder patients, this has to be considered descriptive and explorative only: our study has not been designed for this purpose, so it does not have the power to detect a potential difference in survival between the two groups.

When investigating potential differences between responders and non-responders, at first we have evaluated NTA results, that did not show any difference between the two groups in terms of EV concentration and size (Figure 11). For EV concentration, this is consistent with other reports, showing that baseline EV concentration was not correlated with disease control rate in PC (57.1% of patients with advanced disease, 51.8% treated with gemcitabine + nab-paclitaxel)[60]; on the other hand, a correlation

between increased baseline concentration of leukocyte-derived EVs (CD45+) and higher disease control rate was reported in advanced PC [60].

With regard to the analysis of EV surface markers with flow cytometry, we have observed in responders a significantly lower expression of CD81 and SSEA4.

CD81 is a member of tetraspanin protein family, considered a typical EV marker [61-63], that has been associated with tumor growth and metastases in several cancers [64]. Differently from our finding, a previous study reported that baseline CD81 exosomal expression was not associated with different response rates (66% vs 33% for high vs low, respectively; p = 0.34)[65]. It has to be taken into account that in that work, the study cohort included 19 patients with advanced PC; of 19 patients, only 11 had been treated with gemcitabine + nab-paclitaxel, and data about correlation of CD81 expression with this subgroup only have not been reported. Moreover, exosome analysis had been performed with ELISA rather than flow cytometry, thus introducing a significant methodological difference compared to the present work.

Interestingly, a role of CD81 in chemoresistance has been showed in acute lymphoblastic leukemia, where it is involved in bone marrow microenvironment-induced chemoprotection; indeed, CD81 knockout induces chemosensitivity, by control of Bruton tyrosine kinase signaling and induction of p53-mediated cell death [66]. Thus, a role of CD81 in chemoresistance of PC too, consistently with our finding, can not be excluded.

As for SSEA4, it is a glycosphingolipid that is a stem cell marker, expressed in pancreatic stem cells also [67,68]. SSEA4 is overexpressed in several cancers and has been associated with disease progression; in tumor cell lines, it induces invasion, through loss of cell-cell interactions and gain of a migratory phenotype, and EMT [69]. SSEA4 is also expressed in PC cell lines but not in normal pancreatic cells, and high expression has been associated with reduced OS, thus being proposed as a potential target for treatment of PC [70]. Therfore, data from previous reports, that suggest SSEA4 as a marker for identification of heterogeneous, invasive subpopulations of tumor cells [69], are consistent with its higher expression in non-responder patients.

With regard to the core of this work, i.e. the analysis of miRNA content of EVs, we report several interesting findings, especially with miRNAs upregulated in responder patients. Of particular interest, we have observed an upregulation, in responders, of members of miR-200 family, that has been previously reported as a regulator of EMT [71]. MiR-200a is able to inhibit PC cell proliferation and metastasis by targeting β-catenin, a pathway dysregulated by interleukin-9 [72]. ZEB1 is a target of miR-200b-3p and miR-200c-3p; it is a zinc-finger enhancer binding (ZEB) transcription factor, involved in EMT, induction of stem-cell properties and gemcitabine resistance [73,74]. On the other hand, members of the miR-200 family induce epithelial differentiation, in a feedback loop whose dysregulation may be a driving force for cancer progression towards metastases [73]. The expression of miR-200b and miR-200c is down-regulated in gemcitabine-resistant PC cells, which show features of EMT, including a lower expression of the epithelial marker E-cadherin, and a higher expression of the mesenchymal marker ZEB1 [75]. On the other hand, up-regulation of miR-200 family, results in down-regulation of ZEB1, increased sensitivity to gemcitabine and reversal of EMT to an epithelial phenotype [74-76]. Indeed, miR-200c has been previously reported as a good prognostic factor in PC; its upregulation is associated with E-cadherin expression and inhibits PC invasion [71].
Among the other dysregulated miRNAs, miR-141 targets TM4SF1, a transmembrane protein highly expressed in PC cells and involved in cell invasion and migration [77]. As for miR-375-3p, it is downregulated in PC tissue and cell lines; its lower expression is associated with lymph node metastases and higher tumor staging [78]. The target of miR-375-3p is PDK1, a kinase in the PI3K-Akt-mTOR pathway, involved in cell proliferation and survival [78,79]; consistently, upregulation of miR-375 inhibits cell growth and induces cell apoptosis of PC cells [79]. As for miR-545, its upregulation inhibits growth of PC cell lines by targeting RIG-I, a protein whose high expression in PC tissues is correlated with lower survival [80]. Finally, upregulation of miR-429 is able to reduce growth of PC cell lines by targeting TANK binding kinase 1 (TBK1), an activator of KRAS and Akt; consistently, lower expression of miR-429 and higher expression of TBK1 in PC tissue are associated with shorter survival [81].

As mentioned in the results, other miRNAs showed a significantly different expression between responder and non-responder patients; for these miRNAs, a role in PC has not been previously shown, and they deserve further investigation.

The present work has some limitations: the small sample size (n = 21), and the retrospective design for the collection of clinical data.

However, we have showed the feasibility of the proposed approach in order to identify EV-derived miRNAs with predictive value in advanced PC. Our findings are further corroborated by the biological correlation with gemcitabine sensitivity previously reported for some of the identified miRNAs.

The identification of predictive factors is an unmet need of paramount importance in PC, especially in first line-therapy: as mentioned above, resistance to therapy is a frequent issue, and progressive disease often comes along with a deterioration in performance status that may not allow to start a second-line therapy. Moreover, chemotherapy has many adverse effects, tackling quality of life. Thus, the identification of predictive factors is critical to improve the risk-benefit ratio and allow a better management of this disease, i.e. to improve outcomes and avoid unnecessary adverse events. Nowadays, the only personalized approach is feasible in presence of *BRCA1/2* or *PALB2* mutations, that account for less than 10% of patients; in all other cases, choice of therapy is based on patient's performance status and a sequential strategy dependent on prescription limitations, especially for nab-paclitaxel.

Thus, a personalized approach, with the identification of reliable predictive factors that would allow to select the right treatment for the right patient, would have tremendous clinical impact, with several advantages: 1) improvement of treatment efficacy and outcomes (higher response rate, clinical benefit, increase in PFS and OS); 2) improvement in patient quality of life (avoidance of unnecessary adverse effects); 3) higher cost-effectiveness of the whole therapeutic strategy, that has to be pursued in a disease with a high social and economic burden (e.g. reduction of toxicity related-hospitalization for ineffective therapies, reduction of costs associated with disease progression).

Our personalized approach is based on liquid biopsy, thanks to the easy isolation of EVs from blood: this would translate in a minimally invasive approach with minimum discomfort for patients, that may be even repeated at different timepoints, e.g. in order to investigate possible early changes of EVs during treatment, or to detect potential alterations associated with acquired resistance, with the aim to adapt treatment strategy to the changing conditions. This approach can be expanded and include the analysis of other potential biomarkers such as circulating tumor cells and ctDNA.

Furthermore, the recent advancements in analytical methods, with an increased availability of high-throughput methods and omics technologies, along with a decreased cost for sequencing, make this approach feasible (e.g. in terms of turnaround time, that is crucial for information needed before starting therapy) and affordable.

In conclusion, we have proposed, and showed the feasibility of, a biology-based approach for personalized medicine in PC. Our approach is based on a comprehensive analysis of EVs in order to analyze the unique phenotype of each patient's disease, with the aim to identify predictive features and to establish the most appropriate treatment for each patient, in order to maximize chances of response and patient's outcome.

Our findings are worthy of further investigation: first, the analysis of a validation cohort in the same disease setting is required. If confirmed, findings would be worthy of further validation in a prospective clinical trial, with patients allocated to treatment based on molecular signature, in order to determine the real impact, in terms of response and survival, of this personalized medicine approach.

Other perspectives need to be addressed: our findings are worthy of investigation also with other chemotherapy regimens (e.g. FOLFIRINOX, PAXG) and in other disease settings such as preoperative treatment. Furthermore, a deeper understanding of mechanisms of drug resistance is required, and it should address the analysis of circulating factors at different timepoints and PC tissue. Finally, although we highlighted a predictive value of several miRNAs, based on the different expression between responder and non-responder patients, we expect that a composite molecular signature (composed of several miRNAs, and possibly of other biomolecules, e.g. by analyzing the

protein content of EVs) may result in a stronger predictive value, with higher impact in clinical choices for the management of PC.

6. REFERENCES

1. AIOM-AIRTUM, I numeri del cancro in Italia 2019. https://www.aiom.it/wp-content/uploads/2019/09/2019_Numeri_Cancro-operatori-web.pdf

2. Fitzmaurice, C. *et al.* Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study. *JAMA Oncol.* **5**, 1749–1768 (2019).

3. Hu, J. X. *et al.* Pancreatic cancer: A review of epidemiology, trend, and risk factors. *World J. Gastroenterol.* **27**, 4298–4321 (2021).

4. Siegel, R. L., Miller, K. D., Fuchs, H. E. & Jemal, A. Cancer statistics, 2022. *CA. Cancer J. Clin.* **72**, 7–33 (2022).

5 Rahib, L., Wehner, M. R., Matrisian, L. M. & Nead, K. T. Estimated Projection of US Cancer Incidence and Death to 2040. *JAMA Network Open* **5** (2021).

6. Kindler, H. L. A Glimmer of Hope for Pancreatic Cancer. *N. Engl. J. Med.* **379**, 2463–2464 (2018).

7. Wang, S. *et al.* The molecular biology of pancreatic adenocarcinoma: translational challenges and clinical perspectives. *Signal Transduct. Target. Ther.* **6** (2021).

8. Burris, H. A. *et al.* Improvements in survival and clinical benefit with gemcitabine as first- line therapy for patients with advanced pancreas cancer: A randomized trial. *J. Clin. Oncol.* **15**, 2403–2413 (1997).

9. Conroy, T. *et al.* FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. *N. Engl. J. Med.* **364**, 1817–1825 (2011).

10. Von Hoff, D. D. *et al.* Increased Survival in Pancreatic Cancer with nab-Paclitaxel plus Gemcitabine. *N. Engl. J. Med.* **369**, 1691–1703 (2013).

11. Reni, M. *et al.* Nab-paclitaxel plus gemcitabine with or without capecitabine and cisplatin in metastatic pancreatic adenocarcinoma (PACT-19): a randomised phase 2 trial. *Lancet Gastroenterol. Hepatol.* **3**, 691–697 (2018).

12. Abrams, T. A. *et al.* Patterns of Chemotherapy Use in a U.S.-Based Cohort of Patients with Metastatic Pancreatic Cancer. *Oncologist* **22**, 925–933 (2017).

13. Golan, T. *et al.* Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. *Br. J. Cancer* **111**, 1132–1138 (2014).

14. Wattenberg, M. M. *et al.* Platinum response characteristics of patients with pancreatic ductal adenocarcinoma and a germline BRCA1, BRCA2 or PALB2 mutation. *Br. J. Cancer* **122**, 333–339 (2020).

15. Golan, T. *et al.* Maintenance olaparib for germline BRCA-mutated metastatic pancreatic cancer. *N. Engl. J. Med.* **381**, 317–327 (2019).

16. Peretti, U. *et al.* Germinal BRCA1-2 pathogenic variants (gBRCA1-2pv) and pancreatic cancer: epidemiology of an Italian patient cohort. *ESMO open* **6** (2021).

17. Heeke, A. L. *et al.* Prevalence of Homologous Recombination–Related Gene Mutations Across Multiple Cancer Types. *JCO Precis. Oncol.* **2018**, 1–13 (2018).

18. Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **7**, 1535750 (2018).

19. Becker, A. *et al.* Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell* **30**, 836–848 (2016).

20. Chen, G. *et al.* Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature* **560**, 382–386 (2018).

21. Qiu, J. *et al.* Extracellular vesicles as mediators of the progression and chemoresistance of pancreatic cancer and their potential clinical applications. *Mol. Cancer* **17**, 2 (2018).

22. Massoumi, R. L., Hines, O. J., Eibl, G. & King, J. C. Emerging Evidence for the Clinical Relevance of Pancreatic Cancer Exosomes. *Pancreas* **48**, 1–8 (2019).

23. Chang, C. H. & Pauklin, S. Extracellular vesicles in pancreatic cancer progression and therapies. *Cell Death Dis.* **12**, (2021).

24. O'Driscoll, L. Expanding on Exosomes and Ectosomes in Cancer. *N. Engl. J. Med.* **372**, 2359–2362 (2015).

25. Zhao, H. *et al.* The key role of extracellular vesicles in the metastatic process. *Biochim. Biophys. Acta. Rev. Cancer* **1869**, 64–77 (2018).

26. Ho, W. J., Jaffee, E. M. & Zheng, L. The tumour microenvironment in pancreatic cancer - clinical challenges and opportunities. *Nat. Rev. Clin. Oncol.* **17**, 527–540 (2020).

27. Deng, D., Patel, R., Chiang, C.-Y. & Hou, P. Role of the Tumor Microenvironment in Regulating Pancreatic Cancer Therapy Resistance. *Cells* **11**, 2952 (2022).

28. Xu, R. *et al.* Extracellular vesicles in cancer — implications for future improvements in cancer care. *Nat. Rev. Clin. Oncol.* **15**, 617–638 (2018).

29. Menck, K. *et al.* Characterisation of tumour-derived microvesicles in cancer patients' blood and correlation with clinical outcome. *J. Extracell. Vesicles* **6**, 1340745 (2017).

30. Melo, S. A. *et al.* Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* **523**, 177–182 (2015).

31. Lai, X. *et al.* A microRNA signature in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer. *Cancer Lett.* **393**, 86–93 (2017).

32. Heredia-Soto, V., Rodríguez-Salas, N. & Feliu, J. Liquid biopsy in pancreatic cancer: Are we ready to apply it in the clinical practice? *Cancers (Basel)* **13** (2021).

33. Kamyabi, N., Bernard, V. & Maitra, A. Liquid biopsies in pancreatic cancer. *Expert Rev. Anticancer Ther.* **19**, 869–878 (2019).

34. Rawat *et al.* MicroRNA in Pancreatic Cancer: From Biology to Therapeutic Potential. *Genes (Basel).* **10**, 752 (2019).

35. Peng, Y. & Croce, C. M. The role of MicroRNAs in human cancer. *Signal Transduct. Target. Ther.* **1**, (2016).

36. Melo, S. A. *et al.* Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis. *Cancer Cell* **26**, 707–721 (2014).

37. Si, W., Shen, J., Zheng, H. & Fan, W. The role and mechanisms of action of microRNAs in cancer drug resistance. *Clin. Epigenetics* **11**, 25 (2019).

38. Mikamori, M. *et al.* MicroRNA-155 Controls Exosome Synthesis and Promotes Gemcitabine Resistance in Pancreatic Ductal Adenocarcinoma. *Sci. Rep.* **7**, 42339 (2017).

39. Patel, G. K. *et al.* Exosomes confer chemoresistance to pancreatic cancer cells by promoting ROS detoxification and miR-155-mediated suppression of key gemcitabine-metabolising enzyme, DCK. *Br. J. Cancer* **116**, 609–619 (2017).

40. Richards, K. E. *et al.* Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene* **36**, 1770–1778 (2017).

41. Daoud, A. Z., Mulholland, E. J., Cole, G. & McCarthy, H. O. MicroRNAs in Pancreatic Cancer: Biomarkers, prognostic, and therapeutic modulators. *BMC Cancer* **19**, 1–13 (2019).

42. Comandatore, A. *et al.* Potential Role of Exosomes in the Chemoresistance to Gemcitabine and Nab-Paclitaxel in Pancreatic Cancer. *Diagnostics* **12** (2022).

43. Eisenhauer, E. A. *et al.* New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur. J. Cancer* **45**, 228–247 (2009).

44. Brocco, D. *et al.* Circulating Cancer Stem Cell-Derived Extracellular Vesicles as a Novel Biomarker for Clinical Outcome Evaluation. *J. Oncol.* **2019**, (2019).

45. Bebelman, M. P., Smit, M. J., Pegtel, D. M. & Baglio, S. R. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol. Ther.* **188**, 1–11 (2018).

46. Noren Hooten, N. Extracellular vesicles and extracellular RNA in aging and age-related disease. *Transl. Med. Aging* **4**, 96–98 (2020).

47. Willms, E., Cabañas, C., Mäger, I., Wood, M. J. A. & Vader, P. Extracellular vesicle heterogeneity: Subpopulations, isolation techniques, and diverse functions in cancer progression. *Front. Immunol.* **9**, (2018).

48. Janiszewska, M., Primi, M. C. & Izard, T. Cell adhesion in cancer: Beyond the migration of single cells. *J. Biol. Chem.* **295**, 2495 (2020).

49. Katsuta, E. *et al.* Pancreatic adenocarcinomas with mature blood vessels have better overall survival. *Sci. Rep.* **9**, (2019).

50. Zhang, Y. Y. *et al.* CD31 regulates metastasis by inducing epithelial-mesenchymal transition in hepatocellular carcinoma via the ITGB1-FAK-Akt signaling pathway. *Cancer Lett.* **429**, 29–40 (2018).

51. Zhu, J., He, J., Liu, Y., Simeone, D. M. & Lubman, D. M. Identification of glycoprotein markers for pancreatic cancer CD24+CD44+ stem-like cells using nano-LC-MS/MS and tissue microarray. *J. Proteome Res.* **11**, 2272–2281 (2012).

52. Grzesiak, J. J. *et al.* Knockdown of the β(1) integrin subunit reduces primary tumor growth and inhibits pancreatic cancer metastasis. *Int. J. cancer* **129**, 2905–2915 (2011).

53. Vassilopoulos, A., Chisholm, C., Lahusen, T., Zheng, H. & Deng, C. X. A critical role of CD29 and CD49f in mediating metastasis for cancer-initiating cells isolated from a Brca1-associated mouse model of breast cancer. *Oncogene* **33**, 5477–5482 (2013).

54. Geng, S., Guo, Y., Wang, Q., Li, L. & Wang, J. Cancer stem-like cells enriched with CD29 and CD44 markers exhibit molecular characteristics with epithelial-mesenchymal transition in squamous cell carcinoma. *Arch. Dermatol. Res.* **305**, 35–47 (2013).

55. Zhang, T. *et al.* miR-431 Promotes Metastasis of Pancreatic Neuroendocrine Tumors by Targeting DAB2 Interacting Protein, a Ras GTPase Activating Protein Tumor Suppressor. *Am. J. Pathol.* **190**, 689–701 (2020).

56. Shi, W. *et al.* Long non-coding RNA LINC00346 promotes pancreatic cancer growth and gemcitabine resistance by sponging miR-188-3p to derepress BRD4 expression. *J. Exp. Clin. Cancer Res.* **38**, (2019).

57. Wang, Y. H. *et al.* BRD4 promotes pancreatic ductal adenocarcinoma cell proliferation and enhances gemcitabine resistance. *Oncol. Rep.* **33**, 1699–1706 (2015).

58. Wu, X. *et al.* MiR-216b inhibits pancreatic cancer cell progression and promotes apoptosis by down-regulating KRAS. *Arch. Med. Sci.* **14**, 1321 (2018).

59. Subramani, R. *et al.* FOXC1 plays a crucial role in the growth of pancreatic cancer. *Oncogenesis* **7**, (2018).

60. Brocco, D. *et al.* High Blood Concentration of Leukocyte-Derived Extracellular Vesicles Is Predictive of Favorable Clinical Outcomes in Patients with Pancreatic Cancer: Results from a Multicenter Prospective Study. *Cancers (Basel).* **14**, 4748 (2022).

61. Karimi, N., Dalirfardouei, R., Dias, T., Lötvall, J. & Lässer, C. Tetraspanins distinguish separate extracellular vesicle subpopulations in human serum and plasma –

Contributions of platelet extracellular vesicles in plasma samples. *J. Extracell. Vesicles* **11**, (2022).

62. Andreu, Z. & Yáñez-Mó, M. Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* **5**, (2014).

63. Mizenko, R. R. *et al.* Tetraspanins are unevenly distributed across single extracellular vesicles and bias sensitivity to multiplexed cancer biomarkers. *J. Nanobiotechnology* **19**, (2021).

64. Vences-Catalán, F. *et al.* CD81 as a tumor target. *Biochem. Soc. Trans.* **45**, 531–535 (2017).

65. Giampieri, R. *et al.* Clinical impact of different exosomes' protein expression in pancreatic ductal carcinoma patients treated with standard first line palliative chemotherapy. *PLoS One* **14**, (2019).

66. Quagliano, A., Gopalakrishnapillai, A., Anders Kolb, E. & Barwe, S. P. CD81 knockout promotes chemosensitivity and disrupts in vivo homing and engraftment in acute lymphoblastic leukemia. *Blood Adv.* **4**, 4393–4405 (2020).

67. Munro, M. J., Peng, L., Wickremesekera, S. K. & Tan, S. T. Colon adenocarcinoma-derived cells that express induced-pluripotent stem cell markers possess stem cell function. *PLoS One* **15**, (2020).

68. Lee, S., Lee, C. M. & Kim, S. C. Adult human pancreas-derived cells expressing stage-specific embryonic antigen 4 differentiate into Sox9-expressing and Ngn3-expressing pancreatic ducts in vivo. *Stem Cell Res. Ther.* **7**, 1–13 (2016).

69. Sivasubramaniyan, K. *et al.* Expression of stage-specific embryonic antigen-4 (SSEA-4) defines spontaneous loss of epithelial phenotype in human solid tumor cells. *Glycobiology* **25**, 902–917 (2015).

70. Lin, C. W. *et al.* Homogeneous antibody and CAR-T cells with improved effector functions targeting SSEA-4 glycan on pancreatic cancer. *Proc. Natl. Acad. Sci. U. S. A.* **118**, (2021).

71. Yu, J. *et al.* MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. *Mol. Cancer* **9**, (2010).

72. Hu, B. *et al.* Interleukin-9 Promotes Pancreatic Cancer Cells Proliferation and Migration via the miR-200a/Beta-Catenin Axis. *Biomed Res. Int.* **2017** (2017).

73. Brabletz, S. & Brabletz, T. The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer? *EMBO Rep.* **11**, 670 (2010).

74. Wang, Z. *et al.* Novel crosstalk between KLF4 and ZEB1 regulates gemcitabine resistance in pancreatic ductal adenocarcinoma. *Int. J. Oncol.* **51**, 1239–1248 (2017).

75. Li, Y. *et al.* Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res.* **69**, 6704–6712 (2009).

76. Funamizu, N., Ray Lacy, C., Kamada, M., Yanaga, K. & Manome, Y. MicroRNA-200b and -301 are associated with gemcitabine response as biomarkers in pancreatic carcinoma cells. *Int. J. Oncol.* **54**, 991–1000 (2019).

77. Xu, L. *et al.* hsa-miR-141 downregulates TM4SF1 to inhibit pancreatic cancer cell invasion and migration. *Int. J. Oncol.* **44**, 459–466 (2014).

78. Song, S. *et al.* Expression levels of microRNA-375 in pancreatic cancer. *Biomed. reports* **1**, 393–398 (2013).

79. Zhou, J. *et al.* MicroRNA-375 targets PDK1 in pancreatic carcinoma and suppresses cell growth through the Akt signaling pathway. *Int. J. Mol. Med.* **33**, 950–956 (2014).

80. Song, B. *et al.* miR-545 inhibited pancreatic ductal adenocarcinoma growth by targeting RIG-I. *FEBS Lett.* **588**, 4375–4381 (2014).

81. Song, B. *et al.* miR-429 determines poor outcome and inhibits pancreatic ductal adenocarcinoma growth by targeting TBK1. *Cell. Physiol. Biochem.* **35**, 1846–1856 (2015).